EXPRESSION OF TAU-LIKE MICROTUBULE-ASSOCIATED PROTEINS IN CALCITONIN-PRODUCING CELLS

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ABSTRACT

The expression of tau proteins in calcitonin-producing cells was examined in rat thyroid and in a calcitonin-producing cell line. An immunohistochemical study revealed tau-like protein expression both in adult rat thyroidal calcitonin-producing cells and in the cell line. Immunoblot analysis showed that the calcitonin-producing cell line produced both low and high molecular weight tau-like proteins, which were phosphorylated in varying degrees.

Tau proteins belong to a family of microtubule-associated proteins (MAPs) that co-purify with tubulin during repeated cycles of assembly and disassembly (1). They are known to promote the assembly and stability of microtubules and to play an important role in the determination of neuronal morphology and plasticity (13). In adult brain, tau is found as a set of 5 to 6 related proteins with apparent molecular masses ranging from 50,000 to 70,000 (13). In contrast, peripheral nervous tissues predominantly express two classes of tau proteins with apparent molecular masses of 90,000-105,000, middle molecular weight (MMW) tau, and 110,000, high molecular weight (HMW) tau (12). HMW tau is also detected in the rat pheochromocytoma cell line PC12 (3) and the mouse neuroblastoma N115 (2). This heterogeneity in tau proteins is attributed to alternative splicing of RNA transcripts from a common gene (13).

Calcitonin-producing cells (C-cells) of the thyroid glands are endocrine derivatives of the neural crest (4), and possess several neuronal properties (8, 11). Although C-cells *in situ* do not display a neuronal appearance, C-cells have the potential to extrude neurite-like processes under culture conditions (8, 10). Our previous studies showed that the microtubular cytoskeleton participated in elongation and maintenance of the neurite-like processes

of C-cells (8). In this study, we examined the expression of tau-like proteins in rat thyroid C-cells and in the rMTC 6-23 C-cell line established from rat medullary thyroid carcinoma (15).

MATERIALS AND METHODS

Wistar rats were purchased from Charles River Japan. Thyroid glands were excised from 3- and 9-week-old rats, and fixed in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight. After being washed with phosphate-buffered saline (PBS), the glands were immersed in 20% sucrose in PBS for 24 h, and embedded in an OCT compound (Miles). Transverse sections were cut serially at 5 µm and mounted on egg white-coated slides. The sections were blocked with 1% bovine serum albumin in PBS for 30 min, and incubated overnight at 4°C with mouse anti-rat tau monoclonal antibody (1:500, Boehringer) or rabbit anti-human calcitonin antiserum (1:1,000, ICN). Immunoreaction was located using a horseradish peroxidase-labeled Histofine SAB kit (Nichirei) according to the supplier's recommendations. The reaction products were developed using 3,3'-diaminobenzidine tetrahydrochloride (0.5 mg/ml) and H₂O₂ (0.01%), and the nuclei were stained with Mayer's hematoxylin solution. In control experiments, mouse ascites fluid or normal rabbit serum was substituted for the

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primary antibody, resulting in negative staining.

The rMTC 6-23 cell line was purchased from American Type Culture Collection via Dainippon Pharmaceutical (Japan), and maintained in Dulbecco's modified Eagle's medium containing 10% horse serum as described previously (11). For the detection of tau immunoreactivity in the cells, they were cultured on glass coverslips for 2 days, and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h. After being washed with PBS, the cells were treated with 0.25% Triton X-100 in PBS for 10 min, and tau immunoreactivity was detected as described above. Negative staining in control experiments was confirmed as described above.

For immunoblot analysis of tau proteins in the calcitonin-producing cell line, rMTC 6-23 cells cultured in plastic flasks (25 cm², Nunclon Delta, Nunc) were scraped with a rubber policeman and precipitated by centrifugation at 700 g for 5 min. Tubulin and MAPs were isolated from the rMTC 6-23 cells (approximately 5×10^8 cells) using taxol according to the method of Vallee (14). In some experiments, isolated proteins were treated with calf intestine alkaline phosphatase (200 units/ml, Boehringer, molecular biology grade) in 0.1 M Tris-HCl buffer (pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml pepstatin and 10 μ g/ml leupeptin at 32°C for 2.5 h prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). As a no-enzyme control, the isolated proteins were incubated with the hydrolysis solution alone. Rat tau proteins were similarly prepared from 9-week-old male rat brain. The protein content was determined by the method of Lowry *et al.* (6).

