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Expression of cell division markers in the hippocampus in Alzheimer's disease and other neurodegenerative conditions

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Abstract Recent studies, showing that cell cycle-related nuclear proteins p105 and Ki-67 are associated with Alzheimer's disease (AD)-related cytoskeletal pathology, suggested that these proteins, in addition to their functions in regulating the cell cycle, may have more specialised functions in the adult nervous system. In order to test this hypothesis we studied the expression of the cell cycle-related proteins Ki-67, pCNA and p53 in the hippocampi of 33 subjects, including some with AD or other neurodegenerative disorders and some with no neurological disease. By immunohistochemistry we found nuclear expression of Ki-67 in all subregions of the hippocampus, with the highest levels in the dentate gyrus. Both neurons and glial cells expressed this protein. The proportion of cells positive for Ki-67 and the distribution pattern varied considerably depending on the pathological diagnosis. Neuronal nuclear expression of Ki-67 was increased in AD but was also elevated in young Down's syndrome subjects and in those with Pick's disease. Expression of this protein was therefore not AD-specific. We did not find nuclear pCNA or p53 expressed in our patient groups. Contrary to previous studies AD-type neurofibrillary tangles were not labelled with any of the cell cycle markers used. The presence of nuclear Ki-67 expression indicates that some hippocampal neurons are not in the quiescent G_0 phase but have re-entered the cell cycle. The absence of nuclear pCNA or p53 suggests that the cycle is arrested in G_1. The significance of our findings and their relationship to the production of neurodegenerative cell death via an apoptotic mechanism are discussed.

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Introduction

Cell proliferation markers and tumour suppressor gene products, such as Ki-67, pCNA, p105 and p53, have been used for the study of malignancy of different tumours, including brain tumours [4, 14, 21, 31], but little attention has been paid to their expression in other disease states.

Ki-67 is a DNA binding protein that plays an important role in the regulation of cell division [8, 28]. It is expressed in the nuclei throughout the entire cell cycle with the exception of the G_0 phase and the early stage of G_1 [8]. It starts to accumulate at the beginning of G_1, reaches a peak at mitosis and gradually diminishes afterwards. In dividing cells, after the breakdown of the nuclear envelope, Ki-67 is localised on the chromosomes and loosely distributed in the cytoplasm [28].

pCNA is a cell cycle-regulated protein co-factor for DNA polymerase δ [24]. It is a nuclear acidic protein and is present throughout the cell cycle with maximum expression during the S phase [7, 8]. It has been demonstrated that pCNA is not only a good immunological marker of the proliferative state of the cell, but also that a decrease in its level correlates directly with cellular differentiation [31]. Although its expression in the cytoplasm is also described, there is no convincing evidence regarding its cytoplasmic functions [14].

p53 protein is a nuclear phosphoprotein involved in DNA repair. It is known to halt the cell cycle in the late G_1 phase before DNA replication can occur in the S phase [15, 17]. In normal circumstances p53 has a short half-life and it is not present in detectable quantities. However, the half-life of the protein can be increased either by mutations or by its stabilisation [32], which in turn results in increased, immunohistochemically detectable, quantities of p53. Some studies have also suggested that the loss of p53 function leads to the over-expression of pCNA, consequently leading to DNA synthesis and cell division [3,

17]. Although the presence of p53 in cytoplasm has been described as a feature of neoplastic cells [4], like pCNA, the possible functions of the cytoplasmic p53 are not known.

Recent studies have suggested that cell proliferation markers such as Ki-67 and p105 are involved not only in the regulation of the cell cycle but that they might also have a role in neuronal plasticity in the adult nervous system. This idea is based on the findings that the expression of Ki-67 and p105 is associated with Alzheimer's disease (AD)-related cytoskeletal pathology [16, 28]. In this study we analyse the expression of Ki-67, pCNA and p53 in the hippocampus in neurodegenerative disorders with special emphasis on AD.

