Inactivation of TNF-α ameliorates diabetic neuropathy in mice

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Abstract

Yamakawa I, Kojima H, Terashima T, Katagi M, Oi J, Urabe H, Sanada M, Kawai H, Chan L, Yasuda H, Maegawa H, Kimura H. Inactivation of TNF-α ameliorates diabetic neuropathy in mice. Am J Physiol Endocrinol Metab 301: E844–E852, 2011. First published August 2, 2011; doi:10.1152/ajpendo.00029.2011.—Tumor necrosis factor (TNF)-α is a potent proinflammatory cytokine involved in the pathogenesis of diabetic neuropathy. We inactivated TNF-α to determine if it is a valid therapeutic target for the treatment of diabetic neuropathy. We effects the inactivation in diabetic neuropathy using two approaches: by genetic inactivation of TNF-α (TNF-α−/− mice) or by neutralization of TNF-α protein using the monoclonal antibody infliximab. We induced diabetes using streptozotocin in wild-type and diabetic mice. We measured serum TNF-α concentration and the level of TNF-α mRNA in the dorsal root ganglion (DRG) and evaluated nerve function by a combination of motor (MNCV) and nerve conduction velocity (NCV). Reduced MNCV and NCV were shown to correlate with the loss of sensory nerve fibers. At 8–12 wk after diabetes induction in mice, ~10% of the neurons in the DRG represent BMDC-neuron fusion cells, which provide a local source of TNF-α. In diabetic mice, the PI/TNFα signaling pathway is abnormally expressed and contributes to nerve dysfunction and death. TNF-α is a potent proinflammatory cytokine, whose level is increased in diabetes (14, 21). TNF-α interacts with AGE/RAGE (13) and stimulates the expression of multiple proteins, e.g., aldose reductase (20), PKC (28), mitogen-activated protein kinases (7, 41, 51), activation of protein kinase C (PKC) (48) and inducible nitric oxide synthase (iNOS) (44). The multiplicity of putative pathogenic factors has prompted the testing of a large number of molecules, including antioxidants, prostaglandins, aldose reductase inhibitors, AGE inhibitors, or PKC inhibitors, for the treatment of diabetic neuropathy. However, to date, few of those agents produced clinically significant improvements in diabetic neuropathy.

Recently, we proposed a new mechanism for diabetic neuropathy. In diabetic mice and rats, abnormal proinsulin/TNF-α-positive bone marrow-derived cells (Pl/TNFα-BMDCs) appear within days of hyperglycemia (22) and contribute to the development of diabetic neuropathy (23, 38). In the presence of diabetes, the Pl/TNFα-BMDCs home to nerve tissues, where they fuse with neurons in the dorsal root ganglia (DRG) and nerve fibers. At 8–12 wk after diabetes induction in mice, ~10% of the neurons in the DRG represent BMDC-neuron fusion cells, which provide a local source of TNF-α production that contributes to nerve dysfunction and death. TNF-α is a potent proinflammatory cytokine, whose level is increased in diabetes (14, 21). TNF-α interacts with AGE/RAGE (13) and stimulates the expression of multiple proteins, e.g., aldose reductase (20), PKC (28), mitogen-activated protein kinases (7, 19), iNOS (30), and poly(ADP-ribose) polymerase (10), all of which play important roles in the pathogenesis of diabetic neuropathy (34). We reasoned that inactivation of TNF-α would be a clinically useful approach that may ameliorate multiple downstream deleterious pathways that contribute to diabetic neuropathy and, as such, may be a powerful therapeutic strategy toward this intractable complication of diabetes mellitus.

The genetic ablation of TNF-α in mouse (TNF-α−/− mice) was produced >15 years ago. TNF-α−/− mice are resistant to obesity-induced glucose intolerance (43) as well as to the development of diabetic macrovascular complications (12). Interestingly, there is no study to date on the susceptibility of these animals to diabetic neuropathy. Because TNF-α regulates important signals in nervous system in various kinds of degenerative diseases (32), it is reasonable to expect that lack of TNF-α signals in the peripheral nervous system may prevent or ameliorate diabetic neuropathy.