The isolated proteins (20 μ g/lane for rMTC cells and 5 μ g/lane for brain) were subjected to SDS-PAGE on 7.5% gel, and electrophoretically transferred to a polyvinylidene difluoride membrane (Clear Blot Membrane P, Atto, Japan). The blots were blocked with 5% lipid-free skim milk (GIBCO), and incubated with the anti-rat tau antibody (1:250) for 1 h. Immunodetection of tau proteins was carried out using a horseradish peroxidase-labeled Vectorstain ABC kit (Vector Lab.) as described previously (11).

RESULTS AND DISCUSSION

In the 9-week-old rat thyroid sections, C-cells were distributed as single cells or small cell clusters at the basal portion of the follicular epithelium (Fig.

1A). An immunohistochemical study on serial sections revealed that the majority of calcitonin-positive cells were also immunoreactive to tau proteins (Fig. 1). Anti-tau antibody specifically bound to the cytoplasm of C-cells, but not to surrounding follicular cells and colloid. Basically, the same results were obtained in the 3-week-old rats.

As shown in Fig. 2, the rMTC 6-23 cells were heterogeneous in morphology: some were spherical in shape, while others had spindle-shaped cell bodies and processes of varying lengths (<100 μ m). Despite their heterogeneous morphology, the cytoplasm of all the cells was found to be immunoreactive to anti-tau antibody (Fig. 2A), as described in thyroidal C-cells.

To examine tau isoforms in the rMTC 6-23 cells, MAPs were isolated from the cells and subjected to immunoblot analysis. Tau proteins from rat brain contained six bands of apparent molecular weights ranging between 49,500 and 68,000 (Fig. 3, lane 3), corresponding to the tau isoforms known in central nervous tissues (12). In the rMTC 6-23 cells, positive signals were observed as broad bands at molecular weights ranging from 62,000 to 78,000 (low molecular weight, LMW, tau) and from 110,000 to 120,000 (HMW tau) (Fig. 3, lane 1). Two bands were clearly observed at molecular weights of 73,000 and 78,000. Weak immunoreaction in the rMTC 6-23 cells suggested a lower level of expression of tau-like proteins than in neurons, because 20 μ g of protein was loaded from the rMTC cells and only 5 μ g from brain.

Pretreatment of solubilized proteins with alkaline phosphatase caused a change in the electrophoretic pattern of tau-immunoreactive proteins. The broad band of LMW tau-like proteins was converted into four sharp bands, accompanied by a downward shift by at most 8,000 molecular mass. HMW tau-like proteins were converted into three separate bands at apparent molecular masses of 103,000, 110,000 and 113,000. These results indicated that rMTC 6-23 cells contained LMW and HMW tau-like proteins that were phosphorylated in varying degrees. This diversity in phosphorylation of tau-like proteins may partly account for the heterogeneous shapes of rMTC 6-23 cells, because tubulin polymerization-promoting activity of tau proteins is affected by the extent of phosphorylation of these molecules (5). A clonal approach is now being used for the purpose of obtaining morphologically homogeneous populations of rMTC 6-23 cells.

In addition to LMW and HMW tau-like proteins,

Fig. 1 Serial sections of 9-week-old rat thyroid gland, immunostained by anti-human calcitonin antiserum (A) and anti-rat tau monoclonal antibody (B). Most of tau-immunoreactive cells topographically correspond to calcitonin-positive cells on serial sections. $\times 400$

Fig. 2 Tau immunoreactivity in calcitonin-producing rMTC 6-23 cells. The cells were immunostained by anti-rat tau monoclonal antibody (A) and control ascites fluid (B). $\times 350$

two faint bands at apparent molecular masses of 83,000 and 86,000 were observed in the dephosphorylated extract from rMTC 6-23 cells (Fig. 3, lane 2). These two proteins presumably correspond to MMW tau, which was found in optic nerve and several other central nervous tissues (12), or to degradative products of HMW tau.

