

Circular Nuclear Alignment in Multinucleate PC12D Cells Produced by Cell Fusion with Polyethylene Glycol

Tomoya Kotani¹, Seiji Sawai¹, Tetsuo Kageyama¹ and Mamoru Sano¹

¹Department of Biology, Kyoto Prefectural University of Medicine, Nishitakatsukasa-cho 13, Taishogun, Kita-ku, Kyoto 603–8334

Received October 29, 2001; accepted May 17, 2002

PC12D cells, a subline of PC12 cells, extend neurites within a few hours in response to nerve growth factor (NGF). Multinucleate PC12D cells were produced by cell fusion with polyethylene glycol (PEG). The syncytia extended large neurites following treatment with NGF. In the syncytia, nuclei were distributed in a circular alignment. Many nuclear rings were found in the culture dish 1 day after treatment with PEG, although they were sometimes seen in syncytia within several hours. Colchicine prevented this alignment or disrupted the clusters when added subsequent to their formation. Cytochalasin B did not prevent the formation of these rings, nor affect the alignment of nuclei. Immunofluorescent staining of γ -tubulin, an integral protein of the centrosome, revealed in-

tense dot-like structures in the center of nuclear rings in syncytia. Twice as many dots, or centrioles, as nuclei were present in each syncytium. However, most microtubules were localized around the nuclei and did not appear to extend from the cluster of centrioles. Fluorescent labeling of mitochondria showed them to mainly be localized inside the nuclear rings. Meanwhile, immunofluorescently stained endoplasmic reticulum was mainly localized along with the circular alignment of nuclei. Therefore, not only nuclei but various cell organelles are situated in each characteristic location in the syncytia. These data suggest that microtubules are involved in the migration and alignment of nuclei and presumably of cell organelles in the syncytia.

Key words: PC12D cell, Cell fusion, Nuclear localization, Centriole, Microtubule

I. Introduction

Multinucleate giant cells have been studied in various tumor lesions, inflammatory lesions and experimentally induced foreign-body granulomas [9, 13, 19, 22]. In these cells, nuclei were found to be concentrated in one area and occasionally arranged in a semicircular manner. In contrast, multiple centrioles have been observed in the nucleus-free area of the cytoplasm of these giant cells [2, 22]. An interesting feature of these cells was seen in the syncytia produced on cell fusion induced artificially by viral infection or PEG treatment [7, 9, 10, 24–26]. It has been shown that infection of baby hamster kidney (BHK21-F) cells with the parainfluenza virus SV5 causes cell fusion and the formation of parallel rows of tightly packed nuclei in the syncytial cytoplasm [4]. During the processes of cell fusion and nuclear migra-

tion, centrioles were transported in the cytoplasm where they formed large clusters [24, 25]. On fusion of BHK cells induced with PEG, it was shown that the aggregated centrosomes were excluded from the clusters of nuclei in the syncytia and the nuclei occasionally encircled the centrosomes in stationary syncytia [8]. When colchicine was added, the translocation and movement of centrosomes and nuclei were blocked in fused BHK21-F cells. Cytochalasin B had little effect on these cytoplasmic movements [7, 8, 24, 25]. These results suggest that microtubules play an important role in the intracellular movements and the positioning of nuclei and centrosomes in multinucleate giant cells. However, little is known about the circular localization of nuclei in these cells.

PC12D cells, a subline of PC12 cells, rapidly extend neurites within a few hours in response to nerve growth factor (NGF), basic fibroblast growth factor (bFGF), dibutyryl cyclic AMP and staurosporine, even in the presence of inhibitors of RNA and protein synthesis [5, 14–16, 18]. The

Correspondence to: Mamoru Sano, Department of Biology, Kyoto Prefectural University of Medicine, Kyoto 603–8334, Japan.

NGF-induced outgrowth of neurites in this cell line does not require the activation of MAP kinase [17]. When multinucleate PC12D cells were made by fusion induced with PEG, large neurites were extended in response to NGF [12], and a circular alignment of nuclei was observed. The formation of such rings has been reported in only a limited number of fibroblasts lines and never before in neuronal cells.