TNF-α−/− mice are a powerful tool to study the role of TNF-α in diabetic neuropathy; however, we need to also test the efficacy of TNF-α inhibition to determine if the maneuver is a useful therapeutic option for diabetic neuropathy. Furthermore, it would be ideal if agents that are in clinical use are
available for these preclinical experiments. Fortunately, infliximab is an Food and Drug Administration (FDA)-approved monoclonal anti-TNF-α-antibody developed for the treatment of a variety of autoimmune disorders in people, including psoriasis, rheumatoid arthritis, Crohn’s disease, ankylosing spondylitis, psoriatic arthritis, and ulcerative colitis (25). Infliximab binds to the soluble form of TNF-α (sTNF-α) and inhibits binding of TNF-α to its cognate receptors (36). Furthermore, it acts as an agonist for transmembrane TNF-α (tmTNFα) and induces “reverse signaling” to deliver costimulatory signals, thereby altering the differentiation state or function of the tmTNF-α-producing cells (18). Therefore, in this study, one of our aims is to determine if the inactivation of both sTNF-α and tmTNF-α would produce therapeutic benefits in diabetic neuropathy.

In the first part of this communication, we examined the role of TNF-α in the development of diabetic neuropathy in streptozotocin (STZ)-induced diabetic wild-type (WT) mice and TNF-α-/-/- mice. Next, we tested the efficacy of infliximab-induced TNF-α inactivation in STZ-treated TNF-α-/-/- mice after they had developed diabetic neuropathy. In addition to therapeutic efficacy, we also examined the mechanism of action of infliximab in the diabetic animals.

**MATERIALS AND METHODS**

*Animals.* Eight-week-old C57BL/6J (WT, TNF-α-/-/-) (Japan CLEA, Osaka, Japan) and TNF-α-deficient (TNFα-/-/-) mice of strain B6.129S6-TNFα(-/-/-)1 in the C57BL/6 background (Jackson Laboratories) were used. The animals were maintained on a 12:12-h light-dark cycle under standard laboratory chow and water. All experimental protocols described in this study were approved by the Animal Care Committees of Shiga University of Medical Science. In the first set of experiments, we produced diabetic mice by injection of STZ (Nacalai Tesque, Kyoto, Japan) (170 mg/kg ip injection) in citrate buffer (pH 4.5) to WT or TNF-α-/-/- mice. Mice injected with citrate buffer only were used as nondiabetic controls. The percent survival of TNF-α-/-/- mice by STZ injection was 75%, and that of TNF-α-/-/- mice was 69%. On day 7 after the injection of STZ, we determined the blood glucose and included in our study only diabetic mice that showed glucose levels of 250 mg/dl and <600 mg/dl. The mice were analyzed by 8 wk after STZ or buffer injection. Next, we evaluated the effect of infliximab on diabetic neuropathy. WT mice were injected with STZ at 8 wk of age and then 8 wk later they randomly received a single injection of saline (100 µ/dose ip) or infliximab (10 µg/kg in 100 µl saline/dose ip). The infliximab dose was adjusted for mice metabolic rates compared with human metabolic rates (in clinical practice, the optimal human dose is 5.0–10.0 mg/kg every 8 wk) (1). As control, WT mice were injected buffer only at 8 wk of age, and 8 wk later, half of them received saline (100 µl ip) and the other half infliximab (10 µg/kg in 100 µl saline ip). Body weight and the blood glucose concentration were measured at 8:00 A.M. weekly. The mice were analyzed at 12 wk after STZ or buffer injection (4 wk after saline or infliximab treatment). Some of the mice were killed during the experiment for histological and genetic analysis. After 8 wk from STZ injection, no mice died until they were killed at the termination of the experiment.

