

Night blindness in Sorsby's fundus dystrophy reversed by vitamin A

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Sorsby's fundus dystrophy (SFD) is an autosomal dominant retinal degeneration caused by mutations in the tissue inhibitor of metalloproteinases-3 (*TIMP3*) gene. Mechanisms of the visual loss in SFD, however, remain unknown. In a SFD family with a novel *TIMP3* point mutation, we tested a hypothesis that their night blindness is due to a chronic deprivation of vitamin A at the level of the photoreceptors caused by a thickened membrane barrier between the photoreceptor layer and its blood supply. Vitamin A at 50,000 IU/d was administered orally. Within a week, the night blindness disappeared in patients at early stages of disease. Nutritional night blindness is thus part of the pathophysiology of this genetic disease and vitamin A supplementation can lead to dramatic restoration of photoreceptor function.

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Sorsby's fundus dystrophy (SFD) is an autosomal dominant retinal degeneration in which patients first complain of night blindness, then experience central visual loss from subretinal neovascularization and haemorrhage, and later lose peripheral vision¹⁻⁶. SFD is a relatively rare disease but it commands considerable interest because it is the only genetic disease in which haemorrhagic macular degeneration occurs in the majority of affected patients (Fig. 1a). Haemorrhagic macular degeneration also occurs in age-related macular degeneration (ARMD), the leading cause of blindness in the western world in individuals over 50 years of age⁷.

Although the exact pathophysiology in SFD remains unknown, some clues have been gathered. One clue came from a morphological study of a retina from an eye donor with SFD which showed an abnormal lipid-containing deposit interposed between the photoreceptors and their blood supply, the choroid⁸. This subretinal deposit, located within Bruch's membrane and present across the entire retina, was suggested as a possible barrier to diffusion of nutrients to the photoreceptors⁹. More recently, the disease-causing gene in two SFD families was discovered to be tissue inhibitor of metalloproteinases-3 (*TIMP3*) and it was speculated that mutations could lead to the abnormal subretinal deposit by disturbing the balance between buildup and breakdown of the extracellular matrix¹⁰.

A testable hypothesis about mechanism in SFD is that the subretinal deposit acts as a diffusion barrier for entry of sufficient vitamin A into the photoreceptors. This could cause a local deficiency of this vitamin and lead to the symptom of night blindness in SFD^{8,9}. Vitamin A is a critical part of the visual cycle¹¹ and deficiency in vitamin

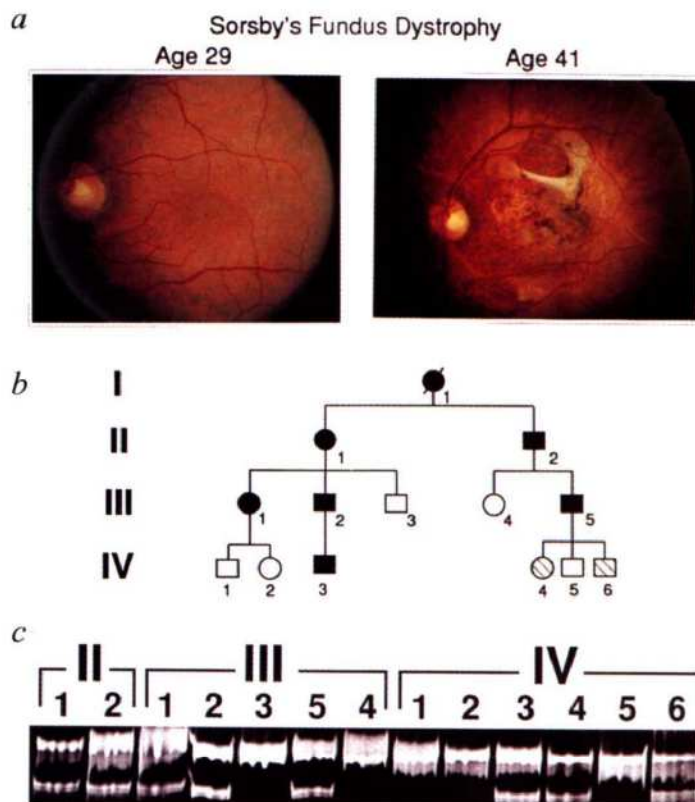
A is well known to cause night blindness and eventual death of photoreceptors¹²⁻¹⁵. Here we present evidence that supplemental vitamin A can restore night vision to SFD patients, thus supporting the notion that nutritional deprivation of photoreceptors may be a part of the pathophysiology of this genetic disease.