This paper describes an investigation into the circular alignment of nuclei in PEG-fused PC12D cells and the effect of colchicine and cytochalasin B on the positioning of nuclei. The localization of centrosomes was also studied by immunofluorescent staining of γ -tubulin under a confocal-laser microscope. The fluorescent staining of microtubules, actin filaments, mitochondria and endoplasmic reticulum was revealed.

II. Materials and Methods

Dulbecco's modified Eagle's medium (DMEM; high glucose) was purchased from GIBCO (Grand Island, NY). Fetal calf serum and horse serum were obtained from M. A. Bioproducts (Walkersville, MD). Mouse NGF (7S form) was purified from submandibular glands of male mice [23]. Polyethylene glycol 1,000 was provided by Nakarai (Kyoto, Japan). Rhodamine phalloidin, Hoechst 33342, propidium iodide (PI), and MitoTracker Green FM were purchased from Molecular Probes (Eugene, OR). Monoclonal antibodies against α -tubulin, β -tubulin, γ -tubulin and streptavidin fluorescein (FITC) were from Amersham (Amersham, UK). Biotinylated antibodies against mouse IgG were from Vector Laboratories (Burlingame, CA). Cytochalasin B, colchicine and poly-L-lysine hydrobromide were purchased from Sigma (St. Louis, MO). Mouse anti-KDEL monoclonal antibody was obtained from StressGen Biotechnologies (Victoria, Canada).

Cell culture and cell fusion

PC12D cells were cultured in 100-mm plastic dishes (Falcon, Plymouth, UK) in DMEM supplemented with 5% fetal calf serum and 10% horse serum in a water-saturated atmosphere of 95% air and 5% CO₂ at 37°C. Coverslips (18×18 mm) were coated with poly-L-lysine solution (0.1%), kept at room temperature for more than one hour, and washed twice with distilled water. DMEM was then added and the coverslips were incubated for more than 30 min. For morphological observation, cells were dissociated by the incubation in phosphate-buffered saline (PBS) with 1 mM EGTA and were plated on coverslips precoated with poly-L-lysine in 35-mm culture dishes. After 20 hr, the medium was changed, and the cultures were used to prepare multinucleate cells. Cell fusion procedures were performed at room temperature. The cells were incubated in DMEM with 50% polyethylene glycol for 40 sec. After being rinsed five times with DMEM, cells were incubated in DMEM with 10% horse serum and 5% fetal calf serum.

Cytochemistry

Staining procedures were performed at room temperature. The cells on coverslips were fixed in 3.7% paraformaldehyde in PBS (pH 7.4), supplemented with 1 mM MgCl₂ and 0.1 mM CaCl₂ for 5 min, and made permeable by treatment with 0.1% Triton X-100 in PBS for 3 min. Cells were rinsed three times with PBS. For fluorescence staining of actin filaments, cells were incubated with rhodamine phalloidin in PBS (1:20 dilution) at 37°C for 30 min, and rinsed in PBS five times for 15 min. For immunofluorescence staining of microtubules, centrioles, and endoplasmic reticula, the detergent-treated cells were incubated with 10% goat serum in PBS, then with a drop of monoclonal antibody in PBS with 1% BSA for 60 min. For staining of microtubules, cells were incubated with the antibodies against α -tubulin and β -tubulin (1:2000). For staining of centrioles, cells were incubated with the antibody against γ -tubulin (1:10,000) for 3 days at 4°C. For staining of endoplasmic reticula, cells were incubated with the antibody against KDEL (1:500) for 60 min [11, 27]. After five washes for 15 min with PBS, cells were incubated with a drop of biotinylated antibody against mouse IgG in PBS with 1% BSA (1:200) for 60 min. After a wash, cells were incubated with streptavidin FITC in PBS (1:200) for 30 min, and rinsed five times in PBS for 15 min. They were then incubated with a drop of 20 μ M Hoechst 33342 or 10 μ M PI for 5 min for nuclear staining. Finally, cells were lightly rinsed in PBS. For staining of mitochondria, living cells were incubated with 100 nM MitoTracker Green FM for 60 min, and observed. Fluorescence microscope (Olympus BH2-RFK), and the confocal laser scanning microscopy (Olympus Fluoview) were utilized.

