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FRDA 1999

FRIEDREICH'S ATAXIA
RESEARCH CONFERENCE

Research, Education, and Advocacy • Friedreich's and related Sporadic Ataxias

Abstracts

Cosponsors *National Institute of Neurological Disorders and Stroke,
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Friedreich's Ataxia Research Alliance (FARA)





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November 16, 1999

Dear Reader,

This package includes abstracts of the 41 presentations made at the Friedreich's Ataxia Research Conference held this Spring at the National Institutes of Health in Bethesda, Maryland. FRDA1999 was primarily sponsored by the National Institute of Neurological Disorders and Stroke, and the Friedreich's Ataxia Research Alliance (FARA), a patient advocacy group which supports research leading to meaningful treatments and therapies for Friedreich's Ataxia and the related sporadic ataxias. The Office of Rare Diseases, the National Institute on Aging, and the National Institute of Child Health and Human Development also provided support.

Friedreich's Ataxia (FRDA) is an autosomal recessive degenerative disorder characterized by ataxia of all four limbs, dysarthria, areflexia, sensory loss, and muscle weakness. In European populations, it appears with a prevalence of approximately 1 in 50,000 live births. Most individuals diagnosed with FRDA have skeletal abnormalities and cardiomyopathy, about 30% have impaired glucose tolerance and/or diabetes mellitus, and some occasionally develop reduced visual acuity and hearing loss. Onset of symptoms usually occurs around puberty, and most individuals with FRDA need to use wheelchairs full-time by their late 20s. Myocardial failure is the most common cause of premature death.

Identification of the FRDA gene in 1996 revolutionized FRDA research. In the three years since, the mutational mechanism has been elucidated and the encoded protein - frataxin - has been localized to mitochondria. Reduced mitochondrial function, secondary to mitochondrial iron accumulation and oxidant damage, appears to underlie the signs and symptoms of FRDA. Data collected from experiments using lower eukaryotes are being confirmed in human cell culture models and in patients themselves. Knockouts of the mouse homolog of the FRDA gene have been made. Other mouse models, including ribozyme transgenics and GAA repeat knock-ins, are also being developed. Therapeutic clinical trials of iron chelators and/or antioxidants are being considered, and in some cases started as pilot studies, based on information unavailable prior to the identification of the disease gene. Various modalities are being developed to follow disease progression and response to therapy.

FRDA1999 provided a unique opportunity to review and discuss the status of knowledge and assess future research options in five key areas: GAA repeat expansions (Session I), Pathology of FRDA (Session II), Frataxin and pathogenesis (Sessions III through VI), Patient/Clinical studies (Session VII), and Therapeutic approaches (Session VIII). The format for the meeting was chosen to maximize information exchange across the various research disciplines relevant to FRDA and to promote interaction and discussion among the participants. Sessions were sequential so that clinicians, clinical investigators, and basic scientists could attend all sessions. Sessions included communications by invited speakers, selected free communications, and round table discussions. Each session opened with a short presentation by the session chair summarizing current knowledge based on published information, followed by 10-minute presentations of original data. Specific questions and a short open discussion followed each presentation.

The speakers invited to participate in FRDA1999 represent the most outstanding scientists and clinicians whose work relates directly to the current issues of most importance to the field of Friedreich's Ataxia research. You will note that three of the abstracts appear twice. Pierre Rustin, Anthony Schapira, and Julie Smith presented their data from a basic-science viewpoint in Sessions III through VI and from an applied viewpoint in Session VIII. The names of all speakers appear in bold. If you are a Scientist with a research interest in FRDA, please contact FARA fara@frda.org for a complete list of addresses.

Sincerely,

A handwritten signature in black ink, appearing to read "Robert B. Wilson".

