

Expression of Tubulin Beta II in Neural Stem/Progenitor Cells and Radial Fibers During Human Fetal Brain Development

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SUMMARY: Recent studies revealed that the “radial glia” in fetal rodent brains are dividing neuronal precursor cells. However, in fetal primate brains, this issue remains unclear, with previous reports indicating that radial glia are a specialized form of astroglia. To investigate the relationship between radial fibers (RFs) and neural stem/progenitor cells in the fetal human brain, we generated polyclonal antibodies to human nestin protein and developed a new mAb, KNY-379, by screening for antibodies that immunostained RFs on paraffin-embedded human fetal brain specimens (12 gestational weeks). The immunostaining for KNY-379 antigen and nestin was seen over the RFs in brains at 8 gestational weeks. Furthermore, KNY-379 antigen and nestin were also detected in human neural stem/progenitor cells in neurosphere cultures. At 12 to 15 gestational weeks, the KNY-379 immunostaining of RFs remained in the periventricular zone and the deep part of the intermediate zone, but it also appeared in outgrowing axons in the cortical plate, in the superficial portion of the intermediate zone, and in apical dendrites in the molecular layer. In the later stages of fetal development (18–40 gestational weeks), this antigen remained in the outgrowing axons and dendrites, but was no longer associated with RFs. Expression cloning and immunoblot analysis demonstrated the antigen to be tubulin beta II, which would thus be a good marker for studying RFs and neural stem/progenitor cells in the early developing human brain. (*Lab Invest* 2003, 83:479–489).

In the developing mammalian brain, young postmitotic neurons leave the periventricular zone (PV) and migrate to the cortical plate (CP) along radial fibers (RFs) that extend from the cells of the periventricular neuroepithelium and terminate in branches at the pial surface of the cortex (reviewed in Bentivoglio and Mazzeo, 1999; Rakic, 1972; Walsh and Cepko, 1992). RFs are the processes of cells known as “radial cells,” which have been termed differently by various researchers (His, 1889; Fujita, 1963; Noctor et al, 2001; Rakic, 1972; Ramon and Cajal, 1911). Radial

cells located in the PV of the fetal monkey have been thought to be a specialized cell type belonging to the astroglial lineage, based on their expression of glial fibrillary acidic protein (GFAP) (Levitt et al, 1981) and thus have been termed “radial glia” (Bentivoglio and Mazzeo, 1999). Accordingly, RFs have been called “radial glial fibers” (Rakic, 1995).

Recent progress in neurobiology has led to a new understanding of the development and cell lineages of mammalian brain cells, specifically that neurons and glial cells derive from common progenitor cells, ie, neural stem cells (McKay, 1997). Neural stem/progenitor cells have been found in the PV in both fetal and adult human brains (Buc-Caron, 1995; Keyoung et al, 2001; Kukekov et al, 1999; Pincus et al, 1997; Roy et al, 2000). Furthermore, many lines of recent evidence suggest that some radial cells may generate neurons and that RFs are the cell processes of neural stem/progenitor cells, at least in fetal mouse brains (Hartfuss et al, 2001; Malatesta et al, 2000; Miyata et al, 2001; Noctor et al, 2001; Parnavelas and Nadarajah, 2001).

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Several immunohistochemical markers for RFs, including intermediate filaments, such as nestin (Hockfield and McKay, 1985; Lendahl et al, 1990) and vimentin (Kamei et al, 1998; Sarnet, 1998; Yachnis et al, 1993), have been reported. Other markers associated with RFs include antigens for the RC1 and RC2 antibodies (Edwards et al, 1990; Misson et al, 1988), brain lipid-binding protein (Feng and Heintz, 1995), bone morphogenetic protein-6 (Schluesener and Meyermann, 1994), receptor-type protein tyrosine phosphatase-beta (Canoll et al, 1993), glutamine synthetase (Akimoto et al, 1993), and 3CB2 antigen (Prada et al, 1995). However, few studies have recently been attempted to characterize the RFs in developing human fetal brain, especially their relationship to the neural stem/progenitor cells.

To address this issue, in the present study we generated polyclonal antibodies to human nestin protein and developed a new mAb, KNY-379, which recognized RFs in situ within developing human fetal brains as well as neural progenitor cells, including neural stem cells, in human neurospheres that are enriched in these cells. Thus, at least some populations of human fetal RF are likely to be neural stem/progenitor cells. We also demonstrated by expression cloning that the antigen of KNY-379 is tubulin beta II. Thus, this study provides new tools with which to study the stem cell biology and developmental biology of the human brain.

Results

mAb KNY-379 and Polyclonal Antibody to Nestin

The antibody KNY-379 was found to belong to the IgG1, κ subclass of immunoglobulins. Both KNY-379 and polyclonal antibody to nestin reacted with paraffin-embedded human and rat tissues and with fresh-frozen sections.

Light Microscopic Immunohistochemistry

mAb KNY-379 and antibodies against vimentin (pretreated by microwaving), nestin (pretreated by incubation in a hot water bath), GFAP (pretreated by microwaving), and tubulin beta III were immunoreactive on both paraffin-embedded and fresh-frozen sections. The mAb KNY-379 demonstrated strong immunoreactivity in paraffin sections, with a longer postmortem interval equal to 12 hours (longest postmortem interval in our study).