III. Results

Multinucleate PC12D cells were produced by treatment with PEG, and a circular nuclear alignment was observed in the fused cells (Fig. 1). In syncytia, immunofluorescently labeled microtubules were localized in neurites and around the nuclei, but rarely seen at the center (Fig. 1A). Stained actin filaments were mainly observed at the center of syncytia, and at the periphery of the cells (Fig. 1B). Nuclear localization was not clearly observed in PEG-fused PC12D cells under phase-contrast microscope (Fig. 2). The observation in some flat syncytia showed the formation of circular nuclear alignment within several hours after PEG-fusion. But many clear rings were observed in the culture dish 1 day after the fusion. The addition of cytochalasin B, an actin filament-disrupting agent, to the culture after cell fusion, did not prevent the formation of a circular alignment of nuclei (Fig. 3). After the treatment, the localization of microtubules was unchanged (Fig. 3A), but actin filaments were degraded and specks were seen in the syncytium (Fig. 3B). The addition of colchicine, a microtubule disrupting agent, after cell fusion prevented nuclei from forming a circular arrangement (Fig. 4). Following the treatment, microtubules were rarely seen, while actin filaments were unaffected. Nuclei in colchicine-

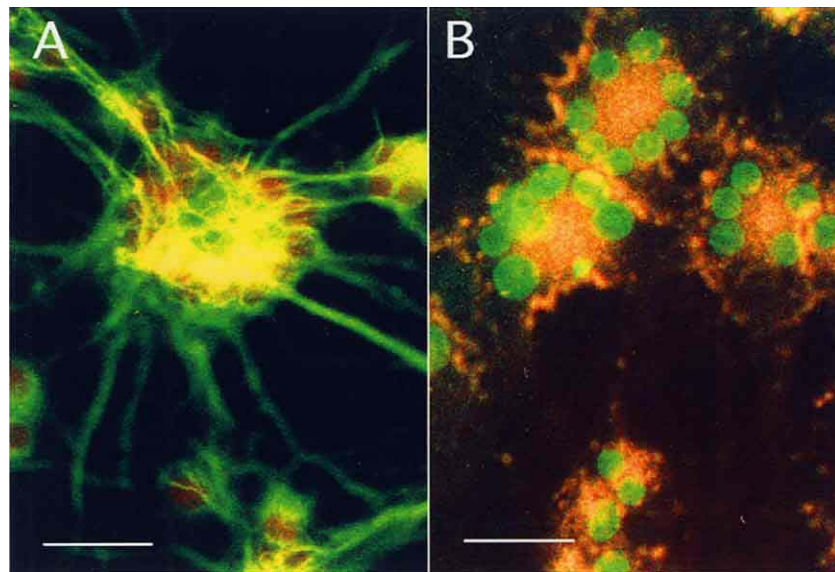


Fig. 1. Circular nuclear alignment in PEG-fused PC12D cells. Multinucleate PC12D cells were produced as described in the text. After 24 hr culture in the presence of NGF, cells were fixed. (A) Immunofluorescently stained α - and β -tubulin appears green. Intense fluorescence is shown as yellow. (B) Rhodamine phalloidin-stained actin filaments and Hoechst-stained nuclei appear orange and green, respectively. Bars=20 μ m.

