An ENU-Induced Mutation in the Mertk Gene (Mertk\textsuperscript{nmf12}) Leads to a Slow Form of Retinal Degeneration

Dennis M. Maddox,\textsuperscript{1,2} Wanda L. Hicks,\textsuperscript{1} Douglas Vollrath,\textsuperscript{3} Matthew M. LaVail,\textsuperscript{4} Jürgen K. Naggert,\textsuperscript{1} and Patsy M. Nishina\textsuperscript{1}

**PURPOSE.** To determine the basis and to characterize the phenotype of a chemically induced mutation in a mouse model of retinal degeneration.

**METHODS.** Screening by indirect ophthalmoscopy identified a line of N-ethyl-N-nitrosourea (ENU) mutagenized mice demonstrating retinal patches. Longitudinal studies of retinal histologic sections showed photoreceptors in the peripheral retina undergoing slow, progressive degeneration. The mutation was named neuroscience mutagenesis facility 12 (nmf12), and mapping normalized the critical region to Chromosome 2.

**RESULTS.** Sequencing of nmf12 DNA revealed a point mutation in the c-mer tyrosine kinase gene, designated Mertk\textsuperscript{nmf12}. We detected elevated levels of tumor necrosis factor (Tnf, previously Tnfα) in retinas of Mertk\textsuperscript{nmf12} homozygotes relative to wild-type controls and investigated whether the increase of TNF, an inflammatory cytokine produced by macrophages/monocytes that signals intracellularly to cause necrosis or apoptosis, could underlie the retinal degeneration observed in Mertk\textsuperscript{nmf12} homozygotes. Mertk\textsuperscript{nmf12} homozygous mice were mated to mice lacking the entire Tnf gene and partial coding sequences of the Lta (Tnfr) and Lib (Tnfc) genes.\textsuperscript{2} B6.129P2-Lth/Tnf/J homozygotes did not exhibit a retinal degeneration phenotype and will, hereafter, be referred to as Tnf\textsuperscript{abc−/−} mice. Surprisingly, mice homozygous for both the Mertk\textsuperscript{nmf12} and the Lib/Tnf/J homozygous (Tnf\textsuperscript{abc−/−}) demonstrated an increase in the rate of retinal degeneration.

**CONCLUSIONS.** These findings illustrate that a mutation in the Mertk gene leads to a significantly slower progressive retinal degeneration compared with all other alleles of Mertk. These results demonstrate that Tnf family members play a role in protecting photoreceptors from Mertk\textsuperscript{nmf12} homozygotes from cell death. (Invest Ophthalmol Vis Sci. 2011;52:4703–4709) DOI:10.1167/iovs.10-7077

Proper function of cells in the retinal pigment epithelium (RPE) is required for normal vision and for the survival of photoreceptor cells. Its importance is underscored by the large number of diseases associated with improper function of the RPE, such as macular edema,\textsuperscript{1} Best’s vitelliform macular degeneration,\textsuperscript{2–4} autosomal recessive macular degeneration,\textsuperscript{5} Leber congenital amaurosis,\textsuperscript{6–8} and Stargardt disease.\textsuperscript{9} Multiple instances of retinitis pigmentosa or allied disorders caused by mutations in the MERTK gene have been reported.\textsuperscript{10–14} The Royal College of Surgeons (RCS) rat is a classic model for assessing photoreceptor-RPE interaction.\textsuperscript{15–17} Mapping efforts determined that a deletion within the Mertk gene (Mertk\textsuperscript{C勁}) led to a frameshift and a termination signal 20 codons after the start codon in RCS rats.\textsuperscript{18,19} Additionally, a targeted mutation of an exon encoding a portion of the Mer tyrosine kinase domain in Mertk\textsuperscript{Gken} mice has been reported.\textsuperscript{20} This murine allele closely recapitulates the RCS rat phenotype of rapid photoreceptor degeneration.\textsuperscript{21} Photoreceptor death in these Mertk mutants is associated with an inability of the RPE to properly phagocytize shed photoreceptor outer segment tips.\textsuperscript{22–25}

The work presented here is the first to describe a missense mutation in the murine Mertk gene (Mertk\textsuperscript{nmf12}) associated with retinal degeneration. This mutation exhibits several hallmarks of degeneration reported in other rodent models of Mertk-mediated disease; however, the rate of degeneration is much slower in Mertk\textsuperscript{nmf12} homozygotes than in RCS rats or Mertk\textsuperscript{C勁} homozygous mice. This slower rate of degeneration offers opportunities to better study the progression of photoreceptor loss under conditions of abnormal MERTK function. Additionally, our studies of the Mertk\textsuperscript{nmf12} mutants suggest that Tnf family members help to protect homozygous Mertk\textsuperscript{nmf12} retinas from degeneration, providing novel insight into mechanisms affecting the rate of photoreceptor degeneration in MERTK-mediated photoreceptor cell death.

