Tenascin-C is Required for Proliferation of Astrocytes in Primary Culture

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Abstract. *Astrocytes in primary culture can be classified morphologically into two types: fibrous astrocytes and protoplasmic astrocytes. To examine the role of tenascin-C (TN) in an in vitro astrocyte culture, primary cultures of astrocytes prepared from the brains of wild-type and of TN-deficient embryonic mice were analyzed. In primary culture of astrocytes from TN-deficient mice fibrous astrocytes did not appear and astrocytes did not become tile-shaped when they came in contact with each other. The rate of 5-bromo-2'-deoxyuridine incorporation in a cell proliferation assay was much lower for astrocytes from TN-deficient mice than for astrocytes from wild-type mice. These results suggest that TN is an essential molecule for maintaining the proliferation and proper morphology of astrocytes in primary culture.*

Tenascin-C (TN) is an astroglia-derived extracellular matrix glycoprotein expressed by immature astroglia and radial glia during development of the central nervous system (CNS) (1-4) and is involved in axonal growth and guidance in the developing CNS (5, 6). Although TN is thought to be an essential molecule for neuron-glia interaction during CNS development, a 1992 study by Saga *et al.* found that TN-deficient mice were viable and showed no abnormalities during development and continuous breeding (7). Detailed analysis of TN-deficient mice in the last ten years has revealed new functions for TN. TN-deficient mice exhibit abnormal behaviors with hyper-locomotion and poor swimming ability caused by

decreased serotonin levels, dopamine transmission and tyrosine hydroxylase activity in the brain (8).

Astrocytes in primary culture are of two morphological types. One type is the fibrous astrocytes, which express TN at high levels, the other is the protoplasmic astrocytes, which express TN at low levels (9, 10). *In vitro* assays of primary cultures of astrocytes from rats have shown that neurite outgrowth is inhibited when neurons are cultured with fibrous astrocytes (9, 10).

Although TN expression is attenuated in the normal adult brain, TN expression is enhanced in astrocytes around lesion sites following CNS injury (11, 12). These reactive astrocytes expressing TN are thought to inhibit neurite outgrowth and axonal regeneration in the injured CNS (13). In the present study, the characteristics of astrocytes in primary culture from TN-deficient mice were investigated to analyze the function of astrocytes expressing TN.

Materials and Methods

Animals were treated in accordance with the Animal Care Committee protocols of The Jikei University School of Medicine. The TN-deficient mice were derived from the C57Bl/6J and CBA strains, and age-matched wild-type mice were C57Bl/6J x CBA F1 (7). The primary cultures of astrocytes were prepared with a previously reported method (14). Briefly, embryos were shelled out of the uterus on embryonic day 15.5, the brains were then excised and trypsinized (0.1% in phosphate-buffered saline) to become a cell suspension, which was then suspended in F-10 medium (Invitrogen Corp., Carlsbad, CA, USA) with 10% newborn calf serum and cultured in a T75 flask. After one week of incubation in a CO_2 incubator at 37°C, the cells were trypsinized, divided into new T75 flasks and incubated for an additional week with several changes of medium. The primary cultures of astrocytes were periodically confirmed to express glial fibrillary acidic protein (GFAP).

Cell proliferation assays using Cell Proliferation Kit (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA) were performed according to the manufacturer's protocols. The culture medium was replaced with labeling medium containing 3 μg/ml of 5-bromo-2'-deoxyuridine (BrdU) every 12 h, and the astrocytes

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were incubated at 37˚C for 24 to 72 h. Labeled astrocytes were fixed with acid-ethanol after 24 or 72 h of labeling, after which immunocytochemical detection was performed with an anti-BrdU mouse monoclonal antibody (1:1000 dilution) supplied with the kit. Peroxidase-conjugated anti-mouse IgG2a was used as the secondary antibody. After immunocytochemical staining, cells were counterstained with eosin and mounted with Softmount solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for microscopic analysis.