Genotype and phenotype of SFD

The four-generation family with SFD in this study is shown in Fig. 1b. Affected patients have a novel single nucleotide substitution in the first position of codon 167 in *TIMP3* which creates a new *Bbv1* recognition site. This allows the mutation to be detected by the appearance of a 148-bp band when exon 5 PCR products are digested with *Bbv1*. All six clinically affected patients carry this mutation as do two clinically unaffected patients (Fig. 1c).

The phenotype of SFD is illustrated in Fig. 2a-e. Visual function test results from representative heterozygotes for this *TIMP3* mutation demonstrate that there is an age-related penetrance of the disease. Patient IV-4 (age 20) has no visual symptoms, a normal map of rod sensitivity across the field of vision (Fig. 2a, left), and a normal profile of rod sensitivity across the central retina (Fig. 2a, right). The map of patient III-5's night vision at age 38 (Fig. 2b, left), however, shows about one log unit of rod sensitivity loss at most tested loci. This patient had a ten year history of night vision complaints. On clinical examination, there were a few drusen-like lesions in the macula but no subretinal neovascularization. The profile test, however, reveals to one side of fixation a profound scotoma (blindspot) for rod function in which sensitivity is reduced by more than four log units (Fig. 2b, right). Patient III-1, age 60, had night blindness and macular

Fig. 1 a, Ophthalmoscopic appearance of a SFD patient (III-5) with the *TIMP3* gene mutation at age 29, when his retina showed only drusen-like lesions in the macula, and at age 41, when it shows scarring and degeneration typical of this disease. **b**, Pedigree of the autosomal dominant SFD family in this study. Symbols: filled, clinically and molecularly affected; shading, molecularly affected but clinically unaffected to date; open, unaffected. **c**, *Bbv1* digestion of exon 5 PCR products. The substitution of T for G in the first position of codon 167 of the *TIMP3* gene creates a new recognition site for the enzyme *Bbv1*. The presence of this mutation can therefore be demonstrated by the appearance of a 148-bp restriction fragment (lower band) in addition to the 178-bp fragment present in unaffected individuals. The pedigree numbers above each gel lane correspond to the drawing in panel **b**. Note that all clinically affected patients have the mutation.



degeneration for decades. Rod sensitivity loss is about one log unit in the peripheral field (Fig. 2c, left) and there is an extensive central scotoma (Fig. 2c, right). The typical abnormality of dark adaptation in SFD⁹ is present in data from Patient III-5 at age 43 (Fig. 2d). His rod-cone break occurs at nearly 35 minutes compared to 12 minutes for Patient IV-4 who had a normal function; baseline pre-bleach dark-adapted rod sensitivity returned by 70 minutes compared to 50 minutes normally. The dashed curve, which closely follows the data from SFD Patient III-5, represents results from a patient with chronic vitamin A deficiency due to liver disease¹⁶. The complaints of night blindness and the rod sensitivity losses on dark-adapted perimetry in the SFD patients are also accompanied by abnormally reduced amplitudes of the rod electroretinogram (ERG) b-wave (Fig. 2e). Only the 20-year-old patient falls within the normal limits for this ERG amplitude parameter and there appears to be a decline of rod ERG b-wave amplitude with age in SFD.

Effect of supplemental vitamin A in SFD

Vitamin A was administered to four heterozygotes with the *TIMP3* mutation from this family (age range, 20–60 years). Patient IV-4, age 20, had no symptoms and a normal eye examination. Patient IV-3, age 27, had a normal eye examination but had recently started to notice night blindness. Patients III-5 and III-1, ages 43 and 60 respectively, had longstanding night blindness and macular degeneration. None of the patients had liver, bowel or other diseases that could cause vitamin A deficiency and their fasting serum levels of vitamin A were normal before the trial began. After baseline measurements of visual function were obtained, a course of 50,000 IU/d of vitamin A (Aquasol A, Astra; Westborough, MA) was given for

one month and visual function was monitored.