**MATERIALS AND METHODS**

Mice were bred and housed in standardized conditions in the Research Animal Facility at The Jackson Laboratory and were monitored regularly to maintain a pathogen-free environment. Mice were provided free access to NIH 6% fat chow and acidified water in a vivarium with a 12-hour light/12-hour dark cycle. All mice were treated in accordance with protocols approved by the Animal Care and Use Committee at The Jackson Laboratory, and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Mouse Production and Mapping**

A C57BL/6J-nmf12/J X DBA/2J F2 intercross was generated by the Speed Expansion IVF (http://jaxmice.jax.org/services /speed expansion html) service at The Jackson Laboratory. DNA isolated from tail tips of 13 affected and 13 unaffected mice were pooled and subjected to a genome-wide scan using 92 SSLP markers distributed throughout the genome. Samples used in the DNA pools were tested individually to confirm the map location. The critical region was established by genotyping additional recombinant F2 mice with commercially available MTF markers and single
mixture of ketamine (70 mg/kg) and xylazine (15 mg/kg), and body temperature (36.5°C-37°C) was maintained with a temperature-controlled heating pad. A gold loop electrode was placed on the surface of the cornea and referenced to a gold wire placed in the mouth. A needle electrode in the tail served as ground. Signals were amplified ($\times$10,000; CP511 AC amplifier; Grass Instruments, Quincy, MA), sampled at 0.8-ms intervals, and averaged. Rod-mediated ERGs were recorded from wild-type and mutant mice that were adapted to darkness overnight and exposed to short-wavelength flashes of light in a Ganzfeld dome (LKC Technologies, Inc., Gaithersburg, MD). Light intensities varied over a 4.0 log unit range up to the maximum allowable by the photopic stimulator (PS3/ Plus; Grass Instruments). Cone-mediated responses were obtained with flashes of white light on a rod-saturating background after 10 minutes of light adaptation. Responses were averaged at all intensities, and up to 50 records were averaged for the weakest signals. A signal-rejection protocol was used to eliminate electrical artifacts produced by blinking and eye movements.

Microscopy and Immunohistochemistry

Retinas were fixed in 37.5% methanol/12.5% glacial acetic acid in (1X) phosphate-buffered saline, embedded in paraaffin, and sectioned at 6-μm intervals. The sections were subsequently mounted on slides (SuperFrost Plus; Fisher Scientific, Pittsburgh, PA) and were stained with hematoxylin and eosin. Light microscopy and fluorescence microscopy were performed (DMR microscope; Leica, Wetzlar, Germany), and images were collected (FireCam; Leitz). Brightness and contrast of images compared between wild-type and mutant mice were equally optimized (Photoshop 7.0; Adobe, Mountain View, CA). Primary antibodies included C57BL/6J control, C57BL/6J homozygotes, and Mertk$^{tm1Gkm}$ homozygotes. Analysis of these sequences reveals an A-to-G mutation. (Figure 2B) Sequence chromatograms from control, C57BL/6J, and Mertk$^{tm1Gkm}$ homozygotes. Analysis of these sequences reveals an A-to-G transition at base pair 2237 (A2237G).

nucleotide polymorphisms (SNPs) and was further refined through the generation of additional mapping markers.

Sequencing

Three pairs of eyes isolated from adult Mertk$^{tm1Gkm}$ homozygous and wild-type mice were homogenized in a tissue homogenizer (VirTishear; VirTis, Gardiner, NY) for 2 minutes on ice with buffer (Trizol; Invitrogen, Carlsbad, CA). RNA was isolated according to the manufacturer’s directions, and the total RNA quality was assessed by running a 5-μL aliquot of each RNA sample on a 1% agarose gel. RNA samples were reverse transcribed into cDNA (RETOscript; Ambion, Austin, TX). The Jackson Laboratory’s Allele Typing and Sequencing Facility performed DNA sequencing (Big Dye Terminator chemistry). Data were analyzed with bioinformatics software (MacVector 7.2.3 [MacVector, Inc., Cary, NC] and Sequencher 4.2 [Gene Codes Corporation, Ann Arbor, MI]).

