Neurturin-Deficient Mice Develop Dry Eye and Keratoconjunctivitis Sicca

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PURPOSE. Neurturin has been identified as a neurotrophic factor for parasympathetic neurons. Neurturin-deficient (NRTN−/−) mice have defective parasympathetic innervation of their lacrimal glands. This study was conducted to evaluate tear function and ocular surface phenotype in NRTN−/− mice.

METHODS. Determined by tail genomic DNA PCR, 25 NRTN−/− mice and 17 neurturin-normal (NRTN+/+) mice aged 6 weeks to 4 months were evaluated. Aqueous tear production, tear fluorescent clearance, and corneal sensation were serially measured.

RESULTS. In comparison to that in age-matched NRTN+/+ mice, aqueous tear production, tear fluorescent clearance, and corneal sensation were significantly reduced in NRTN−/− mice, whereas corneal permeability to AFD was significantly increased. Immunoreactive MUC-4 and -5AC mucin and goblet cell density (P < 0.001) in the conjunctiva of NRTN−/− mice were lower than in NRTN+/+ mice. The expression of MUC-1 and 2 mRNA by the corneal epithelium was reduced in NRTN−/− mice. There were a significantly greater number of IA antigen-positive conjunctival epithelial cells in NRTN+/+ mice than NRTN−/− mice. Tear fluid IL-1β and MMP-9 concentrations and the expression of IL-1β, TNF-α, macrophage inflammatory protein (MIP)-2, cytokine-induced neutrophil chemotactant (KC), and MIP-2 mRNA by the corneal epithelium were significantly increased in NRTN−/− mice, compared with NRTN+/+ mice.

CONCLUSIONS. Neurturin-deficient mice show phenotypic changes and ocular surface inflammation that mimic human keratoconjunctivitis sicca. This model supports the importance of a functional ocular surface-central nervous system-lacrimal gland sensory-autonomic neural network in maintaining ocular surface health and homeostasis. (Invest Ophthalmol Vis Sci. 2003;44:4223–4229) DOI:10.1167/ iovs.02-1319

Dry eye is a common condition that affects 10% of the population between the ages of 30 and 60 years, increasing in prevalence to 15% of the population aged 65 years or more.1,2 Dry eye results from dysfunction of the integrated ocular surface-secretory glandular functional unit. This may result from disease of the sensory afferent nerves innervating the ocular surface, the autonomic efferent nerves innervating the tear-secreting glands, or the tear-secreting glands themselves. Dysfunction of the integrated functional unit leads to an unstable tear film, ocular surface inflammation, and epithelial disease, termed keratoconjunctivitis sicca (KCS).3–5 The pathogenesis of the tear film instability and KCS in dry eye is not well understood. A dry eye animal model with dysfunction of the integrated functional unit mimicking human dry eye disease would be a useful tool for investigating these mechanisms. Animal models of dry eye disease have been created by various means, including surgical removal of the tear-producing glands, ocular surface desiccation by mechanically inhibiting blinking, and pharmacologic inhibition of tear secretion.6–9 We have reported that inhibiting tear secretion in mice by systemic administration of the muscarinic cholinergic antagonist scopolamine produces KCS that mimics human dry eye disease.9 This model is labor intensive and difficult to continue for prolonged periods; however, it suggests that mice with parasympathetic autonomic nerve dysfunction due to disease or gene deletion may be relevant models for the study of dry eye.

Neurturin is a member of the transforming growth factor-β family that functions as a neurotrophic factor for various classes of neurons.10,11 Neurturin acts through a two-component receptor system consisting of the ligand-specific GFRα-2 receptor and the common receptor tyrosine kinase c-Ret.12 Neurturin is essential for the development of specific postganglionic parasympathetic neurons. It has also been shown to support the development and maintenance of cutaneous trigeminal sensory nerves. Neurturin-deficient (NRTN−/−) mice generated by homologous recombination are viable and fertile, but have defects in their autonomic, enteric, and sensory nervous systems.13 Parasympathetic innervation of the lacrimal gland and submandibular salivary glands is dramatically reduced in NRTN−/− mice, as is the number of GFRα2-expressing neurons in the trigeminal ganglion.11,13 The purpose of this study was to evaluate tear function and ocular surface phenotype in NRTN−/− mice. Markers of human KCS (corneal epithelial permeability, conjunctival goblet cell density, and ocular sur-
face inflammation) in neurturin-deficient and wild-type mice were investigated.

