Connective tissue and its growth factor CTGF distinguish the morphometric and molecular remodeling of the bladder in a model of neurogenic bladder

Cengiz Z. Altuntas,1 Firouz Daneshgari,1 Kenan Izgi,1,2 Fuat Bicer,1,2 Ahmet Ozer,1,3 Cagri Sakalar,4 Kerry O. Grimberg,1 Ismail Sayin,1,5 and Vincent K. Tuohy4

1Urology Institute, University Hospitals Case Medical Center and Department of Urology, Case Western Reserve University School of Medicine, Cleveland, Ohio; 2Department of Chemistry, Cleveland State University, Cleveland, Ohio; 3Department of Genetics, Case Western Reserve University School of Medicine, Cleveland, Ohio; 4Department of Immunology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio; and 5Department of Biology, Case Western Reserve University, Cleveland, Ohio

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Altuntas CZ, Daneshgari F, Izgi K, Bicer F, Ozer A, Sakalar C, Grimberg KO, Sayin I, Tuohy VK. Connective tissue and its growth factor CTGF distinguish the morphometric and molecular remodeling of the bladder in a model of neurogenic bladder. Am J Physiol Renal Physiol 303: F1363–F1369, 2012. First published September 19, 2012; doi:10.1152/ajprenal.00273.2012.—We previously reported that mice with experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS), develop profound urinary bladder dysfunction. Because neurogenic bladder in MS patients causes marked bladder remodeling, we next examined morphometric and molecular alterations of the bladder in EAE mice. EAE was created in female SJL/J mice by immunization with the p139–151 encephalitogenic peptide of myelin proteolipid protein in complete Freund’s adjuvant, along with intraperitoneal injections of Bordetella pertussis toxin. Seventy days after immunization, mice were scored for the level of neurological impairment and then killed. Spinal cord sections were assessed for demyelination, inflammation, and T cell infiltration; the composition of the bladder tissue was measured quantitatively; and gene expression of markers of tissue remodeling and fibrosis was assessed. A significant increase in the bladder weight-to-body weight ratio was observed with increasing neurological impairment, and morphometric analysis showed marked bladder remodeling with increased luminal area and tissue hypertrophy. Despite increased amounts of all tissue components (urothelium, smooth muscle, and connective tissue), the ratio of connective tissue to muscle increased significantly in EAE mice compared with control mice. Marked increases in mRNA expression of collagen type I α2, tropoelastin, transforming growth factor-β3, and connective tissue growth factor (CTGF) were observed in EAE mice, as were decreased levels of mRNAs for smooth muscle myosin heavy chain (SMMHC) isoforms and decreased total SMMHC expression were demonstrated in a rat model of partial bladder outlet obstruction (31). Connective tissue growth factor (CTGF; also known as CCN2) has been recognized as a promoter of epithelial-mesenchymal transition (EMT) and fibrosis (5, 7, 10, 16), and its expression can be induced by all three isoforms of transforming growth factor-β (TGF-β) (6).

Following our published report demonstrating the utility of EAE as a model of NGB (2), we have begun a series of studies to address the long-standing research questions related to NGB. In the current study, we aimed to understand the impact of NGB on the gross and targeted molecular phenotype of the bladder.

MATERIALS AND METHODS

Generation of EAE mice. Female SJL/J mice (n = 130; Jackson Laboratory, Bar Harbor, ME) were immunized for induction of EAE at 8–10 wk of age. The encephalitogenic p139–151 peptide of myelin proteolipid protein (PLP 139–151, HSLGKWLGHPDKF; serine substituted for cysteine at residue 140) was synthesized at our institution using standard solid phase methodology and FMOC side chain protected amino acids (2). The peptide was purified >97% by reverse-phase high-pressure liquid chromatography, and amino acid composition was confirmed by mass spectrometry.

