Pathophysiology of the Optic Neuropathy Associated With Friedreich Ataxia

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Objectives: To describe the optic neuropathy associated with the genetic defect in Friedreich ataxia and suggest a pathophysiologic mechanism.

Methods: An experimental model of retinal ganglion cell death in the presence of metal chelation was used to test a hypothetical mechanism for the optic neuropathy of Friedreich ataxia.

Results: Study of cultured rat retinal ganglion cells suggests that abnormal regulation of intracellular iron levels could increase sensitivity to reactive oxygen species and lead to cell death in these metabolically active tissues.

Conclusion: We hypothesize that decreased expression of frataxin, the mutated gene in Friedreich ataxia, could cause an optic neuropathy by increasing the sensitivity of retinal ganglion cells to oxidative stress.

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FRIEDREICH ATAXIA is an autosomal recessive neurodegenerative disease characterized by progressive limb and gait ataxia, loss of deep tendon reflexes, weakness of the legs, dysarthria, and hypertrophic cardiomyopathy. Ophthalmic manifestations of Friedreich ataxia include optic neuropathy and, less commonly, a retinitis pigmentosa–like syndrome.1–3 Patients with this disease also have an increased incidence of diabetes mellitus. Symptoms usually begin during childhood and worsen with age. These patients are most often wheelchair bound by the third decade of life and usually do not survive past early adulthood. The incidence of this disease is about 1 in 50000 live births. The genetic defect in most patients with Friedreich ataxia was discovered in 1996 to be a trinucleotide expansion in frataxin, a gene on chromosome 9.4

Increased numbers of intronic GAA repeats reduce frataxin messenger RNA transcription, eventually leading to abnormal flux of intracellular iron, particularly, elevation of intramitochondrial ferric ion.5,6 One possible mechanism by which abnormal iron processing could cause cell death is if elevated iron levels in retinal ganglion cell (RGC) mitochondria increased levels of reactive oxygen species and consequent apoptosis.7 A second possibility is that the decrease in intracytoplasmic iron is responsible for increased susceptibility to oxidative stress. The latter mechanism would accord with studies in transgenic animals, which show that the accumulation of iron is a late event in the pathogenesis of cardiac and central nervous system abnormalities.8 To study this, we perturbed iron levels in cultured rat RGCs. Based on their susceptibility to oxidative stress experimentally induced by iron chelation in vitro, we suggest a pathophysiologic mechanism for the optic neuropathy of Friedreich ataxia.

METHODS

RGC LABELING AND CULTURE

Newborn litters from Long-Evans rats were used for cell culture experiments, which were performed in accordance with Association for Research in Vision and Ophthalmology and institutional, federal, and state guidelines regarding animal research. Retinal ganglion cells were labeled and cultured using previously described methods.6 Briefly, ganglion cells were retrogradely labeled by stereotactic injection of the fluorescent tracer, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI), dissolved in dimethylformamide into the superior colliculi of anesthetized postnatal day

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2 to 4 Long–Evans rats. At postnatal day 7 to 9, the animals were killed by decapitation, the eyes enucleated, and the retinas dissected free in Hank's balanced salt solution. After 2 incubations in Hank's balanced salt solution containing papain (12.5 U/mL), each for 30 minutes at 37°C, the retinas were gently triturated with a Pasteur pipette and plated on poly-L-lysine–coated 96-well flat-bottom tissue culture plates (0.32-cm² surface area per well) at a density of approximately 2000 cells/mm². The cells were cultured for 24 hours in either serum-containing medium (Eagle’s minimal essential medium with 0.7% methylcellulose, 2 mM of glutamine, 1 µg/mL of gentamicin, 22.5 mM of glucose, and prescreened fetal calf serum) or serum-free medium (neurobasal-A containing 0.7% methylcellulose, 2% B27 supplement without antioxidants, and 1 µg/mL of gentamicin).