Maps of rod sensitivity losses before and after supplementation of vitamin A in patients IV-3 and III-5 are shown in Fig. 3a. Pre-treatment baseline examination of both patients (left panels) showed rod sensitivity losses in the peripheral field; patient III-5 also had a large central scotoma and a retinal scar secondary to subretinal haemorrhage. At the end of a month of supplementation, peripheral rod sensitivity was normal or nearly normal in both patients (Fig. 3a, right panels). The more detailed time course of this improvement is shown in Fig. 3b. Average rod sensitivities across the field are plotted pre- and post-treatment for all four patients. After only four days on supplemental vitamin A, patient III-5 showed an increase in rod sensitivity to within the normal limits. For patient IV-3, the results were normal by day eight, the first time a measurement was made post-treatment. Both patients III-5 and IV-3 had a sensitivity difference of 0.8 log units between pre- and one month post-treatment measurements and this was significantly different in both cases ($P < 0.001$; paired t-test). Eight normal subjects were tested between two and four times using the same protocols as those used in the patients. A variance component analysis¹⁷ determined that 0.09 log units was the upper 95% limit of the standard deviation due to intertest variability. A chi-squared test¹⁸ demonstrated that the change observed in patients III-5 and IV-3 could not be due to normal intertest variability. Patient IV-4 had normal results at baseline and showed no greater variability between visits than normals. Intervisit variability is represented with bars on the last data point of the three patients who were normal after supplementation. Patient III-1, the patient with most longstanding disease, showed greater than normal intervisit variability with both

increases and decreases in sensitivity while on treatment. However, her peripheral rod sensitivity did not improve while on vitamin A; the 0.06 log unit increase in peripheral sensitivity that she achieved after 28 days on the supplemental vitamin A was not significantly different from the baseline measurement ($P=0.08$). Her serum levels of vitamin A increased while on the supplement indicating that she was absorbing the vitamin into the circulation.

Dark adaptation was measured before and after vitamin A supplementation in patients IV-3 and IV-4 (Fig. 3c), the two patients with steady central fixation and the ability to perform the test reliably. The characteristic adaptation abnormality of SFD (Fig. 2d) was present in patient IV-3 and when tested again after eight days on vitamin A, the function had improved. The rod-cone break became 30 rather than 40 minutes and time to final dark-adapted sensitivity improved from nearly 80 minutes to about 60 minutes. Repeated measurements at days 9, 10, 11, 13, 15, 17, 20 and 31 while on therapy showed no further change. As a comparison, patient IV-4, who had normal dark adaptation, showed no difference in measurements made

pre- and post-vitamin A supplementation. Rod ERGs recorded at different intensities of light before and after treatment in Patients IV-3 and IV-4 indicate that there was an increase in maximum b-wave amplitude post-treatment in patient IV-3 but not in patient IV-4 (Fig. 3d). This ERG result in patient IV-3 is consistent with recovery of sensitivity in a proportion of rod photoreceptors, as was evident on the perimetric testing¹⁹.

Macular rod dysfunction and vitamin A dosage

We noted in patient IV-3 that despite the rapid increase in peripheral rod sensitivity to normal while on vitamin A, a deep rod scotoma persisted in the central field (Fig. 4a). This observation was prompted by the patient's complaints of a persistent defect in his 'straight ahead' night vision. After about 20 days on the treatment, however, the scotoma decreased in size and finally disappeared (Fig. 4a), as did all symptoms of night blindness. Reducing the dosage of vitamin A to 5,000 IU/d for a month led to a reappearance of the central rod scotoma and the symptoms (Fig. 4b, day 0). In contrast, peripheral sensitivity was maintained on this lower dose. After a further course of 50,000 IU/d for