Electroretinography

Full-field ERG recordings were performed as previously described.$^{21}$ Briefly, anesthesia was induced by intraperitoneal injection of a...
mary antibodies were used at the concentration of 1:200. Appropriate secondary antibodies conjugated to either CY3 (Jackson Immunochemicals, West Grove, PA) or Alexa-488 (Molecular Probes, Eugene, OR) were applied at a dilution of 1:500.

**Western Blot Analysis**

Six eyes each from C57BL/6j and C57BL/6-J/Mertk<sup>mmf12/j</sup> homozygous mice were homogenized in 300 μL RadiolImmuo Precipitation Assay (RIPA) buffer containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Protein concentrations were determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA), and 40 μg total protein for each sample was loaded with sample buffer (NuPAGE LDS; Invitrogen) and sample reducing agent (NuPAGE; Invitrogen). Samples were run on a 10% Bis-Tris gel with MOPS/SDS running buffer (NuPAGE LDS; Invitrogen) and sample reducing agent (NuPAGE; Invitrogen). Samples were transferred overnight onto a polyvinylidene difluoride (PVDF) membrane (Roche Diagnostics), and the membrane was blocked for 1 hour in Tris-buffered saline plus 5% milk powder before being probed with a polyclonal antibody against MERTK (af591) (R&D Systems, Minneapolis, MN). After washing, the membrane was probed with peroxidase-conjugated donkey anti-rabbit IgG (1:40,000; AffiniPure; Jackson ImmunoResearch, Inc.) and signal was detected with Western Lightning Chemiluminescent Detection Reagent (Perkin Elmer Life Sciences, Inc., Waltham, MA).

**RESULTS**

**Mapping and Identification of the Mertk<sup>mmf12</sup> Mutation**

By in vitro fertilization, we generated a C57BL/6J-<sup>nmf12</sup>/<sup>nmf12</sup> × DBA/2J F1 intercross, simultaneously producing 468 mice (representing 936 meioses) for mapping. A total of 32 F2 mice were identified with genetic crossovers between flanking markers D2Mit397 (122.56 Mb) and D2Mit307 (130.10 Mb). These 32 crossover mice were genotyped with a combination of MIT markers and SNPs (Fig. 1A), and the critical region was reduced to a 3.65-Mb region between markers D2Mit398 (125.46 Mb) and D2Mit224 (129.11 Mb; Fig. 1B). Within this critical region were 56 annotated transcripts, one of which encodes the Mertk gene. Sequencing of Mertk CDNA from Mertk<sup>mmf12</sup> homozygotes revealed a G-to-A substitution at base pair 2237 (Ensembl build 42: OTTMSU0000016359; Fig. 1C). As further confirmation that the mutation in Mertk was causative of the observed retinal degenerative phenotype in Mertk<sup>mmf12</sup> homozygotes, we performed a complementation test between homozygous Mertk<sup>mmf12</sup> mice and mice carrying a targeted deletion (Mertk<sup>tm1Gkm</sup>) in the Mertk gene. 20 Mertk<sup>mmf12</sup> is not capable of complementing Mertk<sup>tm1Gkm</sup> (Fig. 2), offering strong evidence that the two mutations are allelic.

**Analysis of Mertk<sup>mmf12</sup> Protein Expression**

The missense mutation found in Mertk<sup>mmf12</sup> mutants causes a substitution at amino acid position 716 (Fig. 3A) and is predicted to alter a highly conserved histidine residue to arginine (H716R; Fig. 3B). This residue is located within the tyrosine kinase domain of the MERTK protein (Fig. 3C). Immunohistochemical studies showed that MERTK protein was present and properly localized to the RPE of homozygous Mertk<sup>mmf12</sup> mice (Fig. 3D).

**Analysis of Mertk<sup>mmf12</sup> Retinal Phenotype**

Mertk<sup>mmf12</sup> mutants were first identified by screening ENU-mutagenized mice by indirect ophthalmoscopy. The peripheral retina of Mertk<sup>mmf12</sup> homozygotes, when viewed by indirect ophthalmoscopy, appeared depigmented as early as postnatal day (P) 30 (data not shown). As the Mertk<sup>mmf12</sup> homozygotes age, patches become evident in the central portion of the retina (Fig. 2). Western blot analysis shows a 2.5-fold reduction in MERTK protein (Fig. 3D).