Robert B. Wilson

HUP

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FRDA1999

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Session I

GAA Repeat Expansions

- DNA structure and transcription
- Mechanism(s) for repeat stability
- Molecular diagnostic testing

Bronya Keats, Chair

Mitotic and meiotic instability of the GAA repeat in the X25 gene

Francesca Cavalcanti¹, Angela Tammaro¹, Antonella Monticelli¹, Luigi Pianese²,
Alessandro Filla², Giuseppe De Michele², Giuseppina Ruggiero¹, Delia Danzi¹,
Luisa Iodice¹, Alma Contegiacomo³, Josée Poirier⁴, Laura Montermini⁴,
Massimo Pandolfo⁴, and **Sergio Cocozza**¹

Ninety-six percent of patients affected by Friedreich's ataxia (FRDA) are homozygous for GAA trinucleotide repeat expansions in the first intron of the frataxin gene. We previously demonstrated intergenerational instability of the expanded GAA repeat with a clear effect of parental gender, age at birth and expansion length on trinucleotide stability. We also demonstrated mitotic instability of the repeats by showing different expansion sizes in different cells from the same individual and extensive heterogeneity of repeat sizes in the central nervous system of patients. Here we present data about mitotic instability in different in vivo and in vitro systems. In particular we studied the instability of FRDA expanded alleles in DNA extracted from peripheral blood leukocytes, from T lymphocytes after mitogen stimulation, from EBV lymphoblastoid cell lines and from primary skin fibroblasts cultures. In addition we studied also X25 normal alleles in DNA extracted from breast carcinoma tissue samples in comparison with normal tissue from the same patients. Thirteen out of 104 (12.5%) FRDA patients showed three or four bands when GAA expansions were tested by long-range PCR in peripheral blood leukocyte DNA. Four out 104 (4%) showed a very faint band corresponding to a normal-size repeat. We determined the GAA repeat size in lymphocytes from 4 FRDA patients after 20-30 days of mitogen stimulation. PCR amplification of the lymphocyte expanded allele yielded a diffuse array of products shorter than the corresponding allele from non stimulated cells. Complete contraction of the expanded alleles was also found. After repeated passages, cells with contracted GAA repeat expansions appeared in primary skin fibroblasts cultures from two out of three patients. In one case the contraction was to a normal size repeat. Information about repeat instability may help in understanding the molecular basis of this disease and may have important impact on the molecular diagnosis.

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The in vitro DNA conformation of the Friedreich ataxia repeat sequence mutation correlates with in vivo inhibition of gene expression and DNA replication

Keichii Ohshima¹, Naoki Sakamoto², José Poirier¹, Malgorzata Labuda¹, Laura Montermini¹, Melinda L. Moseley³, Laura P. Ranum³, Robert D. Wells², and Massimo Pandolfo¹

The expanded intronic GAA triplet repeat sequence (TRS) of Friedreich ataxia (FRDA) is thought to inhibit transcription of the target frataxin gene. We cloned GAA*TTC repeats containing 9 to 270 triplets into the intron of a two-exon reporter gene contained in the plasmid pSPL3. Transient transfection experiments in Cos-7 cells showed that transcription and replication of these plasmids are inhibited by the TRS in a length- and orientation-dependent manner, i.e. long repeats that transcribe rGAA cause the highest inhibition. Polypurine*polypyrimidine (R*Y) DNA sequences as GAA*TTC are known to form triple helical structures. Here we show that a non-B DNA structure, very likely a triple helix, is a characteristic of GAA*TTC repeats and not of a similar R*Y repetitive sequence. Transcription and DNA replication are inhibited only by repeats able to form the non-B DNA structure.

