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Alteration of Inflammatory Cytokine Production in the Injured Central Nervous System of Tenascin-deficient Mice

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Abstract. *Although tenascin-C (TN) is highly up-regulated during the proliferation of reactive astrocytes, little is known about the function of TN at injury sites in the central nervous system (CNS). Here, the function of TN-expressing astrocytes in the injured brain was investigated by analyzing TN-deficient mice with stab-wound injuries of the cerebral cortex. Glial fibrillary acid protein expression after injury was down-regulated earlier in TN-deficient mice than in wild-type (WT) mice. To evaluate immune responses in the injured CNS in the absence of TN, inflammatory cytokine production was examined after unilateral stab injuries of the cerebral cortex in TN-deficient and WT mice. The expression of interleukin (IL)-1β, tumor necrosis factor-α and IL-6 was higher in TN-deficient mice, whereas levels of IL-4 and granulocyte colony-stimulating factor were lower in TN-deficient mice than WT mice. Our findings suggest that TN helps to regulate production of inflammatory cytokines in the injured brain.*

Tenascin-C (TN) is an astroglia-derived extracellular matrix glycoprotein, expressed by radial glia and immature astroglia in the developing brain, which plays a role in neuron-glia interaction (1). Expression of TN occurs at only very low levels in adult brains but is up-regulated at injury sites in the central nervous system (CNS) (2). *In vitro* cultures of primary astrocytes from embryonic mouse or rat brains show two subsets of primary astrocytes: a regeneration-inhibiting subset with high TN expression (fibrous astrocytes) and a regeneration-promoting subset without TN expression (protoplasmic astrocytes) (3-5). TN plays a role in the proliferation of astrocytes in primary culture. In our previous study of astrocytes from TN-deficient mice in primary culture, fibrous astrocytes did not appear and primary astrocytes proliferated at a low rate (6).

There are many reports of high TN expression around injury sites in the CNS (7, 8); however, there are no reports of TN having neuroprotective or neurotoxic functions around injury sites. In the present study, we produced stab wounds in the cerebral cortices of TN-deficient mice to examine the functional roles of TN-expressing astrocytes surrounding lesion sites in the CNS. Immunostaining for glial fibrillary acidic protein (GFAP) was used to verify astrogliosis around lesion sites. Furthermore, to analyze immune responses in the injured brains of mice in the absence of TN, we compared expression of several inflammatory cytokines after stab injuries of the brain between TN-deficient mice and wild-type (WT) mice.

Materials and Methods

Animals were cared for 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 x CBA strains, and age-matched wild-type (WT) mice were C57Bl/6J x CBA F1 (9). The TN-deficient mice and their WT littermates were anaesthetized with sodium pentobarbital (50 mg/kg). An incision was then made in the scalp of the occipital region, a hole was made in the right side of the skull with an 18-G needle, and a stab wound was made with a 21-G needle along the rostrocaudal axis. All mice were anaesthetized with ether, ice-cold 4% paraformaldehyde in 0.1 M of phosphate buffer (PB, pH 7.4) was perfused through the left ventricle. The brains were removed and embedded in an OCT compound (Tissue-Tek, Sakura Finetek USA, Inc., CA, USA), and frozen sections were prepared.

For lacZ staining, sections were incubated in a working solution (2% X-gal in 50 mM of $K_4[Fe(CN)_6]$ and 50 mM of $K_3[Fe(CN)_6]$