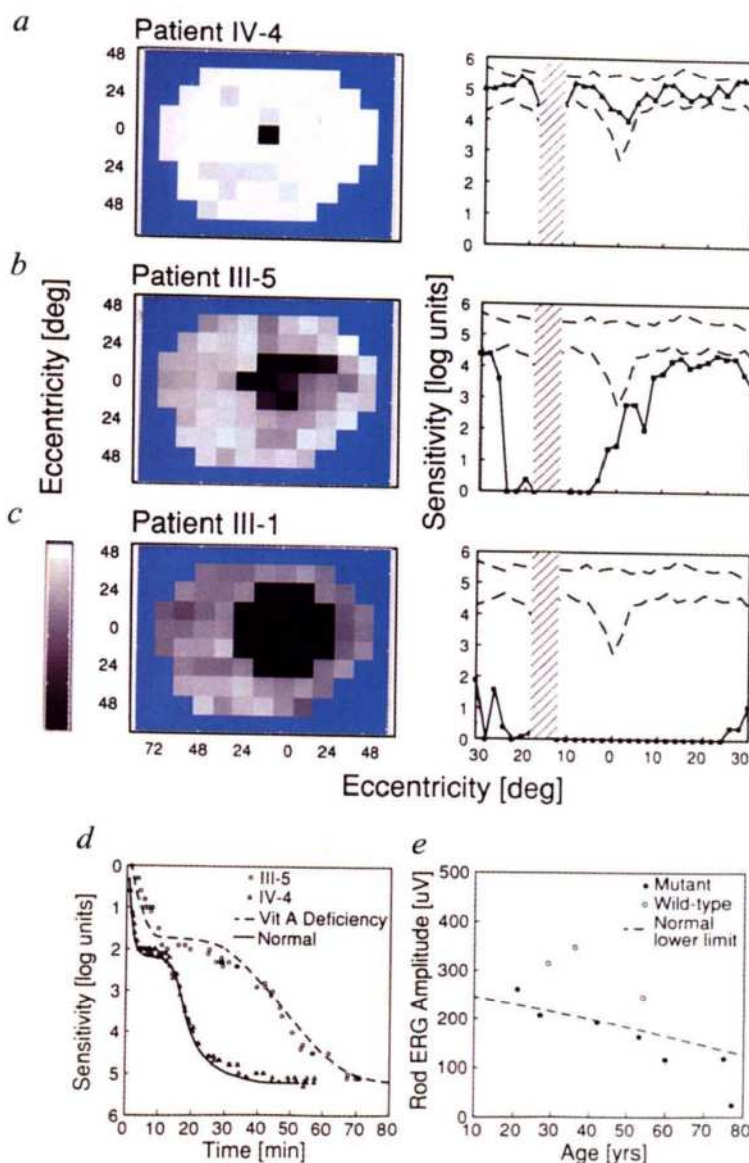


Fig. 2 Phenotype of heterozygotes with the codon 167 *TIMP3* mutation. **a**, Patient IV-4, age 20, shows no significant rod sensitivity loss in the visual field (left) and normal central rod function on the profile test (right). **b**, Patient III-5, at age 38, has not yet developed subretinal neovascularization; there is about 1 log unit of rod sensitivity loss in the peripheral visual field (left) but far greater deficits centrally. The profile shows a greater than 4 log unit rod scotoma temporal to fixation (right). **c**, Patient III-1, age 60, had macular subretinal haemorrhage and scarring years before; there is an extensive central scotoma and the peripheral field shows about 1 log unit of rod sensitivity loss (left). The profile test shows almost no detection of stimuli in the central 60 degrees (right). Grey scales have 16 shades representing sensitivity losses of 0 to 4.0 log units for rods. Dashed lines in the profiles indicate ± 2 s.d. from the mean normal sensitivities for the 500-nm stimulus. Physiological blindspot is shown as a black square at 12 degrees in the temporal field in the maps and as the shaded bar on the profiles. **d**, Dark adaptation after a full bleaching exposure in two SFD patients (symbols) with the *TIMP3* gene mutation compared with results from a patient with vitamin A deficiency from chronic liver disease (dashed line¹⁹) and normal controls (solid line). **e**, Rod ERG amplitude as a function of age for seven heterozygotes for the *TIMP3* mutation (filled circles) in the SFD family and three of their mutation negative family members (unfilled circles). The dashed line represents the lower limit of the 95% prediction interval for a population of 37 normal subjects.