In the cerebrum at 8 gestational weeks (GW), the distribution of the immunopositive staining with KNY-379 was similar to that seen with the antibodies to vimentin (Fig. 1, A and B) and to nestin (Fig. 1, D and E) in the PV and intermediate zone (IZ), suggesting the presence of the KNY-379 antigen along the RFs in PV and IZ. This colocalization was confirmed by double immunostaining with KNY-379 and anti-vimentin or anti-nestin antibodies (Fig. 1, C and F). Staining for tubulin beta III was negative in the PV and along the RF. The immunoreactivities with GFAP and neurofilament protein (NF) were completely negative at 8 GW.

In the cerebrum at 10 to 15 GW, positive immunostaining with KNY-379 occurred in a fibrous pattern in the PV and CP. In the IZ, the pattern of positive staining was fibrous and reticular in the deep zone of the IZ and horizontal in the superficial IZ (Fig. 2A). The pattern of immunoreactivity with anti-vimentin was similar to that of KNY-379 in the PV, but it was different in the IZ. The immunoreactivity with anti-vimentin became weak in the superficial IZ and CP (Fig. 2B). The immunoreactivity with anti-nestin was similar to that of anti-vimentin except for positive staining in the molecular layer (ML) (Fig. 2C). Tubulin beta III immunoreactivity was negative in the PV but positive in the IZ and CP. The immunoreactivities with GFAP and NF were negative between 10 and 15 weeks.

In the cerebrum at 18 to 40 GW, positive fibrous immunoreactivity with KNY-379 was confined to the CP (Fig. 3A). In the IZ, diffuse positive immunoreactivity was seen that diminished gradually. Immunoreactivity, which was probably a result of the invading ends of dendrites and axons, was strong in the ML over the cerebral convexities but somewhat weak in the sulcal depths. The positive immunoreactivity with anti-vimentin remained along the RFs as a bead-like pattern in the fading PV and in the deep zone of the IZ; but immunoreactivity was negative in the superficial IZ, CP, and ML (Fig. 3B). Nestin-positive staining was seen in the RFs of the deep IZ up to 18 GW; thereafter, the RFs were negative for nestin. The RFs were also positive with GFAP antiserum. A small number of the vimentin-positive glial progenitor cells/glioblasts were immunopositive for KNY-379. Mature astrocytes showed positive immunostaining with antibodies to vimentin and GFAP but were negative with KNY-379 and nestin on serial sections. Galactocerebroside-positive oligodendrocytes were also negative with KNY-379 and nestin on serial sections (data not shown).

Postnatally, the positive staining with KNY-379 had weakened in all layers except for the ML (not shown). The vimentin/GFAP-positive RFs disappeared after 6 months of age (not shown). The immunoreactivity with KNY-379 emerged again in some apical dendrites and/or axons of cortical neurons after 1 year of age (Fig. 4A) and in some axons in the white matter. These dendrites and axons were also positively stained with the antibody to tubulin beta III and NF (Fig. 4B). The distribution of immunoreactivity to the different antibodies is summarized in the Table.

In the cerebellum, the ML was immunopositive with KNY-379 throughout the fetal period and some apical dendrites and/or axons in the ML, the granular layer, and the white matter became positive with KNY-379 postnatally. In the brain stem and spinal cord, nerve tracts were positive for KNY-379. Positive immunoreactivity in the brain stem was present throughout the fetal, postnatal, adolescent, and adult periods. Cilia of the ependymal cells lining the ventricular system were also strongly positive with KNY-379. No positive immunoreactivity was found when the primary antibody

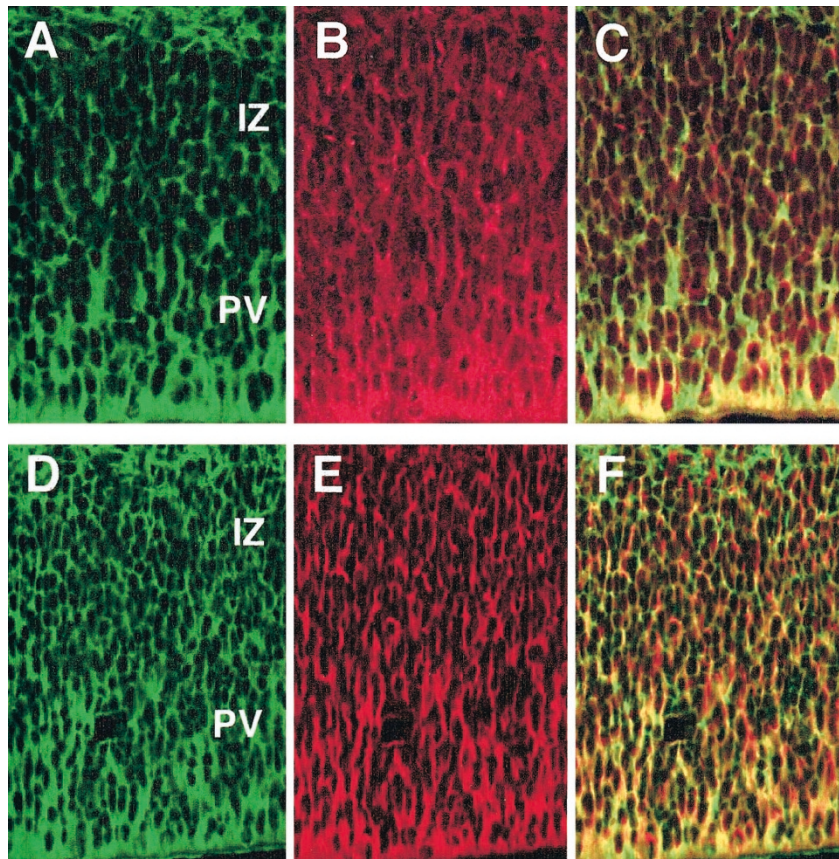


Figure 1.