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with 0.1 M of PB) at 37˚C overnight and counter-stained with nuclear fast red. For immunostaining, sections were incubated with a polyclonal antibody against GFAP (1:3 dilution of prediluted serum; Dako, Denmark) for the first antibody and incubated with anti-rabbit immunoglobulin (Ig) G-Alexa Fluor 488 (1:400 dilution; Molecular Probes, Invitrogen Corp., CA, USA) for the second antibody. For IgG immunostaining, sections were incubated with biotinylated goat anti-mouse IgG (1:200 dilution; Vector Laboratories, Inc., CA, USA), color-developed with a Vectastain Elite ABC kit (Vector Laboratories, Inc.) and mounted with Softmount solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Inflammatory cytokine expression was examined in the wounded hemisphere and, as a control, in the contralateral hemisphere with a real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) method using an ABI Prism 7700 sequence detection system (Applied Biosystems, CA, USA). RNAs were extracted from brain tissue surrounding a stab wound or tissue from a corresponding area of an uninjured brain using Sepasol (Nacalai Co., Kyoto, Japan). The PCR cycle was 95˚C for 10 minutes, 40 cycles of 95˚C for 15 seconds, and 60˚C for 1 minute. Primer sequences were interleukin (IL)-1β: forward, 5'-GCA CAC CCA CCC TGC AG-3' and reverse, 5'-AAC CGC TTT TCC ATC TTC TTC TT-3'; tumor necrosis factor (TNF)-α: forward, 5'-ACA GAA AGC ATG ATC CGC G-3' and reverse, 5'-GCC CCC CAT CTT TTG GG-3'; IL-6: forward, 5'-CTG CAA GAG ACT TCC ATC CAG TT-3' and reverse, 5'-GAA GTA GGG AAG GCC GTG G-3'; IL-6 receptor (R): forward, 5'-AAT GAT GAC CCC AGG CAC TG-3' and reverse 5'-GCC TGA AGT CCT GAG ATC GAA-3'; IL-10: forward, 5'-TGC AGC AGC TCA GAG GGT T-3' and reverse, 5'-TGG CCA CAG TTT TCA GGG AT-3'; IL-4: forward, 5'-ACG GAG ATG GAT GTG CCA AAC-3' and reverse, 5'-GCA CCT TGG AAG CCC TAC AGA C-3'; and granulocyte colony-stimulating factor (G-CSF): forward, 5'-CAT GCC AGC CTT CAC TTC TG-3' and reverse, 5'-TAC GAA ATG GCC AGG ACA CC-3'. An 18S rRNA primer set (Applied Biosystems) was used for internal control.

Results

A stab wound was made on the cerebral cortex of WT mice and TN-deficient mice to determine the role of TN-expressing astrocytes in the injured CNS (Figure 1).

To examine whether TN expression can be detected around the lesion sites as previously reported (10), lacZ staining was performed with frozen sections. LacZ expression was detected in astrocytes in place of TN expression surrounding lesion sites in the cerebral cortex according to a targeting vector construct for establishment of TN-deficient mice (Figure 2).

To determine the time course of astrogliosis in the injured brains of TN-deficient mice, GFAP immunostaining was performed to produce an index of astrogliosis on frozen sections and compared with results in WT mice. The expression of GFAP in WT mice was high around the injury site during the first week after wounding (Figure 3 A-C). In contrast, in TN-deficient mice, expression of GFAP was high 3 days after wounding then decreased 1 week after wounding (Figure 3 D-F).

With stab wounds of the brain, IgG leaks from blood vessels into the brain parenchyma around lesion sites through breakdown of the blood-brain barrier (BBB) (11). Therefore, changes in the permeability of the BBB after wounding were analyzed in both TN-deficient and WT mice with frozen sections by IgG immunostaining. The leakage of IgG peaked 1 day after wounding in WT mice; in contrast, IgG leakage was greatest 3 days after wounding in TN-deficient mice. The BBB began to recover from breakdown 3 days after wounding in WT mice, but 7 days after wounding in TN-deficient mice. Seven days after wounding, BBB recovery from breakdown was nearly complete in WT mice, whereas several more days were needed for complete recovery in TN-deficient mice (Figure 4).

To evaluate immune responses in the injured CNS without TN, the mRNA expression of several inflammatory cytokines in the wounded hemisphere were compared with WT mice and TN-deficient mice. The expression level of IL-1β, TNF-α and IL-6 was higher in TN-deficient mice than in WT mice (Figure 5 A-C), whereas expression levels of IL-4 and G-CSF were lower in TN-deficient mice than in WT mice (Figure 5 F, G). There were no differences in the expression levels of IL-6R or IL-10 between WT mice and TN-deficient mice (Figure 5 D, E).