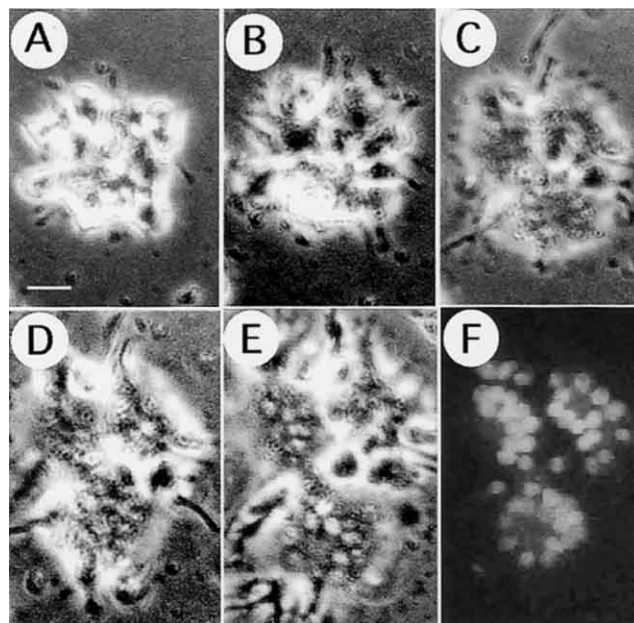


Fig. 2. The formation of circular nuclear alignment in PEG-fused PC12D cells. Phase-contrast photomicrographs show the time course of formation of nuclear rings before (A), and 3 hr (B), 8 hr (C), 14 hr (D), and 24 hr (E) after PEG treatment. Then, cells in (E), were fixed, nuclear-stained with Hoechst 33342, and were presented in (F) for demonstrating the nuclear rings. Bars=20 μ m.

treated syncytia did not align, but rather, were scattered throughout the cytoplasm (Fig. 4A). At the same time, the extended neurites were lost and no microtubules could be seen within them. When colchicine was added to syncytia in which a circular arrangement of nuclei and extended neurites had formed, the nuclear pattern changed (Fig. 4B). When colchicine was removed from the culture, the circle of nuclei re-formed within 24 hr (data not shown). This result

rules out the possibility that the nuclei were prevented from aligning by the cytotoxic effects of the inhibitor. From these experiments, it is suggested that microtubules play a crucial role in the localization and retention of nuclei in the syncytia.

Three-dimensional reconstruction of the syncytium by confocal laser microscopy revealed a cross section of the syncytium to be shaped like a mountain, and that intense

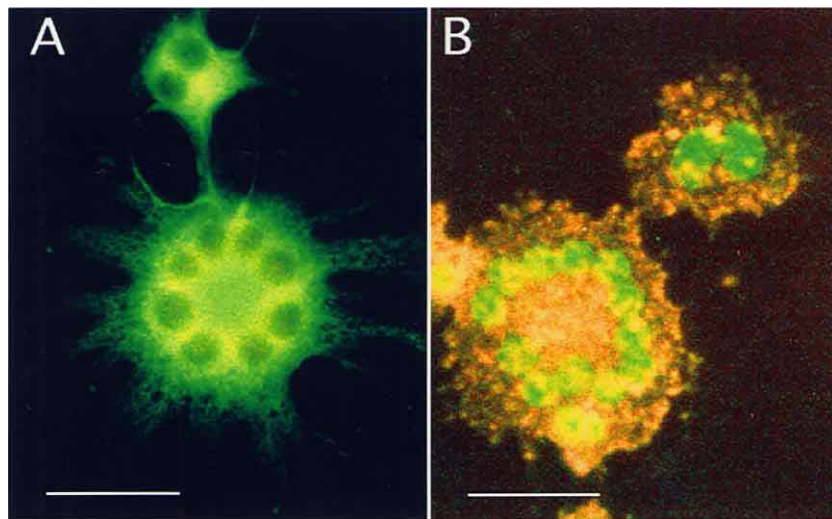


Fig. 3. Effect of cytochalasin B on the localization of nuclei in fused PC12D cells. Cytochalasin B (40 μ M) was added to the culture after the fusion of PC12D cells with PEG. After 3 days of culture with the inhibitor and NGF, cells were fixed. (A) Immunofluorescently stained α - and β -tubulin is green. Intense fluorescence of microtubules is shown as yellow. (B) Rhodamine phalloidin-stained actin filaments and Hoechst-stained nuclei appear orange and green, respectively. Bars=20 μ m.