Immunohistochemical distribution of tubulin beta II, vimentin, and nestin in the cerebrum at 8 gestational weeks (GW). A, Positive immunoreactivity with tubulin beta II (green) was found in the periventricular zone (PV) and intermediate zone (IZ) as a fibrous pattern. B, Positive staining with vimentin (red) was similar to that of tubulin beta II. C, Double immunostaining with tubulin beta II and vimentin suggests that both proteins were associated with radial fibers (RFs). D, Immunohistochemical distribution of tubulin beta II (green). E, Immunohistochemical distribution of nestin (red). F, Double immunostaining with tubulin beta II and nestin suggests that both proteins were associated with RFs (without counterstaining, $\times 100$).

ies were omitted or primary antibodies preadsorbed with excess homogenate or normal sera were used.

Expressional Cloning

Three positive plaques were obtained by expression cloning, using a cDNA library from fetal brains and KNY-379, and 600 to 700 bp of each entrapped cDNA was sequenced. We searched for the cDNA sequence using the BLASTN 2.0.11 program, and a homology search was also made. All three cDNAs were identified (99–100%) as tubulin beta II. Thus, antibody KNY-379 recognizes tubulin beta II.

Immunoprecipitation and Immunoblotting

Standard Western blot showed double bands with a major band at 53 kDa and a minor band at 29 kDa in all samples (21, 27, 31, and 40 GW and adult brain). KNY-379 detected a single band at 53 kDa in immunoprecipitates in all samples (Fig. 5). The single band at 53 kDa precipitated with KNY-379 should thus correspond to tubulin beta II as reported previously (Moody et al, 1996). The bands found in standard Western blot and a single band in immunoprecipitates did not react with anti-vimentin or anti-nestin antibod-

ies (not shown), indicating that this anti-tubulin beta II antibody did not cross-react with vimentin or nestin. A minor band at 29 kDa found in standard Western blot could be the cross-reactivity with a related protein of tubulin beta II.

Immunocytochemical Study on Human Cultured Neural Cells

The majority of cells within neurospheres stained positively with KNY-379 (tubulin beta II) as well as Musashi1 and nestin antibodies (Fig. 6). After differentiation was induced, the distribution of the immunoreactive tubulin beta II was similar to that of PSA-NCAM, indicating that it was expressed in the neural differentiated lineage (Fig. 7). A small number of tubulin beta II-positive cells also appeared to be positively immunoreactive with GFAP (Fig. 7).

Discussion

Tubulin is a major component protein of microtubules and exists as a heterodimer of two subunits designated alpha and beta. In vertebrates, the alpha and beta tubulins are encoded by small multigene families (Cleveland et al, 1980). In mammals, there are multiple

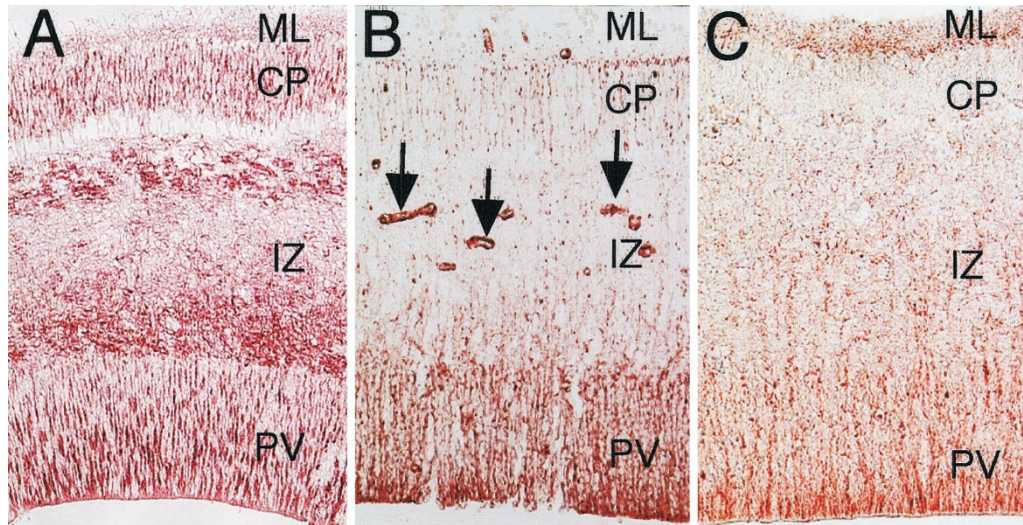


Figure 2.

Immunohistochemical distribution of tubulin beta II, vimentin, and nestin in the cerebrum at 12 GW. A, Positive immunoreactivity with tubulin beta II was fibrillary in the PV and cortical plate (CP), fibrillary/reticular in the deep zone of the IZ, and horizontal in the superficial IZ. B, Positive immunostaining for vimentin was fibrillary, but weak in the superficial IZ and CP. Arrows show the positive immunoreactivity in vascular walls. C, The immunoreactivity to nestin was similar to that of vimentin except for positive staining in the molecular layer (ML) (without counterstaining, $\times 25$).