Discussion

We have clearly demonstrated that TN, rather than lacZ, is present around the lesion site in TN-deficient mice (Figure 2), as has been found in earlier studies (10, 12). These finding indicates that TN plays an important role, other than a neuroprotective or neurotoxic role, in injured areas of the CNS.

A model of CNS injury was prepared by producing unilateral stab wounds of the cerebral cortex in mice to examine the role of TN expression around the lesion site. Expression of GFAP in the cerebral cortex was high for 3 days after wounding in both WT mice and TN-deficient mice (Figure 3). Thus, the early, acute phase response was not altered by TN deficiency. One week after wounding, GFAP expression was lower in TN-deficient mice, but remained high around the lesion site in WT mice. Thus, TN might be involved in the late acute phase response in injured brains. Our previous data indicate that TN is required for the proliferation of astrocytes in primary culture from embryonic mouse brains (6); therefore, TN may be also required for astrogliosis around sites of injury in the mouse brain.

Breakdown of the BBB due to stab wounds was examined with immunostaining for IgG leakage from blood vessels. In TN-deficient mice, IgG leakage persisted much longer after wounding than in WT mice (Figure 4). Thus, TN might be involved in maintenance of the BBB where astrocytic end-feet adhere to blood vessels, because TN is spatially and temporally related to neovascularization in the CNS (13).

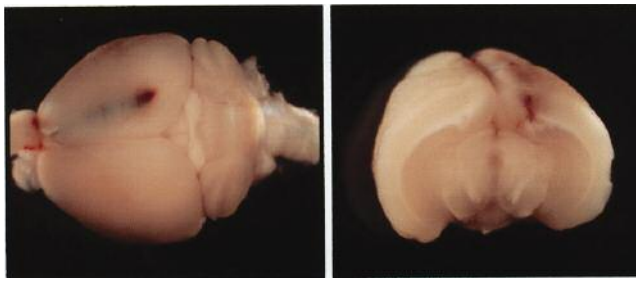

Figure 1. *The entire brain after a stab wound was produced in one hemisphere of the cerebral cortex of an adult mouse. Adult WT or TN-deficient mice were anaesthetized, and stab wounds were produced in the right cerebral cortex along the rostrocaudal axis (left). After several days, 4% paraformaldehyde was perfused through the left ventricle, and the brains were removed for frozen sectioning (right).*

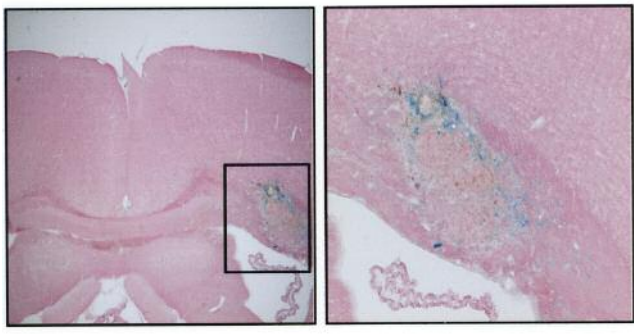

Figure 2. *LacZ expression, instead of TN expression, was detected around the lesion site in the cerebral cortex of TN-deficient mice according to a gene-targeting vector construct (9). Frontal frozen sections were postfixed and incubated in working solution contained X-gal. Inset is a higher-magnification view of the wounded region with lacZ staining. Representative experimental findings are shown (n=3). ×10.*