Materials and Methods

Animals

Neurturin gene–deficient mice were generated by homologous recombination.13 All studies were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-five neurturin gene knockout mice (NRTN−/−) and 17 wild-type control mice (NRTN+/+) were used in the study. Genotypes of 1-month-old pups from heterozygous parents were tested by using genomic DNA isolated from their tails (Genomic DNA Isolation kit; Sigma-Aldrich, St. Louis, MO). PCR amplification was performed on a thermal cycler (DNA Thermal Cycler 480; GeneAmp PCR kit; Applied Biosystems, Foster City, CA) with the neurturin gene primer pair 4743: GAGATCAGCAGCCTCTGTTCCACATAC (yielding a 200-bp fragment from the neurturin gene knockout mice (NRTN−/−), and both bands from the heterozygotes (+/−)).

Aqueous Tear Production

Aqueous tear production was measured with a phenol-red–impregnated cotton thread (Zone-Quick, Oasis, CA), which was placed in the lateral canthus for 20 seconds. Wetting of the thread was measured on a millimeter scale.

Fluorescein Clearance Test

The fluorescein clearance test is a measure of total tear production, tear spread, and tear drainage.14 This test was performed by instilling 1 μL 2% sodium fluorescein into the conjunctival sac. After 15 minutes, 1 μL of phosphate-buffered saline (PBS) was instilled, and the fluorescein-stained tear fluid was collected atraumatically from the lateral tear meniscus for 20 seconds under a surgical microscope by using a porous 1 × 8-mm polyester rod (America FiltroTech Co., Richmond, VA). The rod was placed into a 10-μL micropipette tip within a 1.5-mL tube, which was then centrifuged for 5 minutes after addition of 99 μL PBS directly onto the polyester rod. The solution was transferred to a single well in a 96-well plate. The fluorescein concentration was measured with a fluorophotometer (CytoFluor II; Perspex Biosystems, Farmingham, MA), using 485-nm excitation and 530-nm emission filters.

Measurement of Corneal Sensation

A stream of CO2 gas at a pressure of 1 psi through a 1-mm diameter plastic catheter tip was delivered perpendicularly to the cornea of mice. The tip of the catheter was slowly advanced toward the corneal surface, beginning 15 mm away from the cornea. The distance in millimeters where a blink or withdrawal was observed was recorded.

Corneal Permeability to AlexaFluor Dextran

The corneal uptake of 10-kDa AlexaFluor dextran (AFD; Molecular Probes, Eugene, OR) was measured by using a modification of a previously reported technique. Briefly, 1 μL of 0.3% AFD was instilled onto the ocular surface 15 minutes before death. Excised corneas were rinsed four times with 200 μL balanced salt solution (BSS; Alkon Lab, Inc., Fort Worth, TX) and placed in 200 μL BSS. The solution containing the corneal tissue was protected from light and placed on an orbital shaker. The concentration of eluted AFD was measured with 485-nm excitation and 530-nm emission filters at 10, 20, and 60 minutes on a fluorophotometer (CytoFluor II; Perspex Biosystems).

Histology and Immunofluorescent Staining

The whole eyeball together with the eyelids and conjunctiva was embedded in a mixture of 75% (vol/vol) OCT compound (Sakura Finetek USA, Inc., Torrance, CA) and 25% (vol/vol) aqueous mounting medium (Immuno-Mount; Thermo-Shandon, Pittsburgh, PA), and then flash frozen in liquid nitrogen. Sections (10 μm thick) were cut and stained with periodic acid–Schiff (PAS) reagent. For immunofluorescent staining, sections were fixed with 100% methanol at 4°C for 10 minutes and blocked with 5% normal goat serum in PBS for 30 minutes. The primary antibody was applied for 1 hour at room temperature. These goat polyclonal antibodies were reactive with MUC-4 ASGP2 C-terminal peptide (C- pep; a gift from Kermit Carraway, University of Miami, Miami, FL), MUC-5AC (a gift from Marcia Jumblatt, University of Louisville, Louisville, KY) or HLA class II antigen (IA; Pharmingen, San Diego, CA). After a wash with PBS, the secondary antibody (AlexaFluor-488 conjugate; 1:100 dilution; Molecular Probes) was applied for 1 hour in a dark incubation chamber. After a wash with PBS, antifade medium (Gel-Mount; Fisher, Atlanta, GA) containing 1 μg/ml Hoechst 33342 dye and a coverslip were applied. Sections were examined and photographed with an epifluorescence microscope (Eclipse 400; Nikon, Tokyo, Japan) and a digital camera (model DMX 1200; Nikon).