EAE was induced as described previously (30). Briefly, SJL/J mice were injected subcutaneously in the abdominal flank on day 0 with 200 μg of PLP 139–151 and 400 μg Mycobacteria tuberculosis...
Table 1. Classification of neurological disability

<table>
<thead>
<tr>
<th>Clinical Score</th>
<th>Observed Neurological Disability</th>
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<tbody>
<tr>
<td>CS1</td>
<td>Paralysis of tail only</td>
</tr>
<tr>
<td></td>
<td>Tail paralysis</td>
</tr>
<tr>
<td></td>
<td>Clumsy gait</td>
</tr>
<tr>
<td>CS2</td>
<td>Poor ability to right body</td>
</tr>
<tr>
<td></td>
<td>Hind limb weakness</td>
</tr>
<tr>
<td>CS3</td>
<td>Incomplete paralysis of hind limbs</td>
</tr>
<tr>
<td>CS4</td>
<td>Complete paralysis of hind limbs</td>
</tr>
<tr>
<td>CS5</td>
<td>Morbidity</td>
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CS, clinical score.

H37RA (Difco, Detroit, MI) in 200 μl of an emulsion of equal volumes of water and complete Freund’s adjuvant (CFA) (Difco). Age-matched control mice were injected with water and CFA only. On days 0, 3, and 7, each mouse was injected intraperitoneally with 0.2 μg of purified Bordetella pertussis toxin (List Biological Laboratories, Campbell, CA). Fifteen mice were weighed and scored daily for signs of neurological impairment according to clinical score (CS) criteria (Table 1) up to 74 days after induction. All protocols were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University.

Tissue procurement. Seventy days after immunization, mice were killed by asphyxiation with CO₂ followed by cervical dislocation, bladders and spinal cords were harvested, and bladders were weighed. For characterization of bladder morphology, bladders were equilibrated for 20 min at 37°C in Krebs buffer aerated with 95% O₂-5% CO₂ to maintain pH 7.4. Bladders were sectioned at the equatorial midline, fixed in 10% neutral formalin, dehydrated, and embedded in paraffin. Serial 5-μm tissue sections were placed on microscope slides, de-waxed, and rehydrated for routine hematoxylin and eosin (H&E) and Masson’s trichrome staining.

Morphometric analysis of spinal cord. Spinal cords were removed and fixed in 10% neutral formalin overnight. Paraformaldehyde-fixed tissue was cut into 5-mm-thick sections and then stained with H&E and luxol fast blue to assess the inflammation and demyelination, respectively (12). The severity of tissue injury and inflammation was analyzed by researchers masked to sample identity. Images were collected using a Leica SCN400 Slide Scanner.

Image processing. Image analysis was done as described previously with modifications (17). In brief, stained slides were scanned with a Leica SCN400 Slide Scanner (Buffalo Grove, IL), and digital images of whole cross sections of spinal cord and urinary bladder were saved for analysis. The images were analyzed with Image-Pro Plus (version 7.0; Media Cybernetics, Bethesda, MD). The software can distinguish regions stained with different colors and quantitatively measure the areas. Inflammatory cell accumulation (H&E) and the demyelination area (luxol fast blue) on the spinal cord sections were measured and expressed as percentages. Masson’s trichrome-stained slides were used to determine the three components of bladder tissues (urothelium, collagen, and smooth muscle). In all cases, the images were processed by the same investigators, who were unaware of treatment group assignments.

Quantitative real-time reverse transcription polymerase chain reaction. Total RNA was extracted from whole bladders of CFA control mice and EAE mice at different CS levels 70 days after immunization, using TRIzol according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). cDNA was synthesized from the total RNA using a Super Script III cDNA Synthesis Kit (Invitrogen). Primers for SMMHC, collagen type I α2 (COL1A2), tropoelastin, NGF, GDNF, purinergic receptor P2X1 (P2RX1), muscarinic acetylcholine receptor 3 (M3), CTGF, TGF-β3, and β-actin were designed using the Universal Probe Library Assay Design Center (Roche, Mannheim, Germany; Table 2). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a Sybr Green PCR Master kit (Foster City, CA) with an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). After confirming that the mean levels of β-actin mRNA did not differ significantly between the EAE and CFA mice, data from the genes of interest were normalized to β-actin and expressed relative to the CFA control using the comparative threshold cycle method.