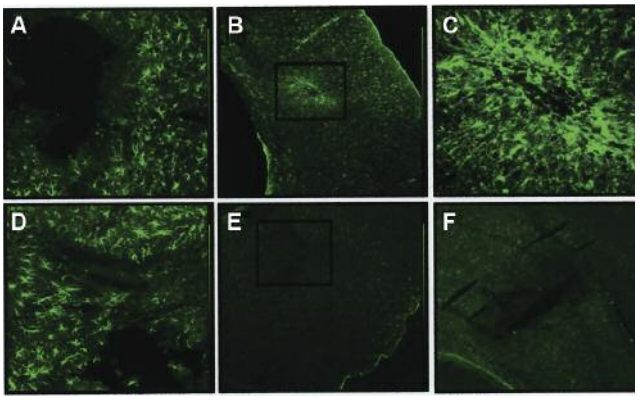

Figure 3. *GFAP expression was detected as a marker of astrogliosis around the lesion site in the cerebral cortex. Frontal frozen sections were immunostained for GFAP in WT mice (A-C) and TN-deficient mice (D-F). Micrographs from 3 days after wounding (A, D) and 7 days after wounding (B-F) are shown. C and F are higher-magnification views of insets in B and E, respectively. Representative experimental findings are shown (n=3). A, C, D, F ×20; B, E ×10.*

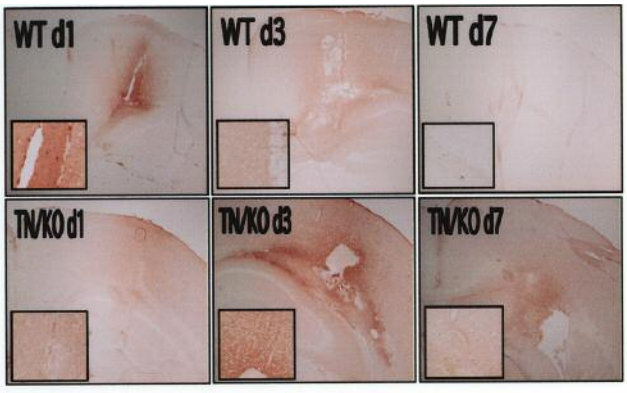

Figure 4. *Immunohistochemical straining for IgG, reflecting impairment of the blood-brain barrier, on frontal frozen sections adjacent to those shown in Figure 3 on days 1 (d1), 3 (d3), and 7 (d7) after wounding of the cerebral cortex in WT mice and TN-deficient mice (TN/KO). Inset in each panel is a higher-magnification view of the wounded region. Representative experimental findings are shown (n=3). ×10; inset ×20.*

Inflammatory cytokine expression was altered around stab wounds in the brains of TN-deficient mice (Figure 5). The cytokines IL-1β, TNF-α, and IL-6 were expressed at high levels in TN-deficient mice and reportedly have neuroprotective functions against excitotoxins in the CNS (14). Thus, TN in the injured brain may play in the role in the modulation of inflammatory cytokine signaling for a precise balance of proinflammatory and anti-inflammatory cytokine production. In fact, TN influences regeneration and inflammation in many tissues and in numerous species (15).

In a previous study, we used a short promoter element of the mouse TN gene to selectively ablate TN-expressing astrocytes in a primary culture of astrocytes from embryonic mouse brains (5). Because most of the surviving astrocytes were TN-negative astrocytes, transplantation of these cells into the injured CNS of mice might be an important tool to clarify the function of TN-negative astrocytes.