Immunocytochemical studies and immunoblotting were performed with anti-α-tubulin mouse monoclonal antibody (1:1000 dilution, Sigma, St. Louis, MO, USA), anti-F-actin-Phalloidin antibody (1:1000 dilution, Sigma), anti- glial fibrillary acidic protein (GFAP) polyclonal antibody (1:3 dilution of prediluted serum, DAKO, Denmark), anti-tenascin (TN) polyclonal antibody (1:1000 dilution, Chemicon, Temecula, CA, USA), anti- chondroitin-6-sulfate proteoglycan (CS-6-PG) monoclonal antibody (1:100 dilution, Seikagaku Corporation, Tokyo, Japan), anti-fibronectin (FN) polyclonal antibody (1:400 dilution, Sigma) and anti-Laminin (LN) polyclonal antibody (1:25 dilution, Sigma). The day before immunocytochemical analysis, the primary cultures of astrocytes were cultured on four-well chamber slides (Nunc, Rockilde, Denmark). The cultured cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature (RT) and made permeable with 0.1% Triton X-100 in PBS for 15 min at RT. Anti-mouse IgG-Alexa Fluor 488 (1:400 dilution, Molecular Probes, Invitrogen Corp.), peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated anti-rabbit IgG (1:1000 dilution, MBL, Nagoya, Japan) was used as the secondary antibody. After immunocytochemical staining, cells were mounted with Softmount solution (Wako Pure Chemical Industries, Ltd.) for microscopic analysis or with PermaFluor Aqueous Mounting Medium (Shandon, Pittsburgh, PA, USA) for fluorescence microscopic analysis. For immunoblotting, ECL Plus Western Blotting Detection Reagents (GE Healthcare Bio-Science Corp.) was used according to the manufacturer's protocols.

Results

After two weeks of incubation of the primary cultures of astrocytes from wild-type mice, protoplasmic astrocytes had become tile-shaped when they came in contact with fibrous astrocytes (Figure 1A). In contrast, after two weeks of incubation of the primary cultures of astrocytes from TN-deficient mice, fibrous astrocytes had not appeared and the cells were unable to come into full contact and did not become tile-shaped (Figure 1B). Primary culture of astrocytes from TN-deficient mice was attempted at both high and low densities, but the cell morphologies were the same as shown in Figure 1B and fibrous astrocytes did not appear in any culture (data not shown). To examine possible cytoskeletal abnormalities in the primary cultures of astrocytes from the TN-deficient mice, immunocytochemical studies were performed with antibodies against the cell structural molecules α-tubulin and F-actin-phalloidin. However, no differences were observed between wild-type and TN-deficient mice in the expression of these molecules by astrocytes in the primary cultures (Figure 3).

The growth rate of the primary cultures of astrocytes from the TN-deficient mice was much lower than that of astrocytes from wild-type mice. In fact, the doubling time of the astrocytes from the wild-type mice was approximately 48 h, whereas that of the astrocytes from the TN-deficient mice was more than 72 h (Figure 2). To examine the proliferative ability of astrocytes in primary culture, BrdU uptake was studied (Figure 1C-F, Figure 2). A few astrocytes from the wild-type mice were stained with an antibody against BrdU after 24 h of labeling (Figure 1C), however, no astrocytes from the TN-deficient mice were stained (Figure 1E). After 72 h of BrdU labeling, many astrocytes from the wild-type mice were clearly stained with BrdU (Figure 1D), whereas very few astrocytes from the TN-deficient mice were stained (Figure 1F). These results suggest that the proliferative ability in primary culture of astrocytes from TN-deficient mice is impaired.

TN has been reported to interact with chondroitin-6-sulfate proteoglycan (CS-6-PG), fibronectin (FN) and laminin (LN) to constitute an interactive network of extracellular matrix components to control cell behavior during morphogenesis (15). The expression of CS-6-PG, FN and LN was analyzed using antisera for astrocytes from the wild-type and TN-deficient mice. Immunocytochemical studies and immunoblotting showed no differences between astrocytes from the wild-type mice and those from the TN-deficient mice in the expression of CS-6-PG, FN, or LN (Figure 4 and 5). Furthermore, GFAP expression was not altered in astrocytes from the TN-deficient mice (Figure 4).

Discussion

To our knowledge, the present study was the first to examine the function of TN by comparative analysis of astrocytes from the wild-type mice and TN-deficient mice in primary culture. Our observations suggest that the expression of TN by astrocytes is essential for maintaining proper cell morphology and cell proliferation in primary culture.

After incubation for two weeks, fibrous astrocytes and protoplasmic astrocytes, which became tile-shaped with cell contact were observed. A similar finding has been reported by Meiners *et al.* (1995), who examined cultures of astrocytes from the cerebral cortices of newborn Sprague-Dawley rats and found that fibrous astrocytes assume "rocky" shapes and express TN at extremely high levels. As fibrous astrocytes did not appear in the primary cultures of astrocytes from the TN-deficient mice it appears that tenascin is required for the development of fibrous astrocytes.