Electrophysiological test. We performed nerve conduction measurements in mice with Medelec Sapphire (Medelec, Surrey, UK) under anesthesia (pentobarbital sodium, 5 mg/kg ip) at temperatures from 30 to 32°C. We exposed the left dorsal femoral, sciatic nerve by opening up overlying skin. The sciatic nerve was stimulated proximally at the sciatic notch, and the compound muscle action potential (CMAP) was obtained distally at the knee. All stimulating and recording electrodes were platinum subdermal needle electrodes with near-nerve temperature kept constant at 37 ± 0.5°C using a heat lamp. The motor nerve conduction velocity (MNCV) was calculated by dividing the distance from the sciatic notch to the ankle by the latency between the distal and proximal wave of CMAP. Next, the sciatic nerve at the left ankle joint level was stimulated, and the sensory nerve action potential (SNAP) was obtained at the proximal site of the sciatic nerve. The sensory nerve conduction velocity (SNCV) was calculated by dividing the distance from stimulation site to the recording site by the initial latency of SNAP.

_Tail flick test._ Thermal stimulation was provided by a beam of high-intensity light (Tail-flick Analgesia Meter MK-330A; Muromachi Kikai, Tokyo, Japan) focused on the root of the tail (8). The heat intensity that we used in this experiment was obtained by 60 volts, and it produced a velocity of skin surface temperature at 3.0°C/s. The response time, defined as the interval between the onset of the thermal stimulation and an abrupt flick of the tail, was measured. The average of two separate readings was taken per animal in a 3-h interval. The thermal stimulation cut-off time in the absence of a response was set at 10 s to prevent tissue injury following the Animal Models of Diabetes Complications Consortium protocol and another study (42).

Therefore, we could not simply show the results by means of seconds. To calculate the statistical significance, the results were translated into a score as follows: latency ≤1 s = score 0; 1 s < latency ≤2 s = score 1; 2 s < latency ≤3 s = score 2; 3 s < latency ≤4 s = score 3; 4 s < latency ≤5 s = score 4; 5 s < latency ≤6 s = score 5; 6 s < latency ≤7 s = score 6; 7 s < latency ≤8 s = score 7; 8 s < latency ≤9 s = score 8; 9 s < latency ≤10 s = score 9; and 10 s < latency = score 10.

**Immunohistochemistry and ELISA.** We examined intraepidermal nerve fiber density (IENFD) in TNF-α-/-/- and TNF-α-/-/- mice 8 wk after STZ or buffer injection and in TNF-α-/-/- mice 4 wk after infliximab or saline treatment in mice that had previously received STZ or buffer injection 12 wk before. The mice were deeply anesthetized with pentobarbital sodium (50 mg/kg ip) and intracardially perfusion-fixed with freshly prepared 4% paraformaldehyde, 0.5% glutaraldehyde, and 0.2% picric acid in 0.1 M PBS (pH 7.4). After perfusion, the footpads were quickly removed from the plantar surface of the hindpaws, postfixed in 4% paraformaldehyde for 2 h, and permeated with 15% sucrose in 0.1 M PBS for 1 day and 15% sucrose in 0.1 M PBS for 2 days with agitation. Three randomly chosen 5-µm sections from each mouse were deparaffinized in xylene, hydrated in decreasing concentrations of ethanol, and washed in water. Endogenous peroxidase activity was blocked by incubation with 0.5% H2O2 in Tris-buffered saline (TBS). Nonspecific binding was blocked by 10% goat serum in TBS for 1 h. We incubated sections in primary antisera, rabbit polyclonal anti-PGP 9.5 (1:1,000; Ultraclone) diluted in TBS and 5% goat serum for 2 h at room temperature after nonimmune serum as a control. After washing, specific immunoreactivity was visualized using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) with dianinobenidine as the chromogen. Sections were counterstained with Gill’s hematoxylin, dehydrated, and mounted in Canada Balsam. We counted immunoreactive nerve fiber profiles in the epidermis and normalized to epidermal length (no./mm). Nerve fibers that branched within the epidermis were counted as one. An average of 3.2 ± 0.2 mm (n = 18) of the sample length was investigated to calculate a number of nerve fiber profiles per millimeter of epidermis (17).