We cloned a (GAAGGA)₆₅ repeat, identified in three related non-FRDA individuals, into pSPL3. Transient transfection of Cos-7 cells showed very little inhibitory effect on transcription and DNA replication. A GAA TRS of similar overall length, (GAA)₁₇₀, had instead a strong inhibitory effect on both processes. Sensitivity to P1 nuclease was shown only for a very short region in the supercoiled plasmid containing (GAAGGA)₆₅, corresponding to a brief poly-A sequence that follows the repeat. A (GAA)₁₅₀-containing plasmid showed instead an extensive single-stranded region spanning the entire TRS. The secondary structure of a supercoiled plasmid containing a R*Y DNA segment of about 400 bp is therefore sequence-dependent. Only the plasmid with the GAA TRS can form what is very likely to be a triplex. In addition, (GAAGGA)₆₅ does not form “sticky DNA” (see abstract by Wells et al. at this meeting), a structure observed in vitro in supercoiled plasmids that is the consequence of triplex formation. We conclude that ability to form a triplex correlates with, and is likely to be necessary for transcription and replication inhibition.

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Do all GAA expansion bearing Friedreich ataxia chromosomes derive from a single Indo-European founder?

Malgorzata Labuda¹, Nadir E. Barucha², and Massimo Pandolfo³

Most patients tested for the FRDA GAA*TTC triplet repeat expansion in our laboratory come from Montreal, a multi-ethnic city with substantial minorities of non-European origin, or from a variety of places in the USA and Canada, ethnically mixed countries. Rather surprisingly, all positive cases (more than 170 in 1997-1999) were of either European or Middle Eastern descent, suggesting that the expansions may be more frequently, if not exclusively found in such individuals and be very rare or absent in other ethnic groups. In particular, no subject with GAA expansions was found among several Chinese patients with a clinical picture suggestive of FRDA. This may relate to a single founder event that originated at-risk alleles for the GAA*TTC expansion. Among Europeans, a founder effect for FRDA chromosomes had been previously suggested by several linkage disequilibrium studies, including one we conducted in 1992 on 140 European (mostly Italian or French) families (Sirugo et al, 1992) using markers at some distance from the gene and a more recent one on French and Spanish families (Monros et al, 1996) using the marker FAD1, which is about 100 Kb from the gene. Further studies indicated that GAA*TTC expansions in the first intron of the frataxin gene derive from a pool of at-risk, large normal alleles containing 12 to more than 30 triplets. Such alleles are found in about 18% of the chromosomes in Caucasians. This conclusion is based on the direct observation of a few new expansion events (reported by Cossée et al, 1997 and Montermini et al, 1997) and on linkage disequilibrium data. Cossée et al in 1997 analyzed five markers spanning the frataxin gene (FAD1, ITR4, F5225, ITR3, and CS2) and found that all chromosomes carrying large normal or expanded alleles contain either a major haplotype (A-T-(2/3)-C-C) or one of a few recombination-derived minor haplotypes, all very rare in chromosomes carrying small normal alleles. They concluded that possibly a single founder event gave origin to large normal alleles, from which expansions are then derived. These authors also tested European, mostly French subjects. We tested eight FRDA patients from India from four families for GAA expansions and for the associated FAD1-ITR4 - F5225 - ITR3 - CS2 haplotype. All were found to be homozygous for GAA expansions and for the A-T-(2/3)-C-C haplotype. This haplotype was not present in any of the normal chromosomes from the same families. They were Hindus or Jains (one family), from Bombay or Madras (one family). We also found the same haplotype in Ecuadorian patients, all reporting European ancestry (Spanish, British or German). It is striking that the same major haplotype is found on expansion-bearing FRDA chromosomes in all populations in which such chromosomes are found. The identification of the haplotype in Indian patients suggest that the mutation had indeed a single Indo-European origin, possibly through an event that gave rise to the pool of large normal alleles.