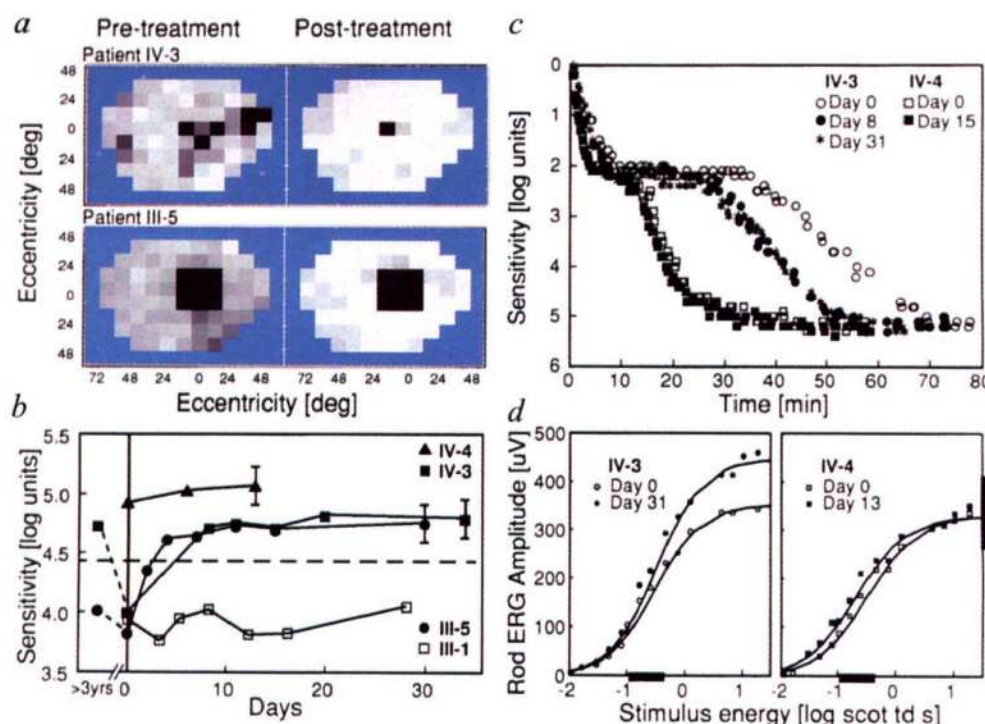


Fig. 3 Effect of vitamin A supplementation on rod function in four SFD family members with the *TIMP3* mutation. **a**, Maps of rod sensitivity loss across the visual field before (left) and after (right) one month of supplementation with 50,000 IU/d of vitamin A in patient IV-3 (upper maps) and patient III-5 (lower maps). Gray scales same as in Fig. 2. **b**, Mean rod sensitivity across the visual field as a function of days on supplementation with 50,000 IU/d of vitamin A in four SFD patients. In Patients IV-3 and III-5, data from tests performed 3–4 years previously were available and are plotted to show the natural history of the disease. Dashed line across the graph is -2 s.d. from mean normal sensitivity, thus defining the lower limit of normal results. Bars on the patient data indicate ± 2 s.d. due to intervisit variability. **c**, Dark adaptometry before and after supplementation with vitamin A at 50,000 IU/d in patients IV-3 and IV-4 at a locus 30 degrees in the peripheral field. Supplementation of patient IV-3 shortens the times to reach rod-cone break and pre-bleach sensitivity while supplementation of patient IV-4 leads to no change in dark adaptation kinetics. **d**, Rod ERG b-wave amplitude versus stimulus energy functions before and after treatment in patients IV-3 and IV-4. Error bar on vertical axis is the mean normal maximum amplitude (V_{max}) minus 2 s.d.; bar on horizontal axis is mean normal stimulus energy at half maximum amplitude (K) ± 2 s.d.

15 days, the central rod scotoma disappeared again and the patient experienced a subjective improvement (Fig. 4b). The time course of change in rod sensitivity in the central retina of patient IV-3 on different dosages of vitamin A is summarized in Fig. 4c. It is of interest that the recovery in macular rod sensitivity appeared to lag behind that of the periphery by several days and, once achieved, could not be maintained on a lower dose of the vitamin. Foveal cone sensitivity was normal throughout this period of more than 80 days while rod function was varying by log units.

Discussion

We have administered supplemental vitamin A to SFD patients with a novel *TIMP3* mutation and monitored their visual function to test the hypothesis that at least part of the visual disturbance in SFD may be due to a form of nutritional deprivation at the level of the photoreceptor. The restoration of rod visual function to normal while on supplemental vitamin A in some of these patients supports the hypothesis. The relatively rapid time course of recovery in the patients resembles the time courses described for recovery of rod function with supplementation in chronic vitamin A deficiency in humans and animals^{12–15,21}. The

pattern of recovery of dark adaptation, albeit incomplete, is also like that in response to vitamin A supplementation in chronic vitamin A deficiency^{13,16,21}. A similarly dramatic and rapid reversal of rod function with vitamin A has also been documented in some patients with abetalipoproteinemia (Bassen-Kornzweig syndrome), which leads to malabsorption of vitamin A and retinopathy^{22,23}.

