

Immunocytochemical Expression of Microtubule-associated Protein-2 (MAP-2) in Small Cell Lung Cancer Cell Lines with Neuronal-like Processes

Kazuhiko Tanaka¹ and Takeo Terasaki²

¹Radiobiology and ²Pathology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045

Five of six human small cell lung cancer (SCLC) cell lines changed morphologically into cells with neuronal-like processes on the extracellular matrix of human lung adenocarcinoma cell line PC-9 cells (PC-9/ECM substrate). The features of the neuronal-like processes of these SCLC cell lines were examined immunocytochemically using monoclonal antibodies against β -chains of tubulin and microtubule-associated protein-2 (MAP-2), which is somatodendritic MAP of neurons. It was observed that β -chains of tubulin and MAP-2 were expressed along the neuronal-like processes of SCLC cell lines. These findings suggest that the β -chains of tubulin and MAP-2 are expressed functionally in SCLC cell lines in association with the development of dendrite-like processes on PC-9/ECM substrate.

Key words: MAP-2 — Small cell lung cancer cell lines — Neuronal-like processes — Immunocytochemistry

Small cell lung cancer (SCLC) is high-grade malignant tumor.^{1–3} SCLC has been shown to have the properties of neuroendocrine cells, such as elevated specific enzyme activities (i.e., aromatic L-amino acid decarboxylase, neuron specific enolase and creatine kinase BB)⁴ and production of peptide hormones (i.e., adrenocorticotrophic hormone, antidiuretic hormone, gastrin releasing peptide, calcitonin and somatostatin).^{5–7}

We have previously reported that a small cell lung cancer cell line Lu-134A, which grows as floating cell aggregates, changed morphologically into cells possessing long neuronal-like processes on the extracellular substrates of human adenocarcinoma cell line PC-9 cells (PC-9/ECM substrate) or polyethyleneimine (PEI substrate).⁸ Expression of microtubule-associated protein-2 (MAP-2)⁹ has been observed immunocytochemically along the neuronal-like processes of these cells.⁸

To know whether the development of the neuronal-like processes occurs similarly in other SCLC cell lines on PC-9/ECM substrate, we examined six SCLC cell lines morphologically and immunocytochemically.

We report here that five of the six SCLC lines developed neuronal-like processes on PC-9/ECM substrate, and MAP-2 and β -tubulin protein were expressed immunocytochemically along these processes.

MATERIALS AND METHODS

Cell lines Six SCLC cell lines were used. Cell lines N-417 and H-82 were kindly supplied by Dr. A. Gazdar, Bethesda, MD.¹⁰ Cell lines Lu-134A, Lu-134B, Lu-130

and Lu-135 were established in our laboratory from tissues obtained at surgery or from xenotransplanted tumors in nude mice.¹¹ Cell lines Lu-134A, Lu-134B, and Lu-130 are classic type and N-417, Lu-135 and H-82 are variant type SCLC cell lines by the published criteria.^{10, 12} Each of these SCLC cell lines grew in suspension as floating cell aggregates. Human adenocarcinoma cell line PC-9¹³ was also used as a source of extracellular substrate. These cell lines were cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS, BIOCELL Co., Carson, CA) (10% FCS/RPMI) or in serum-free HITES medium,¹⁴ which contained RPMI1640 medium supplemented with 10 nM hydrocortisone, 30 nM sodium selenite, 10 nM 17 β -estradiol, 10 μ g/ml transferrin and 5 μ g/ml insulin.

Extracellular substrata PC-9/ECM substrate and PEI substrate were prepared by the reported methods.⁸

Cultivation of SCLC cell lines in the presence of dB-cAMP SCLC cell lines were cultured on PC-9/ECM substrate for 3 days, then dibutyryl cyclic adenosine 3',5'-monophosphate (dB-cAMP) (Sigma Chemical Co., St. Louis, MO) was added to the culture medium at a concentration between 0.1 and 1.0 mM. The medium containing dB-cAMP was changed every 3 days.

