Development of Astrocytes in the Mouse Embryonic Cerebrum Tracked by Tenascin-C Gene Expression*

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Summary. The development of astrocytes in the rodent cerebrum has been considered to occur mainly in the perinatal stage after the end of neurogenesis, and the mode of generation and migration of the astroglial precursors in the embryonic cerebrum remains poorly understood. Tenascin-C (TN-C) is an astroglia-derived extracellular matrix molecule, and its expression can be traced to the ventricular germinative zone of the embryonic cerebrum. TN-C expression may therefore be used as a marker of astroglial precursors prior to the expression of the glial fibrillary acidic protein (GFAP), a marker for the late stage of astroglial differentiation. The development of astrocytes in the mouse embryonic cerebrum was examined by *in situ* hybridization histochemistry for TN-C mRNA as an early marker. Observations showed that TN-C-positive cells were aligned in the ventricular germinative zone and began to migrate radially at E (embryonic day) 15. They were dispersed into the cortex as early as E17, and some were still in the DNA synthetic phase (S phase) even after lodgement in the cortex. TN-C-positive cells differentiated into GFAP-immunopositive astrocytes in the cortical and subcortical areas at the neonatal stage. The distribution of their active generation sites in the ventricular germinative zone was not homogeneous but displayed a restricted pattern. The mode of their migration towards the cortical and subcortical areas also showed a characteristic pattern that correlated to the radial glial arrangement. These findings indicate that TN-C-positive cells represent a new subset of astroglial precursors which arise directly from the ventricular germinative zone, and that astrogliogenesis from this precursor occurs as early as the period of neurogenesis at the embryonic stage.

Cerebral astrocytes are considered to be generated from multiple origins after neurogenesis in the ventricular germinative zone has finished. Subsets of astrocytes are generated from radial glia (CHOI and LAPHAM, 1978; CULICAN et al., 1990; EDWARDS et al., 1990; MARIN-PADILLA, 1995) and non-radial glial precursors in the ventricular germinative zone (GRESSENS et al., 1992) at the late embryonic stage, and other subsets are generated from progenitors in the subventricular zone at the early postnatal stage (LEVISON et al., 1993; LEVISON and GOLDMAN, 1993; KAKITA and GOLDMAN, 1999). Glial fibrillary acidic protein (GFAP), a widely used astroglial marker, is expressed from the late stage of astroglial differentiation. However, little is known about the development of astrocytes in the embryonic cerebrum due to the lack of specific markers for astroglial precursors in the process of generation and migration.

The tenascin (TN) family of extracellular matrix glycoproteins (TN-C, TN-R, TN-W, TN-X and TN-Y) displays highly restricted and dynamic patterns of expression during neural development. The TN family contains the same linear arrangement of four protein domains: TN assembly domain, epidermal growth factor-like repeats, fibronectin type III domains, and fibrinogen domains. The numbers of each domain are altered in the five isoforms (JONES and JONES, 2000). TN-C is an astroglia-derived extracellular matrix protein (BARTSCH et al., 1992) which is already expressed in the ventricular germinative zone of the embryonic brain (DOERRIES and SCHACHNER, 1994; BARTSCH et al., 1995). TN-C gene expression in the postnatal development of the mouse cerebral neocortex has been reported, and a correlation between TN-C gene-expressing cells and GFAP-immunoreactive

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astrocytes has been demonstrated (MITROVIC et al., 1994). It has been also reported that astrocytes express TN-C in the cerebellum of early postnatal and adult mice (BARTSCH et al., 1992) and that cerebellar Bergmann glia express this gene in embryonic development (YUASA, 1996). These results suggest that TN-C may be used as an early marker for the astroglial precursors prior to the expression of GFAP.

This study examined the mode of generation and migration of astroglial precursors in the mouse embryonic cerebrum using TN-C as an early marker. Based on the results obtained, the significance of TN-C-positive cells is discussed in relation to the origins of astrocytes.

MATERIALS AND METHODS

Animals

ICR strain mice were used. The day on which the vaginal plug was confirmed was defined as EO, and the day of birth was defined as PO.

All animal experiments were carried out following the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985). The pregnant mice were deeply anesthetized by an intraperitoneal administration of Nembutal (Abbott Laboratories, USA) and embryos were removed by cesarean section. Neonates were anesthetized by hypothermia during the fixation process.

Experiments were performed in four to six animals at each developmental stage.