After perfusion, the DRG at the level of L2-L4 was quickly removed and postfixed in 4% paraformaldehyde for 2 h and permeated with 15% sucrose in 0.1 M PBS for 1 day and 15% sucrose in 0.1 M PBS for 2 days with agitation; the blocks were then cut into 3-µm-thick paraffin sections. Endogenous peroxidase, avidin, and biotin were blocked appropriately, and 0.1% Tween 20 in Tris buffer was used. Sections were incubated with the primary antibody overnight at 4°C. After washing, specific immunoreactivity was visualized using a Vectastain Elite ABC kit (Vector Laboratories) with dianinobenidine as the chromogen and mounted in Canada Balsam.
We used the following antibodies: anti-phospho-NF-κB p65 serine-276 antibody (rabbit polyclonal; Cell Signaling Technology, Tokyo, Japan) and anti-cleaved caspase-3 (rabbit monoclonal; Cell Signaling Technology). Three levels of one DRG tissue were assayed at 50-μm intervals. Total cell count was 350–500 cells/one mouse. We obtained digital images of the entire tissue section. Neurons with visible nuclei were used for counting. For anti-phospho-NF-κB p65 serine-276 antibody, we determined the number of positively stained nuclei per cell. The analysis was performed on five mice in each group, and we calculated the mean positive percentage. Serum TNF-α concentrations were determined in samples using ELISA kits from R&D Systems (Minneapolis, MN).

Quantitative real-time RT-PCR and mRNA analyses. We freshly isolated L1-L5 DRGs from diabetic and nondiabetic mice. After tissue sampling, we synthesized cDNA by using Superscript III RT (Invitrogen, Carlsbad, CA) and performed quantitative real-time PCR with the LightCycler Fast Start DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). The PCR mixture contained 200 ng of cDNA and the primers. Emitted fluorescence for each reaction was measured three times during the annealing extension phase, and amplification plots were analyzed using the LIGHTCYCLER® software, version 1.5 (Roche Diagnostics). Potential genomic DNA contamination was controlled by the use of intron-encompassing primers. The relative value in each sample was calculated by the standard curve obtained from control samples (TNF-α from 100 mg/kg of lipopolysaccharide-treated liver) and standardized as quotient divided by the value simultaneously obtained in β-actin in the same experiment. The primers used in the real-time PCR were as follows: mouse TNF-α sense 5'-GGCCTCTTTCTGACCTGTGAG-3', antisense 5'-CTGATGAGAAGGCGGCAT-3'; mouse iNOS sense 5'-TTGGACGGAGTGTGGAATGGTTG-3', antisense 5'-GTAGGTTGAGGTTGGCTGTCGA-3'; mouse IL-6 sense 5'-ACGGCCTTCCTCCTACTTACA-3', antisense 5'-CATTTCCAGATGTTTCCCGAG-3'; and mouse β-actin sense 5'-GCGCTGAACTGACATCAAGAGAAC-3', antisense 5'-TGGATGCAAGAGTTCCCAT-3'. PCR reaction was performed under conditions described previously (38) except that the annealing temperature was at 61°C for TNF-α, 65°C for iNOS and IL-6, or 60°C for β-actin.

Statistical methods. Data obtained by tail flick test score were evaluated by the nonparametric relationship with Mann-Whitney U-test. Other data were evaluated by t-testing or ANOVA testing with multiple comparisons of independently assessed samples and groups performed as appropriate in all cases with Scheffé’s corrections as needed. Data were expressed as means ± SE. P < 0.05 was considered significant.

RESULTS

Mice with genetic inactivation of TNF-α fail to develop diabetic neuropathy. We monitored body weight and blood glucose every week and performed nerve conduction study and tail flick test (Fig. 1) at baseline (pre-STZ) and 8 wk after STZ or buffer injection in four groups of animals: nondiabetic and diabetic WT and TNF-α−/− mice. In both TNF-α+/+ and TNF-α−/− groups, mice with diabetes were significantly smaller than nondiabetic mice at 8 wk and showed significantly higher blood glucose concentration compared with nondiabetic mice (Table 1).