Patients and methods

Material from 33 patients was included in the study (Table 1). In order to discriminate AD patients we used the Tierney A1 inclusion criteria for AD [29]. According to these criteria patients with one or more neurofibrillary tangles (NFTs) labelled with antibody AT8 and one or more neuritic plaques per field in their hippocampi (mangification × 250) were considered to have AD. Patients who had neither AT8-positive tangles nor plaques in the hippocampus and did not present any other nervous system pathology were considered to be controls. Patients who had some AD-related pathology, but not sufficient to meet the above criteria, were considered as "pre-AD".

According to these criteria our cohort included 6 patients with AD, 2 of whom were adult Down's syndrome (DS) sufferers and 4 of whom had sporadic AD. The "pre-AD" category contained 19 patients – 3 also had DS, 2 were epileptics, 5 had Parkinson's disease (PD) and 9 patients had only AD-related pathology. Besides the mild AD-related pathology, epileptic patients had non-specific hippocampal sclerosis (cell loss in the CA1 and CA4 regions of the hippocampus), while PD patients had cortical Lewy bodies. Finally, we also had 8 patients who did not have any AD-related pathology. Of these 2 patients were diagnosed as Pick's disease, 2 had DS and 4 were considered as controls (Table 1). Subjects with DS were included because in this condition the pathological features of AD invariably develop by late middle age.

Immunohistochemistry

The tissue was fixed in 10% formalin for at least 4 weeks. Approximately 1-cm-thick tissue blocks were taken from the hippocampus at the level of, or anterior to, the lateral geniculate body. Ten-micron-thick serial sections were cut from the paraffin-embedded blocks and processed for silver impregnation and immunohistochemistry.

For the assessment of plaque pathology we used the methanamine silver stain [20]. AT8 antibody [12, 13] was used for the detection of AD-related cytoskeletal pathology (neuritic plaques and NFTs) using the standard avidin-biotin complex (ABC) technique. Adjacent sections were incubated with pCNA, Ki-67 and p53 antibodies. The AT8 and pCNA antibodies did not require any pretreatment. Microwave antigen retrieval was necessary for the Ki-67 and p53 antibodies. Rehydrated sections were placed in citrate buffer (0.01 M tri-sodium-citrate; pH = 6.0) and heated until boiling twice with a 5-min interval. p53 labelling was also performed using the standard ABC method, while for pCNA and Ki-67 we used the direct detection method using horseradish-peroxidase-conjugated primary antibodies (DAKO, High Wycome, UK). The antibodies used are listed in Table 2. As a positive control for all antibodies we used a glioma; the results for Ki-67 and pCNA are shown in Fig. 1 a, b. As a negative control we used the EPOS control serum provided by DAKO (Fig. 2).

Quantification

Quantification of the immuno-positive cells and nuclei was performed with an image analysis system (VIDAS 21). Neuronal nuclear and cytoplasmic labelling were recorded as the percentage of positive cells in the area examined [labelling index (LI)]. The LI was derived for each hippocampal subfield in each case using Ki-67 and AT8 antibodies. In methenamine silver-stained sections, diffuse and neuritic plaques were quantified in both the hippocampus and the adjacent temporal neocortex and expressed as number per square millimetre.

Statistical analysis

This was carried out using Statgraphics software. The differences in protein expression between the different diagnostic groups and different areas of the hippocampus were examined using the one-way analysis of variance (ANOVA) test. Differences between groups were regarded as significant if $P \leq 0.05$. However, since the

Table 1 Patient groups, number and age of patients included in the study (*AD* Alzheimer's disease)

Diagnosis	Abbreviations	No. of patients	Age years mean (range)
Control	C	4	46.2 (18–79.4)
Down's syndrome (without AD-related pathology)	DS	2	13 (13–13)
All AD patients	AD	7	
Down's syndrome	DS/AD	3	45 (12–65)
Sporadic AD patients	AD	4	80.2 (74–90.1)
Patients with AD-related pathology insufficient for the diagnosis of AD	Pre-AD	18	
Down's syndrome	DS/pre-AD	2	41 (40–42)
No other pathology	pre-AD	9	83.7 (77.1–90.6)
Epileptic patients	E/pre-AD	2	74 (71–77)
Lewy body disease	pre-AD+	5	80.7 (61–93.3)
Pick's disease	Pick's	2	63.5 (59–68)
Total		33	