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Somatic sequence variation at the Friedreich ataxia locus

Sanjay I. Bidichandani, Smita M. Purandare, Ellen E. Taylor, Glenice Gumin, Hazem Machkhas, Yadollah Harati, Richard A. Gibbs, Tetsuo A. Ashizawa, and Pragna I. Patel

The most common mutation in Friedreich ataxia is an abnormal GAA trinucleotide repeat expansion in intron 1 of the X25 gene. Here we present several lines of evidence of somatic sequence variation within and immediately flanking the GAA triplet repeat sequence (GAA-TRS). We have identified a rare Friedreich ataxia patient with a complete contraction of the expanded allele(s). While the contracted allele was detectable by PCR (in two independent samples), Southern blots showed only two expanded alleles. Cloning and sequencing of the contracted product revealed a heterogeneous mix of clones containing 9 to 29 triplets. Haplotype analysis was consistent with the patient having inherited both expanded alleles, indicating that the contraction was a somatic event.

Southern blot analysis of DNA from serially passaged lymphoblastoid cell lines containing a wide range of expansions revealed a few cases with significant changes in repeat length. A contraction and an expansion were seen in two separate heterozygous cell lines. Additionally, in one homozygous cell line both expanded alleles showed identical contractions followed by re-expansions, suggesting an interallelic mechanism. With subsequent passaging, the changes in repeat length tended to become fixed without persistence of the original or any intermediate alleles.

On investigating a 135-bp sequence immediately upstream of the GAA-TRS, we found evidence for a three-fold enhanced point mutation rate in expanded versus normal chromosomes. The poly(A) sequence immediately upstream of the GAA-TRS was also found to be somatically unstable.

These phenomena are likely to have important mechanistic, diagnostic and clinical implications.

Sticky DNA: Self-association properties of long GAA*TTC repeats in R*R*Y triplex structures from Friedreich's ataxia

Robert D. Wells¹, Naoaki Sakamoto¹, Paul D. Chastain², Pawel Parniewski¹, Keiichi Ohshima³, Massimo Pandolfo³, and Jack D. Griffith²

A novel DNA structure, sticky DNA, will be described for lengths of (GAA*TTC)_n found in intron 1 of the frataxin gene (X25) of Friedreich's ataxia patients. Sticky DNA is formed by the association of two purine*purine*pyrimidine (R*R*Y) triplexes in negatively supercoiled plasmids at neutral pH in the presence of divalent metal ions. The intermolecular complexes are remarkably thermostable and survive treatment at 70° for 1 hour. We propose that the marked stability found for this structure is due to the exchange of the Y strands between the two R*R*Y triplexes to generate an interlocked complex. Sticky DNA formation is sensitive to both the length of the (GAA*TTC) tract, since repeats shorter than 59 are inert (and are not found in FRDA patients), and the sequence since (GAAGGA*TCCTTC)₆₅ does not adopt this unusual conformation. An excellent correlation was found between the lengths of (GAA*TTC) found (a) in FRDA patients, (b) required to inhibit transcription in vivo and in vitro, and (c) required to adopt the sticky conformation. Fourth, (GAAGGA*TCCTTC)₆₅, also found in intron 1, does not form sticky DNA, inhibit transcription, or associate with a disease. Hence, R*R*Y triplexes and/or sticky DNA may be involved in the etiology of FRDA. To appear in *Molecular Cell*, 3, 1-20 (1999).

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Incipient GAA repeats in the primate Friedreich's ataxia homologous genes

Pilar Gonzalez-Cabo¹, María Isabel Sanchez¹, Joaquín Cañizares¹, José M. Blanca¹, Rosa Martinez-Arias², **Marisol De Castro**³, Jaume Bertranpetit², Francesco Palau^{1,3}, M. Dolores Moltó¹, and Rosa de Frutos¹

Friedreich's ataxia (FRDA) represents the first autosomal recessive inherited trinucleotide disease, the primary cause of which is an unstable GAA repeat expansion within an Alu sequence in the first intron of the FRDA/X25 gene. The GAA motif is polymorphic with bimodal size distribution in the normal human population (83% of normal alleles have 6-12 repeats and 17% have 13-36 repeats). The presence of microsatellite repeats associated with both the 3' oligo(A) tails and the central A-rich region of Alu elements has already been reported. Alu elements have also been proposed as a source of primate microsatellite repeats. In order to understand the evolutionary context of the FRDA GAA triplet repeat, we have analyzed the Alu-X25 element.