In terms of nerve functional measurements, TNF-α+/+ mice displayed a reduction in both MNCV and SNCV and decreased 8 wk after STZ. In contrast, in TNF-α−/− mice, both MNCV and SNCV remained in the normal range and did not change after STZ-induced diabetes (Fig. 1, A and B). It is interesting, however, that the basal tail flick test score in TNF-α−/− mice before STZ diabetes induction was significantly higher than that in TNF-α+/+ mice. TNF-α expression correlates with the development of thermal sensation through the Schwann cell function that can envelop axons efficiently in peripheral sensory bundles (9). Therefore, absence of TNF-α expression in mice is associated with a delay in the latency of the hot plate test, a result that is totally consistent with that reported previously (9). In TNF-α+/+ mice, the tail flick test score doubled 8 wk after STZ-induced diabetes. However, in TNF-α−/− mice, there was no change in latency in the tail flick test at 8 wk after STZ-induced diabetes (Fig. 1C). These functional changes were further corroborated by the immunohistochemical analysis of IENFD. Consistent with the data from the tail flick test, nondiabetic TNF-α−/− mice showed a significant decrease in IENFD compared with that in nondiabetic TNF-α+/+ mice. On the other hand, STZ diabetes induction did not lead to any worsening in this parameter in TNF-α−/− mice but produced a marked reduction in IENFD in TNF-α+/+ mice (Fig. 1, D and E).

Next, we examined by immunohistochemistry the presence of the phosphorylated form of NF-κB p65 at serine-276, as well as cleaved caspase-3 to evaluate apoptosis, in DRG neurons of TNF-α+/+ and TNF-α−/− mice 8 wk after STZ-induced diabetes. In TNF-α+/+ mice, the proportion of DRG neurons that stained positive with phosphorylated NF-κB p65 serine-276 was increased significantly in diabetic (6.40 ± 0.70%) vs. nondiabetic (1.33 ± 0.22%) animals (Fig. 2, A and B). In contrast, DRG neurons that stained positive for phosphorylated NF-κB p65 serine-276 were very rare in either diabetic or nondiabetic TNF-α−/− mice; the proportion of neurons positive for phosphorylated NF-κB p65 serine-276 in diabetic TNF-α−/− mice (1.12 ± 0.07%) was not significantly different from that in nondiabetic animals (0.89 ± 0.07%) (Fig. 2, A and B).

The proportion of DRG neurons that displayed cleaved caspase-3 was significantly higher in diabetic (3.56 ± 0.80%) vs. nondiabetic TNF-α−/− mice (0.33 ± 0.21%) (Fig. 2, C and D). However, among TNF-α−/− mice, the number of cleaved caspase-3-positive DRG neurons remained very low in diabetic animals (0.63 ± 0.07%) and was not different from that in nondiabetic animals (2.32 ± 0.60%) (Fig. 2, C and D).

A single injection of infliximab leads to marked improvement in diabetic neuropathy. Eight weeks after buffer or STZ injection (producing diabetes) in WT mice, we injected infliximab or saline in diabetic and nondiabetic mice 8 wk later at a time when diabetic neuropathy was clearly evident by nerve function measurements in the STZ diabetic mice. We followed these mice to determine the effect of infliximab treatment at 12 wk after STZ (4 wk after infliximab treatment). STZ diabetic mice were significantly smaller than nondiabetic mice at 8 or 12 wk after STZ, whether they received saline or infliximab, which did not cause any body weight changes compared with saline controls (Table 2). Furthermore, infliximab had no effect on blood glucose levels in either diabetic or nondiabetic mice (Table 2).
between nondiabetic and infliximab-treated diabetic mice (Fig. 3, A and B). Nondiabetic mice showed no change of SNCV and MNCV throughout the experimental periods for 12 wk either treated with infliximab or with saline (Fig. 3, A and B). The tail flick test score in diabetic mice increased 8 wk after the STZ injection compared with the value before STZ injection or the 8-wk value of saline-treated mice (Fig. 3C). Diabetic mice treated with saline 8 wk after diabetes development showed a persistently increased tail flick test score 8 and 12 wk after STZ. In contrast, diabetic mice treated with infliximab showed significant improvement in the tail flick test score at 12 wk after STZ injection (4 wk after infliximab treatment) compared with that at 8 wk after STZ (before infliximab treatment) or the diabetic controls treated with saline. The score did not, however, return completely to the basal level in nondiabetic mice. Nondiabetic mice showed no change of the tail flick test score throughout the experimental periods for 12 wk whether they were treated with infliximab or with saline only (Fig. 3C).

