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Histological Markers in Nasal Mucosa of Patients with Alzheimer's Disease

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Abstract

Neuropathological changes such as dystrophic neurites and the presence of abnormal τ protein in the olfactory system, including primary sensory cells and nerve fibres have previously been demonstrated in nasal mucosa tissue of patients with Alzheimer's disease (AD). These changes were detected in autopsy-derived material from histopathologically confirmed AD cases as well as in biopsy tissue from clinical severely ill AD patients. To investigate the potential usefulness for the early diagnosis of AD, we obtained biopsy tissue from olfactory mucosa from 5 clinically mild to moderate AD patients and stained for the presence of τ or β-amyloid by immunocytochemistry using a panel of specific antibodies. No positive staining was found in any of the cases. For comparison, post-mortem olfactory tissue from AD patients with severe neuropathological changes (widespread neurofibrillary tangles and amyloid in the brain) was investigated. In these severe cases, τ immunoreactivity was found in fine nerve fibres in the lamina propria and in a few olfactory epithelial cells. These results are consistent with other reports showing that cytoskeletal changes and τ pathology in the olfactory epithelium are not primary (or specific) features of AD and may occur predominantly in late stages of the disease.

Key Words
Biological markers
τ protein
Immunocytochemistry
Nasal mucosa

Introduction

Alzheimer's disease (AD) is a common neurodegenerative disorder that leads to dementia and death. Consistent pathological hallmarks of AD are the formation of brain amyloid, consisting mainly of amyloid β-peptides (Aβ), and neurofibrillary tangles (NFTs, and their constituent paired helical filaments), with the microtubule-associated protein τ as the major component [for a review, see 1]. The clinical manifestation of the disease may be preceded by cerebral amyloid deposition for years, if not decades [2, 3]. Therefore, neurodegeneration in AD is regarded as a long-term process that may be eliminated only by preventive therapeutic strategies. The availability of valid markers for early diagnosis is a major prerequisite for establishing preventive or therapeutic strategies in AD. Several studies have suggested that olfactory deficiencies may be one of the early signs of neurodegeneration in AD [4]. Because human olfactory mucosa contains neuronal structures of ontogenetic central origin, it is suspected

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that signs of neurodegeneration might be detected in this area in early clinical or even preclinical stages of AD. Talamo et al. [5] reported dystrophic cytoskeletal changes in olfactory epithelium of AD post-mortem tissue. τ protein immunoreactivity using affinity-purified antisera was detected in autopsy [6] as well as in biopsy-derived olfactory epithelium from AD patients [7]. As the latter study was restricted to severely demented patients (Mini Mental State, MMS [8], scores ranging from 0 to 11 of max. 30), we investigated whether specific histopathological changes (τ and/or β-amyloid deposition) in the olfactory epithelium may be detectable in AD patients with beginning dementia. We therefore obtained biopsy tissue from olfactory mucosa from 5 clinically mild to moderate AD patients and stained for the presence of τ or β-amyloid by immunocytochemistry using a panel of specific antibodies.

Methods and Materials

Nasal mucosa biopsy samples were obtained from patients with mild to moderate probable AD according to the National Institute of Neurological and Communicative Disorders Association (NINCDS-ADRDA) criteria [9] (n = 5, age 46–82 years, only males, MMS ± SD 21 ± 3, MMS range 16–24, duration of the disease: mean ± SD 3.2 ± 1.3 years, range 2–5 years). The Structured Interview for the Diagnosis of Dementia [10] score was 33 ± 10 (max. 55). Two patients were CDR stage 1 (mild dementia), 3 patients were CDR stage 2 (moderate dementia; Clinical Dementia Rating [11]). Informed consent was given by each patient and their caregivers before each investigation. The biopsy patients underwent brief olfactory testing and were able to recognize coffee, faeces, lilac, menthol, acetic acid, pyridine and chloroform. The study was approved by the local ethics committee. Autopsy samples of the olfactory mucosa from non-demented controls of similar age (n = 5) were obtained from the Institute of Forensic Medicine, University of Munich, Germany. Brain tissue and olfactory mucosa from 2 AD patients served as positive controls for immunocytochemistry. Both patients were clinically diagnosed as probable AD according to the NINCDS criteria and were neuropathologically confirmed. The first AD case was an 88-year-old woman. The post-mortem delay was 2 h. The patient was clinically described as severely demented. The duration of the disease was 3 years. No familial history of dementia was reported and the patient suffered from minor depression. Other disorders included chronic heart failure, sick sinus syndrome, diabetes mellitus II and polyneuropathy. The neuropathological investigation showed numerous senile plaques and neurofibrillary tangles in the hippocampus, parahippocampus, entorhinal cortex and cortex. The Braak [12] staging was V. No other CNS disease was detected. Pneumonia caused by aspiration was indicated as cause of death. Other internal disorders included diabetic retinopathy, diabetic nephropathy and systemic amyloidosis (liver, heart, lung). The second AD case was an 86-year-old woman. The post-mortem delay was 38 h. The patient was clinically described as severely demented and the duration of the disease was 7 years. No familial history of dementia was reported. The patient suffered from chronic pain and chronic right heart failure. The neuropathological investigation showed numerous senile plaques and NFTs in the hippocampus, parahippocampus, entorhinal cortex, amygdala and cortex. The Braak staging was V. No other CNS disease was detected. Acute right heart failure and pulmonary embolism were documented as causes of death. Other disorders included emphysema, pericarditis and deep-vein thrombosis (right leg).