Immunocytochemistry Mouse monoclonal antibodies against MAP-2 (280 kDa clone M13, recognizing a phosphate-independent epitope) and β -tubulin were purchased from Zymed Laboratories, Inc. (South San Francisco, CA). We used these antibodies as supplied (indicated as ready-to-use, do not dilute). SCLC cell lines cultured on PC-9/ECM substrate or adhering to PEI substrate were briefly rinsed in calcium-magnesium-free Dulbecco's PBS (PBS⁻). These cells were fixed in 95% ethanol for 30 min at 0°C

for MAP-2. For β -tubulin, these cells were fixed in 4% paraformaldehyde in microtubule-stabilizing buffer (MTSB)¹⁵ for 30 min at room temperature, then held in MTSB containing 0.1% Triton X-100 for 4 min, and washed with PBS⁻. Immunocytochemical staining was performed in a moist chamber using a HistoScan Monoclonal Detector Kit (06-601A). Normal mouse IgG₁ was used as the control.

Immunoprecipitation and SDS-PAGE We used the IMMUNO-catcher system (CytoSignal Research Products, Irvine, CA) for protein immunoprecipitation. Cell pellets (1×10^7 cells) of SCLC cell lines (Lu-134A, N-417, H-82) were resuspended in lysis solution containing protein inhibitors, sonicated for 10 s on ice and centrifuged. The supernatants were precleared by using protein A/G resin which had been treated with preimmune serum (isotype control mouse IgG₁-purified, Leino Technologies, Inc., Manchester Rd, UK). The precleared cell lysates (100 μ l) were immunoprecipitated using mouse monoclonal anti MAP-2 antibody (4 μ g) (280 kDa, clone M12, recognizing a phosphate-independent epitope, Zymed Laboratories, Inc., South San Francisco, CA) overnight at 4°C. The protein A/G resin suspension (10 μ l) was added to the immune complex and rotated for 2 h at 4°C. After rinsing, the immune complex was eluted with the sample buffer. Immunoprecipitated proteins were subjected to 5% SDS-PAGE under non-reducing conditions and the gel was stained using silver stain II kit (Wako Pure Chemical Industries, Ltd., Osaka). Molecular weight marker DAI-ICH II was used (Daiichi Pure Chemicals Co., Ltd., Tokyo) as an indicator of molecular size.

RESULTS

Development of neuronal-like processes in SCLC cell lines cultured on PC-9/ECM substrate SCLC cell lines (Lu-134A, Lu-130, Lu-134B, N-417 and Lu-135) grew as floating cell aggregates (Fig. 1, A–E). When these cell lines were cultured on PC-9/ECM substrate, floating cell aggregates attached to the substrate, spread and then developed neuronal-like processes after 1 to 20 days. To develop effectively these neuronal processes, we used dB-cAMP, a traditional differentiating agent.¹⁶ Accelerated development and elongation of neuronal-like processes were observed in Lu-134A, Lu-134B, N-417 and Lu-135 cell lines after addition of dB-cAMP, within 5 to 10 days (Fig. 1, F, H, I and J). The length of these processes was from three to more than ten times the cell body length. The addition of dB-cAMP to Lu-130 cells cultured on PC-9/ECM substrate did not accelerate development or elongation of neuronal-like processes (data not shown). These processes appeared to be dendrite-like features. The morphologies of neuronal-like processes were different among these SCLC cell lines. In Lu-134A cells, obvious branch-

ing of elongated processes was observed in the presence of dB-cAMP (Fig. 1F). In N-417 cells, thick elongated processes were observed in the presence of dB-cAMP, but there were few branching processes (Fig. 1I). Lu-130 cells adhered and spread most strongly on PC-9/ECM substrate among the examined cell lines, as observed under a phase-contrast microscope (Fig. 1G). The length of neuronal-like processes was about five times the length of the cell body and flat features in the distal tip of processes were characteristically observed (Fig. 1G). In Lu-135 cells, bipolar long neuronal-like processes were observed in the presence of dB-cAMP (Fig. 1J). In Lu-134B cells, the length of neuronal-like processes was about three to five times the length of the cell body (Fig. 1H). H-82 cells showed neither cell adherence nor development of neuronal-like processes on PC-9/ECM substrate (data not shown).