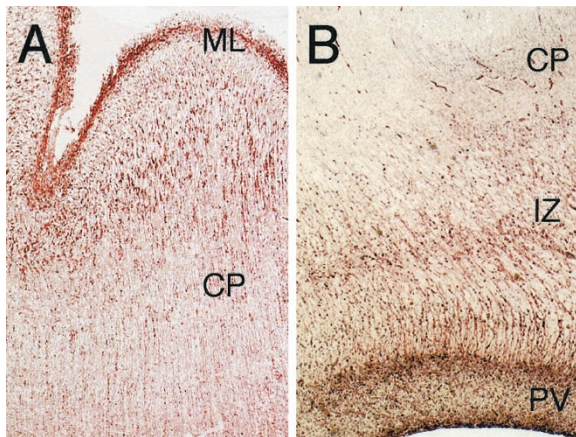


Figure 3.

Immunohistochemical distribution of tubulin beta II and vimentin in the cerebrum at 25 GW. A, positive fibrillary immunoreactivities to tubulin beta II remained only in the CP. B, Positive immunoreactivity with vimentin was found along the RF in the PV and the deep zone of the IZ but not in the superficial IZ and CP (without counterstaining: A, $\times 25$; B, $\times 12$).

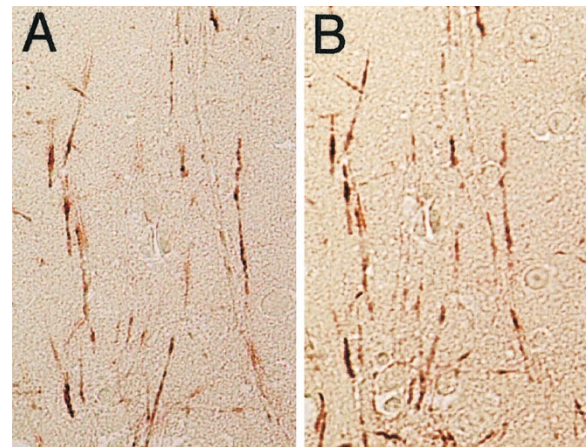


Figure 4.

Immunohistochemical distribution of tubulin beta II and neurofilament protein (NF) in the serial sections of cerebral cortex at 10 years of age. The positive immunoreactivity of tubulin beta II is seen in some apical dendrites and axons. B, Positive reactivity of NF follows the same pattern (without counterstaining, $\times 100$).

isotypes of alpha and beta tubulin encoded by six α -tubulin and seven β -tubulin genes (Cleveland, 1987). Among the seven β -tubulins, tubulin beta I is expressed constitutively in many tissues. Beta II is the major form of tubulin beta found in neurons and is also present in many other tissues. Beta III is neuron specific, beta IVa is expressed in the nervous system and other tissues, beta IVb is expressed mainly in the testis, beta V is expressed in many tissues except for neurons, and beta VI is specific for hematopoietic tissues (Sullivan, 1988). The different β -tubulin types are highly homologous throughout most of their sequences. A major variable region exists at the carboxyl terminus (Sullivan, 1988).

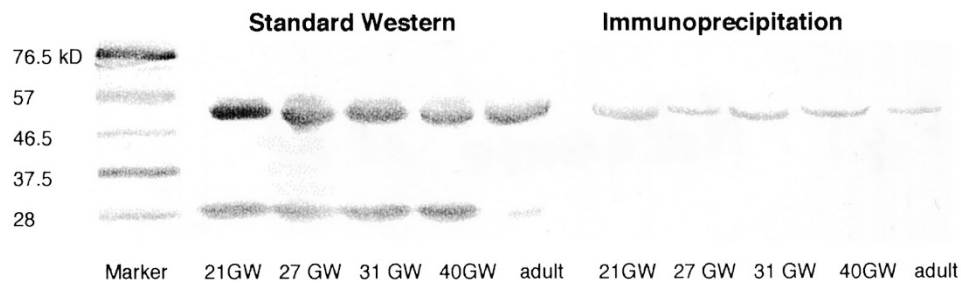
The mAb KNY-379, raised against the brains of 14-day-old rat embryos, which correspond to human

fetuses of 12 to 16 GW, recognizes tubulin beta II in human and rat tissues. Purified bovine brain tubulin contains tubulin beta that is comprised of 3% type I, 58% type II, 25% type III, and 13% type IV (Banerjee et al, 1988). Thus, tubulin beta II is the major form of tubulin beta in bovine brain, although it is not specific for the nervous system and is also expressed in lung, chick embryo fibroblasts, and Schwann cells (Lewis et al, 1985; Lopata and Cleveland, 1987; Robertson et al, 1992). Tubulin beta III, which is specific for neurons, has been studied extensively. The initial expression of tubulin beta III begins during or immediately after the completion of mitosis in neuroblasts (Lee et al, 1990). However, in frog embryos, tubulin beta II is the neuron-specific form and is expressed in embryonic

Table 1. Immunohistochemical Distribution of Studied Antigens

	Nes	Vim	Tub II	GFAP	Tub III	NF
8 GW						
Neural stem/progenitor cell	+	+	+	-	-	-
Radial fiber	+	+	+	-	-	-
10 to 15 GW						
Neural stem/progenitor cell	+	+	+	-	-	-
Radial fiber	+	+	+	-	-	-
Migrating neuroblast	-	-	-	-	-	-
Neuronal cells in CP	-	-	+	-	+	-
18 to 40 GW						
Radial fiber	+/-	+	-	+	-	-
Glial progenitor cells	-	+	+/-	+	-	-
Astrocyte	-	+	-	+	-	-
Oligodendrocyte	-	-	-	-	-	-
Neuronal cell	-	-	+	-	+	+
Adult brain						
Astrocyte	-	+/-	-	+	-	-
Oligodendrocyte	-	-	-	-	-	-
Neuronal cell	-	-	+	-	+	+

GW, gestational weeks; Nes, nestin; Vim, vimentin; GFAP, glial fibrillary acidic protein; Tub II, tubulin beta II; Tub III, tubulin beta III; NF, neurofilament protein; CP, cortical plate.