Biopsies of the olfactory mucosa were performed by using the technique of Lovell et al. [13]. During local anaesthesia with 4% lidocaine an approximately 2 mm^3 small piece of tissue was removed with a cup forceps from the superior and medial nasal turbinate using a rhinoscope. The tissue samples were immediately placed in 4% phosphate-buffered paraformaldehyde and fixed for 24–48 h. For immunohistochemical experiments the samples were cut into 10-μm sections with a freezing microtome. After blockage of endogenous peroxidase, the serial sections were incubated overnight with a monoclonal antibody (mAb) against τ, called AT8 [14] or a polyclonal antibody (pAb; Dako, Hamburg, Germany). The mAb AT8 recognizes an epitope including Ser202 when phosphorylated, the pAb from Dako recognizes the C-terminal epitope of human τ. Additional antibodies used for staining included affinity-purified polyclonal anti-β-amyloid (β-A4, Boehringer Mannheim) and polyclonal anti-neuron-specific enolase (NSE, Dako) to visualize nerve fibres. All antibodies were used in a concentration of 10 μg/ml in Tris-phosphate-buffered saline, containing 0.3% Triton X-100 and 1% normal goat serum. Routine immunocytochemistry was performed with the peroxidase-antiperoxidase method [15], using diaminobenzidine as substrate for visualizing the immunoreactivity. Sections were slightly counterstained with haematoxylin and analysed and photodocumented with a Zeiss axiophot microscope.

Results

The results are summarized in figures 1–7. Brain tissue of patients with AD shows very bright immunohistochemical staining of τ in NFTs and in nerve fibres within neuritic plaques with each of the anti-τ antibodies (fig. 4). In the nasal tissue, nerve fibres were identified by positive NSE staining (fig. 5 and 6).

In postmortem tissue from neuropathologically confirmed AD cases, τ immunoreactivity was detected in fine nerve fibres in the lamina propria and in a few olfactory epithelial cells (fig. 1–3). Using the mAb AT8 and the pAb from Dako gave similar results. In contrast, neither the autopsy samples from non-demented controls nor the biopsy specimens from probable AD patients showed any τ-immunoreactive neurites (fig. 7). β-Amyloid was not detectable in any of the tissue samples from nasal mucosa (data not shown).