**GANGLION CELL TREATMENT, IDENTIFICATION, AND COUNTING**

Cultures were treated with 1 or more of the following at the time of plating: FeSO₄ (2–10 mM), FeCl₃ (2–10 mM), CuSO₄ (5 mM), CoCl₂ (5 mM), MnCl₂ (5 mM), MgCl₂ (5 mM), ascorbate (450 µM), the ferric ion chelator, deferoxamine (150 µM), the copper chelator, 2,3,2 tetramine (10 µM), or combinations thereof. Ascorbate was used to indirectly generate reactive oxygen species stress. Concentrations were chosen based on published use in cultured cells or our previous experience in RGC cultures. Twenty-four hours later, RGC viability was determined. Cells were incubated in a 1-µM solution of calcine-AM in phosphate-buffered saline for 20 minutes, after which the medium was replaced with fresh phosphate-buffered saline. Retinal ganglion cells were identified by the presence of retrogradely transported cytoplasmic DiI, which appears red-orange when viewed with rhodamine filters. Cell viability was determined by metabolism of calcine-AM, which produces green fluorescence when viewed with fluorescein filters. The survival of RGCs was determined by identifying the percentage of DiI-positive cells that were also calcine-positive in 5 low-power fields. Survival of retinal cells other than RGCs was determined by counting 50 DiI-positive cells per well. Wells were counted in duplicate. Results are expressed as mean±SEM, based on counts of all fields per condition. Statistical comparison of 2 group means was by the unpaired *t* test, based on counts of all fields per condition. Statistical comparison of 2 group means was by the unpaired *t* test, and of more than 2 group means by analysis of variance.

**RESULTS**

**RGC VIABILITY IN THE PRESENCE OF FERROUS OR FERRIC IRON**

Decreased frataxin levels result in the elevation of intramitochondrial ferric ions. Abnormally elevated iron in RGC mitochondria could cause increased levels of reactive oxygen species and consequent apoptosis. Alternately, a decrease in intracytoplasmic iron could be responsible for the increased susceptibility to oxidative stress. Because it was not possible to directly perturb the intramitochondrial iron levels, we studied the effect of elevated extracellular iron levels on RGC death. To do this, we cultured RGCs in the presence of reduced Fe⁺⁺ and oxidized Fe+++ ions, as well as other transition metals (Cu++, Co++, or Mn+++). We used calcine-AM as a viability indicator, which does not depend on whether the cell dies by apoptosis or necrosis. We found that RGC survival in the presence of 2 to 10 mM Fe⁺⁺ and Fe+++ was significantly reduced (Figure 1). Retinal ganglion cell survival was significantly greater than the survival of other (non-RGC) retinal cells with either Fe⁺⁺ (P = .006 by analysis of variance) or Fe+++ (P = .007). There was no RGC survival with 5 mM of CuSO₄, CoCl₂, or MnCl₂.

**OXIDATIVE STRESS AND INTRACELLULAR FERRIC ION**

To test the second hypothesis, ie, a decrease in intracytoplasmic iron could be responsible for increased susceptibility to oxidative stress, we exposed mixed rat retinal cultures containing retrogradely labeled RGCs to oxidative stress in the presence of the cell-permeable ferric ion chelator, deferoxamine. We first showed that iron chelation alone did not cause RGC death (Figure 2). We then used ascorbate in the presence of serum-containing medium to generate an oxidative stress, which
we had previously shown to occur via intracellular generation of peroxide.9 We found decreased RGC viability with ascorbate alone, compared with controls (50.8% ± 6.9% vs 100.0% ± 2.6%; P = .003) (Figure 3). This was in accordance with our previous observations and in contrast with the complete lack of viability of ascorbate-treated non-RGC retinal cells (data not shown). However, there was no RGC survival in the presence of ascorbate and defereroxamine (150 µM) compared with ascorbate alone (0.0% ± 0.0% vs 50.8% ± 6.9%; P = .002). There was also no RGC survival when cells were cultured in ascorbate and the cell-permeable copper ion chelator, 2,3,2-tetramine (10 µM). Similar results were seen with the copper chelator, diethyldithiocarbamate (1 µM; data not shown).

**COMMENT**

Friedreich ataxia results from a trinucleotide repeat expansion within a region (X25) of the frataxin gene, located on chromosome 9q13. Normal alleles of the frataxin gene have less than 80 GAA repeats in the first intron of the frataxin gene. The GAA repeat expansion within this region leads to DNA structural alterations that interfere with transcription,10 resulting in low levels of frataxin messenger RNA. The severity of the disease and the age at onset are correlated with the number of repeats. The greater the expansion of the smaller allele, the earlier the onset and the greater the severity of the disease.11,12