Table 2 Antibodies used in the study (*IgG* immunoglobulin, *HRP* horseradish peroxidase, *DAB* diaminobenzidine, *AEC* aminoethylcarbazde)

Antibody		Concentration	Second layer	Third layer	Chromogen
AT8	Innogenetics	1:50	Anti-mouse IgG, Amersham 1:200	Streptavidin HRP, DAKO 1:200	DAB
pCNA	DAKO-EPOS[a]	As supplied			AEC
Ki-67	DAKO-EPOS[a]	As supplied			DAB
p53	Santa-Cruz	1:100	Anti-rabbit IgG, Amersham 1:200	Streptavidin HRP, DAKO 1:200	DAB

[a] Negative control serum also provided by DAKO

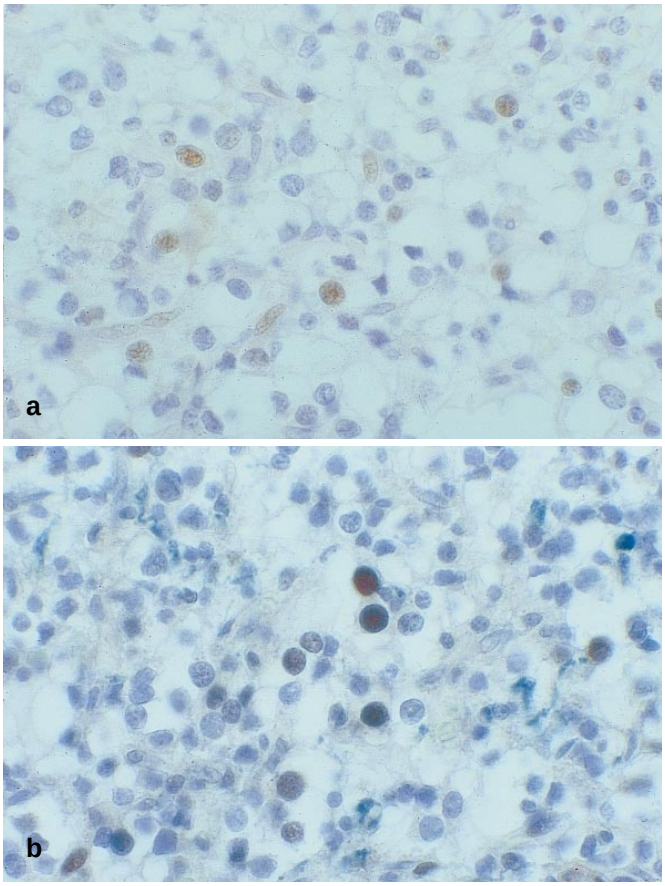


Fig. 1 a, b Expression of cell division markers in glioma. **a** Nuclear Ki-67 expression. **b** Nuclear pCNA expression