Statistical analysis. Morphometric analyses in Table 3 and qRT-PCR experiments comparing two groups were evaluated with unpaired, two-tailed Student’s t-tests. For other experiments comparing CS1–4 and CFA mice, including some qRT-PCR results, one-way ANOVA with Tukey’s post hoc test for comparisons between groups was used. The mean and SD of each group was calculated. P < 0.05 was considered significant.

RESULTS

EAE mice showed signs of neurological deficits beginning on day 10 after immunization, with cycles of remission and relapse with varying CS levels during the remaining observation period (Fig. 1 shows the mean CS progression of 15 mice through day 74). Control SJL/J mice immunized with CFA alone showed no impairment throughout the entire time period. Seventy days after immunization, the spinal cords of the CFA-immunized control group showed no inflammatory cell infiltration, whereas those of the EAE mice consistently showed conventional signs of CNS inflammation that correlated with clinical deficit (Fig. 2C), often marked by large perivascular inflammatory cell infiltrations of dorsal and ventral columns along with peripheral infiltrations (Fig. 2, A and B). We previously showed by CD4 immunostaining that inflammatory cells infiltrating the spinal cord in a similar EAE model were predominantly CD4⁺ T cells (12). In addition,
spinal cords of EAE mice demonstrated increased demyelination that correlated with clinical deficit (Fig. 3).

The early mean body weight was similar for all groups. By day 70 postimmunization, the bladder weight-to-body weight ratio had increased significantly with increasing CS of EAE mice compared with the CFA-immunized group (Fig. 4A). Histological examination showed bladder hypertrophy and lumen dilation in the EAE mice relative to the CFA control mice, corresponding with increasing CS (Fig. 4B). Automated digital imaging was used to quantify the cross-sectional area and composition of bladder tissue. The total cross-sectional wall and lumen areas of the bladder (at the equatorial midline) increased significantly with the increase of CS from one to four in EAE mice compared with CFA controls (Table 3).

Collagen and urothelium areas were significantly elevated in EAE mice compared with CFA controls. However, the percentage of smooth muscle area in EAE mice bladders decreased with increasing CS from CS2 to CS4 (Table 3). A gradual decrease of smooth muscle thickness, which was calculated as smooth muscle area divided by the length of the outer perimeter of cross-sectional area, was

Fig. 1. Clinical score (CS) evaluation of experimental autoimmune encephalomyelitis (EAE) up to 74 days after induction. EAE was induced by immunization of 6-wk-old female SJL/J mice with PLP 139–151. After immunization, mice were examined daily for neurological deficit on a score of 1 to 5 (Table 1). Data are mean CS over time in 15 mice with actively induced EAE. Error bars show ± SD.

Fig. 2. A: enlarged image of hematoxylin and eosin (H&E)-stained dorsal column section from an EAE CS4 mouse showing infiltrating cells. B: H&E-stained spinal cord section from an EAE CS4 mouse (left) and captured inflammatory cells in the same section colored red (right) by using image-Pro Plus (version 7.0; Media Cybernetics). C: quantification showed increasing inflammatory cell infiltrations that correlated with clinical deficit in EAE mice. Values are means of 5 mice/group ± SD. \( P < 0.0001 \) by 1-way ANOVA. Tukey’s post hoc test revealed significant differences \( P < 0.05 \) in comparisons of complete Freund’s adjuvant (CFA) control mice vs. each CS level (*), as well as CS1 vs. CS2–4, CS2 vs. CS4, and CS3 vs. CS4.