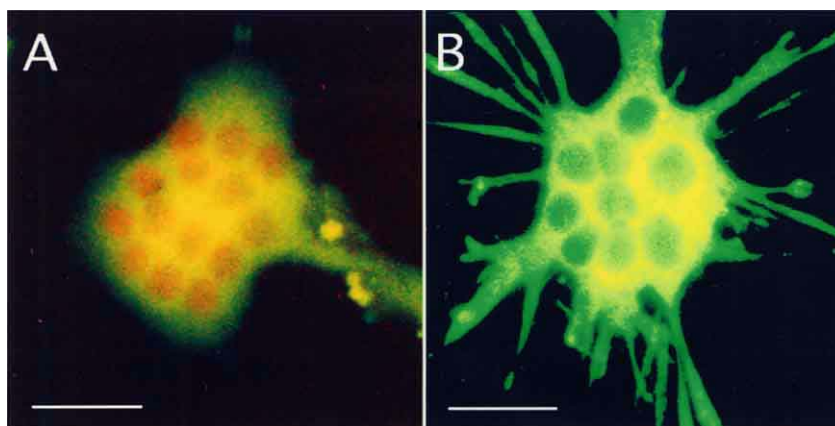


Fig. 4. Effect of colchicine on the localization of nuclei in fused PC12D cells. (A) Colchicine (100 μ M) was added to a cell culture after the fusion of PC12D cells with PEG. After 3 days of culture with NGF, cells were immunofluorescently stained with anti- α - and β -tubulin (green, and intense fluorescence was shown as yellow) and with PI (red). (B) Colchicine (100 μ M) was added to 3 day-cultures of fused cells with NGF in which a circular nuclear alignment had formed. After further culture for 24 hr, the syncytia were immunofluorescently stained with anti- α - and β -tubulin. Bars=20 μ m.

dot-like structures of immunofluorescence for γ -tubulin, which indicate centrioles [3, 6, 20], had aggregated at the center of the ring of nuclei. Twice as many centrioles as nuclei were observed in each cell and syncytium (Fig. 5A). Colchicine blocked not only the alignment of nuclei but the aggregation of centrioles (not shown). Confocal laser microscopy clearly revealed the microtubules to be localized in neurites and around the nuclei in tangles (Fig. 5B). Microtubules were not accumulated at the center of the circular arrangement. Therefore, it is difficult to consider that microtubules extended from the aggregation of centrosomes or the central region of the nuclear alignment.

Fluorescent staining of mitochondria (green, and in-

tense fluorescence appears yellow) was found throughout the syncytium (Fig. 6A). Intense yellow labeling was observed at the center of the circular alignment of nuclei. However, the cross-section of the syncytium was mountain-shaped (not shown), indicating that mitochondria would be uniformly distributed in the syncytium. Immunofluorescent staining of the endoplasmic reticulum showed a uniform labeling in the cytoplasm but no accumulation at the center of the arrangement of nuclei (Fig. 6B). Therefore, not only nuclei but various organelles like centrosomes and endoplasmic reticulum are distributed in each characteristic location in the syncytium.

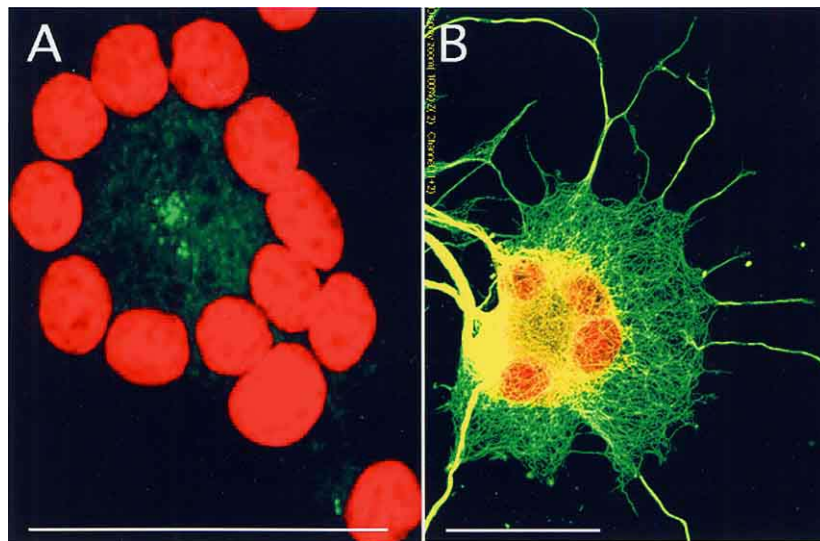


Fig. 5. Confocal laser microscopy of fused PC12D syncytia. (A) Immunofluorescently stained γ -tubulin (green) and PI-stained nuclei (red) in syncytia cultured for 3 days with NGF. (B) Microtubules (green, and intense fluorescence was observed as yellow) and PI-stained nuclei (red) in syncytia cultured for 3 days with NGF. Bars=20 μ m.