Tau proteins are specifically expressed in neuronal cells in central and peripheral nervous systems (13), including the neuron-like cell lines PC12 (3) and neuroblastoma N115 (2). Tau proteins are known to be enriched in axons in neurons, and to promote *in vitro* polymerization of tubulin into microtubules (7, 13), suggesting that neuronal tau

Fig. 3 Immunoblot analysis of tau proteins in rMTC 6-23 cells. Partially purified fractions of microtubule-associated proteins and tubulin were prepared from rMTC 6-23 cells (lanes 1 and 2) and adult rat brain (lane 3). Intact (lanes 1 and 3) and dephosphorylated (lane 2) samples were subjected to SDS-PAGE on 7.5% polyacrylamide gel. Polyvinylidene difluoride membrane transfer was immunostained with anti-rat tau monoclonal antibody. In lanes 1 and 2, pyronin Y was run with the sample proteins for indicating the dye front (arrow). Molecular weights ($\times 10^{-3}$) of marker proteins are indicated on the right.

proteins play a physiological role in the formation and maintenance of axons. The present results first demonstrated the expression of tau-like proteins in rat thyroidal C-cells and in a calcitonin-producing endocrine cell line. Although thyroidal C-cells *in vivo* do not extrude neurite-like processes (Fig. 1), thyroidal C-cells *in vitro* retain the potential to extrude neurite-like processes in response to a laminin substratum (8) or protein kinase inhibitor H-7 (9). In contrast, nanomolar concentrations of okadaic acid, a potent inhibitor of protein phosphatase, completely suppress the process outgrowth from C-cells induced by H-7 (9). Thus, it is possible that process outgrowth from C-cells is partly regulated by the phosphorylation-dephosphorylation of tau proteins. Further biochemical experiments will shed light on the physiological role of the low-level expression of neural-specific tau proteins in C-cells.

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REFERENCES

1. CLEVELAND D. W., HWO S. Y. and KIRSCHNER M. W. (1977) Purification of tau, a microtubule associated protein that induces assembly of tubulin from purified tubulin. *J. Mol. Biol.* **116**, 207-225
2. COUCHIE D., MAVILIA C., GEORGIEFF I. S., LIEM R. K. H., SHELANSKI M. L. and NUNEZ J. (1992) Primary structure of high molecular weight tau present in the peripheral nervous system. *Proc. Natl. Acad. Sci. USA* **89**, 4378-4381
3. GOEDERT M., SPILLANTINI M. G. and CROWTHER R. A. (1992) Cloning of a big tau microtubule-associated protein characteristic of the peripheral nervous system. *Proc. Natl. Acad. Sci. USA* **89**, 1983-1987
4. LE DOUARIN N. (1982) *The Neural Crest*, Cambridge University Press, Cambridge
5. LINDWALL G. and COLE R. D. (1984) Phosphorylation affects the ability of tau protein to promote microtubule assembly. *J. Biol. Chem.* **259**, 5301-5305
6. LOWRY O. H., ROSEBROUGH N. J., FARR A. L. and RANDALL R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275
7. MATUS A. (1988) Microtubule-associated proteins: Their potential role in determining neuronal morphology. *Annu. Rev. Neurosci.* **11**, 20-44
8. NISHIYAMA I. and FUJII T. (1992) Laminin-induced process outgrowth from isolated fetal rat C-cells. *Exp. Cell Res.* **198**, 214-220
9. NISHIYAMA I. and FUJII T. (1993) H7-induced neuritic process outgrowth from cultured fetal rat C-cells. *Jap. J. Pharmacol.* **61**, 203P
10. NISHIYAMA I., OOTA T. and OGISO M. (1994) Neuron-like morphology expressed by perinatal rat C-cells *in vitro*. *Zool. Sci.* **11**, 441-444
11. NISHIYAMA I., SEKI T., OOTA T., OHTA M. and OGISO M. (1993) Expression of highly polysialylated neural cell adhesion molecule in calcitonin-producing cells. *Neuroscience* **56**, 777-786
12. TALEGHANY N. and OBLINGER M. M. (1992) Regional distribution and biochemical characteristics of high molecular weight tau in the nervous system. *J. Neurosci. Res.* **33**, 257-265
13. TUCKER R. P. (1990) The roles of microtubule-associated proteins in brain morphogenesis: a review. *Brain Res. Rev.* **15**, 101-120
14. VALLEE R. B. (1982) A taxol-dependent procedure for the isolation of microtubules and microtubule-associated proteins (MAPs). *J. Cell Biol.* **92**, 435-442
15. ZEYTINGLU F. N., DELELLIS R. A., GAGEL R. F., WOLFE H. J. and TASHJIAN A. H. (1980) Establishment of a calcitonin-producing rat medullary thyroid carcinoma cell line. I. Morphological studies of the tumor and cells in culture. *Endocrinology* **107**, 509-515