### Table 3. Histological observations in bladder cross sections 70 days after immunization

<table>
<thead>
<tr>
<th>Tissue area, mean mm² (SD)</th>
<th>CFA ( n = 6 )</th>
<th>CS1 ( n = 6 )</th>
<th>CS2 ( n = 7 )</th>
<th>CS3 ( n = 10 )</th>
<th>CS4 ( n = 9 )</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wall area</td>
<td>2.3 (0.07)</td>
<td>2.6 (0.13)</td>
<td>3.1 (0.54)</td>
<td>3.5 (0.58)</td>
<td>4.0 (1.39)</td>
<td>0.0015</td>
</tr>
<tr>
<td>Lumen area</td>
<td>0.2 (0.1)</td>
<td>0.4 (0.09)</td>
<td>0.4 (0.16)</td>
<td>0.7 (0.05)</td>
<td>0.8 (0.29)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>1.5 (0.05)</td>
<td>1.6 (0.17)</td>
<td>1.9 (0.41)</td>
<td>2.0 (0.48)</td>
<td>2.2 (0.7)</td>
<td>0.0434</td>
</tr>
<tr>
<td>Collagen</td>
<td>0.5 (0.03)</td>
<td>0.6 (0.05)</td>
<td>0.8 (0.11)</td>
<td>0.9 (0.12)</td>
<td>1.1 (0.13)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Urothelium</td>
<td>0.3 (0.02)</td>
<td>0.4 (0.06)</td>
<td>0.5 (0.03)</td>
<td>0.5 (0.04)</td>
<td>0.7 (0.22)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Collagen</td>
<td>64.8 (1.5)</td>
<td>60.4 (4.7)</td>
<td>60.3 (2.6)</td>
<td>57.6 (2.1)</td>
<td>56.6 (2.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lumen area</td>
<td>21.9 (1.6)</td>
<td>24.4 (2.5)</td>
<td>24.7 (1.6)</td>
<td>26.4 (2.9)</td>
<td>25.7 (1.9)</td>
<td>0.0071</td>
</tr>
<tr>
<td>Urothelium</td>
<td>13.3 (0.9)</td>
<td>15.2 (2.4)</td>
<td>15.0 (2.2)</td>
<td>16.0 (2.1)</td>
<td>17.5 (2.3)</td>
<td>0.0097</td>
</tr>
<tr>
<td>Thickness of bladder compartments, mean (SD)</td>
<td>0.16 (0.01)</td>
<td>0.14 (0.02)</td>
<td>0.12 (0.03)</td>
<td>0.11 (0.02)</td>
<td>0.08 (0.03)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

\( n \), No. of animals. CFA, complete Freund’s adjuvant. *P value from 1-way ANOVA. Significant differences \( (P < 0.05) \) from pairwise comparisons by Tukey’s post hoc test were as follows: wall area, CFA vs. CS3&4, CS1 vs. CS4; lumen area, CFA vs. CS3&4, CS1 vs. CS4, CS2 vs. CS3&4; collagen area, CFA vs. CS2–4, CS1 vs. CS2–4, CS2 vs. CS4, CS3 vs. CS4; smooth muscle %, CFA vs. CS3&4, collagen %, CFA vs. CS3&4; urothelium %, CFA vs. CS4; smooth muscle thickness, CFA vs. CS2–4, CS1 vs. CS4, CS2 vs. CS4; urothelium thickness, CFA vs. CS3&4, CS1 vs. CS3&4, CS2 vs. CS3&4.
significant in CS2–4 relative to the CFA control group (Table 3). A decrease of urothelium thickness, calculated as urothelium area divided by inner perimeter length, was significant in CS3 (P < 0.05) and CS4 (P < 0.001) mice.