Tear Collection

PBS (1.5 μL containing 0.1% bovine serum albumin (BSA) was instilled into the conjunctival sac. The tear fluid and buffer were collected with a 1-μL volume glass capillary tube (Drummond Scientific Co., Broomhall, PA) by capillary action from the tear meniscus in the lateral canthus. The tear solution was stored at −80°C until zymography and ELISA were performed.

IL-1β ELISA and Gelatin Zymography

The IL-1β concentration in tear samples was assayed with ELISA (Quintikine M Murine ELISA kit; R&D Systems, Minneapolis, MN), according to the manufacturer’s protocol.

The level of gelatinolytic enzymes in the tear fluid was measured by SDS-PAGE gelatin zymography, according to a previously reported method. A 2-μL tear sample (from both eyes of each mouse) was added to SDS-PAGE sample buffer and fractionated by electrophoresis on an 8% polyacrylamide gel containing gelatin (0.5 mg/mL). The gels were soaked in 0.2% Triton X-100 for 30 minutes at room temperature to remove the SDS and incubated in a digestion buffer containing 50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl2, 2 mM ZnSO4, 0.01% Brij-35, and 5 mM phenethylsulfonyl fluoride (PMSF), a serine protease inhibitor, at 37°C overnight, to allow proteinase digestion of its substrate. The gels were rinsed in distilled water and stained with 0.25% Coomassie brilliant blue R-250 in 40% isopropanol for 2 hours and destained with 7% acetic acid.

RNA Isolation and Semiquantitative RT-PCR

Total RNA from the corneal epithelium was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method, and
stored at −80°C before use. The gene expression was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) with a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal control. In brief, first-strand cDNAs were synthesized from 0.5 μg of total RNA with murine leukemia virus (MuLV) reverse transcriptase. PCR amplification of the first-strand cDNAs was performed with specific primer pairs for murine cDNA of matrix metalloproteinase (MMP)-9, IL-1β, TNF-α, macrophage inflammatory protein (MIP)-2, cytokine-induced neutrophil chemoattractant (KC), MUC-1, MUC-5AC, or GAPDH (Table 1). Semiquantitative RT-PCR was achieved by terminating reactions at intervals of 24, 28, 32, 36, and 40 cycles for each primer pair to ensure that the PCR products formed were within the linear portion of the amplification curve.

**Statistical Analysis**
Depending on the normality of the data distribution, the t-test or Mann-Whitney test was used for statistical comparison of assay results between groups.

**RESULTS**

**Aqueous Tear Production and Clearance**

Aqueous tear production was reduced in NRTN−/− mice (n = 25) compared with age-matched NRTN+/+ mice (n = 17). The difference did not reach statistical significance at 6 weeks (P = 0.126), but did at 8 weeks and in older mice (P < 0.05–0.01, Fig. 2). Similarly, NRTN−/− mice from 8 weeks forward had significantly delayed tear fluorescein clearance compared with age-matched NRTN+/+ mice (P < 0.001, Fig. 3).

**Corneal Sensation**

Corneal sensation was assessed in both groups of 10-week-old mice. The distance of the CO2 stream from the mouse cornea that triggered a blink reflex was significantly less in NRTN−/− mice (mean = 2.40 ± 1.13 cm; n = 34 eyes) than in NRTN+/+ mice (mean = 7.62 ± 2.0 cm, n = 28 eyes; P < 0.001; Mann-Whitney test).

**Corneal Epithelial Permeability to AFD**

Decreased corneal epithelial barrier function is a key feature of human KCS. Corneal epithelial barrier function was assessed by measuring corneal permeability to AFD. The corneas of 10-week-old NRTN−/− mice were more permeable to AFD (36.11 ± 5.1 U, n = 9) than age related NRTN+/+ mice (22.6 ± 4.1 U, n = 9; P < 0.05 by the Mann-Whitney test). This finding suggests that the corneal epithelial barrier function is altered in NRTN−/− mice.