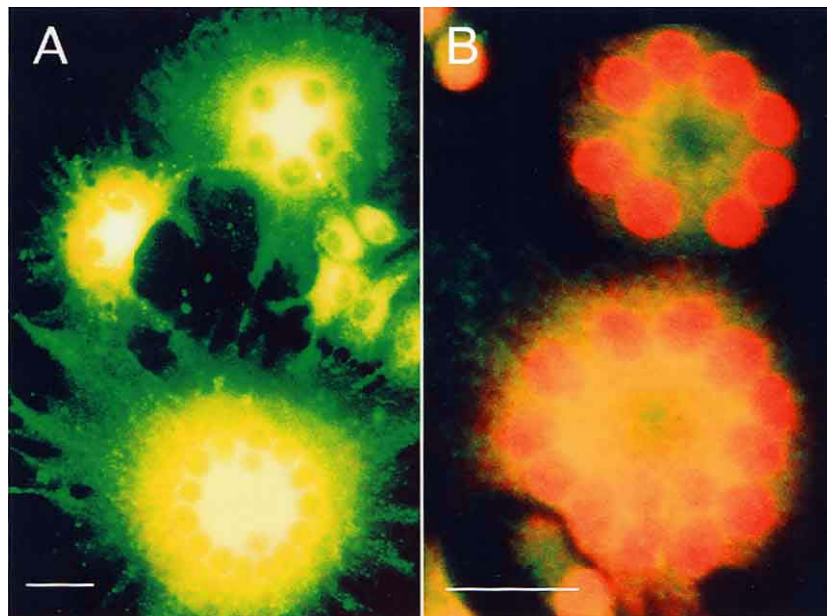


Fig. 6. Fluorescent staining of mitochondria and endoplasmic reticula in fused PC12D cells. After 3 days culture in the presence of NGF, cells were fixed. (A) Mitochondria in the syncytia were stained with MitoTracker Green FM (intense fluorescence was observed as yellow). (B) Endoplasmic reticula in the syncytia were stained with anti-KDEL protein antibody (yellow). Nuclei were stained with PI (red). Bars=20 μ m.

IV. Discussion

Multinucleated clonal fibroblasts were produced through cell fusion induced by viral infection or treatment with PEG. Watt *et al.* (1980) observed after fusion of African green monkey kidney (Vero) cells that nuclei were often arranged around a large centrosome [26]. Lyass *et al.* (1984) reported a wide nuclear ring in the syncytia of transformed mouse L cells [9]. On the fusion of viral infected baby ham-

ster kidney fibroblasts (BHK21-F), nuclei migrated in the syncytial cytoplasm and aligned in tightly packed rows [24, 25]. Studies on BHK syncytia produced by PEG reported that the nuclei encircled the cluster of centrosomes in stationary syncytia but were seen adjacent to the cluster in motile syncytia [7, 8]. So, a circular arrangement of nuclei around a large centrosome has been observed in several cultured syncytia of fused fibroblasts. The present study also showed clear nuclear circles around the aggregation of

centrioles in syncytia on cell fusion in a neuronal clonal line, PC12D. The formation of these circles was reproducible. As shown in Fig. 4, immunofluorescence for γ -tubulin showed a cluster of centrioles at the center of the circle. A similar localization of clusters of centrioles has been reported in electron microscopic studies [1, 2, 19] and in immunostaining experiments with anti-centriole antisera [25]. In the present study, twice as many centrioles as nuclei were visualized in the center of syncytia by confocal laser microscopy. A giant centrosome made of diplosomes (pairs of centrioles) in equal number to that of the nuclei was also observed in multinuclear cells of giant cell tumors of long bones [21].