Genomic DNA was obtained from the following species: chimpanzee (*Pan troglodytes*) (2), gorilla (*Gorilla gorilla*) (2), orangutan (*Pongo pygmaeus*) (1), drill (*Mandrillus leucophaeus*) (2), and rhesus macaque (*Macaca mulatta*) (1). Multiple alignment of the Alu-X25 sequences showed the following divergence values: human-gorilla 1.77%, human-chimpanzee 2.12%, human-drill 10.95%, human-rhesus 9.19%, gorilla-chimpanzee 2.47%, gorilla-drill 10.95%, gorilla-rhesus 8.83%, chimpanzee-drill 11.31%, chimpanzee-rhesus 9.89%, and drill-rhesus 3.18%. These values are in accordance with the established phylogenetic relationships. Old World monkey analyzed sequences showed an incipient GAA repeat (2 repeats in gorilla and 3 repeats in chimpanzee) within a complex repetitive pattern; thus, its origin has to predate the Old World monkey radiation. Due to Alu-X25 belongs to the Sx subfamily, the structure of the linking region of 608 Alu-Sx human sequences has been analyzed. 36.8% of them maintain the canonical A5TACA5, but in any case GAA repeats have been found. In respect to the origin of the Alu-X25 element a likely first step could be a A@G transition giving a G(A)n structure suitable at multiplication by polymerase slippage. This work is funded by CICYT grant no. SAF97-0082

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Molecular diagnosis of Friedreich ataxia: Perspective from the clinical laboratory

Myra J. Wick¹, Vickie. L. Matthias Hagen², and Ronald C. McGlennen¹

The identification of the GAA repeat expansion as the causative mutation in the majority of Friedreich Ataxia patients was quickly followed by the availability of molecular FRDA testing in the clinical molecular diagnostics laboratory. GeneTests' (formerly Helix), an on-line directory of medical genetics laboratories, currently lists 23 clinical laboratories which offer FRDA testing; 19 of these laboratories are located in North America, and the four remaining laboratories are located in New Zealand, Denmark, Italy, and Germany. Despite the number of laboratories which offer FRDA testing, the molecular diagnosis of FRDA has proved to be technically challenging. Initially, many clinical laboratories attempted to exclusively employ long range PCR protocols for FRDA testing. This is a departure from "traditional" protocols for the detection of large trinucleotide repeat expansions (e.g., Fragile X, myotonic dystrophy), which typically require the use of both PCR and Southern analysis. However, difficulties with long range PCR has caused many clinical laboratories to incorporate the use of Southern analysis into their FRDA testing protocols. An additional concern associated with the molecular diagnosis of FRDA, which was discussed in a recent survey by the College of American Pathologists, is the use of assays which allow for accurate sizing of expanded alleles. Thus, for many molecular diagnostic laboratories, the FRDA protocol remains in a developmental phase. The Ataxia Molecular Diagnostic Testing Group (AMDTG) is in the process of conducting an FRDA proficiency test and technical survey. The results of this survey will be presented and discussed at the 1999 American Society of Human Genetics Meeting. It is anticipated that the data resulting from this survey will provide direction for the continued evolution of Molecular FRDA testing.

