Disturbed Assembly of Human Cerebral Microtubules in Alzheimer's Disease

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It is shown for the first time that microtubular proteins isolated from the brain of patients with Alzheimer's disease can *in vitro* polymerize into microtubules with abnormal structure.

Key Words: *Alzheimer's disease; microtubules*

Despite ample data on biological bases of the pathogenesis of Alzheimer's disease (AD), the etiology of this disease remains unknown. About 20 etiological factors promoting AD development were identified and several hypothesis on its pathogenesis were put forward [1,4]. However, none of these hypotheses can explain gradual and stubborn progress of the disease and variety of its clinical manifestations, describe numerous neuronal and other than neuronal disorders in the brain in AD.

According to one hypothesis, disorders in the structure and functions of microtubules (MT) of brain neurons can explain the emergence of the majority of neuropathological signs of AD [3,11].

Microtubule is a rigid hollow cylinder with external diameter of 25 nm and inner diameter of 12-13 nm, about 0.3-0.9 μ long [9]. On the other hand, MT are labile and dynamic intracellular structures capable of rapid assembly and disintegration into protein components at a needed site of the extracellular compartment. The main structural unit of MT is tubulin capable of rapid exchange with tubulin subunits dissolved in the cytoplasm [9,13]. The range of intracellular functions performed by MT is extremely wide and ranges from provision of neuronal and neurite structure and intracellular transport to regulation of a variety of enzymatic systems, activity of membrane receptors, and genetic apparatus [10,14]. In addition, regulation of MT assembly/disintegration processes can prevent neurodegeneration in neuronal culture caused by β-amyloid protein, characterized by a neurotoxic effect, a possible factor of AD pathogenesis [1,12].

Alzheimer's disease can be associated with disordered autoregulation of the microtubular system in human brain cells, which can lead to structural defects of MT and presumably, to stubborn destabilization of the functions of the priority intracellular metabolic systems. Therefore, studies and monitoring of cerebral MT assembly and structure is an important problem. Our aim was *in vitro* assembly of MT obtained by polymerization of tubulin and microtubule-associated proteins (MAPs) isolated from the brain of AD patients and electron microscopy of the resultant MT samples.

MATERIALS AND METHODS

Isolation of tubulin and MAPs from the brain of AD patients and MT assembly were carried out by a previously described method [15] in our modification. Cerebral tubulin and MAPs were isolated from autopsy material from 2 AD patients (men, aged 78 and 81 years) 7 and 8 h postmortem. Since typical morphological sign of AD is disseminated

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degeneration of the temporofrontal compartments of the cerebral cortex, we used specimens of the frontal cortex from AD patients [1].

Brain tissue (200 g) was homogenized 1:1 (w/v) at 4°C in buffer A (pH 6.9): 50 mM Tris-HCl, and 2 mM EGTA. The homogenate was centrifuged at 10,000*g* for 30 min (4°C). Supernatant was centrifuged at 100,000*g* for 60 min (4°C). Protein concentration in the supernatant was measured [2].

Supernatant (extract of water-soluble proteins containing, among other components, tubulin and MAPs) was used for MT assembly. Protein concentration in the extract was brought to 30 mg/ml with buffer A and diluted 2-fold with buffer B (pH 6.9) containing 50 mM Tris-HCl, 0.2 mM guanosine triphosphate, and 12 mM $MgCl_2$. After incubation (6 h at 37°C) the sample was centrifuged at 100,000*g* for 60 min (28°C). The precipitate containing MT was resuspended in buffer A at 4-6°C in a 10-fold lesser volume than the volume of water-soluble protein extract in which MT polymerization was carried out. The suspension was incubated at 4-6°C for 30-60 min for MT depolymerization and centrifuged at 100,000*g* for 30 min (4°C). After measuring protein concentration in the supernatant it was frozen in liquid nitrogen and stored at