Lewis and Albrecht-Buehler (1987) observed a circular alignment of nuclei in multinuclear BHK cells fused by PEG [7]. However, this study has focused on the relationship between cell motility and the location of nuclei. In fact, a few studies have focused on why nuclei localize in a circular arrangement in multinucleate cells [24, 25]. These studies have shown that colchicine blocks the migration and alignment of nuclei in the syncytial cytoplasm of BHK cells. Colchicine also prevents the translocation and aggregation of centrioles, but cytochalasin B has little effect on this process. We obtained similar results in fused PC12D cells: the migration and alignment of nuclei were blocked by the addition of colchicine upon the treatment of cells with PEG. The nuclear circles were readily degraded by colchicine. But they re-formed on removal of the inhibitor. Furthermore, cytochalasin B did not block the alignment. These results suggest that microtubules but not microfilaments are involved in the formation and maintenance of the circular alignments of nuclei in the syncytia of PC12D cells. In mononuclear PC12D cells, mononuclear BHK cells, and syncytia of BHK cells, the centrosomes seemed to serve as microtubule-organizing centers. In syncytia of PC12D cells, microtubules were congregated around the nuclei and in the neurites and spread out along the margin of the syncytium. In the syncytia, a cluster of centrioles apparently do not contribute to the organization of microtubules, although centrioles did not assemble in the presence of colchicine. On the basis of this information, we speculate that centrioles assemble in the center of the cytoplasm by means of microtubules and that the nuclei avoid the centrioles. Microtubules were entangled around nuclei, and extended to the periphery of the cytoplasm and to the neurites. Consequently, microtubules may contribute to the circular alignment of nuclei. However, we can not rule out the possibility that the assembled centrosomes control the alignment as a microtubule-organizing center. The specific localization of nuclei and centrosomes in the syncytia may be based on the mechanisms to locate the nucleus and cell organelles. Further study of syncytia may reveal the mechanism that controls the localization of the nucleus and organelles in the cytoplasm.

V. Acknowledgments

This study was supported by Grant (C-13680848) from the Ministry of Education, Culture and Science of Japan.

VI. References

- Baba, T., Shiozawa, N., Hotchi, M. and Ohno, S.: Three-dimensional study of the cytoskeleton in macrophages and multinucleate giant cells by quick-freezing and deep-etching method. *Virchows Arch. Cell Pathol.* 61; 39–47, 1991.
- Cain, H., Kraus, B., Fringes, B., Osborn, M. and Weber, K.: Centrioles, microtubules and microfilaments in activated mononuclear and multinucleate macrophages from rat peritoneum: Electron-microscopic and immunofluorescence microscopic studies. *J. Pathol.* 133; 301–323, 1981.
- Dictenberg, J. B., Zimmerman, W., Sparks, C. A., Young, A., Vidair, C., Zheng, Y., Carrington, W., Fay, F. S. and Doxsey, S. J.: Pericentrin and γ -tubulin form a protein complex and are organized into a novel lattice at the centrosome. *J. Cell Biol.* 141; 163–174, 1998.
- Holmes, K. V. and Choppin, P. W.: On the role of microtubules in movement and alignment of nuclei in virus induced syncytia. *J. Cell Biol.* 39; 526–543, 1968.
- Katoh-Semba, R., Kitajima, S., Yamazaki, Y. and Sano, M.: Neuritic growth from a new subline of PC12 pheochromocytoma cells: Cyclic AMP mimics the action of nerve growth factor. *J. Neurosci. Res.* 17; 36–44, 1987.
- Khodjakov, A., Cole, R. W., Oakley, B. R. and Rieder, C. L.: Centrosome-independent mitotic spindle formation in vertebrates. *Curr. Biol.* 10; 59–67, 2000.
- Lewis, L. and Albrecht-Buehler, G.: Distribution of multiple centrospheres determines migration of BHK syncytia. *Cell Motil. Cytoskel.* 7; 282–290, 1987.
- Lewis-Alberti, L.: Altering the vector of polarity of BHK syncytia changes their motile behavior. *Cell Motil. Cytoskel.* 14; 187–193, 1989.
- Lyass, L. A., Beshadsky, A. D., Gelfand, V. I., Serpinskaya, A. S., Stavrovskaya, A. A., Vasiliev, J. M. and Gelfand, I. M.: Multinucleation-induced improvement of the spreading of transformed cells on the substratum. *J. Cell Biol.* 81; 3098–3102, 1984.
- Marin, G. and Argenton, F.: Centrosome competition: A possibility? *Exp. Cell Res.* 187; 1–3, 1990.
- Oda, K., Wada, I., Takami, N., Fujiwara, T., Misumi, Y. and Ikehara, Y.: Bip/GRP78 but not calnexin associates with a precursor of glycosylphosphatidylinositol-anchored protein. *Biochem. J.* 316; 623–630, 1996.
- O' Lague, P. H. and Huttner, S. L.: Physiological and morphological studies of rat pheochromocytoma cells (PC12) chemically fused and grown in culture. *Proc. Natl. Acad. Sci. U S A* 77; 1701–1705, 1980.
- Papadimitriou, J. M. and Rigby, P. J.: The detection of a contractile apparatus in murine multinucleate giant cells. *J. Pathol.* 129; 91–97, 1979.
- Sano, M. and Iwanaga, M.: Requirement for specific protein kinase activities during the rapid redistribution of F-actin that precedes the outgrowth of neurites in PC12D cells. *Cell Struct. Funct.* 17; 341–350, 1992.
- Sano, M. and Kitajima, S.: Activation of microtubule-associated protein kinase in PC12D cells in response to both fibroblast growth factor and epidermal growth factor and concomitant stimulation of the outgrowth of neurites. *J. Neurochem.* 58; 837–844, 1992.
- Sano, M. and Kitajima, S.: Inhibition of the nerve growth factor-induced outgrowth of neurites by trichostatin A requires protein synthesis *de novo* in PC12D cells. *Brain Res.* 742; 195–202,