**Figure 5.**

Western blotting analysis of tubulin beta II expression in developing brains at 21, 27, 31, and 40 GW and in adult brain. Standard Western blot with KNY-379 showed double bands with a major band at approximately 53 kDa and a minor band at approximately 29 kDa in all brain samples. Immunoprecipitates obtained with KNY-379 showed a single band at approximately 53 kDa in all samples.

neurons but not in radial glia (Moody et al, 1996). In animal brains, tubulin beta II (Hoffman and Cleveland, 1988; Jiang and Oblinger, 1992; Paden et al, 1995) is induced significantly during development and axonal regeneration and is distributed mainly in neuronal components, but its expression in developing human brains has been little studied.

The present study clearly demonstrated that tubulin beta II, as well as vimentin and nestin, the markers for RFs, are expressed over the RFs in the human cerebrum at 8 GW. At 10 to 15 GW, the presence of tubulin beta II in the PV and the deep zone of the IZ remained the same as at 8 weeks, but the superficial zone of the IZ and CP showed a more complex pattern of positive staining, possibly reflecting tubulin beta II in outgrowing axons in addition to RFs. In the CP, although weak positive staining was seen for vimentin and nestin, staining for tubulin beta II was strongly positive as a fibrillary pattern. It was recently reported that the cortical RFs could generate neurogenesis based on a study in Pax 6 mutant mice (Heins et al, 2002). The positive signal for tubulin beta II could be attributed to

dendrites and to outgrowing axons in the CP and IZ or to changes in the antigenicities of RFs themselves. The findings that the immunoreactive GFAP and NF were completely negative in developing brains during 8–15 weeks gestation may suggest that RFs and outgrowing axons are in the progenitor state without markers for mature glial and neuronal differentiation. After 18 GW, the positive fibrillary immunoreactivity of tubulin beta II remained only in the CP and had faded in the IZ. It is reported that RFs themselves remain in these structures even in the postnatal period (Yachnis et al, 1993). This study showed that vimentin and GFAP are present in RFs after 18 GW and up to 6 months after birth. RFs in this stage could be called radial glial fibers because of differentiation into glia expressing GFAP. Although vimentin was seen in astrocytes and glial progenitor cells/glioblasts, tubulin beta II was only found in some glial progenitor cells/glioblasts and not at all in mature astrocytes.

It is not clear why the positive reactivity of tubulin beta II was reduced in the cerebrum postnatally, but it may be important for the development and mainte-

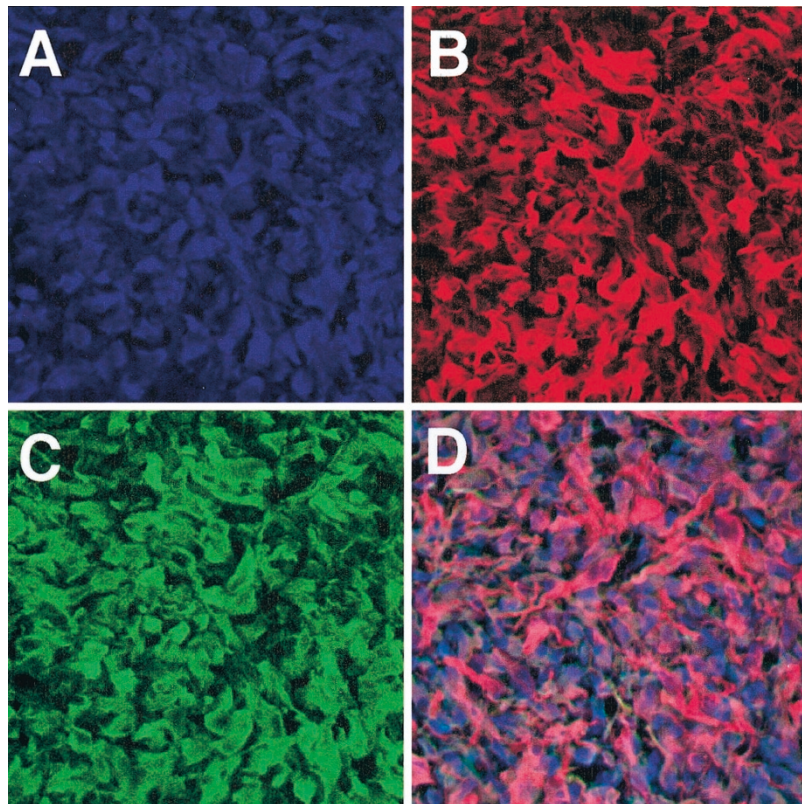


Figure 6.