The immunocytochemical and immunoblotting for CS-6-PG, FN and LN findings, in the present study, are in good agreement with those of previous immunohistochemical studies of the brains of wild-type and TN-deficient mice (7).
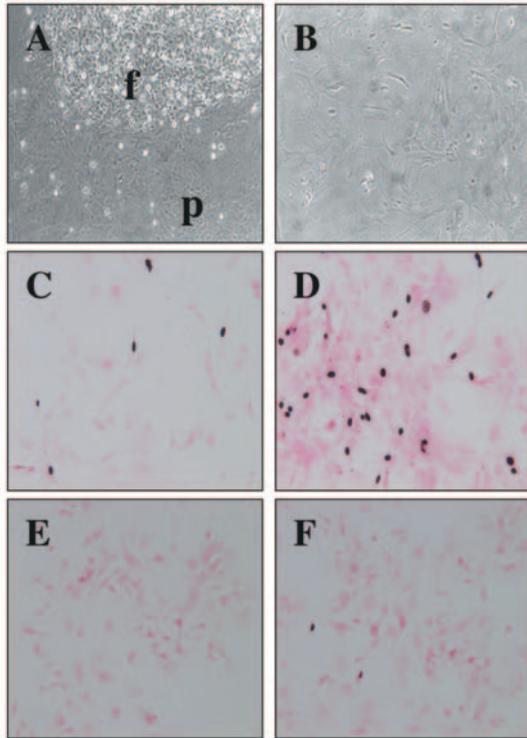
Figure 3. *Immunocytochemical studies of α-tubulin and F-actin-phalloidin staining in primary culture of astrocytes from wild-type mice and TN-deficient mice. There were no differences in the α-tubulin (A, B) or F-actin-phalloidin (C, D) staining pattern in primary culture between astrocytes from wild-type mice (A, C) and those from TN-deficient mice (B, D).*

Figure 1. *(A) After two weeks of primary cultures of astrocytes from wild-type mice, protoplasmic astrocytes (p; monolayer cells) became tile-shaped with cell contact and some fibrous astrocytes (f; uneven surface cells) appeared. (B) In primary culture of astrocytes from the TN-deficient mice, fibrous astrocytes with uneven surfaces did not appear and the astrocytes did not become tile-shaped, even when the cells were in contact with each other. (C-F) Cell proliferation assays for primary cultures of astrocytes from the wild-type and TN-deficient mice. The astrocytes were cultured with labeling medium containing BrdU for 24 h (C, E) or 72 h (D, F). The primary cultures of astrocytes were prepared from the wild-type (C, D) or TN-deficient (E, F) mice. Labeled astrocytes were fixed with acid-ethanol, and the proliferation rate was immunohistochemically determined with a monoclonal antibody against BrdU.*
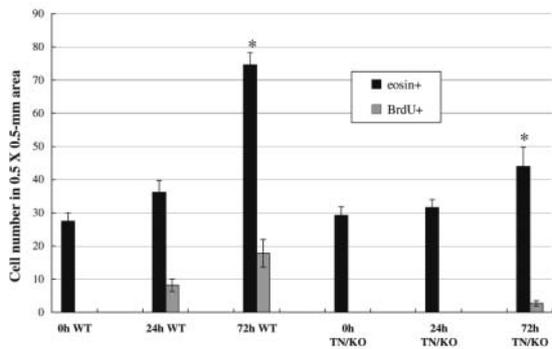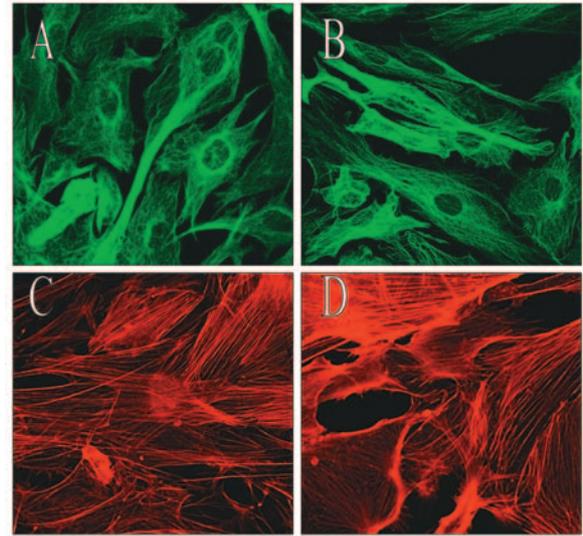