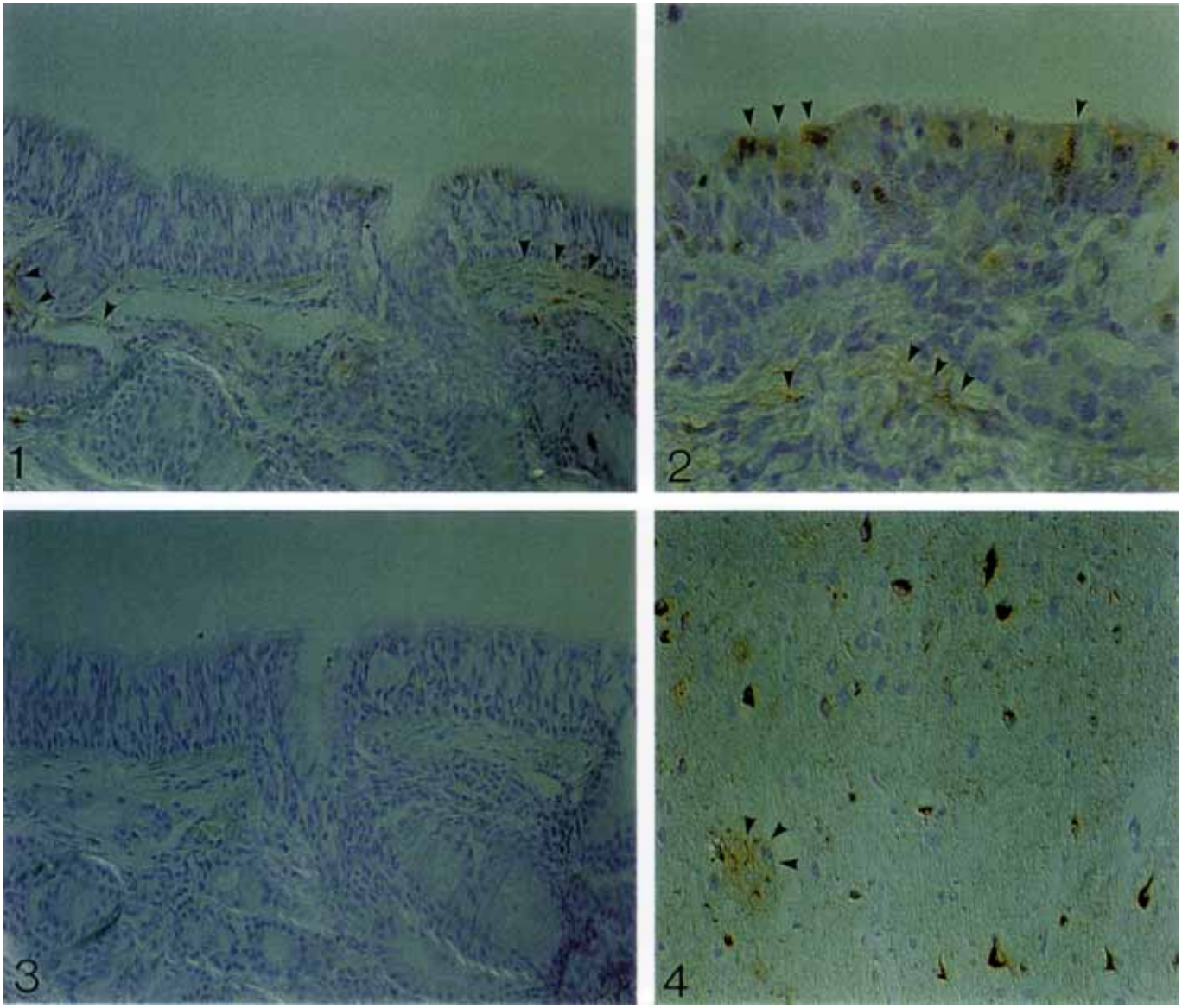
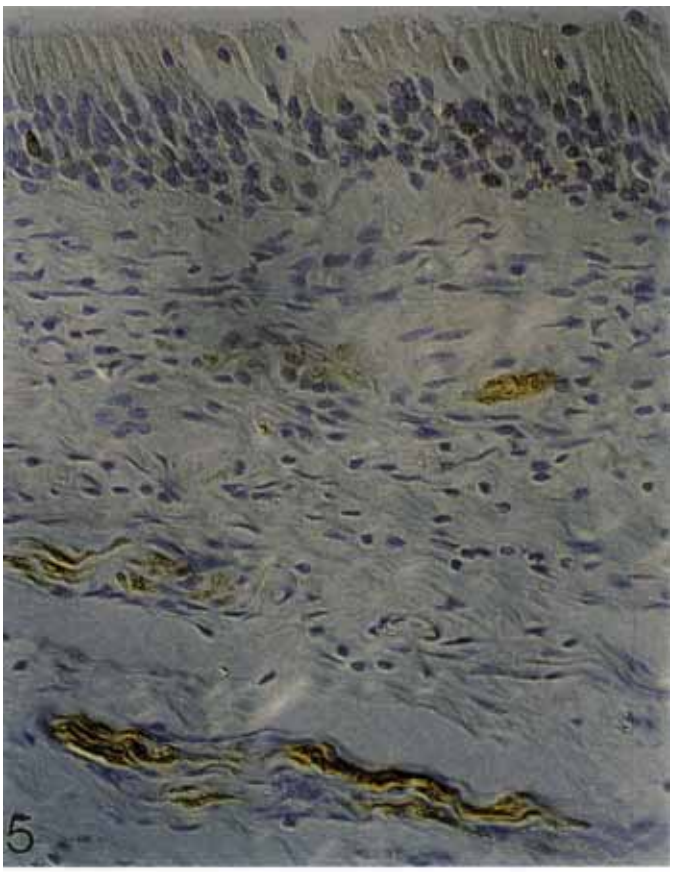