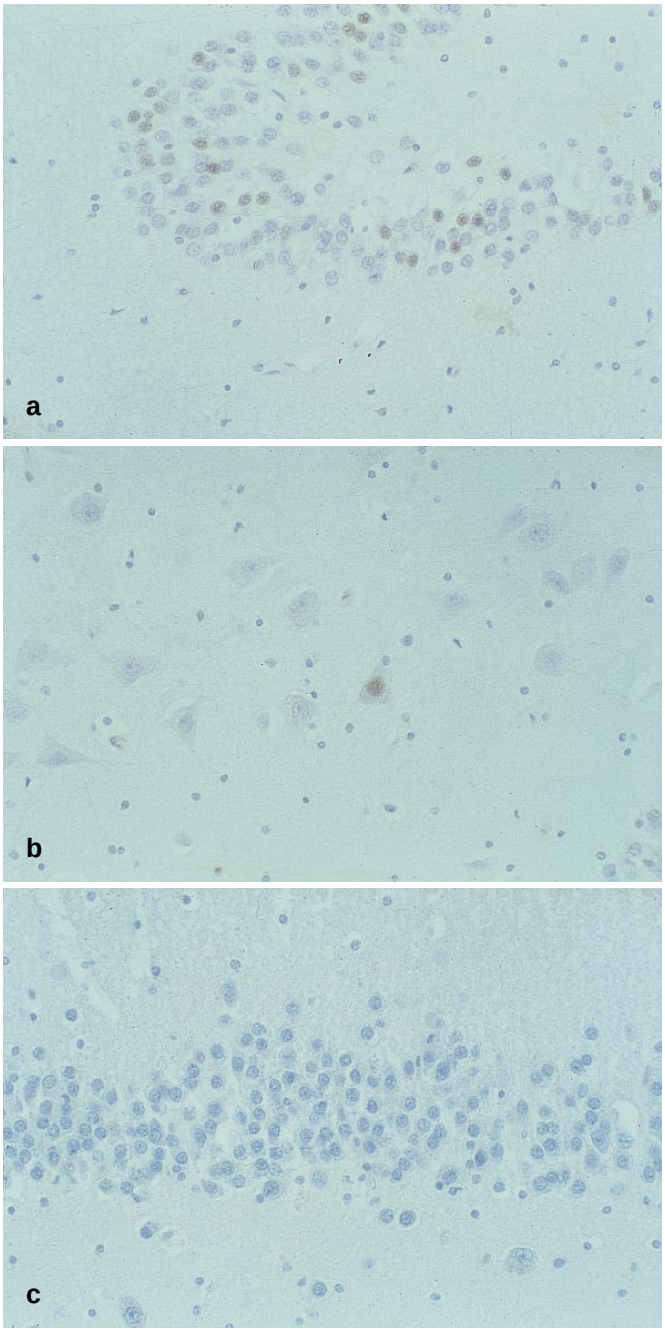
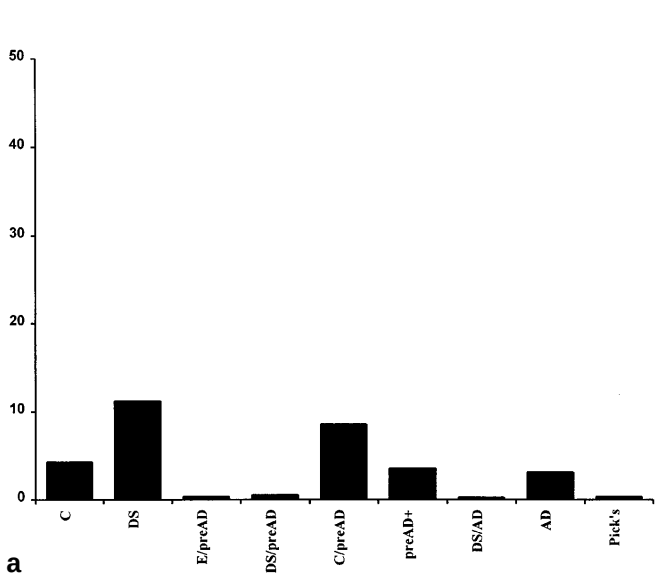