Immunocytochemical expression of β -tubulin and MAP-2 in SCLC cell lines with neuronal-like processes

To analyze cytoskeleton components of neuronal-like processes in SCLC cell lines which developed on PC-9/ECM substrate, we examined immunocytochemically the expression and distribution of β -tubulin and MAP-2 using monoclonal antibodies.

Both the cytoplasm and neuronal-like processes of Lu-134A, N-417 and Lu-135 cells reacted intensely with monoclonal antibody against β -chains of tubulin (Fig. 2A, data for Lu-134A and Lu-135 not shown). The thick long processes in Lu-134A and N-417 cells reacted especially intensely with this antibody (Fig. 2A, data for Lu-134A not shown). The cytoplasm of Lu-130 cells with neuronal-like processes reacted with monoclonal antibody against β -chains of tubulin, but the processes reacted weakly with this antibody as compared with other cell lines (Fig. 2B). Both the cytoplasm and neuronal-like processes of Lu-134A, N-417 and Lu-135 cells reacted intensely with monoclonal antibody against MAP-2 (Fig. 2, C, D and E). The cytoplasm and neuronal-like processes of Lu-130 cells reacted weakly with this antibody (Fig. 2F). Both the cytoplasm and processes of Lu-134B cells with neuronal-like processes reacted intensely with monoclonal antibodies against β -tubulin and MAP-2 (data not shown). The controls did not react with the antibodies (data not shown).

Immunocytochemical expression of MAP-2 in SCLC cell lines without processes on PEI substrate

To examine the expression of MAP-2 in Lu-134A, N-417 and H-82 cells grown as floating cell aggregates without processes, we studied these cell lines attached to polyethyleneimine-coated cover slips (PEI substrate).

Cell lines Lu-134A, N-417 and H-82 attached to PEI substrate within 10 min without forming processes, and we examined immunocytochemically the expression of MAP-2 in these cell lines without processes.

Both the cytoplasm of N-417 and Lu-134A cells reacted intensely with monoclonal antibody against MAP-2 (Fig.

