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Extracellular matrix alterations, accelerated leukocyte infiltration and enhanced axonal sprouting after spinal cord hemisection in tenascin-C-deficient mice

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ABSTRACT

The extracellular matrix glycoprotein tenascin-C has been implicated in wound repair and axonal growth. Its role in mammalian spinal cord injury is largely unknown. *In vitro* it can be both neurite-outgrowth promoting and repellent. To assess its effects on glial reactions, extracellular matrix formation, and axonal regrowth/sprouting *in vivo*, 20 tenascin-C-deficient and 20 wild type control mice underwent lumbar spinal cord hemisection. One, three, seven and fourteen days post-surgery, cryostat sections of the spinal cord were examined by conventional histology and by immunohistochemistry using antibodies against F4/80 (microglia/macrophage), GFAP (astroglia), neurofilament, fibronectin, laminin and collagen type IV. Fibronectin immunoreactivity was significantly down-regulated in tenascin-C-deficient mice. Moreover, fourteen days after injury, immunodensity of neurofilament-positive fibers was two orders of magnitude higher along the incision edges of tenascin-C-deficient mice as compared to control mice. In addition, lymphocyte infiltration was seen two days earlier in tenascin-C-deficient mice than in control mice and neutrophil infiltration was increased seven days after injury. The increase in thin neurofilament positive fibers in tenascin-C-deficient mice indicates that lack of tenascin-C alters the inflammatory reaction and extracellular matrix composition in a way that penetration of axonal fibers into spinal cord scar tissue may be facilitated.

Introduction

Tenascin-C (TNC), an extracellular matrix (ECM) glycoprotein, plays an important role during development, wound repair and axonal growth (see for review: Faissner, 1997; Talts et al., 1999; Jones and Jones, 2000; Brellier et al., 2009; Midwood and Orend, 2009). TNC is highly expressed in embryonic tissues, where it regulates cell proliferation, migration and differentiation during organogenesis (Mackie, 1997; von Holst, 2008; Dityatev et al., 2010; Karus et al., 2011; Garwood et al., 2012). In adults, TNC is only found in the central nervous system (CNS), bone marrow (Mackie et al., 1988) and T-cell-dependent areas of lymphoid organs (Chilosi et al., 1993). However, during tissue repair and remodeling, TNC becomes re-expressed, playing an important role in ECM formation. It is, for example, expressed during wound healing (Mackie et al., 1988), in inflamed human corneas (Maseruka et al., 1997) and in the injured brain (Dobbertin et al., 2010), suggesting a key role in inflammation and tissue repair. After spinal cord compression injury (Hashimoto et al., 2005), transection of the dorsal funiculi (Zhang et al., 1997) and incision of the ventral funiculus (Deckner et al., 2000), TNC mRNA and immunoreactivity are upregulated at the lesion site and around the lesion, where it is mainly secreted by reactive astrocytes (Wiese et al., 2012), displaying highest intensity during the first two weeks after the injury. The pivotal role of TNC in CNS development is illustrated by studies performed on TNC-deficient (TNC−/−) mice (Saga et al., 1992; Forsberg et al., 1996; Evers et al., 2002) which show morphological alterations in the cerebral cortex (Irintchev et al., 2005), impaired memory in the step-down passive avoidance task (Strekalova et al., 2002) as well as delayed onset of neonatal olfactory responses (de Chevigny et al., 2006) and alterations in cell proliferation and migration during development (Garcion et al., 2001). In addition, TNC−/− mice display enhanced novelty-induced exploration, reduced anxiety, delayed resynchronization of circadian activity and reduced muscle strength (Morellini and

Abbreviations: CNS, central nervous system; CST, corticospinal tract; ECM, extracellular matrix; GFAP, glial fibrillary acidic protein; H&E, hematoxylin and eosin; IOD, integrated optical density; NF, neurofilament; PBS, phosphate buffered saline; PFA, paraformaldehyde; TNC, tenascin-C; TNC−/−, tenascin-C-deficient; TNC+/+, tenascin-C wild type; U, arbitrary unit.

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Schachner, 2006). However, the influence of TNC on inflammation and axonal growth remains unclear. *In vitro*, TNC may facilitate or inhibit neurite outgrowth, depending on how the molecule is presented as a substrate (Dorries et al., 1996; Meiners et al., 1999; Rigato et al., 2002; Liu et al., 2005; Ahmed et al., 2006; Andrews et al., 2009).

Until now, early changes, in particular ECM deposition and glial response have not yet been analyzed. To elucidate the function of TNC after CNS injury *in vivo*, we performed a lumbar spinal cord hemisection in inbred TNC−/− and TNC+/+ control mice. Glial response, ECM formation and axonal outgrowth were analyzed. Since the process of peripheral wound healing can be subdivided into four different periods (Clark, 1996), *i.e.* early inflammatory response (0–24 h post-injury), late inflammatory response (2–3 days post-injury), proliferation phase (3–7 days post-injury) and maturation phase (1 week to months after injury), animals were studied 1, 3, 7 and 14 days post-surgery. CNS macrophage reaction and astroglial scarring were examined by immunohistochemistry using anti-F4/80 (microglia and recruited blood monocytes) and anti-GFAP (astrocytes) antibodies. Moreover, the distribution of the ECM molecules fibronectin, laminin and collagen type IV was studied immunohistochemically. These molecules interact with TNC and also influence axonal growth. Fibronectin is expressed during development, plays an important role in the formation of connective tissue (Lorke and Moller, 1985) and is differentially expressed in response to injury, suggesting a role in CNS repair processes (Pires Neto et al., 1999; Priestley et al., 2002; Tate et al., 2002). Laminin and collagen type IV are the major components of ECM formation following injury (Hermanns et al., 2001; Klapka and Muller, 2006; Brazda and Muller, 2009) and have been associated with the success or failure of neurite outgrowth (Sosale et al., 1988; Grimpe and Silver, 2002; Liesi and Kauppila, 2002; Onose et al., 2009). To elucidate the role of TNC with regard to axonal regrowth/sprouting, neurofilament (NF) immunoreactivity was used as an axonal marker. Immunoreactivity was evaluated morphologically and quantitatively by densitometry. In addition, hematoxylin and eosin (H&E) as well as combined Masson trichrome and Verhoeff stains were used for a detailed morphological analysis of tissue damage, leukocyte infiltration and scar formation.