Immunocytochemical distribution of Musashi1 (A, blue), nestin (B, red), and tubulin beta II (C, green) in human neurosphere cells. All three proteins were expressed in undifferentiated human neurosphere cells. D, Triple-colored immunofluorescence staining revealed the three antigens were distributed similarly in human neurosphere cells ($\times 800$).

nance of progenitor-state RFs, outgrowing axons, and dendrites in the developing brain. After cells differentiate into glia and neurons, tubulin beta II may no longer be expressed. Strong positive staining for tubulin beta II in the ML of the cerebrum and cerebellum and differences in the staining intensity in cerebral sulci suggest the important and unique roles of the ML in the developing brain. One microtubule-associated protein (MAP), MAP 5, is expressed strongly in the cerebral and cerebellar ML (Ohyu et al, 1997). MAP 2 and a phosphorylated MAP 1B are found in the developing brain and considered important for axonal outgrowth and maintenance (Honig et al, 1996; Nothias et al, 1996).

In this study, we confirmed that tubulin beta II is expressed in Musashi1 and nestin double-positive cells within neurospheres. Previously, the expression of Musashi1 and nestin was carefully studied by the infection of adenoviruses bearing the gene for green fluorescence protein in neural stem/progenitor cells, and the results showed that cells that bore both proteins were able to self renew and cogenerated neurons and glia (Keyoung et al, 2001). Neural stem cells can be selectively expanded in a serum-free defined medium containing epidermal growth factor and/or fibroblast growth factor 2, in which they give rise to a floating cell mass called a "neurosphere" (Reynolds et al, 1992; Svendsen et al, 2001). Cells within neurospheres derived from human fetal brain,

which include a population with self-renewing ability and multipotency, are mostly immunopositive against anti-nestin and anti-Musashi1 antibodies. Moreover, we also observed that tubulin beta II was expressed in neuronal progenitor cells and developing neurons that express PSA-NCAM (Seki and Arai, 1993). These findings indicate that tubulin beta II is expressed in human neural stem/progenitor cells and/or neuronal lineage cells, consistent with the results from our immunohistochemical analysis.

On the other hand, in developing human brain tissue in situ after 18 GW and in differentiation-induced neuronal cells in vitro, some cells were immunopositive for both GFAP and tubulin beta II. These findings suggest that tubulin beta II is transiently expressed in glial progenitor cells (glioblasts).

The results of the present study indicate that tubulin beta II may function as a temporary constituent of progenitor-stage RFs that guide neuroblast migration and that it may be important for axonal and dendrite outgrowth thereafter, with other associated proteins such as MAP 1B and 2. We conclude that tubulin beta II is a useful marker for RFs and as such should reveal valuable information for understanding human brain development. However, the function of the tubulin beta II in neural stem/progenitor cells or RFs remains to be elucidated. Future studies using targeted disruption of the mouse tubulin beta II gene should unequivocally reveal the in vivo function of this gene product.

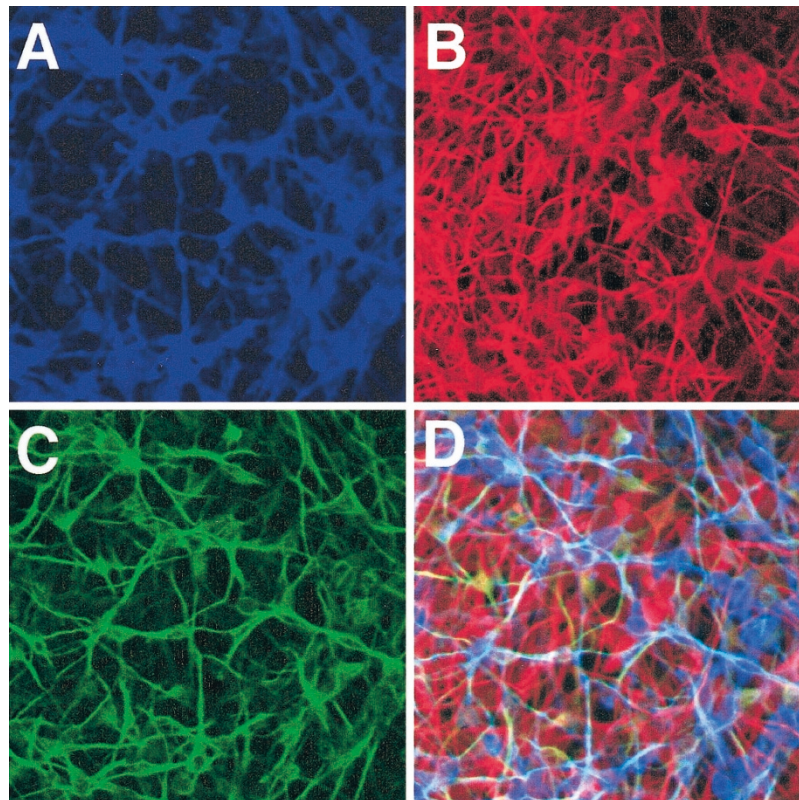


Figure 7.

Immunocytochemical distribution of PSA-NCAM (A, blue), glial fibrillary acidic protein (GFAP) (B, red), and tubulin beta II (C, green) in differentiated neural stem cells. The distribution of immunoreactivity against PSA-NCAM and tubulin beta II was quite similar. D, Triple-colored immunofluorescence staining confirmed a similar distribution of PSA-NCAM and tubulin beta II on differentiated cells, indicating neuronal differentiation. Some similarity to the distribution of GFAP was also found, suggesting that tubulin beta II might be expressed in the progenitor stage (or glioblast) of glial differentiation ($\times 800$).