Although the exact mechanisms leading to a vitamin A deficiency in SFD are unknown, it may be the cumulative effect of decades of impaired transport to the photoreceptors and/or deficient local storage of the vitamin, possibly related to the extracellular matrix disturbance caused by the *TIMP3* mutation^{8,10}. Photoreceptors without available vitamin A to combine with the protein opsin lose sensitivity to light and cannot regenerate functional visual pigment (the vitamin A-opsin complex) at the normal rate after light exposure, hence the slowing in dark adaptation. With increased availability of vitamin A to the receptor, there may be increased chance for transport into the receptor and recombination with opsin with attendant increase in sensitivity and rate of adaptation.

Based on this hypothetical scheme, the following explanations can be offered for the patterns of results in the four SFD patients examined. The 20-year-old patient must still retain sufficient ocular stores of vitamin A to be asymptomatic and have normal vision tests. It is of interest that patient IV-3 at age 24 also had normal results (Fig. 3c). The 27- and 43-year-old patients had become vitamin A deficient; their photoreceptor sensitivity losses in peripheral retinal regions, however, could be reversed suggesting that any hypothesized barrier was at least partially permeable to the vitamin. Retained sensitivity in these patients after reducing the dose of vitamin for a possible. On the other hand, central retinal function in the 27-year-old took a few days longer to return to normal in response to vitamin A and normal function was not maintained after the higher dose of vitamin was stopped. Such results could be related to the fact that the central retina has a greater density of photoreceptors with increased demand for the vitamin and higher utilization of stores. The macular retinal pigment epithelium may well be subclinically abnormal in this patient and thereby less efficient at transporting vitamin to the photoreceptors. A rod-cone rivalry theory has been invoked to explain slower rod than cone recovery in the very central retina during treatment of vitamin A deficiency²¹. It is conceivable that such a competition between receptors for available vitamin A is also occurring in our 27-year-old patient

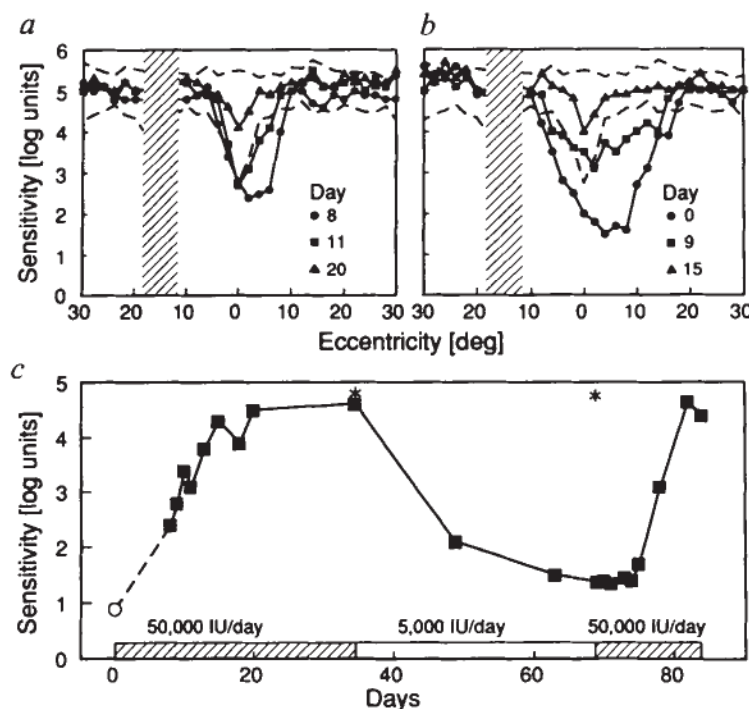


Fig. 4 Effect of different doses of vitamin A supplementation on rod function in the central visual field of Patient IV-3. *a*, Profiles of rod sensitivity in the central field on days 8, 11 and 20 after starting vitamin A supplementation at 50,000 IU/d. *b*, Following a 30 day period of 5,000 IU/d of vitamin A, the scotoma had reappeared. Restarting 50,000 IU/d led to its disappearance by day 15 on the higher dose. *c*, Lowest rod sensitivity in the central scotoma (between 2–8 degrees eccentricity in the nasal field) as a function of days on vitamin A at different dosages. All data points except day 0 (of the first trial) were taken from profile tests, which were performed beginning on day eight. The first datum represents an average of thresholds at 4–6 degree loci from another test strategy (circle connected by dashed lines). The asterisks indicate the mean rod sensitivities across the entire visual field after the first period of vitamin A 50,000 IU/d and the trial at 5,000 IU/d.