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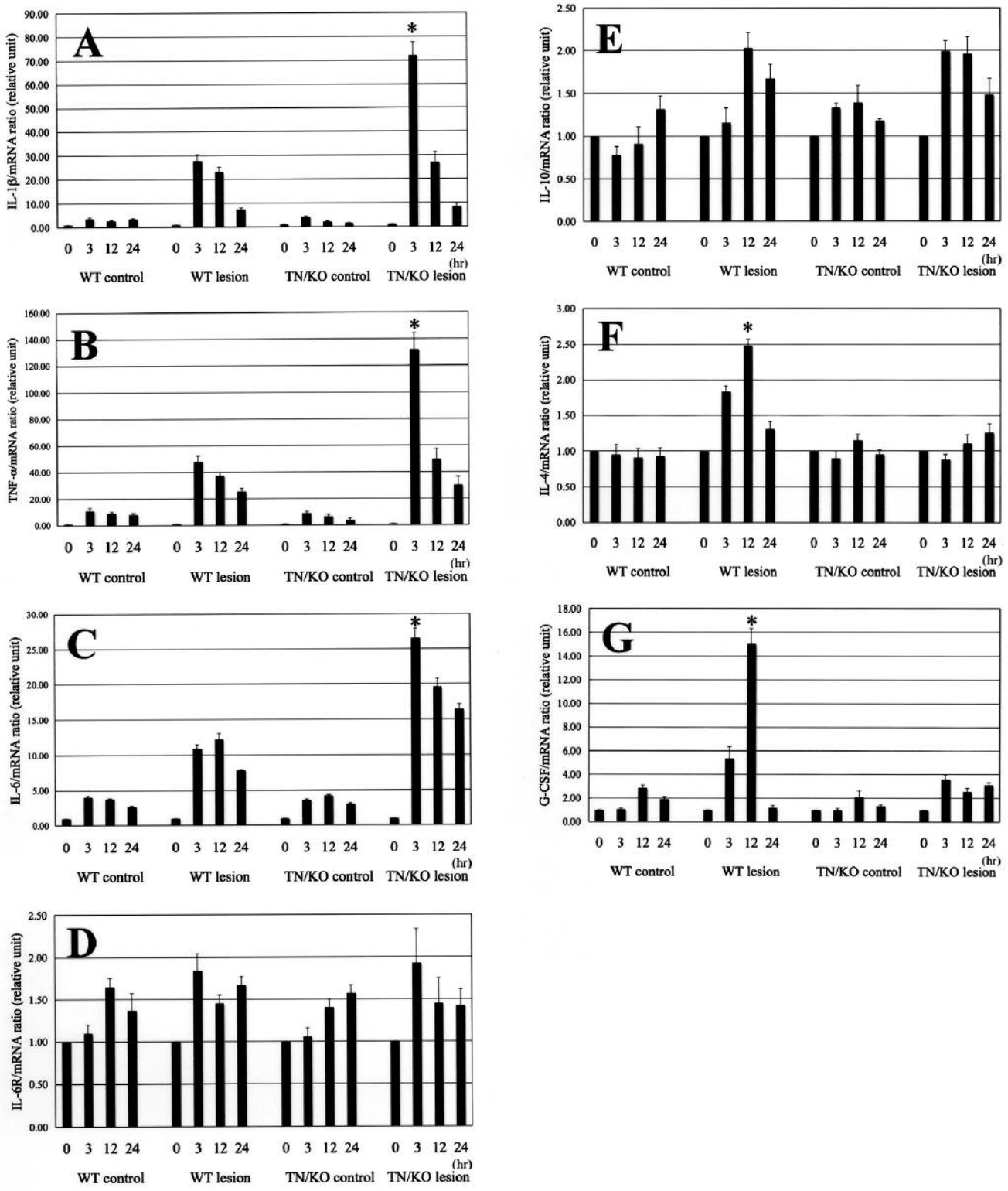

Figure 5. *Inflammatory cytokine expression surrounding the lesion site was determined 0, 3, 12 and 24 hours after wounding in WT mice (n=3) and TN-deficient mice (TN/KO, n=3). A quantitative real-time RT-PCR method was used to measure expression level of the cytokines IL-1β (A), TNF-α (B), IL-6 (C), IL-6R (D), IL-10 (E), IL-4 (F) and G-CSF (G). Values from the unwounded hemisphere (0 hour) were set to "1" for comparison. RNA was compared between the wounded right hemisphere (lesion) and the control, sham-operated contralateral hemisphere (control). Values shown are means ± S.E.M. from at least 6 different experiments per group. *p<0.05 by Student's t-test.*

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