Coats’ disease of the retina (unilateral retinal telangiectasis) caused by somatic mutation in the NDP gene: a role for norrin in retinal angiogenesis

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Coats’ disease is characterized by abnormal retinal vascular development (so-called ‘retinal telangiectasis’) which results in massive intraretinal and subretinal lipid accumulation (exudative retinal detachment). The classical form of Coats’ disease is almost invariably isolated, unilateral and seen in males. A female with a unilateral variant of Coats’ disease gave birth to a son affected by Norrie disease. Both carried a missense mutation within the NDP gene on chromosome Xp11.2. Subsequently analysis of the retinas of nine enucleated eyes from males with Coats’ disease demonstrated in one a somatic mutation in the NDP gene which was not present within non-retinal tissue. We suggest that Coats’ telangiectasis is secondary to somatic mutation in the NDP gene which results in a deficiency of norrin (the protein product of the NDP gene) within the developing retina. This supports recent observations that the protein is critical for normal retinal vasculogenesis.

INTRODUCTION

Coats’ disease of the retina, so-called ‘retinal telangiectasis’ (1,2) is characterized by a defect of retinal vascular development which results in vessel leakage, subretinal exudation and retinal detachment. On retinal fluorescein angiography, areas of capillary non-perfusion and dilatation together with fusiform aneurysms are observed (Fig. 1). Initially the condition is seen within a sector of the retina and at this stage may be associated with normal vision. However, the consequent retinal detachment often leads to progressive visual loss. In its classical form Coats’ disease is almost invariably seen in males and unlike other forms of retinal telangiectasis, such as idiopathic juxtafoveal telangiectasis (3), unilateral. Coats’ disease is isolated in the majority of cases although associations with facioscapulohumeral muscular dystrophy, Senior–Loken syndrome and multiple glomus tumours have been described (4–6).

Norrie disease is an X-linked recessive disorder characterized by congenital blindness, deafness and, in approximately one-third of patients, central nervous dysfunction (7). The ocular findings are characterized by retinal dysplasia and the development of a white, vascularized retrolental mass which may lead to phthisis bulbi (a shrunken appearance of the eyeball). Through a classical positional cloning approach a gene for Norrie disease was identified on Xp11.4. The gene, NDP, comprises three exons, of which the second and third encode a 133 amino acid protein whose tertiary structure is similar to transforming growth factor β (TGF-β) (8–10). The orthologous mouse gene has been identified and encodes a protein which shows 94% similarity to the human polypeptide (11). Investigation of a mutant mouse line demonstrated retinal disorganization with abnormalities of both retinal and hyaloid vasculature (12–14).

A female with a unilateral variant of Coats’ disease gave birth to a son affected by Norrie disease. Both carried a missense mutation within the NDP gene. We subsequently analysed the retinas of enucleated eyes from males with Coats’ disease and in one have demonstrated a somatic mutation which is not present within non-retinal tissue. We suggest that Coats’ telangiectasis is a consequence of localized deficiency of norrin (the protein product of the NDP gene) within the developing retina; this strongly supports the hypothesis that norrin is an important factor in normal retinal vasculogenesis.

RESULTS

Analysis of Norrie disease family ‘OE’

Patient II.2 was diagnosed at the age of 11 years as having a unilateral variant of Coats’ disease with retinal telangiectasis, secondary fibrosis and exudation in a pattern consistent with unilateral ‘peripheral nodular retinal telangiectasis’ (15). Now aged 27 years, she is blind in her right eye and has a secondary cataract that precludes retinal examination. Her left eye and retina remain entirely normal. The Coats’ eye has not been removed. Her second child, a son (III-2), is affected by...
classical Norrie disease and is the first child in her extended family with that diagnosis (Fig. 2). He is blind as a result of bilateral leucocoria and retinal detachment and now aged 2 years has normal hearing and intellectual development.