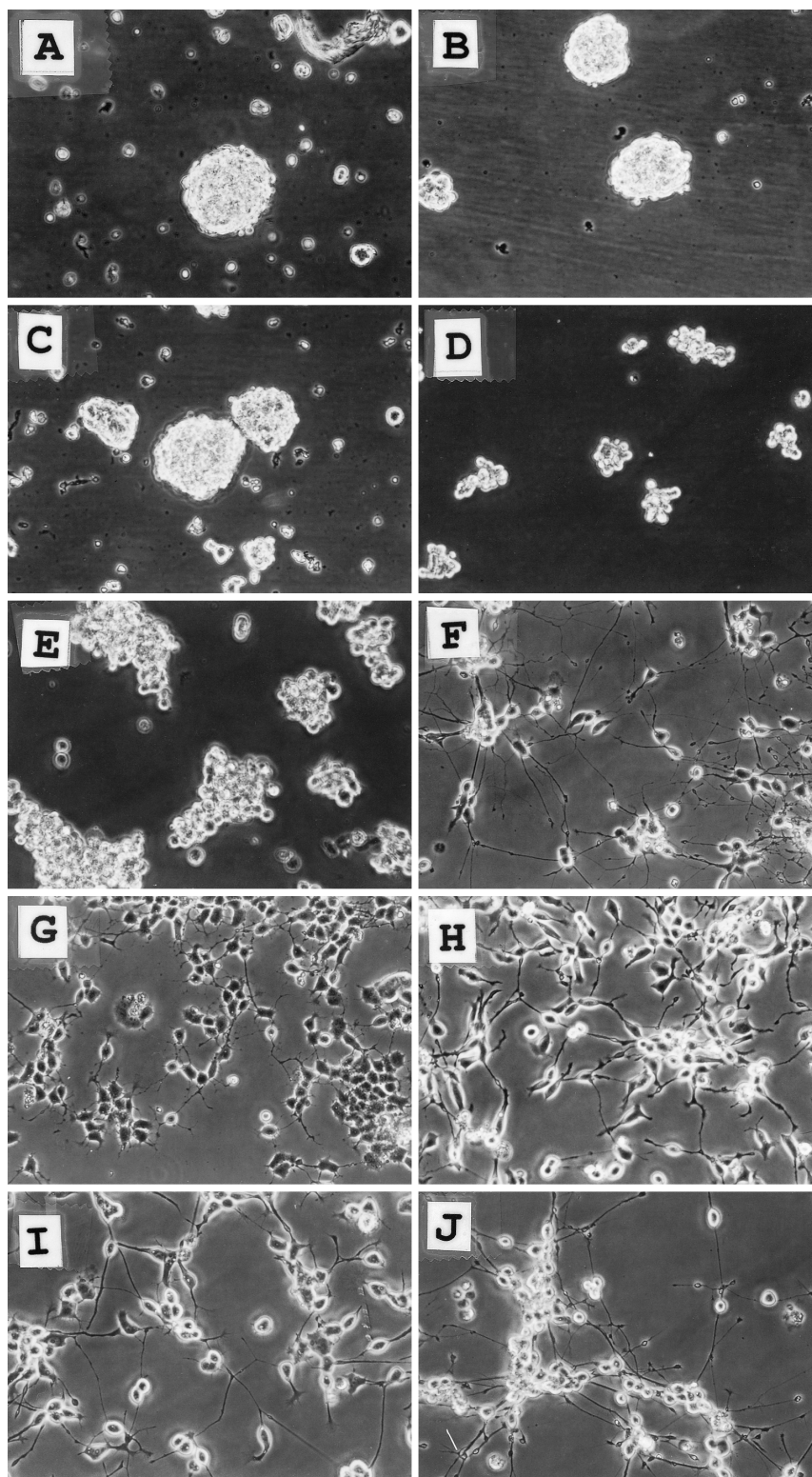


Fig. 1. Development of neuronal-like processes in SCLC cell lines. A–E: Cell lines grew as floating cell aggregates on non-coated cover glass. A: Lu-134A cells (×150), B: Lu-130 cells (×150), C: Lu-134B cells (×150), D: N-417 cells (×150), E: Lu-135 cells (×200). F, H–J: Cell lines were cultured on PC-9/ECM substrate in the presence of 0.25 mM dB-cAMP. F: Lu-134A cells, after 8 days of culture, clear branching of elongated processes was seen (×200). G: Lu-130 cells were cultured on PC-9/ECM substrate in the absence of dB-cAMP. After 9 days of culture, cells adhered and spread most strongly as compared with other cell lines. The branching of the thin processes and flattened distal tip of processes were characteristic (×200). H: Lu-134B cells, after 6 days of culture, the length of processes was about five times the length of the cell body (×200). I: N-417 cells, after 3 days of culture, long thick processes were observed. There were few branching processes (×200). J: Lu-135 cells, after 9 days of culture, the development of bipolar processes was characteristic (×200).