**Histology and Epithelial Mucin Expression**

The corneal epithelium of 10-week-old NRTN−/− mice was noted to be markedly thickened, approximately eight epithelial cell layers thick compared with five epithelial cell layers in wild-type mice (Fig. 4, top). The corneal epithelial cells in NRTN−/− mice had a more basal cell phenotype and often contained PAS-positive granules. RT-PCR showed that the expression of MUC-1 and -4 mRNA by the corneal epithelium was lower in NRTN−/− than in NRTN+/+ mice (Fig. 5). Differences in mucin expression were observed in the conjunctiva. Immunofluorescent staining for the cell membrane mucin MUC-4 was much less in the bulbar and tarsal conjunctiva.

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**Figure 2.** Comparison of the tear production between wild-type (+/+) and neurturin gene knockout (−/−) mice at different ages (6–20 weeks). Data show mean and SD (error bars). *P < 0.05, **P < 0.01; Mann-Whitney test.

**Figure 3.** Comparison of tear fluorescein clearance between wild-type (+/+) and neurturin gene knockout (−/−) mice at different ages (6–20 weeks). Data show mean and SD (error bars) fluorescence units. **P < 0.01; Mann-Whitney test.

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**Table 1. Mouse Primer Sequences Used for RT-PCR**

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<th>Antisense Primer</th>
<th>PCR Product (bp)</th>
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tiva of NRTN\(^{-/-}\) mice than in NRTN\(^{+/-}\) mice (Fig. 6). Conjunctival goblet cell density was measured over 100-mm lengths on the tarsal and bulbar conjunctiva (Fig. 4, bottom). The number of goblet cells in the bulbar (0.68 ± 0.25) and tarsal (10.25 ± 2.3) conjunctiva of NRTN\(^{-/-}\) mice was significantly lower than in the bulbar (10.5 ± 3.7) and tarsal (51.6 ± 2.1) conjunctiva of NRTN\(^{+/-}\) mice \((n = 3, P < 0.001\) for both sites; Mann-Whitney test). This was supported by reduced expression of the goblet cell mucin MUC-5AC by the NRTN\(^{-/-}\) mice (Fig. 7). Leukocytes were observed in the epithelium and surface of the tarsal conjunctiva in many sections of NRTN\(^{-/-}\) mice, but rarely in the NRTN\(^{+/-}\) mice. These findings suggest that NRTN\(^{-/-}\) mice undergo conjunctival phenotypic changes similar to those in human KCS.

### IA Expression in the Conjunctiva

Aberrant HLA class II (IA) antigen expression by the conjunctival epithelium has been reported to increase in human KCS. Scattered stromal cells and epithelial dendritic cells in the conjunctiva of NRTN\(^{-/-}\) mice were immunofluorescent for IA antigen. In contrast, numerous IA-positive conjunctival epithelial cells (Fig. 8, arrow) were observed in NRTN\(^{-/-}\) mice. This finding indicates there is immune activation of the conjunctival epithelium in NRTN\(^{-/-}\) mice.

### Tear Fluid IL-1β ELISA and Gelatin Zymography

Elevated concentrations of the proinflammatory cytokine IL-1β and the protease MMP-9 have been detected in the tear fluid of humans with KCS. IL-1β and MMP-9 were detected in tear fluid of wild-type and NRTN\(^{-/-}\) mice by ELISA and gelatin zymography, respectively. Compared with that in NRTN\(^{+/-}\) mice, the IL-1β concentration in tear fluid of NRTN\(^{-/-}\) mice was slightly
higher in 2-month-old mice (44.74 ± 9.94 pg/mL vs. 57.79 ± 14.93 pg/mL, n = 10, P > 0.05), and was dramatically increased in 4-month-old mice (29.83 ± 16.16 pg/mL vs. 189.57 ± 67.10 pg/mL, n = 10, P < 0.05 by the t-test, Fig. 9). Murine MMP-9 (105 kDa digestion band) was observed in the tear fluid of 88% (22/25) of NRTN−/− mice aged 8 to 12 weeks, compared with a weak MMP-9 band in the tear fluid of 29.4% (5/17) of NRTN+/+ mice at similar ages (Fig. 10).