Materials and Methods

Human Brain Samples

Human brain samples for immunohistochemical study were obtained from abortions and at autopsy from 32 cases ranging from 8 GW to 82 years (2 at 8 GW, 1 at 10 GW, 1 at 11 GW, 2 at 12 GW, 1 at 13 GW, 1 at 14 GW, 1 at 15 GW, 6 at 18–30 GW, 5 at 30–40 GW, 2 at 7–28 days after birth, 2 at 3 or 6 months of age, 2 at 1 year, 1 at 5 years, 1 at 10 years, and 4 at 30–82 years). All fetus samples before 20 GW were from spontaneous or electively performed abortions and were fixed in formalin. Other samples were obtained at autopsy. All cases had no abnormalities in the central nervous system. For immunohistochemistry, buffered formalin-fixed and paraffin-embedded sections were also prepared from the brains. Some samples obtained at autopsy were placed in liquid nitrogen for immunoprecipitation and Western blot preparations.

For cell culture preparations, forebrain tissue from human fetuses at 7 to 10 GW were obtained from routine legal terminations performed at the Osaka National Hospital with the approval of the ethical committee of the hospital.

Approval to use human fetal neural tissues was obtained from the ethical committees of each institution. Tissue procurement was in accordance with the Declaration of Helsinki (1964) and in agreement with

the ethical guidelines of the European Network for Transplantation (NECTA) and the Japan Society of Obstetrics and Gynecology.

mAbs

For the preparation of monoclonal antibodies, homogenates were obtained from the brains of 14-day embryonal rat fetuses. Monoclonal antibodies were produced by the fusion of FO1 mouse myeloma cells with inguinal lymph node B lymphocytes from female BALB/c mice immunized with three subcutaneous injections of 200 mg of homogenate each, with the Ribi adjuvant system (Funakoshi, Tokyo) (Orlik and Altaner, 1988). Approximately 10^8 lymphocytes were obtained from the inguinal lymph nodes of 8 to 10 mice by rubbing the tissue between two frosted glass slides. The obtained lymphocytes were fused with 2 to 4×10^7 FO-1 cells by adding 50% polyethylene glycol (Sigma-Aldrich Japan, Tokyo, Japan). HAT (Sigma-Aldrich) medium selection was performed in 5×96 -well plates with feeder cells (mice thymocytes) and growth factors (Bryclone, Dainippon-pharm, Osaka, Japan). Hybridoma culture supernatants from 480 wells were screened by incubating them with paraffin-embedded sections of developing human brain (12 GW). Approximately one fifth of supernatants were immunoreactive on the paraffin-embedded sections.

Only one clone produced antibody that reacted with RFs. It was named KNY-379 after the well number. The hybridoma cells were subcloned three times by limiting dilution and injected into the peritoneal cavity of pristane-pretreated mice. The ascites fluid was obtained approximately 1 week later. The Ig subclass was determined using a kit (Amersham Pharmacia Biotech, Sweden) according to the instructions of the manufacturer. The IgG fractions were purified on a protein G-Sepharose column. For the procedures using animals, we followed the Guide for the Care and Use of Laboratory Animals (1985).

Creation of Antibodies Against Human Nestin Protein

Antibodies against human Nestin protein were prepared as described previously (Nakanishi et al, 2001). Briefly, rabbits were immunized with a synthetic polypeptide corresponding to the C-terminal portion (1596–1610 amino acids) of the human Nestin protein (Dahlstrand et al, 1992) (GenBank accession no. X65964). To enhance its antigenicity, the peptide was covalently attached to activated keyhole limpet hemocyanin (Pierce Chemical, Rockford, Illinois) before immunization. The same Nestin peptide was immobilized on CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) for affinity purification of the peptide-specific antibodies. Immunoglobulins adsorbed to the peptide resin were eluted with 0.2 M glycine-HCl buffer (pH 2.7) and then neutralized by adding 2 M Tris-HCl buffer (pH 8.0). The specificity of the antibody was confirmed by ELISA with the peptide used for the immunization and by immunohistochemical reaction to fetal human brains (based on the characteristic staining of the RFs (Fig. 2C) and human neurosphere cells (Fig. 6B).

Light Microscopic Immunohistochemical Study

Paraffin-embedded specimens were used for immunohistochemistry according to previously reported methods (Nakamura et al, 1998) with 3-amino-9-ethylcarbazole as the chromophore. For immunohistochemical double staining, the methods are described under "Immunocytochemical Analysis" using mouse mAb KNY-379 and goat polyclonal vimentin antibody or rabbit polyclonal nestin antibody and confocal microscopy. Serial sections were used for the study of colocalization of tubulin beta II and NF or tubulin beta III or galactocerebroside. In this study, the antibody KNY-379 and antibodies against vimentin (mouse monoclonal [DAKO, Carpinteria, California] and goat polyclonal [Chemicon, Temecula, California]), nestin, GFAP (DAKO), tubulin beta III (mouse monoclonal [BABCO, Richmond, California]), NF (70 and 200 kDa; mouse monoclonal [DAKO]), and galactocerebroside (mouse monoclonal [Chemicon]) were used. In some cases, the slides were pretreated (antigen rescue) by microwaving them for 15 minutes in 10 mM citrate buffer at pH 6.0 or incubating them in a hot water bath (95°C, 40 minutes) in the same buffer. The dilutions of primary antibodies were 1:1000

(KNY-379), 1:500 (anti-tubulin beta III and nestin), and 1:200 (anti-vimentin, GFAP, galactocerebroside, and NF). For controls, the primary antibodies were omitted or primary antibodies preadsorbed with excess homogenate or normal sera were used.