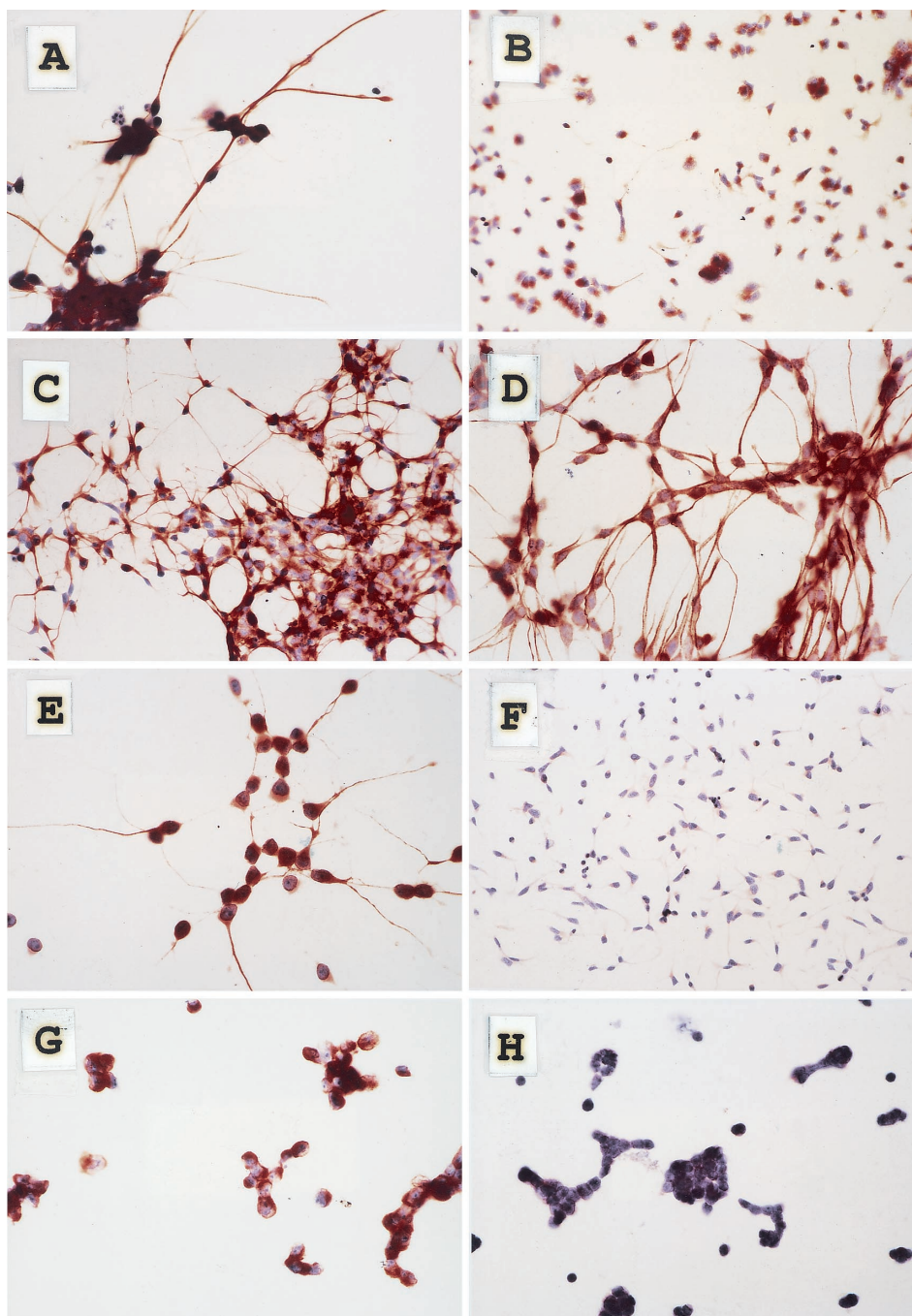


Fig. 2. Immunocytochemical expression of β -tubulin and MAP-2 in SCLC cell lines. A–F: SCLC cell lines developed neuronal-like processes on PC-9/ECM substrate in the presence (Lu-134A, N-417 and Lu-135 cells) or absence (Lu-130 cells) of 0.25 mM dB-cAMP. A, B: Reactivity with anti β -tubulin antibody. The cytoplasm and neuronal-like processes of N-417 cells (A, $\times 400$) reacted intensely with the antibody. Lu-130 cells (B, $\times 200$), the cytoplasm reacted intensely with the antibody, while the processes reacted weakly. Counterstained with hematoxylin. C–F: Reactivity with anti MAP-2 antibody. The cytoplasm and processes of Lu-134A cells (C, $\times 200$), N-417 cells (D, $\times 400$) and Lu-135 cells (E, $\times 400$) reacted intensely with the antibody. The cytoplasm and processes of Lu-130 cells (F, $\times 200$) reacted weakly with the antibody. Counterstained with hematoxylin. G, H: Reactivity with anti MAP-2 antibody of SCLC cell lines without processes, attached to PEI substrate. The cytoplasm of N-417 cells (G) reacted intensely with the antibody. The cytoplasm of H-82 cells (H) did not react with the antibody. Counterstained with hematoxylin ($\times 400$).