The mechanism by which decreased frataxin messenger RNA expression leads to pathologic changes in a restricted group of tissues is the subject of active investigation. The frataxin protein product is targeted to mitochondria by an N-terminus domain.13 Yeast that are engineered to be deficient in a frataxin homologue (YFH1) accumulate iron in their mitochondria.5,6 Patients with Friedreich ataxia have elevated levels of iron in their hearts.14 It is therefore believed that the frataxin gene product directly or indirectly regulates intracellular iron levels. Transgenic mouse models have confirmed that decreased activity of iron- and sulfur-dependent enzymes occurs early, followed by mitochondrial iron accumulation. Although knockout of the frataxin gene causes embryonic lethality,15 conditional gene knockouts have established that experimental animals with frataxin deficiency in the heart or nervous system have decreased electron transport complex activity, which leads to an accumulation of iron in the mitochondria.8

Several lines of evidence support a role for frataxin in mitochondrial respiration and the propensity for oxidative damage. Phosphorus magnetic resonance spectroscopy of skeletal muscle from patients with Friedreich ataxia shows decreased mitochondrial adenosine triphosphate production.16 Overexpression of frataxin in cultured cells increases mitochondrial respiration.17 Yeast that are deficient in a frataxin homologue (YFH1) are more sensitive to oxidative stresses and damage5,18 as are fibroblasts from patients with Friedreich ataxia.19 The mechanism by which mitochondrial iron overload could lead to increased sensitivity to oxidative stress is probably through the Fenton reaction, in which reduced transition metals, eg, ferrous (Fe²⁺) or cuprous (Cu⁺) ions donate electrons to hydrogen peroxide to yield hydroxyl free radicals. Friedreich ataxia fibroblasts are more sensitive to ferric ions than are control fibroblasts, but equally sensitive to ferrous ions.20 Also, Friedreich ataxia lymphoblasts are very sensitive to oxidative stress, and they are rescued by transfection with the normal frataxin gene.21

However, this mechanism by itself would not explain why there is a type of optic neuropathy associated with Friedreich ataxia.22 An alternative mechanism is suggested by our experimental findings, which demonstrate the sensitivity of RGCs to iron or copper depletion in the setting of ascorbate-induced oxidative stress. We hypothesize that frataxin deficiency decreases intracellular iron and indirectly activates apoptosis by shifting the redox potential. Retinal ganglion cells are relatively resistant to peroxide-induced oxidative stress but are highly redox sensitive.7 Deferoxamine has been shown to increase oxidative stress in erythrocytes via an extracellular mechanism.23 The mechanism by which this occurs is not clear, although there is evidence that chelating copper ions can also induce oxidative stress.24 The presence of the extended GAA repeat, leading to reduced frataxin expression, will decrease intracytoplasmic iron levels25 and shift the RGC redox state out of its optimum set point, thereby leading to early death and consequent optic neuropathy. Because RGCs, like other cells, have mechanisms for managing reactive oxygen species (eg, reduced glutathione), it is not surprising that patients would be relatively normal early in life but that cumulative damage in the retina would gradually occur, leading to loss of vision. This theory is partly supported by the known occurrence of retinopathy in patients taking deferoxamine to achieve systemic iron chelation.20 Although in this clinical setting, retinal damage is not specific to RGCs, and the time course is typically much more rapid than in Friedreich ataxia, possibly accounting for the relatively greater outer retinal damage.

It is unclear why overt optic neuropathy is not more commonly reported in patients with Friedreich ataxia.
We have found that patients with Friedreich ataxia and optic neuropathy have had very large numbers of GAA repeats, and it is possible that patients with fewer repeats have frataxin levels that are associated with less RGC death and, therefore, less apparent visual dysfunction. Alternatively, it is plausible that optic neuropathy is a frequent occurrence in Friedreich ataxia but that the mildness and gradual nature of its onset, coupled with concurrent maculopathy, has led to infrequent diagnosis by physicians.

In summary, optic neuropathy can occur in genetically identified individuals with Friedreich ataxia. The mechanism by which this occurs is uncertain but may involve redox changes driven by decreases in intracytoplasmic iron, increases in intramitochondrial iron, or both. Therapy for the optic neuropathy of Friedreich ataxia may turn out to be similar to that of other end organs, eg, reactive oxygen species scavengers, but iron chelators could theoretically be harmful. Alternatively, specific features of RGC iron biochemistry may lead to therapies that are tissue specific and possibly more efficacious.

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REFERENCES


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