with the cones being at an advantage by their density and structure; this could help explain the difference we observed between the rate of peripheral versus central rod recovery and the persistence of normal cone function at the foveal locus during the presence of profound central rod scotomas. The lack of response of the peripheral retina of the 60-year-old patient to the treatment may be due to inadequacy of dose or time on that dose, lesser permeability of the barrier with age and disease progression, or the presence of a photoreceptor degenerative stage that cannot be reversed.

Our study represents a starting point on metabolic intervention in this genetic disease. It demonstrates that a genetic disease can be accessible to pharmacological intervention and that a dramatic short-term reversal in visual dysfunction can occur in a subset of patients selected for their genotype and at a specific stage of disease. It must be emphasized, however, that further *in vivo* and *in vitro* studies of *TIMP3*-associated disease should be performed before the results of the current study can be translated into practical treatments for these and other patients. Among the clinical studies required for vitamin A are trials of different doses between the two extremes used in this study. Long-term use of 50,000 IU/day, for example, has potential toxicity²⁴; lower doses should be studied for effectiveness. Still, given the uniformly poor visual prognosis for patients with SFD, it is quite encouraging that any drug is capable of causing the degree of visual restoration observed in this study, even in the short term.

In future studies, it will be important to determine whether the effects we observed also occur in patients with other *TIMP3* mutations and other inherited retinopathies that may have a similar pathogenesis to SFD (for example, Kuntz, C.A. *et al.*, *Invest. Ophthalmol. Vis. Sci.* 35, S64; (1995)). Once the lowest effective dose of vitamin A is determined, it should be explored whether the effects are long lasting and can alter the natural history of the disease. The ability to detect *TIMP3* gene mutations in

presymptomatic heterozygotes allows prophylactic treatment to be considered, if proven useful.

It is of interest that rod sensitivity and adaptation abnormalities such as those found in SFD have recently been described in some patients with ARMD²⁵. This raises the possibility that SFD and ARMD share some pathophysiological features and that some form of nutritional therapy could eventually prove useful in this extremely common cause of visual loss in the elderly. Unfortunately, it is likely that the constellation of clinical findings recognized as ARMD is caused by many different mechanisms and that no single therapy will be successful in all patients. As the various aetiologies of ARMD are discovered and as patients with each type become reliably identifiable with molecular testing, one or more may share sufficient similarities with SFD to warrant trials of those treatments that are eventually found useful and safe in SFD.

Methods

Clinical examination. The family was diagnosed as having SFD on clinical criteria and followed by one of us (SGJ) with routine ophthalmic examinations and specialized tests of vision for a decade. Blood was collected from 13 family members in generations II–IV for this study. Informed consent was obtained from all participants in this study for all procedures.

SSCP analysis. DNA was prepared from whole blood using a non-organic procedure. Oligonucleotide primers flanking *TIMP3* exon 5 (ref. 10) were synthesized using standard phosphoramidite chemistry (Applied Biosystems model 391 DNA synthesizer). 30 ng of each patient's DNA was used as template in a 8.35 µl PCR containing: 1.25 µl 10× buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, 15 mM MgCl₂, 0.01% w/v gelatin), 200 µM of each dCTP, dATP, dGTP and dTTP, 1 pmole of each primer and 0.25 U *Taq* polymerase (Boehringer Mannheim). Samples were incubated in a DNA thermocycler (OmniGene) for 35 cycles under the following conditions: 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s. After amplification, 5 µl of stop solution (95% formamide, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to each sample. Samples were denatured and electrophoresed on fan-cooled nondenaturing 6%

polyacrylamide gels (5% glycerol and .05X TBE) at 20 W for approximately 4 h at room temperature. Following electrophoresis, gels were silver stained as described^{26,27}. Permanent records were created of all gels with EDF film (Kodak).