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Session II

Pathology of FRDA

- Biochemistry and immunochemistry
- Iron accumulation and oxidative stress
- In situ hybridization analyses

Arnulf Koeppe, Chair

The uniform neuropathological phenotype of Friedreich's ataxia

Arnulf H. Koeppe

The neuropathology of the spinal cord in Friedreich's ataxia (FA) is quite characteristic and includes atrophy of the dorsal columns and the spinocerebellar and lateral corticospinal tracts. The dorsal nuclei of Clarke reveal neuronal loss and atrophy of surviving nerve cells. Fiber depletion of the anterior corticospinal tracts is variable. The dorsal roots are thin due to progressive destruction of the dorsal root ganglia. Supraspinal lesions are variable and include atrophy of the dentate nuclei and their efferent fibers, fiber loss in the medullary pyramids, optic atrophy, and loss of Betz cells. The lesion of the dentate nucleus has gained recent attention. Neuronal destruction may be a direct consequence of frataxin deficiency in a gray matter nucleus that normally contains abundant iron. A review was made of 10 archival cases of FA. Ages of onset ranged from 1 to 34 years, and ages of death from 18 to 87 years. The commensurate disease duration ranged from 16-53 years. The cross-sectional area of the thoracic spinal cord was reduced in all cases and ranged from a minimum of 13.5 mm² to 23 mm² (normal: 28-30 mm²). Regression analysis revealed no convincing relationship between spinal cord atrophy and age of onset and death, or disease duration. However, the early onset cases were more likely to show myocardial iron collections. The lengths of the GAA trinucleotide repeats were known in 3 patients. The long survivor (age at death: 87 years) had minor expansions (170/104 repeats). It is likely that the correlation of neuropathological phenotype and GAA expansion will require a new morphometric approach at several levels of the central and peripheral nervous systems.

Iron in the central nervous system of Friedreich ataxia patients

Lidia Cova¹, Michael Babcock², **Julie C. Smith**², Stephen J. Kish³, Arnulf Koeppen⁴, Sarn Jiralerspong¹, Laura Montermini¹, Jerry Kaplan², and Massimo Pandolfo¹

We obtained evidence of iron accumulation in selected areas of the central nervous system (CNS) of patients with Friedreich ataxia (FRDA). Iron was directly measured in several CNS areas from FRDA patients and controls., including cervical cord, pons, cerebellar cortex, dentate nucleus, putamen, caudate, pallidus, and cerebral cortex. Fifty to sixty milligrams of tissue were dissected from frozen autopsy samples and analyzed by atomic absorption spectroscopy. Patients were homozygous for expanded repeats in the frataxin gene, as determined in DNA samples extracted from the CNS samples. Two cases were also analyzed by western blot with anti frataxin monoclonal antibodies (1G2) and found to have an important decrease in the level of frataxin in the CNS. Total iron levels were similar in patients and controls in areas that do not show severe pathological changes in FRDA. We could test a severely affected structure, the dentate nucleus, only in one patient and one control and iron content was three times higher in the patient.

Staining with Prussian Blue and direct detection of redox active iron (II) through its peroxidase activity were performed in dorsal root ganglia histological sections (formalin fixed and paraffin-embedded) from two FRDA cases. Several surviving DRG neurons presented a punctate blue staining and endogenous peroxidase activity compatible with iron deposits. Similar findings had previously been interpreted to represent lipofuscin accumulation, which was noticed to be very high in DRG neurons from FRDA patients.

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Pathology of “sporadic” degenerative ataxias

Georg Auburger

Patients manifesting with degenerative ataxia without family history can suffer from recessive mutations such as FRDA or FIVED, true sporadic disease such as MSA, or dynamic dominant mutations such as SCA2 or SCA7. The role of oxidative stress is well established in FRDA and FIVED. Since the vulnerable neuronal populations in MSA and in SCA2 are strikingly similar, we investigated SCA2 brains for the presence of cytoplasmic aggregates of alpha-synuclein, which are a characteristic feature of MSA. Alpha-synuclein was found accumulated in fiber tracts within the striatum of SCA2 patients. Both MSA and SCA2 thus are similar to Parkinson’s disease, where aggregates of alpha-synuclein form both in patients with the dominant A53T mutation and under conditions of oxidative stress.