Expression Cloning

A cDNA library from whole fetal human brain (male/female, 19–23 weeks, pooled) was purchased from Stratagene (La Jolla, California; catalog no. 937227). Using appropriately prepared host bacteria, XL1-Blue MRF strain, titrating of lambda phages was performed according to the manufacturer's instructions. Tubes containing titered phages and host bacteria were incubated for 15 minutes at 37°C and 5 ml of LB top agarose was added. The plating culture was poured onto LB agar plates, which were then inverted and incubated overnight at 42°C. Isopropylthiogalactoside-saturated filters were placed on the surface of the agar in each plate and incubated at 37°C for 3 to 4 hours. The filters were removed, rinsed, and placed in block solution for 1 hour, then placed in KNY-379 1:1000 solution for 1 hour, rinsed, incubated with peroxidase-conjugated second antibody (anti-mouse Ig, Dako, Carpinteria, California) at a 1:1000 dilution for 1 hour, rinsed, and finally developed with 3,3'-diaminobenzidine. Positive plaques with KNY-379 antibodies were picked and subcloned three times. The cDNA was amplified by PCR with T1 and T3 primers and subcloned into pT7Blue™ T vectors (Novagen, Madison, Wisconsin), transfected into *Escherichia coli*, and sequenced.

Immunoprecipitation and Immunoblot Analysis

Tissues stored in liquid nitrogen were pulverized, washed with PBS, homogenized with lysis buffer, and immunoprecipitated using a kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions, using KNY-379. Both nonimmunoprecipitated and immunoprecipitated proteins were separated by electrophoresis on an SDS-polyacrylamide gel (5~20%), transferred to a nitrocellulose membrane, and blotted with KNY-379. Immunopositive bands were detected with an immunoperoxidase Western blotting kit (Amersham). To determine whether there was cross-reactivity with vimentin or nestin, antibodies against vimentin or nestin were also applied to the immunoblots.

Cell Culture from Human Fetal Brain Tissues

Fetal brain tissue samples were mechanically dissected in DMEM/Ham's F-12 (1:1). After dissection, the tissue samples were enzymatically digested with 0.05% trypsin/0.53 mM EDTA (Invitrogen, Carlsbad, California) for 20 minutes at 37°C. The trypsin activity was then stopped by adding soybean trypsin inhibitor (2.8 mg/ml; Beringer). Cell suspensions were grown using the neurosphere method (Reynolds et al, 1992) in defined DMEM/F-12 (1:1)-based growth medium supplemented with human recombinant epidermal growth factor (20 ng/ml; Invitrogen), human recombi-

nant fibroblast growth factor 2 (20 ng/ml; PeproTech Inc., Rocky Hill, New Jersey), human recombinant leukemia inhibitory factor (10 ng/ml; Chemicon), heparin (5 ng/ml; Sigma, St. Louis, Missouri), B27 supplement (Invitrogen), 15 mM HEPES, penicillin (100 U/ml), streptomycin (100 ng/ml), and amphotericin B (250 ng/ml). To induce differentiation, neurospheres were plated on polyornithine-coated glass coverslips and cultured in the same medium lacking the three growth factors and supplemented with 1% fetal bovine serum. The cells were cultured for 14 days before fixation for immunocytochemistry.

Immunocytochemical Analysis

Human neurospheres and coverslips with differentiated cells were fixed for 20 minutes at room temperature with 4% paraformaldehyde in PBS. After fixation, the neurospheres were dipped in 30% sucrose/PBS at room temperature for 30 minutes, embedded in OCT compound, sectioned on a cryotome at 12- μ m thickness, and mounted on coverslips. The sections of neurospheres were incubated with three primary antibodies: anti-human nestin (rabbit polyclonal, 1:500); anti-Musashi1 (rat monoclonal, 14H1, 1:500) (Kaneko et al, 2000), which is an evolutionally conserved neural RNA-binding protein that is selectively expressed in neural stem cells and neuronal progenitors, astroglial progenitors, and mature astrocytes (Kaneko et al, 2000; Kanemura et al, 2001; Sakakibara and Okano, 1997; Sakakibara et al, 1996); and KNY-379 in PBS/0.1% Triton-X/10% normal goat serum at 4° C overnight. The coverslips with differentiated cells were then incubated with another set of three primary antibodies: KNY-379 (1:1000), anti-PSA-NCAM (mouse IgM, 1:500; a kind gift from Dr. Seki, Department of Anatomy, Juntendo University School of Medicine) (Seki and Arai, 1993), and anti-GFAP (rabbit poly, 1:80; Sigma), under the same reaction conditions. After 3 washes, the neurosphere sections and coverslips with differentiated cells were incubated with the appropriate secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 568 goat anti-rabbit IgG, Alexa Fluor 647 goat anti-rat IgG, and Alexa Fluor 647 goat anti-mouse IgM; Molecular Probes Inc., Eugene, Oregon) at room temperature for 1 hour. The sections were then examined using a laser scanning microscope (Carl Zeiss LSM510).

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