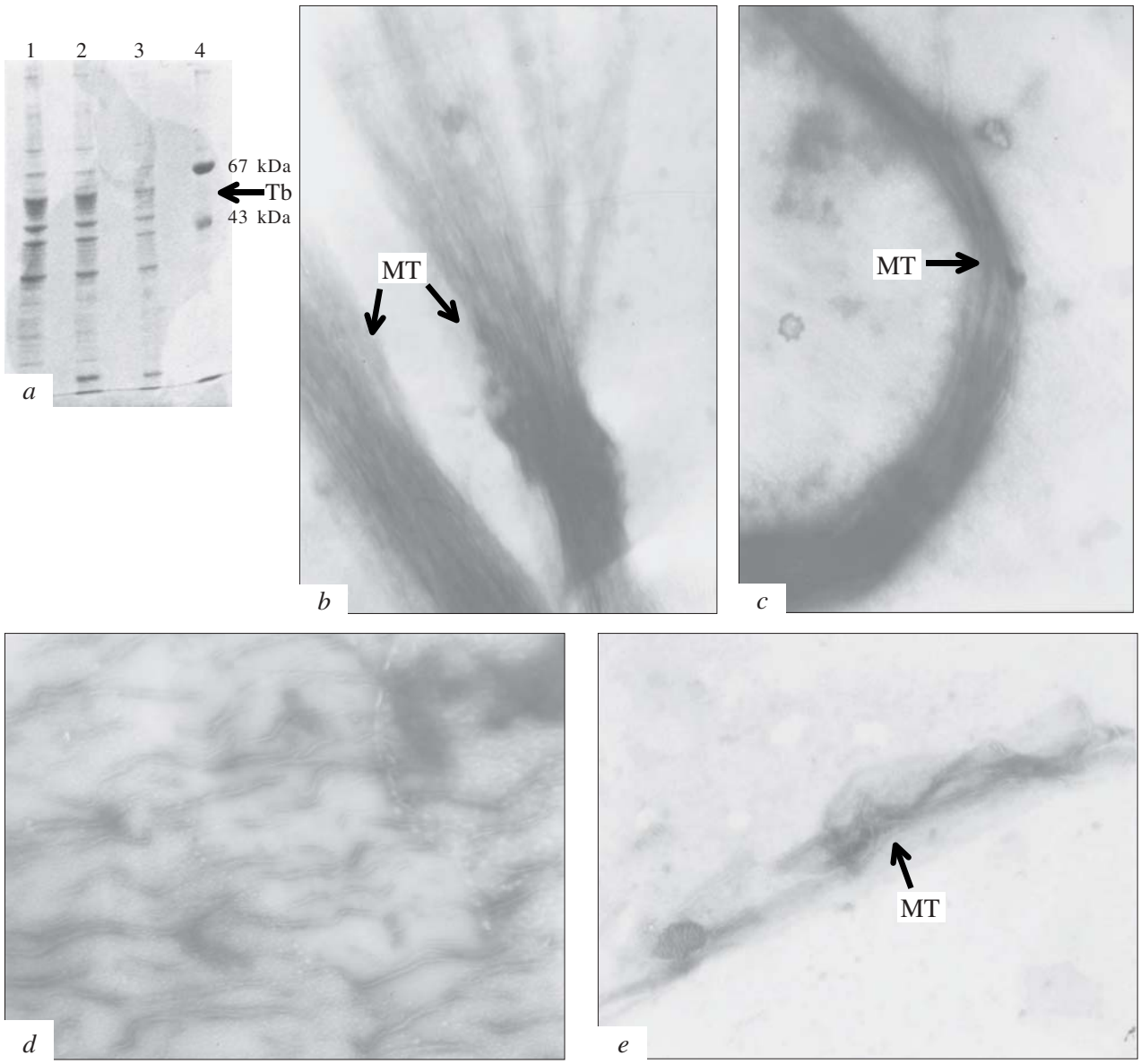

Fig. 1. Electrophoregram of purified preparation of tubulin (Tb) and MAPs isolated from the brain of patients with Alzheimer's disease (*a*) and microtubules (MT) assembled *in vitro* from Tb and MAPs preparation (*b*-*e*); ×20,000. *1*) 60 μg; *2*) 40 μg; *3*) 20 μg; *4*) molecular weight markers. *b*) normally oriented MT; *c*) MT twisted into a compact bundle; *d*) solitary shortened MT twisted in pairs; *e*) amorphous microtubular bundle.