These functional changes were also corroborated by immunohistochemical analysis of IENFD. Diabetic mice treated with saline showed decreased IENFD, but those treated with infliximab showed complete restoration of nerve fiber density at 12 wk after STZ injection. In contrast, nondiabetic mice showed no change in nerve fiber density throughout the experimental periods for 12 wk whether they were treated with infliximab or saline (Fig. 3, D and E).

At 12 wk after STZ or buffer injection (4 wk after infliximab or saline treatment), we examined the DRG neurons by immunocytochemistry for the presence of phosphorylated NF-κB p65 serine-276 or cleaved caspase-3 (Fig. 4). In nondiabetic mice, we found extremely low levels of phosphorylated NF-κB p65 serine-276 by immunostaining and detected no significant difference between mice injected with saline (0.91 ± 0.33%) or infliximab (0.78 ± 0.22%) (Fig. 4, A and B). However, treated with saline 8 wk after diabetes development showed a persistently increased IENFD during the experimental period for 12 wk (Fig. 4C).

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<th>Nondiabetic TNF-α+/+ mice</th>
<th>Body Wt, g</th>
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<td>Before</td>
<td>After 8 wk</td>
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<td>17.9 ± 0.4</td>
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<td>134.6 ± 3.7</td>
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<td>Diabetic TNF-α+/+ mice</td>
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<tr>
<td>Nondiabetic TNF-α−/− mice</td>
<td>16.7 ± 0.3</td>
<td>19.5 ± 0.6</td>
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<tr>
<td>Diabetic TNF-α−/− mice</td>
<td>16.7 ± 0.3</td>
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Data are means ± SE; n, no. of mice. Before, before buffer or streptozotocin (STZ) injection. TNF, tumor necrosis factor; after 8 wk, 8 wk after buffer or STZ injection. *Significance at P < 0.05 vs. nondiabetic mice with the same strain.
diabetic mice treated with saline displayed an increase in the number of phosphorylated NF-κB p65 serine-276-positive cells among DRG neurons (9.79 ± 3.41%), whereas infliximab treatment significantly reduced the level to nondiabetic control levels (1.78 ± 1.13%) (Fig. 4, A and B).

By immunocytochemistry, we found very low levels of immunoreactive cleaved caspase-3 in DRG neurons of nondiabetic mice. Saline or infliximab injection did not affect the immunostaining pattern in nondiabetic mice (0.19 ± 0.11% for saline and 0.32 ± 0.15% for infliximab; Fig. 4, C and D). Mice with diabetes showed an increased proportion of cleaved caspase-3-positive cells in DRG neurons (4.81 ± 1.58%), which was reduced significantly down to normal by infliximab (1.03 ± 0.16%) (Fig. 4, C and D).

Infliximab normalized serum TNF-α level and the TNF-α mRNA in DRG. We determined the serum concentration of TNF-α in mice treated with saline or infliximab 12 wk after STZ or buffer injection (4 wk after the infliximab or saline treatment). Diabetic mice that received saline only showed a significant increase in serum TNF-α concentration compared with nondiabetic mice. However, infliximab completely reversed the elevated serum concentration of TNF-α in diabetic mice (Fig. 5).

We further examined the levels of TNF-α, iNOS, and IL-6 mRNA in RNA extracted from the DRG of mice treated with saline or infliximab at 12 wk after STZ or buffer injection (4 wk after the infliximab or saline treatment). In the absence of infliximab treatment, diabetic mice showed a 12-fold increase in TNF-α mRNA expression in the DRG compared with nondiabetic mice. Furthermore, compared with nondiabetic mice, diabetic animals showed increased iNOS and IL-6 mRNA expression in the DRG by 2.4- and 2.4-fold, respectively. Interestingly, infliximab treatment brought the elevated level of TNF-α, iNOS, and IL-6 mRNA expression completely down to normal nondiabetic levels (Fig. 6).