Fig. 2 a, b Expression of Ki-67 in Alzheimer's disease. **a** Ki-67-positive nuclei in the dentate gyrus of an Alzheimer's disease patient. **b** Ki-67-positive pyramidal cell in the CA3 region of the hippocampus of an Alzheimer's patient. **c** Negative control section for the EPOS antibodies provided by DAKO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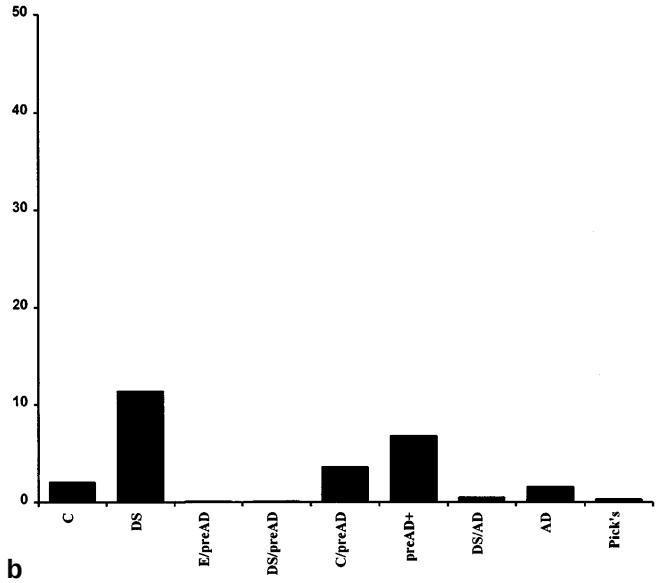
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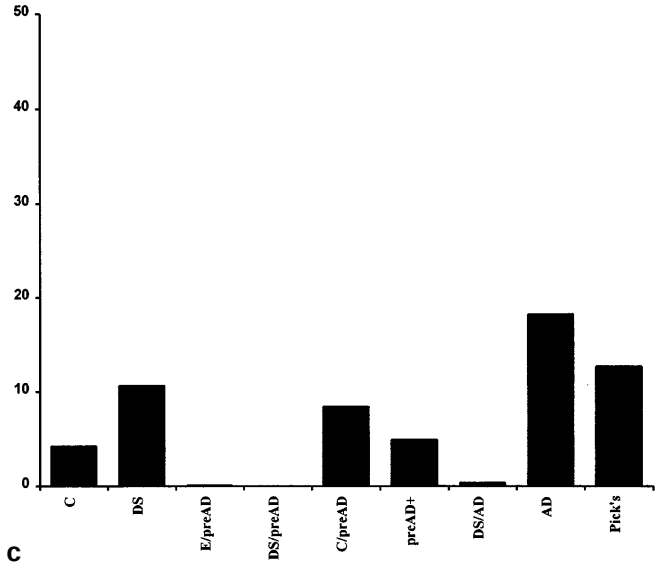
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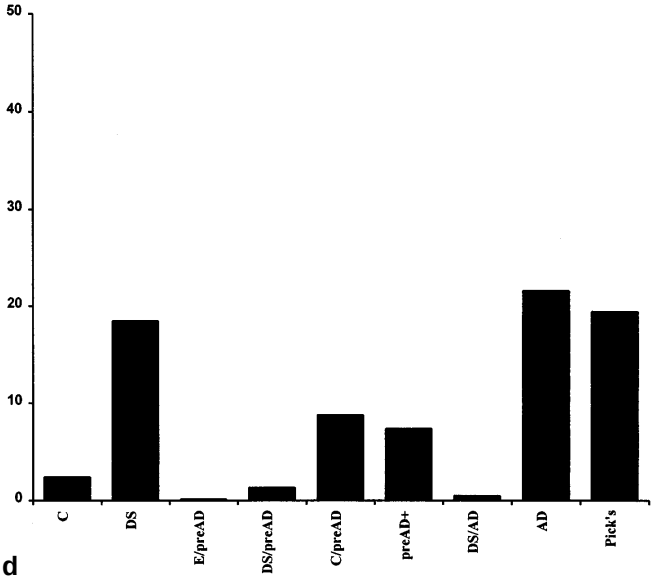
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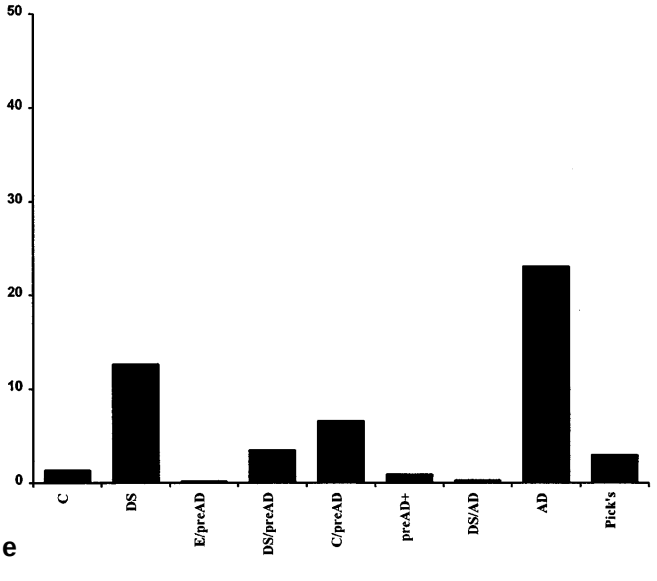
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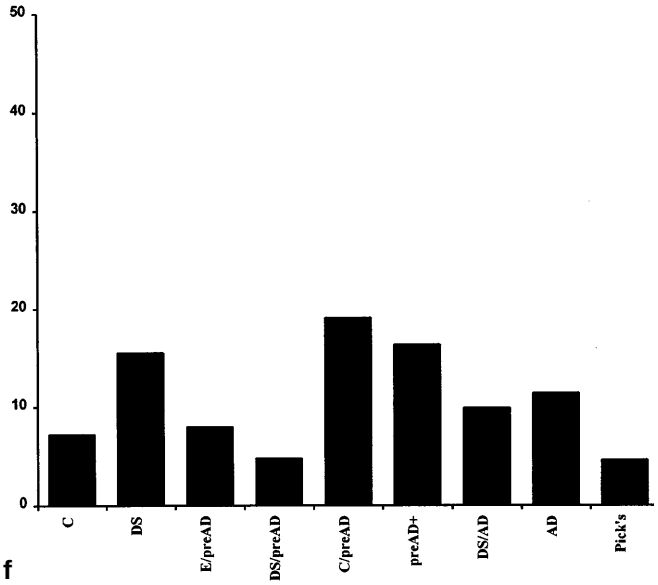
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number of cases in each patient group were relatively small, the results of the analysis are interpreted as trends only. Relationships between the different markers were analysed using the Spearman rank correlation.