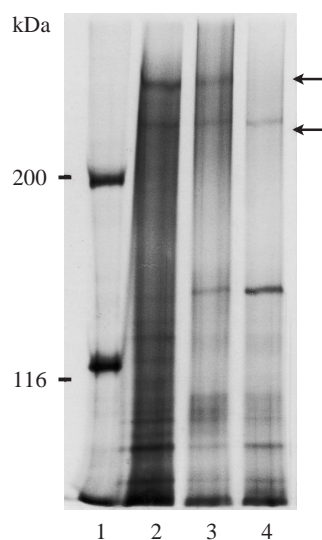


Fig. 3. Immunoprecipitation of MAP-2 in N-417, Lu-134A and H-82 cell lysates. Cell lysates were prepared as described in "Materials and Methods." Following immunoprecipitation with anti MAP-2 antibody, proteins were subjected to 5% SDS-PAGE under non-reducing conditions, followed by silver staining. Lane 1: Molecular weight marker (200 kDa, 116 kDa), lane 2: N-417, lane 3: Lu-134A, lane 4: H-82. MAP-2: ~280 kDa (upper arrow), ~220 kDa (lower arrow).

2G, data in Lu-134A not shown), but the cytoplasm of H-82 cells did not react with this antibody (Fig. 2H). The controls did not react with this antibody (data not shown). **Immunoprecipitation/SDS-PAGE analysis** To confirm the specificity of the expression of MAP-2 in N-417 and Lu-134A cells with neuronal-like processes, we performed immunoprecipitation and SDS-PAGE analysis of cell lysates of SCLC cell lines (Lu-134A, N-417, H-82) using anti MAP-2 antibody. MW ~280 kDa and weak MW ~220 kDa protein bands were observed in lysates of N-417 and Lu-134A cells (Fig. 3, lane 2, 3), while the MW ~280 kDa band was not observed, but a weak MW ~220 kDa band was observed in the lysate of H-82 cells (Fig. 3, lane 4) under non-reducing conditions.

Table I shows a summary of the formation of neuronal-like processes, and immunocytochemical expression of MAP-2 and β -tubulin in six SCLC cell lines on PC-9/ECM substrate.

DISCUSSION

The attachment of floating cell aggregates and the development of neuronal-like processes were observed in five (Lu-134A, Lu-134B, Lu-130, N-417 and Lu-135) of six SCLC cell lines on PC-9/ECM substrate. These results were more marked when the cells were cultured in serum-

Table I. Immunocytochemical Expression of MAP-2 and β -Tubulin in SCLC Cell Lines Which Developed Neuronal-like Processes on PC-9/ECM^{a)} Substrate

Cell lines	Neuronal-like processes ^{c)}	MAP-2 ^{d)}	β -Tubulin ^{e)}
Lu-134A (C) ^{b)}	+++	+	+
Lu-134B (C)	+	+	+
Lu-130 (C)	+	+	+
Lu-135 (V)	++	+	+
N-417 (V)	+++	+	+
H-82 (V)	-	-	-

a) Extracellular matrix derived from PC-9 cells (human adenocarcinoma cell line).

b) C, classic type; V, variant type.

c) Development of neuronal-like processes in SCLC cells on PC-9/ECM substrate 10 days after culture. + + +, processes more than ten times the length of the cell body; + +, processes five to ten times the length of the cell body; +, processes about three times the length of the cell body (floating cell aggregates attached to the substrate); -, no neuronal-like process formation (floating cell aggregates did not attach to the substrate). 0.25 mM dB-cAMP was added to the culture of Lu-134A, Lu-134B, Lu-135 and N-417 cells.

d) Immunocytochemical reactivity with anti MAP-2 antibody. +, positive; -, negative.

e) Immunocytochemical reactivity with anti β -tubulin antibody. +, positive; -, negative.