**DISCUSSION**

In this study, we examined the role of TNF-α in diabetic neuropathy using two different experimental approaches. First, we found that genetic ablation of TNF-α gene expression completely prevents the development of diabetic neuropathy in STZ-induced diabetic mice. Second, infliximab, a monoclonal antibody that targets and inactivates TNF-α, suppresses the elevated serum TNF-α concentration associated with STZ diabetes and reverses established diabetic neuropathy in TNF-α-/- mice. Moreover, infliximab treatment completely suppresses the elevated TNF-α mRNA in the DRG of these mice. These results implicate the elevated TNF-α protein expression in serum and mRNA expression in the DRG as playing a key role in the development of diabetic neuropathy, and their suppression by infliximab may be an efficacious way to treat diabetic neuropathy. This study was performed in a type 1 diabetes model. Although we believe that the therapeutic effect of the approach may extend to animals with type 2 diabetes, the efficacy of infliximab cannot be assumed until the treatment has been tested in a type 2 model.

Absence of functional TNF-α produces no overt abnormality in murine embryonic development (29). However, in the peripheral nervous system, TNF-α expression appears to be required for normal development of thermal sensory perception through Schwann cell function that enables them to envelop axons efficiently in peripheral sensory bundles. Lack of TNF-α

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<th>Body Wt. g</th>
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Data are means ± SE; n, no. of mice *Significance at P < 0.05 vs. nondiabetic mice with the same treatment.

**Table 2. Changes in body weight and blood glucose in nondiabetic or diabetic mice treated with infliximab or saline**
expression in mice leads to a delay of the latency by hot plate test (9). We found that the tail flick test score and IENFD were both abnormal under basal conditions in nondiabetic TNF-α−/− mice, corroborating the findings of Dhadialla et al. (9). Interestingly, however, STZ-induced diabetes led to an increase in latency in the tail flick test in WT mice but not in TNF-α−/− mice. In contrast, the MNCV and SNCV in nondiabetic TNF-α−/− mice fell within the normal range in another study (46), which was confirmed in our study. Furthermore, both MNCV and SNCV were impaired 8 wk after STZ-induced diabetes in TNF-α−/− mice, but these parameters remained normal in diabetic TNF-α−/− mice. These observations support a central role for TNF-α in the development of diabetic neuropathy.

Fig. 3. Changes in electrophysiological records, tail flick test score, and immunohistochemical analysis of intraepidermal nerve fiber density in nondiabetic and diabetic mice treated with infliximab or buffer injection. A: SNCV in both nondiabetic and diabetic mice treated with infliximab or buffer injection. B: MNCV in nondiabetic and diabetic mice treated with infliximab or buffer injection. C: Tail flick test score in both nondiabetic and diabetic mice treated with infliximab or buffer injection. D: Representative images of intraepidermal nerve fiber profiles in control and diabetic mice with or without infliximab treatment. The arrowheads indicate nerve fibers. Control, nondiabetic mice; Diabetes, diabetic mice; Buffer, saline injection; Infliximab, infliximab injection. E: Skin fiber density in control and diabetic mice after treatment with buffer or infliximab. Data are means ± SE. *P < 0.05. Bar = 25 μm; n = 5/group.

Fig. 4. Immunohistochemical analysis of NF-κB p65 serine-276 phosphorylated and cleaved caspase-3 in DRG neurons. A: Immunohistochemical analysis of NF-κB p65 serine-276 phosphorylated in DRG neurons in control mice (top) and diabetic mice (bottom). Arrowheads, cells positive for NF-κB p65 serine-276 phosphorylated in the nucleus. B: Percentage of the cells positive for NF-κB p65 serine-276 phosphorylated in control and diabetic mice. C: Immunohistochemical analysis of cleaved caspase-3 in DRG neurons in control (top) and diabetic (bottom) mice. Arrows, cells positive for cleaved caspase-3. D: Percentage of the cells positive for cleaved caspase-3 in control and diabetic mice. Data are means ± SE. *P < 0.05. Bar = 50 μm; n = 5/group.
neuropathy and suggest that the absence of functional TNF-α may protect against this common complication of diabetes.