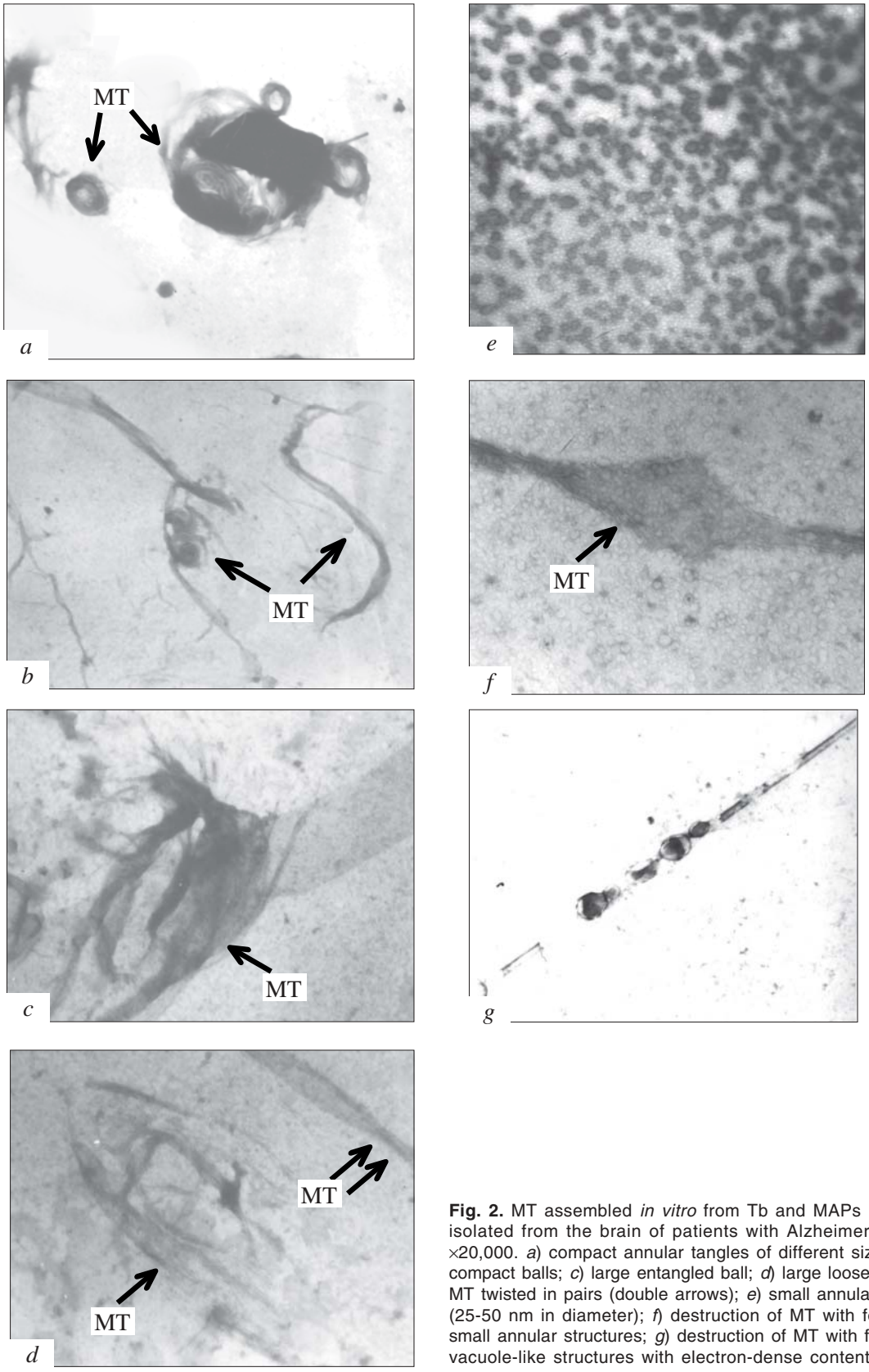

Fig. 2. MT assembled *in vitro* from Tb and MAPs preparation isolated from the brain of patients with Alzheimer's disease; ×20,000. *a*) compact annular tangles of different size; *b*) small compact balls; *c*) large entangled ball; *d*) large loose tangle and MT twisted in pairs (double arrows); *e*) small annular structures (25-50 nm in diameter); *f*) destruction of MT with formation of small annular structures; *g*) destruction of MT with formation of vacuole-like structures with electron-dense contents.