---

**Figure 3. Analysis of Mertk<sup>mmf12</sup> protein expression and function.** (A) Depiction of protein alignment between C57BL/6j and Mertk<sup>mmf12</sup> homozygotes (amino acids presented in randomly selected colors). A substitution of arginine for histidine is evident at amino acid 716 (H716R; arrow). This residue is conserved across species (B). (C) Schematic showing that the mutated amino acid falls within the tyrosine kinase domain of the Mertk gene. Western blot analysis shows a 2.5-fold reduction of MERTK protein in Mertk<sup>mmf12</sup> homozygous retinas (D). The blot was stripped and probed again with an antibody against β-actin as a loading control (E).
retina in addition to those observed in the periphery (Figs. 4A, 4B). When viewed by histology at P30, wild-type mice have normal, well-structured retinas (Fig. 4C). Mertknmf12 homozygous retinas, when viewed at P30, demonstrate thinning of the peripheral outer nuclear layer (ONL; Fig. 4D). As time progresses, photoreceptor cell death increases and the ONL becomes increasingly thinner (Fig. 4E). As the majority of photoreceptors die, a single layer of photoreceptor nuclei is left adjacent to the RPE in the peripheral retina (Fig. 4F). Although the death of photoreceptor nuclei is most dramatic in the peripheral retina, presence of apoptotic nuclei is evident throughout the mutant retina at P45 (Figs. 4G, 4H). Most photoreceptors in the central retina, however, are spared (data not shown), and though electroretinographic (ERG) recordings do become progressively attenuated, ERG a- and b-waves are detectable in Mertknmf12 homozygotes as old as 2 years of age (Fig. 5).

Analysis of TNF Expression in Mertknmf12 Eyes

Studies involving Mertktm1Gkm homozygotes have demonstrated that serum levels of TNF are increased because of an inability of macrophages to phagocytize apoptotic nuclei.20,25 The increased TNF levels are thought to cause cellular damage and lethal tissue injury, and the small intestines of Mertktm1Gkm homozygotes were shown to be damaged as a result of excess TNF. Therefore we investigated whether TNF levels were increased in retinas of Mertknmf12 homozygotes. Immuno-histochemistry with an antibody directed against TNF (ab9739; Abcam) was performed on wild-type (Figs. 6A, 6C) and Mertk nmf12 homozygous (Figs. 6B, 6D) retinas. A marked increase in staining was observed in retinal sections from the Mertknmf12 homozygotes compared with controls.

Genetic Manipulation of Tumor Necrosis Factor Levels in Mertknmf12 Mutant Eyes

To ascertain the effect of increased TNF levels in the retinas of Mertknmf12 homozygotes, we performed a genetic experiment in which Mertknmf12 homozygotes were crossed to Tnfabc−/− homozygotes, and the resultant F1 offspring were intercrossed. Mertknmf12/Tnfabc−/−; Tnfabc−/− double homozygous mice exhibited an increased rate of photoreceptor cell degeneration compared with littermate mice homozygous for the Mertknmf12 mutation alone. At P45, when examined by indirect ophthalmoscopy, a marked decrease in photoreceptor cell density was observed in the peripheral retina of double homozygous mice compared with controls.
moscopy, the central retinas of Mertk<sup>mnf12/mnf12</sup>; Tnfabc<sup>−/−</sup> double homozygotes (Fig. 7B) demonstrated an abnormal appearance across the entire retina, whereas the central retinas of homozygous Mertk<sup>mnf12</sup> mice appeared normal (Fig. 7A). Histologically, at 1 month of age, the thicknesses of Mertk<sup>mnf12</sup> homozygous ONL in the central (Fig 7C) and peripheral (Fig 7D) retina were similar to those observed for Mertk<sup>mnf12/mnf12</sup>; Tnfabc<sup>−/−</sup> mice (Figs. 7E, 7F). At 3 months of age, the central retina of Mertk<sup>mnf12</sup> homozygotes appeared relatively normal (Fig. 7G), whereas slight thinning of the ONL was evident in the peripheral retina (Fig. 7H). Demonstrating an accelerated rate of degeneration, the ONL of Mertk<sup>mnf12/mnf12</sup>; Tnfabc<sup>−/−</sup> double mutant mice was markedly thinned pan-retinally at 3 months of age (Figs. 7I, 7J), with an additional loss of cells in the inner nuclear layer in the peripheral retina.