TNF-α activates two different downstream signaling pathways: the activation of caspase-3 for apoptosis or the activation of NF-κB for inflammatory and antiapoptotic gene expression (15). Caspase-3, in addition, is also involved in a number of nonapoptotic events, including proliferation and differentiation of many other types of cells (27). However, in this study in DRG, the number of cleaved caspase-3-positive cells was found to be increased in diabetic TNF-α mice compared with nondiabetic mice, recapitulating what was reported in rats (6, 16, 35). In contrast, the number of cleaved caspase-3-positive cells in the DRG of diabetic TNF-α mice was the same as that in nondiabetic mice. In addition, although the number of NF-κB p65 serine-276 phosphorylated-positive cells went up in DRG of diabetic TNF-α−/− mice was the same as that in nondiabetic mice. In addition, although the number of NF-κB p65 serine-276 phosphorylated-positive cells went up in DRG of diabetic TNF-α−/− mice compared with nondiabetic mice, the number of NF-κB p65 serine-276 phosphorylated-positive cells also failed to increase in the DRG of diabetic TNF-α−/− mice. NF-κB interacts with RAGE/AGE in diabetes (13) and is intimately involved in the pathogenic actions of RAGE in diabetic neuropathy (2, 41). Moreover, NF-κB is involved in the transcriptional control of a large number of genes, including endothelin-1, cyclooxygenase-2, lipoxygenase, iNOS, and IL-6 (5). The fact that TNF-α−/− mice are protected against diabetic neuropathy supports a central role for TNF-α in the abnormal cellular signaling that underlies diabetic neuropathy.

Infliximab treatment suppressed the elevated serum TNF-α concentration completely back to normal, and one could postulate that normalization of serum sTNF-α underlies the preservation of normal nerve function in diabetic mice. However, infliximab could be beneficial via actions not directly related to circulating TNF-α. We found that infliximab completely normalized the mRNA levels for not only TNF-α but also those for iNOS and IL-6 in the diabetic DRG. We speculate that, in addition to neutralizing tmTNF-α by blocking its interaction with cells that express TNF receptor (TNFR) I and/or TNFRII, infliximab may also act as an agonist to produce reverse signaling to the tmTNF-α-expressing cells (18). In studies on endotoxin-mediated Toll-like receptor signaling in vitro, reverse signaling of infliximab through tmTNF-α has been shown to induce endotoxin resistance and suppression of the expression of cytokines, including TNF-α, IL-1β, IL-10, and IL-12 (37). Furthermore, previous reports showed that TNF-α promotes its own production via activation of NF-κB (47), and Fig. 5. Serum TNF-α concentrations in nondiabetic and diabetic mice treated with infliximab or buffer. Data are means ± SE. *P < 0.05; n = 10/group.

Fig. 6. Quantitative real-time RT-PCR of TNF-α, inducible nitric oxide synthase (iNOS), and interleukin (IL)-6 mRNA expression in DRG. The value for the reference RNAs is arbitrarily assigned a value of 1.0 in nondiabetic mice treated with saline. We standardized as quotient divided by the value simultaneously obtained in β-actin. Data are means ± SE. *P < 0.05; n = 4–5/group.
TNF-α acts on the secreting cells in an autocrine or local paracrine manner in the DRG (11). Therefore, it is possible that these additional actions of infliximab amplify the beneficial action of infliximab on the activated tmTNF-α in the DRG of diabetic mice.

In conclusion, we showed that TNF-α plays a central role in diabetic neuropathy and its inhibition by infliximab leads to the amelioration of this major complication of diabetes. Multiple TNF-α antagonists, including infliximab, have been approved by the FDA for the treatment of different chronic inflammatory diseases in people. Our findings suggest that clinical trials of such agents for the treatment of early diabetic neuropathy may be warranted.

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DISCLOSURES

The authors have nothing to disclose.

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