Comparison of gene expression by qRT-PCR in whole bladders of EAE CS3 mice relative to CFA control mice 70 days after immunization revealed a significant decrease in SMMHC level, whereas the connective tissue markers COL1A2 and tropoelastin were increased significantly (P < 0.001; Fig. 5). Levels of the mRNAs for neurotrophic factors NGF and GDNF were significantly downregulated in remodeled CS3 bladders compared with controls, suggesting that EAE results in decreased autonomic innervation of the bladder smooth muscle (P < 0.001; Fig. 6, A and B). Gene expression of purinergic and muscarinic receptors P2RX1 and M3, respectively, key mediators of neurologically controlled bladder contraction, were also significantly lower in CS3 mice bladders relative to controls, which could result in altered bladder contractile function in EAE mice.

Gene expression levels of two signaling proteins for fibrosis, CTGF and TGF-β3, were significantly upregulated in EAE mice compared with CFA controls, corresponding with increased clinical deficit in the EAE mice (P < 0.0001 by 1-way ANOVA; Fig. 6C). The levels of TGF-β1 and TGF-β2 mRNAs were not significantly different in EAE mice relative to CFA control mice (data not shown).

**DISCUSSION**

MS and other neurological deficits such as spinal cord injury and spina bifida cause LUTD, collectively referred to as NGB. It is not entirely understood how disturbances in neurological pathways result in NGB. However, patients with MS plaques interrupting the neural pathways connecting the pontine micturition center to the sacral micturition center develop a combination of storage and voiding problems, or a condition specific to neurogenic conditions, detrusor sphincter dyssynergia (DSD) (15). DSD is among the most difficult conditions to treat and causes severe bladder complications in MS patients that may lead to other complications such as overflow incontinence, vesico-ureteral reflux, and renal failure.

The remarkable changes frequently observed in patients with NGB include marked thickening of the bladder wall, formation...
and in urothelium, but decreased in smooth muscle, indicating that collagen and urothelium contributed larger proportions to the increased mass in the remodeled bladder in EAE. In addition, the decreased ratio of smooth muscle to collagen correlated with the level of CS of the animals. These findings differ from previous studies of the bladder in other disease models such as spinal cord injury, diabetes mellitus, and obstruction, in which bladder hypertrophy and, particularly, an increase of detrusor smooth muscle in response to elevated bladder burden and distension were reported (11, 14, 18, 25).

In our exploration of the molecular portfolio of the bladder remodeling, we found decreased expression of SMMHC mRNA in EAE CS3 mice relative to CFA control mice, whereas COL1A2 and tropoelastin were increased significantly. Thus, those alterations in gene expression, as the first step in alterations of the protein activities and thus tissue remodeling, confirmed the morphometric analyses of the bladder remodeling in which the increase in the amount of connective tissue was greater than that of muscle. Gene expression of neurogenic components, including NGF, GDNF, muscarinic receptors, and purinergic receptors, all decreased in EAE CS3 mice relative to CFA controls, which may indicate neurological function impairment in the bladder. Although our results differ from the increased NGF and GDNF expression observed in the bladders of rats with spinal cord injury, cyclophosphamide-induced cystitis, and other sources of bladder inflammation (9, 27), we assessed gene expression at a later time point than in those studies; thus, we cannot rule out increased neurotrophic factor expression in the acute phase of EAE in our model.

Our findings of increased expression of CTGF and TGF-β3 mRNAs in EAE mice in direct proportion to CS level parallel the correspondence of increased connective tissue and bladder remodeling with CS. Those results further support our contention and that of others that a significant part of NGB is reflected in increased fibrosis and present an interesting possible pathophysiologic pathway. CTGF has been recognized as a promoter of fibrosis, neovascularization, embryonic processes, wound healing, bone development, and, more recently, EMT, which may be a driver of fibrosis (5, 10, 20). Interestingly, an increase in CTGF expression has also been reported in rat urinary bladder after surgical outlet obstruction (7). TGF-β is a potent inducer of EMT and CTGF expression, and its effects may be mediated by CTGF (4, 29). Although most of those studies have involved the TGF-β1 isoform, TGF-β3 has been shown to induce CTGF expression through Smad3 in intervertebral discs of rats and mice (23). Clarification of the functional roles of CTGF and TGF-β3, as well as identification of additional signaling proteins and pathways related to activation of bladder remodeling in NGB, have the potential to open a gate to several lines of new research that may subsequently lead to untangling of the complexities related to bladder remodeling and LUTD seen in NGB.