Analysis of the NDP gene was performed by SSCP/heteroduplex analysis and demonstrated a band of abnormal mobility in both II-2 and III-2. Subsequent analysis confirmed its presence in individual I-2, the mother of patient II-2 (Fig. 2). The abnormally migrating band was not observed in 60 normal control chromosomes and has not been observed by others (16). Direct sequencing demonstrated a missense mutation within the coding region of exon 3, a C→G transversion at position 704 resulting in

Figure 1. Coats’ disease: clinical manifestations. (a) Fundus photograph through a pupil of classical Coats’ disease: total retinal detachment associated with retinal telangiectasis and subretinal exudate. (b) Fundus photograph of peripheral nodular variant of Coats’ disease: tortuous retinal vasculature shows areas of focal dilatation and hemorrhage. (c) Fluorescein angiogram of (b): abnormal retinal vasculature shows areas of capillary non-perfusion and dilatation together with fusiform aneurysm formation. (d) Histopathological section from enucleated eye of patient E317: section shows opposed retina from base of funnel detachment. Retina (r) shows areas of microcystoid degeneration. Grossly dilated retinal capillaries visible in the mid-periphery are not shown. Foamy macrophage (m) infiltration of subretinal space is seen with widespread retinal exudation (e) and development of characteristic cholesterol clefts (c). (H&E stain.)
undertaken to cover the entire NDP mutation screening using small target fragments was Retinal and non-retinal tissue were separated by dissection and Nine enucleated eyes from males with unilateral, classical and painful, at which point enucleation may be considered. Coats' disease can result in an eye becoming blind, unsightly NDP mutation analysis of archival Coats' disease tissue (Fig. 3a), a residue for which another pathogenic missense a cysteine to tryptophan substitution at amino acid 96 (C96W) analysis of the entire eye. No bands of abnormal mobility were documented from an tion confirmed the presence of the mutation within the ampli-

Family OE. This causes the removal of an NaeI site, and diges-

vestibular schwannoma tumour blocks. enucleated eyes, 10 breast cancer tumour blocks and 10 controls were analysed from the retinas of 10 normal male female carriers of, NDP mutations (17–20). Three possible

NDP mutation analysis of archival disease tissue

Coats’ disease can result in an eye becoming blind, unsightly and painful, at which point enucleation may be considered. Nine enucleated eyes from males with unilateral, classical Coats’ disease were identified from our archives (Fig. 1d). Retinal and non-retinal tissue were separated by dissection and mutation screening using small target fragments was undertaken to cover the entire NDP gene. Somatic tissue controls were analysed from the retinas of 10 normal male enucleated eyes, 10 breast cancer tumour blocks and 10 vestibular schwannoma tumour blocks.

In one eye (removed from patient E317), DNA was extracted from two regions of retina as well as non-retinal tissue. SSCP/ heteroduplex analysis demonstrated bands of both normal and abnormal mobility within one of the retinal samples. The abnormal band was not found within non-retinal tissue. The abnormal band was purified, re-amplified and sequenced to reveal a C96W missense mutation (Fig. 3a), identical to that in Family OE. This causes the removal of an NaeI site, and diges-
tion confirmed the presence of the mutation within the amplified product of an independent PCR reaction (Fig. 3b). The mutation was not present within non-retinal tissue of the same eye. No bands of abnormal mobility were documented from an analysis of the entire NDP coding sequence for the remaining Coats’ disease eyes or from the control tissues. Amplification with novel, flanking primers was performed on retinal and non-retinal tissue from eye E317 in the presence of new PCR reagents: this confirmed the presence of the mutation.

FIGURE 2 Pedigree of Family OE. Proband II-2 has unilateral Coats’ disease. Her son (III-2) has classical Norrie disease. Female I-2 is also a carrier of the mutant NDP allele. Half-filled circle, carrier female; quarter-filled circle, Coats’ disease; filled square, affected, Norrie disease.