In situ hybridization histochemistry

All the procedures were as previously reported (YUASA, 1996). Briefly, a cDNA fragment coding the sequence unique for TN-C among the TN family (SAGA et al., 1991), extending from nucleotide 131 to 706 (GenBank accession number D90343), was digested with *Sac* I and *Bgl* II and cloned into the *Sac* I/*Bam* H I site of the pBluescript II/KS⁻ vector. The clone was digested with *Sma* I or *Ecl* 136 II (*Sac* I site) and used as a DNA template to synthesize TN-C antisense or sense cRNA probe. The TN-C antisense or sense digoxigenin-labeled cRNA probe was prepared employing T7 or T3-RNA polymerase using a DIG-RNA-labeling kit (Boehringer-Mannheim).

Mouse embryos and neonates were fixed by transcardiac perfusion with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.4). Cryostat sections (10 μ m thick) were digested with 0.8% pepsin (DAKO, USA) in 0.2 M HCl for 15 min at 37°C. Subsequently, sections were hybridized with cRNA probe (1.2 μ g/ml) in a hybridization solution containing 10

mM Tris-HCl (pH 7.6), 0.6 M NaCl, 50% formamide, 10% dextran sulfate, 0.025% SDS, 1 $\times$ Denhardt's solution, 1 mM EDTA, and 0.2 mg/ml yeast tRNA at 50°C for 18 h. The sections were then rinsed with 2 $\times$ SSC containing 50% formamide at 50°C for 30 min, and treated with RNase A (Sigma, USA: 10 μ g/ml in a solution of 0.5 M NaCl, 1 mM EDTA and 10 mM Tris-HCl, pH 7.4) at 37°C for 30 min. They were next washed with 2 $\times$ SSC, and after a final washing in 0.2 $\times$ SSC at 50°C for 20 min each, the hybridized probe was detected with a DIG-nucleic acid detection kit (Boehringer-Mannheim) by using an alkaline phosphatase-labeled anti-digoxigenin antibody. Sense cRNA probe was employed as a control, and no signals were detected in the brain.

Three-dimensional reconstruction of the distribution of TN-C gene-expressing cells was carried out with a computer assisted system OZ (Olympus, Japan).

Double labeling by in situ hybridization and immunohistochemistry

Following the development of the deep blue color for TN-C *in situ* hybridization, the sections were rinsed with 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA (TE), and immunohistochemical staining was performed using rabbit anti-glial fibrillary acidic protein (GFAP) antiserum (prediluted, DAKO) as the primary antibody and horseradish peroxidase (HRP)-labeled anti-rabbit IgG (Fab'-fragment, 1:100 dilution, MBL, Japan) as the secondary antibody. The localization of the antigens was detected with diaminobenzidine (DAB) and H₂O₂ by the development of a brown color.

For double labeling with TN-C *in situ* hybridization and bromodeoxyuridine (BrdU) immunostaining, BrdU (Sigma, 100 mg/kg body weight) was administered intraperitoneally to the pregnant mice and the embryos were fixed after 3 h by transcardiac perfusion with 4% paraformaldehyde in PB. *In situ* hybridization for TN-C mRNA was carried out on cryostat sections and the deep blue color was developed. The sections were rinsed with TE and then incubated with 2 M HCl for 1 h at room temperature. After the rinse with PB containing 0.9% NaCl, immunohistochemical staining was performed using mouse anti-BrdU monoclonal antibody (1:50 dilution, Becton-Dickinson, USA) as the primary antibody, HRP-labeled anti-mouse IgG (Fab'-fragment, 1:100 dilution, MBL) as the secondary antibody, and the brown color was developed with DAB and H₂O₂.

Fig. 1. Generation and migration of TN-C-positive cells in the embryonic cerebrum. **A.** At E15 in a parasagittal section of the parietal cortex, most of the labeled cells are distributed in the ventricular germinative zone, while a small number have begun to migrate into the intermediate zone. **B.** At E17 in a parasagittal section of the parietal cortex, the labeled cells migrate radially towards the cortex. **C.** Higher magnification of the lower cortical plate in **B.** Some of the labeled cells display a leading process (*arrows*). **D.** The site of active generation is restricted to the dorsolateral aspect of the lateral ventricle in a cross section at E17 (left hemisphere). **E.** The site of active generation is restricted to the rostral aspect of the anterior horn of the lateral ventricle in a horizontal section at E17 (right hemisphere). *A* anterior, *D* dorsal, *L* lateral, *ah* anterior horn, *cc* corpus callosum, *cp* cortical plate, *imz* intermediate zone, *LV* lateral ventricle, *st* striatum, *vz* ventricular zone, *wm* white matter. Scale bars=100 μ m (**A**, **B**, **D**, **E**) and 20 μ m (**C**)