Results

Neuritic and diffuse plaque densities were used as a reference for the evaluation of AD-related pathology (data not shown). The expression of AT8 antigen in neurons was by definiton higher in AD patients than in controls or the pre-AD categories (data not included).

Ki-67 expression

Ki-67 labelling of neuronal and glial nuclei was found in all patient groups (Fig. 2 a, b). The percentage of neurons with labelled nuclei in individual subjects varied from 0 to 41.2%, depending on patient category and hippocampal subfield.

Analysis of the regional distribution of Ki-67 labelling showed that in most of our patient categories the dentate gyrus was significantly more involved than any other region of the hippocampus (Fig. 3). The exceptions were young subjects with DS subjects with AD alone and those with Pick's disease, in which the LI was higher in CA4 or CA3 than in the dentate gyrus. In most disease categories, CA1 and subiculum had the lowest LIs.

Comparing the Ki-67 LIs in the hippocampus (all subfields pooled together) in the different patient categories we found that AD patients had significantly higher values than control subjects, DS cases with AD-related pathology (both DS/AD and DS/pre-AD groups) and epileptic patients (with AD-related pathology insufficient to make a pathological diagnosis of AD) (Fig. 3). The control group had significantly lower LIs than patients with some AD-type pathology insufficient to make the diagnosis of AD. LIs in young subjects with DS without any AD-related pathology were significantly higher than those found in DS cases with AD and DS patients with pre-AD. LIs in DS fell progressively as pre-AD and AD pathology supervened (Fig. 3).