free HITES/RPMI medium than in 10% FCS/RPMI medium. The development of neuronal-like processes in Lu-134B cells on PC-9/ECM substrate was observed in the present study, but not in the previous report.⁸⁾ This might be attributed to the use of 10% FCS/RPMI medium, which contained lysophosphatidic acid (LPA) in serum. It has been reported that neurite retraction in neuronal tumor cells was evoked by LPA in serum.¹⁷⁾ To avoid the effects of serum, we used serum-free HITES/RPMI medium for the culture of SCLC cell lines in this study. In the previous report, Lu-139, Lu-140 and Lu-165 cell lines also did not show the development of neuronal-like processes on PC-9/ECM substrate in 10% FCS/RPMI medium. We examined whether these cell lines develop neuronal-like processes in serum-free HITES/RPMI medium, and found that they did not. Non-development of neuronal-like processes in these cell lines did not depend on the effects of serum.

The expression of MAP-2, which is the somatodendritic MAP of neurons,⁹⁾ has been observed immunocytochemically on neuronal-like processes in the SCLC cell line Lu-134A on PC-9/ECM substrate.⁸⁾ We examined whether the expression of MAP-2 also occurs in other SCLC cell lines with neuronal-like processes on PC-9/ECM substrate, and whether MAP-2 is expressed in these cells grown as floating cell aggregates without processes.

Immunocytochemical studies using antibodies against β -tubulin and MAP-2 showed that these proteins were also expressed on neuronal-like processes of four cell lines, Lu-134B, Lu-130, N-417 and Lu-135 on PC-9/ECM substrate. It also appeared that considerable numbers of neuronal-like processes in these cell lines contained bundles of microtubules (MTs) and MAP-2, indicating that they were dendrite-like processes. The result that MAP-2 was expressed in the cytoplasm of Lu-134A and N-417 cells without processes on PEI substrate suggests that these cells originally expressed MAP-2. These findings indicate that MAP-2 is expressed functionally in these SCLC cell lines to form MTs, that are a major component of neuronal cell processes. Low expression of MAP-2 in Lu-130 cells with neuronal-like processes may correlate with the short extension of neuronal-like processes in this cell line. Non-development of neuronal-like processes in H-82 cell line on PC-9/ECM substrate may be due to deficient adhesion to this substrate and low or deficient expression of MAP-2 in this cell line. It is suggested that the expression of MAP-2 in human SCLC cell lines is a requirement for the development of dendrite-like processes on PC-9/ECM substrate. We also performed immunoprecipitation/SDS-PAGE analysis to confirm the immunocytochemical expression of MAP-2 in SCLC cell lines which developed neuronal-like processes on PC-9/ECM substrate. N-417 and Lu-134A cells expressed MAP-2 of MW ~280 kDa, but H-82 did not express this protein. The expression of lower-molecular (MW ~220 kDa) MAP-2 was detected weakly in N-417, Lu-134A and H-82 cells. This molecule might be an alternatively spliced variant of this gene.

These findings support the immunocytochemical expression of MAP-2 in SCLC cell lines with neuronal-like processes on PC-9/ECM substrate. It was also suggested that the expression of MW ~280 kDa MAP-2 is related to the development of these neuronal-like processes.

Recently, it has been reported that several SCLC cell lines adhered on thrombospondin-1 (TSP1) substrate and one of them, OH1, extended neurite-like outgrowths containing F-actin without organization of actin on this substrate.¹⁸⁾ It has also been reported that the extracellular matrix proteins protect SCLC cells against apoptosis by chemotherapeutic agents.¹⁹⁾ These reports suggest that the biological properties of SCLC are influenced by the extracellular matrix (microenvironment).

Although most SCLC cell lines grow anchorage-independently as floating cell aggregates on tissue culture substrates, five of the six SCLC cell lines adhered to PC-9/ECM substrate and developed long neuronal-like processes (dendrite-like processes) containing β -tubulin and MAP-2. *In vivo*, a certain population of SCLC cells might differentiate into neuronal-like cells with neuronal-like processes under the influence of the specific type of extracellular matrix secreted from SCLC cells or normal cells.

Interactions between SCLC cells and extracellular matrix (microenvironment) should be further examined to improve our understanding of the biological properties of SCLC, and for the development of differentiation therapy and chemotherapy for SCLC.

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