Figure 2. *The proliferation rate for BrdU-positive cells in eosin-positive cells. BrdU-positive cells and eosin-positive cells were counted in a 0.5 x 0.5-mm area on culture dishes. Labeling medium containing BrdU was changed every 12 h. The term "TN/KO" refers to TN-deficient mice. Values represent the means±S.E.M. from at least five different areas per group. The results clearly demonstrate a difference in the proliferation rate in primary culture between astrocytes from wild-type mice and those from TN-deficient mice. The difference between WT (wild-type) and TN/KO cells cultured for 72 h was significant (*p<0.05).*
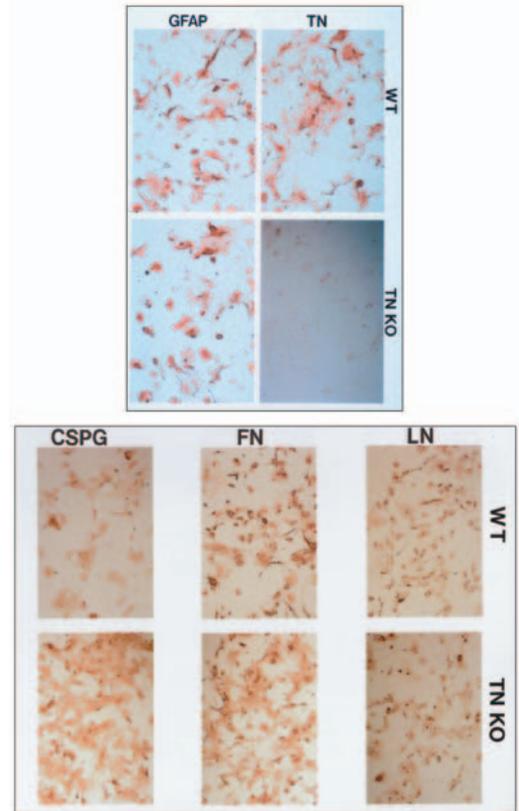
Figure 4. *Immunocytochemical studies of glial fibrillary acidic protein (GFAP), tenascin (TN), chondroitin-6-sulfate proteoglycan (CS-6-PG), fibronectin (FN) and laminin (LN) in the primary culture of astrocytes from wild-type mice (WT) and TN-deficient mice (TN/KO).*
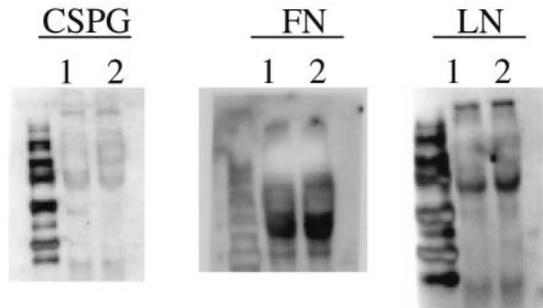
Figure 5. *Immunoblotting studies of chondroitin-6-sulfate proteoglycan (CS-6-PG), fibronectin (FN) and laminin (LN) in the primary culture of astrocytes from wild-type mice (lane 1) and TN-deficient mice (lane 2).*

The proliferative ability in primary culture of the astrocytes from the TN-deficient mice was impaired compared with that of astrocytes from the wild-type mice. Functional blocking with an anti-TN antibody has also been shown to decrease cell proliferation and induce morphological change after subculture and serum deprivation/re-addition treatment in primary cultures of astrocytes from the cerebral cortex of newborn rats (16). TN expression was also found to be up-regulated during proliferation and migration of cultured astrocytes in a scratch wound assay (17). Our findings of the function of TN are consistent with their data. Interactions of TN with some members of the integrin family might be involved in the proliferation of astrocytes in primary culture, as has been reported for colon carcinoma cells (18). We speculate that the presence of TN-expressing astrocytes in primary culture is essential for the proliferation of both protoplasmic and fibrous astrocytes. TN may act as a growth factor for both adjacent astrocytes and, when secreted into the extracellular matrix, for nonadjacent astrocytes.

Garcion *et al.* have found that TN-deficient mice show increased rates of oligodendrocyte precursor migration along the optic nerve and reduced rates of oligodendrocyte precursor proliferation in the CNS, but show a normal phenotype at later developmental stages (19). They demonstrated that in knockout mice with normal phenotype for the molecules that have important roles in early developmental stages, such as TN, their loss of function could be compensated for by other molecules at later developmental stages.

In an *in vivo* study the GFAP expression pattern was not altered in brain sections of adult TN-deficient mice (data not shown). However, in the present *in vitro* study involving astrocytes from embryonic mice morphological and functional differences between TN-deficient mice and wild-type mice were apparent. Therefore, an analysis of the cell proliferation rate of astrocytes during early development in the brains of TN-deficient mice is now being undertaken. The proliferation rates of astrocytes during CNS development might differ between wild-type and TN-deficient mice.

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