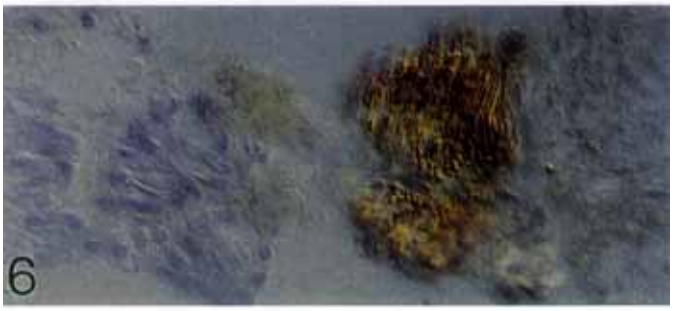
Fig. 1. Cross-section through the nasal mucosa (autopsy tissue) of a neuropathologically confirmed AD case (stage V-VI in Braak's classification). Arrowheads indicate mAb AT8 τ-immunoreactive nerve fibres in the lamina propria and a thin fibre surrounding a subepithelial gland. Magnification: × 200.

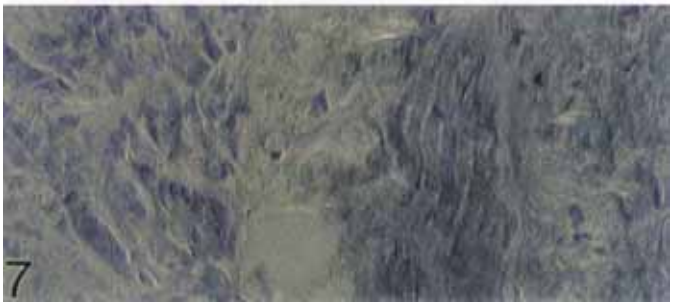
Fig. 2. Cross-section through the nasal mucosa (autopsy tissue) of a neuropathologically confirmed AD case (stage V-VI in Braak's classification). Arrowheads indicate mAb AT8 τ immunoreactivity in the olfactory epithelium and fine fibres of the lamina propria. Magnification: × 500.

Fig. 3. Cross-section through the nasal mucosa (neighbouring to 1, autopsy tissue) of a neuropathologically confirmed AD case incubated with non-immune IgG. Note the absence of staining clearly indicating the negative control used in these experiments. Magnification: × 200.

Fig. 4. Anti-τ AT8 immunoreactivity in frontal cortex (post-mortem tissue) of an AD case. This tissue served as positive control and clearly showed dense staining of abnormal cytoskeletal elements in neurons and neurofibrillary structures in a plaque (arrowheads). Magnification: × 400.







Discussion

Histopathological changes in the olfactory epithelium have been discussed as potential diagnostic markers of AD. However, to date, τ immunoreactivity has been demonstrated only in autopsy material [6] or biopsy-derived nasal mucosa from severely demented AD patients [7]. These findings were confirmed in the two late-stage AD cases of this study, which showed severe neurofibrillary changes throughout the limbic (i.e. entorhinal cortex) and isocortical areas (i.e. frontoparietal, temporal cortex). In these cases τ immunoreactivity was demonstrated in fine submucosal nerve fibres and in a few primary sensory cells of the olfactory epithelium.

To serve as a tool for early diagnosis in AD, a potential marker should be present during early progression of AD. Although brain areas belonging to the olfactory system are consistently affected during the neurodegenerative process, i.e. plaques, NFTs and cell loss in the olfactory bulb, olfactory nuclei and amygdala [16–18], it is presently unknown at what stage during the disease pathological neuronal changes spread to the peripheral olfactory epithelium and related nerve fibres. Using a panel of highly specific monoclonal antibodies, we were unable to detect the presence of τ or β-amyloid in olfactory mucosa in any of the 5 AD patients with beginning dementia. The patients had been carefully diagnosed using standard clinical criteria and were shown to have mild to moderate AD. Due to the consistently negative results and because of the invasive diagnostic procedure, the study was not expanded beyond this point. Although the number of patients was relatively small, the identical results in all patients suggest that olfactory biopsies may not be useful

Fig. 5. NSE staining of nerve fibres in the subepithelial connective tissue of nasal mucosa. Tissue was obtained from a non-demented control autopsy. Magnification: × 500.