Fig. 2. GFAP immunoreactivity and BrdU labeling of TN-C-positive cells. Colocalization of the TN-C gene expression and GFAP immunoreactivity is evident in the cortical (A) and subcortical (B) astrocytes of the P2 cerebrum. TN-C-positive cells extend GFAP-immunoreactive processes and show a stellate morphology. BrdU incorporation into TN-C-positive cells is seen in the ventricular germinative zone (C) and cortex (D) at E17. TN-C mRNA appears as deep blue, and immunoreactivity for GFAP and BrdU as brown. LV lateral ventricle, vz ventricular zone. Scale bars=20 μ m

RESULTS

Generation and migration of TN-C-positive cells

In parasagittal sections of the parietal cortex, TN-C gene expression was restricted to the ventricular germinative zone at E15 (Fig. 1A). At E17, the labeled cells actively migrated through the intermediate zone and reached the cortex (Fig. 1B). Some of the labeled cells possessed a leading process extending in the migratory direction (Fig. 1C).

In cross sections of the cerebrum at E17 (Fig. 1D), the site of active generation of TN-C-positive cells was restricted to the dorsal to dorsolateral part of the ventricular germinative zone surrounding the lateral ventricle, and the stream of labeled cells extended laterally along the lower margin of the forming corpus callosum. In horizontal sections (Fig. 1E), the stream of labeled cells originated from the

ventricular germinative zone surrounding the anterior horn of the lateral ventricle and extended along the inner margin of the forming white matter.

TN-C-positive cells as astroglial precursors

A double-labeling study demonstrated the colocalization of TN-C mRNA and GFAP immunoreactivity in the cortical (Fig. 2A) and subcortical (Fig. 2B) astroglial cells at P2. About 80% of the TN-C-positive cells in the P2 cerebrum showed GFAP immunoreactivity. Most of the double-labeled cells extended processes and displayed a stellate morphology. In contrast, about 20% of the GFAP-immunoreactive cells were negative for TN-C gene expression. GFAP immunoreactivity was not found in the TN-C-positive cells at the embryonic stage. TN-C-positive cells situated in the ventricular germinative zone (Fig. 2C) as well as in the cortex (Fig. 2D) showed the incorporation of

FIG. 3. Three-dimensional distribution of TN-C-positive cells in the embryonic cerebrum. The images were reconstructed from cross sections at E15 (**A**) and E17 (**B**), and from horizontal sections at E15 (**C**) and E17 (**D**). **A** and **B** show the left hemisphere; **C** and **D** show the right hemisphere. At E15, the labeled cells are densely distributed in the dorsal to dorsolateral aspect of the lateral ventricle in **A**, and at the rostral and caudal ends of the lateral ventricle in **C**. At E17, the labeled cells migrate from the site of active generation and are dispersed dorsally and ventrolaterally in the frontal plane (**B**). In the horizontal plane (**D**), the labeled cells migrate from the rostral end of the lateral ventricle and are dispersed anteriorly and caudolaterally, and also migrate posteriorly from the caudal end of the ventricle. *A* anterior, *D* dorsal, *L* lateral, *ah* anterior horn, *cg* cingulate cortex, *par* parietal cortex, *pir* piriform cortex, *ph* posterior horn, *st* striatum, *te* temporal cortex, *LV* lateral ventricle. Scale bars=500 μ m

BrdU into the nuclei at E17 by double labeling with TN-C *in situ* hybridization and immunohistochemical detection of BrdU.

Developmental changes in the three-dimensional distribution of TN-C-positive cells

At E15, TN-C-positive cells were distributed in the ventricular germinative zone surrounding the lateral ventricle as observed in three-dimensional images reconstructed from cross sections (Fig. 3A) and from horizontal sections (Fig. 3C). In the cross sections, the labeled cells were densely distributed in the dorsal to dorsolateral aspect of the lateral ventricle (Fig. 3A). In the horizontal sections, the dense distribution of labeled cells was restricted to the ventricular germinative zone surrounding the anterior horn (Fig. 3C).