**DISCUSSION**

We have demonstrated that the nmf12 mutation maps to Chromosome 2 and that the observed phenotype segregates with a point mutation in the Mertk gene. This mutation leads to the replacement of a conserved histidine residue, within the MERTK tyrosine kinase domain, with arginine. The failure to complement the Mertk allele and the reduced levels of MERTK protein in Mertk<sup>mnf12</sup> homozygotes provide strong evidence that the missense mutation detected in the Mertk gene causes the observed retinal phenotype.

The ONL of Mertk<sup>mnf12/mnf12</sup> homozygotes is almost completely normal at P20. Soon afterward (P25), the ONL becomes significantly thinned throughout the entire retina. Most photoreceptor nuclei are missing by P45, and no ERG activity is readily discernible after P40.21 In contrast, retinal thinning proceeds slowly from the periphery toward the central retina in Mertk<sup>mnf12</sup> homozygotes, and normal ONL thickness is preserved in the central retina of Mertk<sup>mnf12</sup> homozygotes as old as 2 years of age. Because of this conservation of the central retina, ERG measurements are also recordable up to 2 years of age in Mertk<sup>mnf12/mnf12</sup> homozygotes.

The Mertk<sup>mnf12</sup> allele was generated by targeting an exon that encodes essential portions of the tyrosine kinase domain of MERTK.20 Whether the difference in the rate of retinal degeneration was due to allelic variance or genetic background is uncertain because the nmf12 allele arose on a mutagenized C57BL/6j background, whereas the Mertk<sup>mnf12/Glem</sup> mutant allele was targeted in a 129/Sv background and was maintained on a mixed genetic background of C57BL/6j and 129/Sv. It is interesting to note as well that the Mertk<sup>mnf12/Glem</sup> allele was not intended to be a null allele.20 Given that the rate of retinal degeneration in Mertk<sup>mnf12/Glem</sup> mutant mice is rapid and comparable to that seen in the rat Mertk<sup>M449</sup> null allele and that no truncated protein is detectable in kidney or retina samples from Mertk<sup>mnf12/Glem</sup> mutant mice, the Mertk<sup>mnf12</sup> allele does...
appear to be null.  The Mertk<sup> tm1Gkm </sup> allele also affects the tyrosine kinase domain of MERTK but is a point mutation. In contrast to what is seen in the Mertk<sup> tm1Gkm </sup> mutant mice, MERTK protein is detectable in retinal samples of Mertk<sup> tm12 </sup> mutant mice, though at a reduced amount compared with wild-type controls. Thus, the Mertk<sup> tm12 </sup> mutant protein may retain some functional activity and thereby cause a slower rate of photoreceptor cell loss than that observed in previously described models. After the work of Camenisch et al., who reported increased serum TNF levels in Mertk<sup> tm1Gkm </sup> and associated this accumulation with tissue damage in the small intestine, we undertook to determine whether TNF levels were increased in the retinas of Mertk<sup> tm12 </sup> homozygous mice. Our immunohistochemical studies suggest that TNF levels are increased in the retinas of Mertk<sup> tm12 </sup> homozygous mice compared with those of wild-type controls. TNF is a cytokine that produces paradoxical effects in various instances. The role of TNF in uveoretinitis has been studied in depth, with increased TNF levels most often preceding tissue damage. Similarly, in many other types of retinal disease, increased levels of TNF are associated with increased retinal degeneration. Because of this, TNF inhibitory antibodies (infliximab, etanercept) have been developed and are being used in trials to treat human diseases such as age-related macular degeneration, Eales disease, neurosarcoidosis, various forms of ocular inflammation, diffuse subretinal fibrosis syndrome, and glaucoma. As has been noted and as our present study underscores, there are instances in which TNF plays a poorly understood protective role in retinal degeneration. It has been posited that in retinas of Mertk<sup> 500 547 </sup> rats, the RPE secretes a factor that might attract phagocytic cells, such as microglia, to help restore an environment more conducive to photoreceptor survival. Our studies suggest that TNF may be this predicted factor. That the loss of TNF function could potentially lead to deleterious effects should certainly be kept in mind when administering TNF inhibitors to human patients, and Mertk<sup> tm12 </sup> mutant mice offer a powerful tool to better illuminate the protective role TNF can play in various forms of retinal degeneration.

Acknowledgments

The authors thank Jeanie Hansen for excellent technical assistance and The Jackson Laboratory’s Fine Mapping Service for performing the initial steps in the mapping process.

References

8. Camenisch TD, Koller BH, Ehrlich A, Mertk<sup> tm1Gkm </sup> mutant mice, our immunohis-