- 1996.
17. Sano, M. and Kitajima, S.: Activation of mitogen-activated protein kinases is not required for the extension of neurites from PC12D cells triggered by nerve growth factor. *Brain Res.* 785; 299–308, 1998.
 18. Sano, M., Iwanaga, M., Fujisawa, H., Nagahama, M. and Yamazaki, Y.: Staurosporine induces the outgrowth of neurites from the dorsal root ganglion of the chick embryo and PC12D cells. *Brain Res.* 639; 115–124, 1994.
 19. Sapp, J. P.: An ultrastructural study of nuclear and centriolar configurations in multinucleated giant cells. *Lab. Invest.* 34; 109–114, 1976.
 20. Schiebel, E.: γ -tubulin complexes: binding to the centrosome, regulation and microtubule nucleation. *Curr. Opin. Cell Biol.* 12; 113–118, 2000.
 21. Vacher-Lavenu, M. C., Louvel, A., Daudet-Monsac, M. and Abelanet, R.: Configuration et position des centrioles des cellules mono et multinucleées des tumeurs a cellules géantes des os longs. *Ann. Anat. Pathol. (Paris)* 25; 331–339, 1980.
 22. van der Rhee, H. J. and van der Burgh-de Winter, C. M. P.: The differentiation of monocytes into macrophages, epithelioid cells, and multinucleated giant cells in subcutaneous granulomas. *Cell Tissue Res.* 197; 355–378, 1979.
 23. Varon, S., Nomura, J. and Shooter, E. M.: The isolation of mouse nerve growth factor protein in a high molecular weight form. *Biochemistry* 6; 2202–2209, 1967.
 24. Wang, E., Cross, R. K. and Choppin, P. W.: Involvement of microtubules and 10-nm filaments in the movement and positioning of nuclei in syncytia. *J. Cell Biol.* 83; 320–337, 1979.
 25. Wang, E., Connolly, J. A., Kalnins, V. I. and Choppin, P. W.: Relationship between movement and aggregation of centrioles in syncytia and formation of microtubule bundles. *Proc. Natl. Acad. Sci. U S A* 76; 5719–5723, 1979.
 26. Watt, F. M., Sidebottom, E. and Harris, H.: Microtubule-organizing centers in binucleate cells and homosynkaryons. *J. Cell Sci.* 44; 123–133, 1980.
 27. Wiest, D. L., Bhandoola, A., Punt, J., Kreibich, G., McKean, D. and Singer, A.: Incomplete endoplasmic reticulum (ER) retention in immature thymocytes as revealed by surface expression of “ER-resident” molecular chaperones. *Proc. Natl. Acad. Sci. U S A* 94; 1884–1889, 1997.