Materials and methods

Ethics statement

During the entire experiment, the principles outlined in the "Guide for the Care and Use of Laboratory Animals" (National Academy of Sciences Institute for Laboratory Animal Research) have been observed. All procedures involving experimental animals were approved by the local governmental body of animal care and welfare of the Freie und Hansestadt Hamburg, Germany.

Animals

Generation of TNC−/− and TNC+/+ mice inbred on the C57BL/6 genetic background have been described previously (Evers et al., 2002). Animals were bred and kept at the animal facilities of the University Medical Center Hamburg-Eppendorf under standard conditions (22 ± 1 °C, 55% humidity, 12 h day–night illumination cycle). Animals had free access to water and rodent chow. Twenty TNC−/− and 20 TNC+/+ littermates of both sexes, aged 3 months ± 2 weeks, were examined.

Surgical procedure

For surgery, animals were anesthetized by an intraperitoneal injection of 0.1 ml Ketamine/Xylazine per 10 g body weight (Ketamin 10, Atarost GmbH & Co., Twistringen, Germany; Rompun 2%, Bayer Vital GmbH, Leverkusen, Germany). The skin was surgically scrubbed, the hair at the surgical field was removed and the eyes were covered with ointment to prevent drying. Under aseptic conditions skin and thoracolumbar fascia were opened in a caudo-cranial direction 12 mm from the lumbar segment L_3/L_4. The medial part of the back muscles was deflected to the side over two segments from the spinal processes, thereby producing as little tissue trauma and bleeding as possible. Laminectomy was performed by searching the intervertebral foramina and cutting both laminae of one vertebral arch. Using a blade (#11 blade, Bruno Bayha GmbH, Tuttlingen, Germany), which was manually advanced in one movement from the medial dorsal septum to the lateral edge, the spinal cord was gently hemitransected on the right side at the L_1/L_2 level. Care was taken not to injure the anterior spinal artery. Then the superficial back muscles of both sides were repositioned and carefully sutured with absorbable Vicryl® (Ethicon GmbH, Norderstedt, Germany). The skin was closed by the same suture. Immediately after the operation, animals received a subcutaneous injection of 0.02 ml Metamizol-WDT (WDT, Garbsen, Germany) as an analgesic. After the operation, mice were kept in individual cages and placed on soft bedding on a warming blanket kept at 37 °C for 24 h to prevent hypothermia. Mice were then transferred to a temperature-controlled (22 °C) room and fed with softened rodent chow *ad libitum*. For analgesia, animals received 0.2 ml Metamizol (Novaminsulfon-ratiopharm®, Ratiopharm GmbH, Ulm, Germany) in 75 ml drinking water until they were sacrificed.

Preparation of tissue

Animals were sacrificed 1, 3, 7 and 14 days after surgery. Mice were deeply anesthetized with Ketamine/Xylazine (0.15 ml/10 g body weight intraperitoneally). Using a Luer Lock 18-gauge needle (G18, B. Braun Melsungen AG, Melsungen, Germany) mice were first perfused through the left ventricle (pressure 90 mmHg) with Ringer solution (Apotheke, University Medical Center Hamburg-Eppendorf, Germany) for 30 s, followed by a transcardial perfusion with 4% paraformaldehyde (PFA) in 0.2 M phosphate buffer for 10 min. Through bilateral laminectomy the spinal cord was removed as a whole and placed in the same fixative for 2 h. Thereafter tissue was left overnight in 30% sucrose at room temperature. The following day spinal cords were placed in plastic dishes, covered with Tissue-Tek® O. C. T. Compound (Sakura Finetek Europe B.V., Zoeterwoude, Netherlands) and frozen to −80 °C in isopentane cooled by liquid nitrogen. Frozen tissue was stored at −84 °C in 1 ml Cryo-tubes (Nunc, Wiesbaden, Germany), which were preserved in Falcon tubes (Greiner, Frickenhausen, Germany) surrounded by frozen distilled water, to prevent tissue from drying. Longitudinal serial sections (16 μm thick) in the frontal plane from the dorsal pial surface to the ventral pial surface were cut using a Leica CM 3050 cryostat (Leica Instruments GmbH, Nussloch, Germany). Sections were collected on SuperFrost®Plus slides (Menzel-Gläser, Braunschweig, Germany) and air-dried for at least 20 min at room temperature. Sections were then fixed with acetone (Merck, Darmstadt, Germany) for 3 min, again air-dried for at least 20 min and stored in boxes at −84 °C until staining was performed.

Spinal cord regions analysis

The incision site was defined as the area between the wound edges. The surrounding area was defined as the ipsilateral region approximately 0.05 mm above and 0.05 mm below the incision site. The ipsilateral distant corticospinal tract (CST) cranial to the incision site was defined as the white matter between the border of the surrounding area and the edge of the section (0.05–1.5 mm cranial of the incision site). H&E staining revealed morphological