Comparison of different subfields of the hippocampus in controls, patients with pre-AD and those with AD showed that pre-AD subjects had LIs in CA2, 3 and 4 intermediate between those of controls and those with AD (differences were not statistically significant). In the subiculum, CA1 and dentate gyrus the LIs were highest in the pre-AD stage of pathology. However, a statistically significant difference was found only when comparing dentate gyrus LIs in control subjects with those found in patients with AD-related pathology insufficient for the diagnosis of AD (Fig. 3). Young DS patients had significantly higher LIs in the CA1 region of the hippocampus than patients with DS-related AD or sporadic AD (Fig. 3).

No significant relationship was found in any disease category between the Ki-67 LI and that of AT8. Similarly Ki-67 LI did not relate to neuritic or diffuse plaque densities (data not shown). Age at death of the patients did not relate to the expression of Ki-67.

Ki-67 reaction with glial cell nuclei showed no clear relationship to disease category or hippocampal region. It seemed to be related to the extent of gliosis occurring in different areas in hippocampus. The expression of Ki-67 in our cases was not influenced by the post-mortem delay (data not included).

pCNA and p53 expression

No nuclear pCNA expression was detected in the hippocampi of any of our patients. However, in some patients varying degrees of cytoplasmic and white matter labelling was found. No nuclear p53 was detected in our cases. NFTs were not labelled by Ki-67, pCNA or p53 antibodies.

Discussion

Until recently the expression of cell proliferation markers, such as Ki-67 or pCNA and tumour suppressor gene products, such as p53 in the CNS were associated only with malignancy [4, 14, 21, 31]. However, some studies have shown the expression of pCNA in reactive glial cells in the human [19] and animal [18, 23] CNS and the report of p105, but not Ki-67, being associated with AD-related neurofibrillary changes drew attention to the possible involvement of these proteins in the plasticity of adult neurons [16]. Recently it was demonstrated that, in contrast to the previous report [16], Ki-67 is also associated with neurofibrillary changes of AD [28].

In the present study we found that Ki-67 is expressed in the nuclei of neurons and of glial cells in the hippocampus of AD patients (sporadic or DS-associated) as well as in patients with other forms of neurodegeneration (i.e. Pick's disease, epilepsy) and controls. The presence of Ki-67 indicates that these cells are not in the quiescent G_0 phase. However, the complete lack of nuclear pCNA labelling suggests that these cells have not passed beyond the G_1 phase of the cell cycle. These findings with respect to glial cells are not entirely unexpected, given the known capacity of glial cells to proliferate in response to damage. However, with respect to neurons the findings were unex-

◀ **Fig. 3 a–f** Distribution of nuclear Ki-67 labelling. Ki-67 labelling index in **a** subiculum, **b** CA1, **c** CA2, **d** CA3, **e** CA4, **f** dentate gyrus (*x axis* patient groups in our study, *y axis* labelling index, i.e. the ratio of labelled cells expressed in percent; *C* control subjects, *DS* patients with Down's syndrome without AD-related pathology, *E/preAD* epileptic patients with AD-related pathology insufficient to make the diagnosis of AD, *DS/preAD* Down's syndrome patients with some AD-related pathology insufficient to make the diagnosis of AD, *C/preAD* patients with no other than AD-related pathology insufficient for the diagnosis of AD, *preAD+* cortical Lewy body disease patients with AD-related pathology insufficient for the diagnosis of AD, *DS/AD* Down's syndrome-related AD, *AD* patients with AD, *Pick's* Pick's disease patients)

pected, since it is generally accepted that differentiated, adult neurons are in the resting G_0 phase of the cell cycle [25]. It is also believed that during development neuronal differentiation and the formation of synaptic connections occurs after cells have ceased to proliferate [25]. In the present study, although some neuronal nuclear Ki-67 expression was seen in controls as well as in disease states, there were interesting differences in the extent and distribution of this expression. Thus, it was low in controls and generally higher in disease states. In relation to AD pathology, the expression of Ki-67 in the subiculum, CA1 and dentate gyrus was higher in pre-AD (i.e cases with AD pathology insufficiently severe to meet the Tierney A1 criteria for AD) than in full-blown AD, while the involvement of CA2, CA3 and CA4 increased with the accumulation of AD-related pathology. In DS, on the other hand, there was more neuronal nuclear Ki-67 expression in young subjects not yet expressing AD-type pathology than in cases with any AD pathology. Overall, the granule cells of the dentate fascia most commonly expressed nuclear Ki-67 and cells of the subiculum and CA1 were less affected, though this varied somewhat between disease groups.