Fig. 6. Cross-section through the nasal mucosa obtained by biopsy from a patient with moderate AD. High-power magnification of nerve fibres stained by anti-NSE pAb. Magnification: × 800.

Fig. 7. Neighbouring section, depicting the same nerve fibres as shown in figure 6, stained with anti-τ. Note the complete absence of immunoreactivity. The finding is representative of all tissue sections and patients investigated. Magnification: × 800.

as a diagnostic procedure in early AD. It is tempting to speculate that, as τ immunoreactivity is detectable in patients with severe dementia [7], the occurrence of abnormal τ proteins in the olfactory mucosa might reflect the progression from mild/moderate to severe stages of the disease. In addition to the poor sensitivity of τ staining in the nasal mucosa of AD patients, recently, the specificity of abnormal τ protein staining has been questioned. Kishikawa et al. [19] described the appearance of abnormal τ proteins in the olfactory epithelium even in postmortem tissue from young patients without dementia. Similar, neuritic changes in the olfactory epithelium are also found in a number of neurodegenerative disorders and in neurologically healthy humans [20].

In contrast to the autopsy procedures, the amount of material obtained by biopsy is much less. However, obtaining more material by biopsy from deeper regions of the nose would increase the risk of side-effects, such as bleeding or a reduction of the olfactory sensorium. Therefore, more invasive procedures than the one described in the present study were not performed. It can therefore not be completely excluded that the absence of τ-reactive neurites in biopsies from living AD patients may be, at least in part, due to limitations of the technique. Nevertheless, it has to be emphasized that the overall goal of this study was to contribute to the development of a clinical diagnostic tool for early diagnosis of AD. Both medical and ethical concerns make it unlikely that any nasal biopsy technique more invasive than the technique applied here would be suitable for widespread use. On the other hand, it is unlikely that the lack of τ immunoreactivity is due solely to technical reasons, as the autopsy cases demonstrated τ immunoreactivity also in the subepithelium. As in the biopsy cases nerve fibres were clearly demonstrated in the subepithelium (fig. 6), one may expect τ immunoreactivity in these samples as well. Finally, there is another argument that supports the assumption that a milder severity of the disease may be the major explanation for τ immunoreactivity lacking in nasal biopsy tissue. It has been shown that central parts of the olfactory system are more affected than peripheral parts (glomeruli and mitral cells of the olfactory bulb) during neurodegeneration in AD and that, within the olfactory system, the anterior olfactory nucleus is predominantly involved [21]. These findings would be consistent with the hypothesis of an anterograde spreading of neurodegenerative changes in the olfactory system. Therefore, the occurrence of τ immunoreactivity in olfactory tissue would be expected in more advanced stages of the disease. Another issue that should be considered is that abnormal τ reactivity was demonstrated in early-stage AD patients in the entorhinal region and olfactory nuclei [12] and that olfactory dysfunction occurs as an early sign of the disease. Since olfactory neurons show a higher potential for regeneration compared to neurons of the CNS, olfactory dysfunction might be due to CNS neuronal degeneration in central olfactory pathways and might not be associated with cytoskeletal abnormalities in the olfactory neurons themselves. All patients were able to complete a brief olfactory standard test. Therefore, it remains unclear whether abnormal τ staining in olfactory mucosa is restricted to patients where olfactory dysfunction can be demonstrated.

In contrast to τ staining in nasal mucosa, ELISA quantitation of τ protein in cerebrospinal fluid (CSF) may corroborate the clinical diagnosis of AD. To date, several groups have demonstrated significantly increased CSF levels of τ protein in AD patients as compared to a variety of age-matched control groups [22, and others].

However, although the staining for τ proteins and amyloid deposits in nasal mucosa may not be useful for the early diagnosis of AD, the investigation of olfactory structures may help to elucidate changes in metabolism and signal transduction occurring in the central nervous system of AD patients, i.e. demonstration of reduced cAMP production in tissue cultures of olfactory epithelium [23]. In addition to the mAb AT8, several phosphorylation-dependent τ antibodies are available. τ in NFTs is hyperphosphorylated at various sites. The use of other phosphorylation-dependent τ antibodies than AT8, in nasal mucosa, may help clarify the diagnostic properties of this method.

In summary, the present findings support the idea that neurodegenerative changes in the olfactory epithelium are not primary or specific characteristics of AD. The investigation of nasal mucosa biopsy samples may provide certain insights into the biochemical and morphological phenomena occurring during the course of AD. It remains to be seen whether it will provide a useful tool for the corroboration of the clinical diagnosis of early AD.

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