mRNA Expression of IL-1β, TNF-α, MIP-2, KC, and MMP-9 by the Corneal Epithelium

The expression of RNA encoding the inflammatory cytokines IL-1β and TNF-α, the chemokines MIP-2 and KC, and the protease MMP-9 was evaluated in the corneal epithelia of wild-type and NRTN−/− mice, according to the results of semiquantitative RT-PCR of pooled total RNA from six to eight corneal epithelia in each group of mice aged 12 to 16 weeks. The corneal epithelia of NRTN−/− mice showed a low level of expression of MMP-9 mRNA, whereas IL-1β, TNF-α, MIP-2, and KC mRNA were barely detectable. The corneal epithelium of NRTN−/− mice expressed higher levels of MMP-9, IL-1β, TNF-α, MIP-2, and KC mRNAs than did wild-type mice (Fig. 11). These findings indicate that the expression of these inflammatory cytokines and chemokines was dramatically stimulated in the corneal epithelia of the NRTN−/− mice.

**DISCUSSION**

In this study we report a naturally occurring and permanent dry eye model in genetically manipulated neurturin-deficient mice. These mice show significantly decreased aqueous tear production and tear fluorescein clearance. Accompanying altered tear function are altered corneal epithelial barrier function, reduced mucin production, and ocular surface inflammation, all phenotypic changes that are observed in human KCS. Based on these findings, we believe that neurturin-deficient mice provide an exciting novel model for the study of the pathogenesis and natural history of dry eye disease.

It is recognized that the ocular surface, the tear secreting glands, the central nervous system and their interconnecting reflex neural pathways function as an integrated functional unit. Tear flow is engendered through stimulation of ocular surface, eyelid, and nasal mucosal trigeminal sensory afferent nerves. Disease or damage of the afferent or efferent arms of the integrated ocular surface lacrimal functional unit are common causes of dry eye in humans. Herpes virus infections and surgical amputation of afferent trigeminal nerves in the

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**FIGURE 8.** Upper panel: representative immunofluorescent staining for IA in the conjunctiva of NRTN+/+ and NRTN−/− mice. Lower panel: conjunctival sections of respective mouse strains counterstained with Hoechst 33342 dye. Abbreviations as in Figure 4.

**FIGURE 9.** IL-1β concentration in tear fluid of NRTN−/− and NRTN+/+ mice aged 2 (n = 10) and 4 (n = 10) months and all ages (n = 20) by ELISA. Data are the mean ± SD.

**FIGURE 10.** A representative zymogram showing MMP-9 protein in the tear fluid of NRTN+/+ and NRTN−/− mice aged 8 to 12 weeks.

**FIGURE 11.** Representative semiquantitative RT-PCR showing increased expression of IL-1β, TNF-α, MIP-2, KC, and MMP-9 mRNA by corneal epithelia in NRTN−/− mice, compared with age-matched NRTN+/+ mice, with GAPDH mRNA as an internal control.
cornea cause dry eye. These include increased concentrations of proinflammatory cytokines in the conjunctival epithelium and tear fluid, and increased concentration and activity of proteases, such as plasmin and MMP-9 in the tear fluid. The proinflammatory cytokines IL-1 and TNF-α are important mediators of inflammation and immunity. IL-1 is a potent inducer of other inflammatory cytokines, such as IL-6 and TNF-α, and of chemokines, such as IL-8. In mice, IL-1 and TNF-α induce the key chemotactic factors, KC and MIP-2. MIP-2 is a homologue of human IL-8 that promotes leukocyte recruitment. IL-1 and TNF-α also stimulate the production of MMP enzymes by epithelial and inflammatory cells. Our results showed that the concentrations of IL-1β and MMP-9 proteins in tear fluid were significantly increased, and the mRNA expression of IL-1β, TNF-α, and MMP-9, as well as chemokines MIP-2 and KC, by the corneal epithelia was dramatically upregulated in NRTN−/− mice, compared with NRNT−/− mice. The increase in these soluble inflammatory mediators was accompanied by increased leukocyte infiltration of the conjunctival epithelium in NRTN−/− mice. These findings indicate that the increased inflammatory mediators in our mouse model parallel those in human dry eye.

In conclusion, neuturin-deficient mice exhibit a phenotype of ocular surface disease and inflammation that mimics human KCS. This model supports the importance of a functional ocular surface-central nervous system-lacrimal gland sensory-autonomic neural network for maintenance of ocular surface health and homeostasis. This model may be a useful tool for studying the mechanism of human dry eye disease.

References