A potential limitation of this study is that we did not attempt to correlate the extent of bladder remodeling with the degree of NGB severity, including measurements of hyperreflexic/overactive vs. areflexic/decompensated bladder, and DSD. In addition to requiring additional techniques specially adapted to the mouse, such as simultaneous cystometry and external urethral sphincter electromyography to identify DSD, such studies will likely require additional time points and frequent CS monitoring of the EAE mice. The latter view stems from our observed correlation of

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**Fig. 5.** A: the collagen area-to-smooth muscle area ratio increased significantly with the increase of neurological deficits in EAE mice compared with CFA controls. Crossbars are means of 6–10 mice/group; error bars indicate ± SD. 

B: the mRNA level of smooth muscle myosin heavy chain (SMMHC) was decreased at EAE CS3, whereas the connective tissue markers collagen type 1 α1 (COL1A2) and tropoelastin were increased significantly relative to CFA control mice. Values are means of 8 mice/group ± SD. *Significantly different from CFA control (P < 0.025) by Student’s t-test with Welch’s correction for unequal variances.

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of trabeculae, outpouching of the wall called “cellulae formation,” and an erythematous urothelium (Fig. 7). However, studies still have not determined why and how these changes occur in the bladder. Furthermore, it is unclear if potentially permanent changes contribute to clinical manifestations of NGB. A translational approach and an animal model that mimics the clinical phenotype are essential to determine the associations between the nervous system and LUTD.

EAE is an established model commonly used in MS research (22). Shortly after immunization, SJL/J mice develop acute EAE and severe multiligament weakness and/or paralysis followed by a remitting-relapsing stage of disease, with ultimate chronic progression (21, 24, 30). This chronic-progressive stage of EAE is characterized by sustained limb paresis or paralysis and marked CNS demyelination and neurodegeneration particularly pronounced in the spinal cord. Our previous study (2) described the development of LUTD during EAE progression that was loyal to the clinical phenotype. In the present study, we report a significant increase in the bladder weight-to-body weight ratio corresponding to the degree of neurological deficit (CS) in this model of NGB. All major components of the bladder wall were increased in the EAE mice 70 days after immunization, leading to a larger bladder with dilation of the lumen, in agreement with the increased bladder volumes detected by ultrasound in another murine EAE model 60 days postimmunization (1). Interestingly, the areas expressed as percentages of total tissue area were also increased in collagen.
bladder remodeling with CS level 70 days after immunization, when all of the EAE mice most likely had undergone one or more remission/relapse cycles. That suggests the possibility of a degree of reversibility of remodeling during remissions that could affect the progression of bladder dysfunction. Our forthcoming functional study takes into consideration the potential fluctuation in NGB progression owing to the remitting/relapsing model.

We conclude that EAE-caused neurological disability in mice contributes to marked bladder remodeling that proportionally worsens with increasing neurological deficit. Although all three components of detrusor smooth muscle, urothelium, and connective tissue contribute to the increased bladder mass, the role of connective tissue is more prominent and potentially detrimental. The prominence of molecular constituents and signaling factors of fibrosis open a gate for further exploration of the role of connective tissue in the bladder remodeling and LUTD of NGB. The EAE mouse is a useful model for translational studies of NGB.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

Fig. 7. Cytoscopic image from a patient with neurogenic bladder due to multiple sclerosis, showing marked remodeling, including trabeculation, outpouching of the wall called "cellulae formation," and an erythematous urothelium.


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