DISCUSSION

The ocular findings in Norrie disease are characterized by congential blindness secondary to retinal dysplasia and detachment associated with a white, vascularized retrolental mass. Coats’ disease is not a recognized feature in either males with, or female carriers of, NDP mutations (17–20). Three possible mechanisms exist for its manifestation in the female carrier II-2: (i) coincidence; (ii) segmental skewed X-inactivation of NDP on the affected side; or (iii) a second ‘hit’ on the other X-chromosome due to somatic mutation. We sought to test the hypothesis that Coats’ disease might be the result of somatic mutation of the NDP gene. We analysed archival tissue from nine enucleated eyes from males with unilateral Coats’ disease and in one, from patient E317, mutation analysis demonstrated both normal and mutant NDP alleles within the same retina but not within non-retinal tissue. Sampling problems are in part likely to explain the lack of demonstrable mutations within the other eyes: Coats’ disease is a segmental retinal disorder which primarily only affects a small region of the retina and analysis of archival tissue from phthisical eyes with total retinal detachment presents enormous difficulties in separating telangiectatic retina from that which was originally normal. In addition the inflammatory component of the disease process, in particular in its end stages, may result in retinal necrosis leading to loss of retinal tissue and DNA. While family OE and male patient E317 carry an identical mutation and it is possible that this may have specific phenotypic implications, the lack of a strong genotype–phenotype correlation amongst other NDP mutations suggests that this may be coincidental.

Our findings are consistent with the hypothesis that Coats’ disease is a mosaic phenotype. We therefore suggest that a somatic NDP mutation causing Coats’ disease occurs within cells of neuroectodermal origin at a stage of development that results in a segment of the retina carrying the mutant allele. The male preponderance suggests that at the cellular level females and males may not show identical NDP expression which might result, for example, from partial escape of X-inactivation (21). In the case of II-2 we propose that Coats’ disease represents a ‘second hit’ inactivating both NDP alleles in the temporal retina of one eye. Skewed X-inactivation might not be expected to result in a sectoral phenotype such as Coats’ disease since the female carrier manifestations of retinal phenotypes (e.g. X-linked ocular albinism), which often show a fine-grained ‘mud-splattered’ pattern of affected and unaffected areas, suggest that differently inactivated clones are finely intermixed within the retina (22).

Since its discovery, the role of norrin in development has remained elusive. The demonstration of NDP mutations in Coats’ disease implicates norrin in retinal developmental vasculogenesis. Several other lines of evidence support this hypothesis. A previous ultrastructural analysis of retrolental and retinal tissue from an infant with Norrie disease demonstrated complete retinal avascularity (23). Using gene targeting Berger et al. established a mutant mouse line as a mouse Norrie disease model (12). Analysis of lectin-stained retinal whole mounts of mice harbouring mutations in the murine Ndph gene have shown abnormalities of the retinal vessels which include telangiectasis, bulb-like dilatations and underdevelopment of the capillary bed (13,24). In situ studies on normal mouse retina showed increased NDP mRNA expression at 2 weeks within the ganglion cell and inner nuclear layers of the retina, the layers in which mesenchymal cells are induced to form the retinal vasculature (12). Furthermore, recent observations of the retinas of Norrie disease mice have demonstrated abnormalities of structure, integrity and form of the retinal vasculature in addition to abnormalities of the hyaloid vessels (13).
To our knowledge this is the first demonstration of functional somatic mosaicism of a single X-linked gene causing a distinct clinical phenotype. Mosaicism involving X-linked genes may also underlie other conditions such as cutaneous pigmentary mosaicism (hypomelanosis of Ito) associated with X-autosome translocations (IP1), the development of the linear skin lesions in incontinentia pigmenti and in Goltz syndrome (25–27). It is interesting to note that unilateral Coats’ disease, as well as potentially similar peripheral vascular abnormalities, have been described in hemizygous females with Turner (45, XO) syndrome (28–29). We suggest that a two-hit mechanism might underlie similar manifestations in a female with Coats’ disease highlighting the potential risk of passing on pathogenic NDP mutations to the offspring of females with Coats’ disease, a condition hitherto presumed to be ‘non-genetic’.

**MATERIALS AND METHODS**

**DNA preparation and extraction**

Retinal and non-retinal material was microdissected aseptically from $5 \times 10 \, \mu m$ sections, provided on microscope slides, from nine male eyes enucleated as a result of Coats’ disease and from tissue from 10 normal control eyes, 10 breast cancer tumour blocks and 10 vestibular schwannoma tumour blocks. Where possible retinal tissue was dissected into two or three segments, representing different regions of the detached retina. Non-retinal tissue was also dissected into two to three sections. For each region tissue from the same anatomical regions was pooled from all slides. This was incubated overnight at 55°C in 20 $\mu l$ restriction buffer (0.05 M Tris–HCl pH 8.0; 0.5 mM EDTA, Tween-20 to an concentration of 0.5%) and 10 U proteinase K (Sigma, Poole, UK). Cell debris was removed by centrifugation (10 min, 13 000 $g$) and the enzyme inactivated at 85°C for 15 min. Following phenol and phenol–chloroform extraction DNA was precipitated with 3 M NaOAc pH 5.3. The yield from Coats’ eye DNA extraction was extremely low and concentrations were not therefore formally measured.