At E17, the labeled cells were migrating from the germinative zone towards the cortex. In cross sections (Fig. 3B), the streams originated from the dorsal aspect of the ventricle and extended in a medial direction to the cingulate cortex, dorsal direction to the parietal cortex, and ventrolateral direction to the temporal and also piriform cortices. The migratory stream also invaded the ventrolateral part of the striatum (Fig. 3B). In horizontal sections (Fig. 3D), the labeled cells originated from the rostral end of the anterior horn of the lateral ventricle and were dispersed towards the cortex in a rostral to caudolateral direction. Another migratory stream originated from the ventricular germinative zone surrounding the posterior horn and extended in a caudal direction (Fig. 3D).

DISCUSSION

The present study demonstrated by double labeling for TN-C mRNA and GFAP immunoreactivity that TN-C-positive cells in the mouse embryonic cerebrum differentiate into astrocytes (Fig. 2A, B), and that they represent a subset of astroglial precursors. TN-C-positive S-phase cells in the ventricular germinative zone therefore appear to retain the potential to proliferate as astroglial precursors, and those in the cortex generate astrocytes even after their final settlement (Fig. 2C, D). The TN-C-positive, GFAP-negative cells in the neonatal cerebral cortex could represent astroglial precursors that are immature and not yet expressing detectable levels of GFAP. On the other hand, the GFAP-immunoreactive TN-C-negative cells might represent astroglial subclasses which express a very low amount of TN-C, as found *in vitro* (MEINERS et al., 1995).

Neurogenesis occurs from E11 to E17 in the mouse cerebrum (CAVINESS, 1982). In contrast, it has been thought that the great majority of astrocytes in the rodent cerebrum are generated in the perinatal period after the end of neurogenesis, and the process of astroglial development in the embryonic cerebrum is not well understood. At present, three pathways of astroglial development have been established. The first one involves the transformation from radial glia to astrocytes in the late embryonic period after neuronal migration (TAKAHASHI et al., 1990). The second one involves the generation of astroglial precursors from the ventricular germinative zone in the late embryonic period after neuronal migration (GRESSENS et al., 1992). The third involves the generation of astroglial precursors from the subventricular zone in the early postnatal period (LEVISON et al., 1993; LEVISON and GOLDMAN, 1993; KAKITA and GOLDMAN, 1999). TN-C-positive cells are generated as astroglial precursors as early as E15 during neurogenesis, earlier than the above three pathways, and thus could represent a new pathway for cerebral astroglial development.

The possibility that TN-C-positive cells are generated in part by the mitosis of radial glial somata in the ventricular germinative zone remains to be examined because the unequal mitotic division of the radial glial somata has been recently demonstrated in the mouse embryonic cerebrum (MIYATA et al., 2000). TN-C gene expression in the subventricular zone of the mouse neonatal cerebrum has also been reported (GATES et al., 1995). Subventricular zone cells in the neonatal rat generate both astrocytes and oligodendrocytes (LEVISON et al., 1993; LEVISON and GOLDMAN, 1993). It therefore remains to be elucidated whether TN-C-positive cells are common progenitors for astrocytes and oligodendrocytes or whether they belong to an astrocyte-specific sublineage.

Sites of the active generation of TN-C-positive cells in the ventricular germinative zone showed a restricted distribution (Fig. 1D, E). Such a mosaic-like pattern of differentiation in the ventricular germinative zone has been demonstrated in the neurogenesis of the diencephalon and hippocampus (ALTMAN and BAYER, 1988, 1990). These findings imply that the ventricular germinative zone is heterogeneously differentiated not only in neurogenesis but also in gliogenesis.

The migratory pattern of TN-C-positive cells from the ventricular germinative zone of the mouse embryonic cerebrum observed in this study was very similar to the developmental pattern of the subventricular zone glial precursors identified by labeling with retrovirus-mediated gene transfer at the postnatal stage (LEVISON et al., 1993; LEVISON and GOLDMAN,

1993; KAKITA and GOLDMAN, 1999). TN-C-positive cells exhibited a radial dispersion towards the medial and dorsal cortex (Fig. 3A, B). On the other hand, the mode of dispersion towards the lateral cortex appeared to circumvent the striatum and to involve ventrolateral migration (Fig. 3A, B). All these migratory patterns coincided with the arrangements of the radial glial processes which have been reported to guide neuronal migration in the embryonic cerebrum (EDWARDS et al., 1990; MISSON et al., 1991). Furthermore, it has been suggested that subventricular zone progenitors for both astrocytes and oligodendrocytes migrate along radial glial processes in neonatal rats (ZERLIN et al., 1995). These results suggest that migratory astroglial precursors in the mouse embryonic cerebrum are also guided by the radial glial processes. Further research is needed to examine the guidance of astroglial migration by contact with radial glia.

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