-70°C. The preparation of purified tubulin and MAPs was used for MT assembly. To this end, the preparation after defrosting was centrifuged at 5000*g* for 10 min (4°C) for precipitation of denatured and aggregated protein molecules. MT assembly was carried out at 37°C for 6 h in 50 mM Tris-HCl buffer (pH 6.9), 0.1 mM guanosine triphosphate, 6 mM $MgCl_2$, with protein concentration in the sample 0.2 mg/ml. Polymerization was recorded by changes in light scattering on a Beckman immunochemical analyzer at λ=450-550 nm. Electron microscopic monitoring was carried out using negative contrasting at ×20,000 [15]. Homogeneity of tubulin and MAPs was evaluated by electrophoresis in PAAG with 0.1% sodium dodecyl sulfate [15].

RESULTS

Until present the assembly of MT after *in vitro* polymerization of tubulin and MAPs isolated from the brain of AD patients was considered impossible. This assumption was based on the results obtained by the classical method developed for polymerization of tubulin and MAPs isolated from rat brain [6,15]. We reproduced this experiment and confirmed the rightfulness of authors' conclusion. However, we consider that experimental conditions did not correspond to brain cell protein composition in AD. The content of tubulin markedly decreased in the brain in AD [11]. We therefore modified the conditions of MT assembly in water-soluble protein extract by increasing the concentration of total protein from 7 to 15 mg/ml (thus expecting to increase tubulin concentration), increasing the concentration of polymerization activator Mg^{2+} from 2 to 6 mM, and prolonging the duration of polymerization from 1 to 6 h. In addition, we did not use DEAE dextran as a polymerization medium component. This polycation is a potent activator of tubulin polymerization and MT assembly even in the absence of MAPs [6].

These modifications enabled *in vitro* polymerization of tubulin and MAPs from the brain of AD patients; electron microphotographs of the resultant MT were made. By the distribution of protein zones the preparation of tubulin and MAPs (Fig. 1, *a*) is close to that obtained by other authors [6]. The process of MT assembly does take place in the brain of AD patients, but is essentially disordered. Normally, MT are strictly orderly packed into a bundle of long parallel cords, while the shape of MT resultant from polymerization of tubulin and MAPs isolated from the brain of AD patients varied from normally oriented (Fig. 1, *b*) to abnormal structures of different degree of complexity (Figs. 1, 2).

After comparison of our results with published data describing cytomorphological changes in the brain of AD patients, but using other microscopic technology, other stains, and less potent magnification (×1000-1200), we think that we also detected MT twisted in pairs (Fig. 1, *d*, 2, *d*) [11]. Presumably, tangles of different shape and different compactness seen in our microphotographs (Fig. 2, *a-d*) are neurofibrillar balls with obligatory MT [5].

Presumably, we observed destruction of MT paralleled by the formation of vacuole-like structures with electron-dense contents from MT (Fig. 2, *g*), which also corresponds to the known pathomorphological signs of AD. Neurodegenerative process in brain cells of AD patients is associated with granulovascular degeneration of neurons leading to the formation of spherical structures containing electron-dense tubulin granules [11].

We detected small annular structures (25-50 nm in diameter; Fig. 2, *e*). These annular structures (Fig. 2, *e*, *f*) can be typical results of MT degradation [7].

Our experiments demonstrated disorders in MT assembly process in the brain cells of AD patients, presumably promoting the development of intracellular disorders associated with AD development. This prompts creation of drugs modulating the dynamics of normal MT assembly.

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