Possibly relevant to these findings are the relationships between the cell cycle and apoptosis [22], and the relationships between the cell cycle and cytoskeletal changes found in neurodegeneration. With regard to apoptosis, a suggested mechanism of cell death in neurodegenerative disorders including AD [27], it has been observed that cells arrested in G_1 phase may subsequently proceed to apoptotic cell death if they are unable to divide [30]. This observation might predict that, in AD, cells in G_1 would be concentrated in the subiculum and CA1, subregions of the hippocampus in which cell death occurs [34]. However, this predicted pattern is not the one we observed in this study, since neurons of the CA4, CA3 and CA2 most frequently expressed nuclear Ki-67 in AD patients, and the fascia dentata was the most affected region in pre-AD cases. Likewise, in epilepsy the distribution of maximal Ki-67 expression in the dentate gyrus did not resemble the distribution of neuron loss, which in maximal in CA1.

A possible alternative explanation of this distribution of Ki-67 that is worth considering is that it is related to loss of hippocampal synaptic connections. In AD, but also in ageing to a much lesser extent, there is a gradual loss of synaptic input to the hippocampus [26, 33]. The first neuronal changes in both AD and ageing appear in the entorhinal cortex [6], probably reducing the entorhinal input to the dentate fascia of the hippocampus [26]. Could this explain why there is most abundant expression of Ki-67 in the dentate fascia of the hippocampus in the pre-AD cases? It would need to be postulated that cells that lose their synaptic input leave G_0 and re-enter G_1 (therefore becoming Ki-67-positive). Their fate thereafter in unclear, but since cell death in AD occurs in hippocampal regions with low Ki-67 expression it would seem that other mechanisms than entry into the cell cycle may lead to cell death in AD. The finding of higher Ki-67 labelling in DS without AD-related changes and DS cases with pre-AD than in DS with full-blown AD might suggest that cell-cycle perturbances may be an early event in the development of AD-related pathology and that as cells die the number of remaining cells in G_1 decreases. This is also supported by the finding that elevated levels of mitogenic factors [1, 9, 10] in AD precede the formation of paired helical filaments. Mitogen-activated protein (MAP) kinase, a likely candidate for tau phosphorylation, has also been found to be elevated early during the course of AD [2]. It has been found that activation of MAP kinases can trigger cells to exit the quiescent G_0 phase and re-enter the cell cycle [5] and it has also been found recently that p21, a protein involved in the arrest of the cell cycle via a p53-dependent pathway, was expressed at high levels in tangle-bearing neurons in AD [11].

It could, then, be speculated that cell death in AD occurring via the apoptotic pathway may involve an aberrant re-entry into the cell cycle that in turn is arrested in a p53-dependent manner. Our observation of the expression of Ki-67 as a marker of re-entry into the cell cycle in other neurodegenerative processes as well as in AD and control patients indicates that the phenomenon is not AD-specific. Nor is it clearly age-related. Moreover, the regional distribution of nuclear Ki-67 in the hippocampus does not parallel neuronal loss. However, it is possible that the phenomenon of neuronal Ki-67 expression is enhanced by any condition, including AD, Pick's disease, ischaemia, epilepsy, etc., leading to functional disconnection of neurons and it is possibly accelerated in AD by the accumulation of mitogenic growth factors in senile plaques. However, for the elucidation of the phenomena involved, further studies looking at other, more specific markers of the cell cycle are necessary. It is also important that such studies should involve more patients and controls to allow a more accurate analysis of the factors that influence or are influenced by the possible re-entry of neurons into the cell cycle.

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