**PCR**

DNA (2 $\mu l$) was amplified using the following primer pairs: (i) exon 2, primers 620 (CTTGCCTGTTTCTGAGGGAA) and 2.3 (CTGCATCCTTTTCTATGCTC), 210 bp product; primers 612 (CCGATCCTGCCCTTTCCT) and 2.4 (CCATTATGAATGCGTGTCC), 200 bp product; (ii) exon 3, primers 613 (CTTGGCAAGGTTGGCAT) and 2.1 (CAGTGCCTTCAGCTTGGAAGTC), 200 bp product; primers 608 (ACAGTTGTCGAGGTTGTGGCAT) and 1.2 (CCGTTCCTCCTGTCACTGC), 190 bp product; for E317, confirmatory amplification of the mutated fragment of exon 3 used primers 1.2 and e3.906 (CCGTTCCTCCTGTCACTGC). All reactions were performed in 25 $\mu l$ containing 3.7 mM MgCl$_2$, 67 mM Tris–Cl pH 8, 16.6 mM (NH$_4$)$_2$SO$_4$, 0.17 mg/ml bovine serum albumin (BSA), 3 mM each dNTP, 10 pmol of each primer and 0.5 U Pfu Turbo polymerase (Stratagene, Cambridge, UK). Samples were overlain with mineral oil and processed through 35 cycles of amplification consisting of 1 min at 94°C (denaturation), 1 min annealing (64–1°C) and 1 min at 72°C (extension). The final extension step was 10 min. SSCP/heteroduplex analysis was performed using 1 vol PCR product and 1 vol formamide loading dye. Gels were run at 350 V overnight at 4°C and silver stained according to standard protocols. Gels were inspected for bands of abnormal migration.

Figure 3. (a) Sequence analysis of NDP exon 3 PCR fragment (primers 2.1/613). (i) Individual III-2 (Family OE) affected by Norrie disease; (ii) retinal tissue from patient E317—purified lower SSCP of abnormal migration; (iii) control. (i and ii) Identical C→G transversion at position 704 resulting in a cysteine to tryptophan substitution. The mutation results in the loss of an NaeI site. (b) Restriction enzyme digestion of NDP exon 3 PCR fragment (primers 1.2/e3.906) with restriction enzyme NaeI (6% acrylamide gel, silver stained). Lane 1, patient II-2 (genomic DNA), family OE; lane 2, patient III-2 (genomic DNA), family OE; lane 3, patient E317, retinal tissue; lane 4, patient E317, non-retinal tissue; lanes 5 and 6, somatic controls (normal eyes). For patient E317 (as for the genomic control from carrier female II-2) the retinal tissue (lane 3) shows incomplete digestion of retinal, but not non-retinal, tissue. The presence of upper and lower bands (251 and 228 bp, respectively) suggests the presence of normal and mutant DNA within the same retina.

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which were then excised, equilibrated for 45 min in 50 mM EDTA and then equilibrated for 48 h in TE buffer at room temperature. Serial dilutions of the eluted DNA were then PCR amplified for the exon region of interest and then sequenced directly using the ABI prism Dye Terminator Cycle Sequencing kit (Perkin Elmer, Applied Biosciences Division, Warrington, UK).

**Restriction digests**

NaeI (10 U; New England BioLabs, Hertfordshire, UK), 2 µl 1× NEBuffer 4 (50 mM KOAc, 20 mM Tris–OAc, 10 mM MgOAc₂, 1 mM DTT pH 7.9 at 25°C), 2 µl 10× BSA and 10 µl PCR reaction were combined in a total volume of 20 µl and incubated at 37°C overnight. The bands were resolved on a 3% agarose gel, stained with ethidium bromide and visualized under UV light.

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