DNA sequencing. Primers used in the screening assay were also used for DNA sequencing. PCR-amplified DNA samples were prepared for sequencing by electrophoresis on low melting point agarose gels. Gels were stained briefly with ethidium and the desired band was cut from the gel. DNA was isolated using a commercially available kit (Promega). Purified DNA was sequenced in a DNA thermocycler using a dideoxy-termination kit (Promega). Following 30 cycles of extension at 95°C for 30 s, and 70°C for 30 s, 3 µl of stop solution was added to each sample. Following denaturation, 3 µl of each sample was loaded onto 6% polyacrylamide gels (19:1 acrylamide:bis and 7 M urea). Following electrophoresis, approximately 3 h, gels were dried and exposed to Kodak Xomat film for 24–48 h.

Restriction enzyme digestion. 300 ng of each patient's DNA was used as template in a 50 µl PCR containing: 7.5 µl 10× buffer (same as in SSCP analysis), 1,200 µm of each DCTP, DATP, DGTP and DTPP, 6 pmole of each primer and 1.5 U *Taq* polymerase (Boehringer Mannheim). Samples were incubated in a DNA thermocycler (Perkin Elmer/Cetus) for 35 cycles under the following conditions: 94°C for 30 s, 58°C for 30 s, 72°C for 30 s. 10 µl of the *TIMP3* exon 5 PCR product, 2 U of *Bbv1* (New England Biolabs) and 2 µl NE1 buffer in a 20 µl reaction was incubated at 37°C for 2 h. Following digestion, 3 µl of load dye was added to each tube. Samples were electrophoresed on a nondenaturing 8% polyacrylamide gels at 20 W for approximately 2 h. The gel was stained with ethidium bromide, placed on a UV transilluminator and photographed with Kodak Tri-X film.

Visual function tests. *Static threshold perimetry* was performed in the dark-adapted state using monochromatic stimuli (500-nm and 650-nm stimuli; diameter, 1.7 degrees). Instrumentation, technique, analysis methods, and normal results have been described^{28,29}. The test strategies used in these patients included: a full field test of 75 loci (12-degree grid) and a profile test (2-degree spacing) in the central

60 degrees along the horizontal meridian. Mean rod sensitivities across the visual field were calculated using the 500-nm data; in the case of two of the patients, the number of data points was less than 75 because of extensive central scotomas. For the maps of rod sensitivity losses, calculations were based on comparison of patient data using the 500-nm stimulus with mean values of normal subjects. Tests were conducted in the same order and at the same time of day on serial visits; dark-adaptation time prior to the tests was the same on every visit. A foveal cone threshold to a 600-nm target in the light-adapted state²⁸ was also measured at each test session. *Dark adaptometry* was tested with 500-nm and 650-nm targets (1.7 degrees) according to methods described^{16,30}. Baseline dark-adapted thresholds were determined after more than 3 h of dark adaptation. Recovery of sensitivity was measured after a retinal exposure of 7.8 log scot.td.s which was estimated to bleach about 99% of the rhodopsin present. *Electroretinography* was performed with full-field stimulation using bipolar Burian-Allen contact lens electrodes and described methods²⁹. Rod electroretinograms (ERGs) were elicited with a supra-threshold blue flash (−0.1 log scot.td.s) in the dark adapted state. A rod intensity series was elicited in the dark-adapted state to different intensities of blue flash stimuli. Waveforms were measured conventionally from baseline (or from a-wave trough, when present) to major positive peak. The Naka-Rushton equation was fitted to the measured b-wave amplitudes from the intensity series¹⁹.

Acknowledgements

We thank W.J. Feuer for statistical consultation; D. Azevedo for help with testing; Dr. X.K. Sun and K. Hummer for data analysis; B. Koernig, D. Suner and J. Lee for study coordination. This work was supported by Public Health Service research grants (EY05627, EY10539, EY10564 and EY10900); the Foundation Fighting Blindness; The Chatlos Foundation, Inc.; the Helen Gass Fund for Retinal Degenerations; the Roy Carver Charitable Trust; the George Gund Foundation; and the Groubeck Family Foundation. Dr. Stone is a Research to Prevent Blindness Dolly Green Scholar.

Received 13 April; accepted 19 June 1995.

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