Pathology of the Friedreich's Ataxia cardiomyopathy

Jacques B. Lamarche

A morphological, morphometric, and electron-microscopic study of the cardiomyopathy in 13 cases of Friedreich's Ataxia will be described. The study includes an X-ray probe analysis, and other measurements, to detect iron.

Increased iron in the dentate nucleus of patients with Friedreich's ataxia

Daniel Waldvogel¹, Peter van Gelderen², and Mark Hallett¹

To test the hypothesis that iron is accumulated in the cerebellum of patients with Friedreich's ataxia, we developed a new multigradient echo MR sequence which allows the three dimensional imaging of brain iron induced contrast.

Clusters of iron tend to be paramagnetic and therefore cause local inhomogeneities in a magnetic field. These microscopic inhomogeneities as well as the scanner related macroscopic inhomogeneities lead to an increase in the decay rate (T2) of the MR signal, which is then called T2*. The macroscopic inhomogeneity can be estimated from the phase information in the images and used to correct the measured relaxation time. The contrast in the corrected images is then solely due to the combination of the T2 relaxation plus the effects of the accumulated iron.

Our newly developed sequence was tested in 23 normal volunteers, ages 5 -35 years. The measured R2* values (the inverse of the T2*) showed an age dependent increase and a regional distribution that corresponded to the histochemically documented age-dependent increase and regional distribution of iron. After this validation of the accuracy of our sequence, we examined twelve patients with FA, ages 5 - 32 years. R2* values in the dentate nucleus of FA patients were significantly higher than in controls ($p < 0.05$), while R2* values in the unaffected globus pallidus were not different. We conclude that the difference in R2* values found in the dentate nucleus of FA patients is most likely due to increased iron.

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Session III

Frataxin & Pathogenesis: Biochemistry

- Protein interactions
- Transport and processing
- Structure
- Functional studies

Pierre Rustin, Chair

Towards a prevention of cardiomyopathy by idebenone in Friedreich ataxia

Pierre Rustin, Agnès Rötig, and Arnold Munnich

Friedreich Ataxia (FRDA) is a frequent autosomal recessive condition causing spinocerebellar degeneration and hypertrophic cardiomyopathy, and resulting from the deficiency of frataxin, a protein involved in regulation of mitochondrial iron content. We have recently reported a combined deficiency of a Krebs cycle enzyme, aconitase, and three mitochondrial respiratory chain complexes in endomyocardial biopsies of FRDA patients. All four enzymes share iron-sulfur cluster containing proteins (ISP) that are damaged by iron overload via generation of oxygen free radicals. We have devised an in vitro system using human heart homogenates both to elucidate the mechanism of iron-induced injury and to test the protective effects of various substances. Under our experimental conditions, reduced iron mimicked the damages observed in heart biopsies of FRDA patients. Ascorbate, which reduced iron and desferrioxamine, increased iron-induced injury in vitro. Conversely, reduced idebenone efficiently protected mitochondrial enzymes from iron-induced damages. Accordingly, three FRDA patients with hypertrophic cardiomyopathy were given idebenone orally (5 mg/kg/d). After 4-9 months, ultrasound evidence of drug efficiency was provided by the reduction of left ventricle hypertrophy in the three patients (mass index reduction: 21, 30, 32%, respectively). Our in vitro data suggest that both iron chelators and antioxidant drugs likely to reduce iron are potentially harmful in FRDA. Conversely, our preliminary in vivo data suggest that idebenone protects heart muscle from iron-induced injury in FRDA. Ongoing nation-wide clinical trial in process in France will hopefully confirm these preliminary observations.

Why do mitochondria accumulate iron?

Gyula Kispal and **Roland Lill**

Mutations in a few mitochondrial proteins cause abnormal loading of these organelles with iron. Examples include i) the yeast mitochondrial ABC transporter Atm1 and its human orthologue hABC7 implicated in a form of sideroblastic anemia, ii) the cysteine desulphurase Nfs1, and iii) frataxin. In the course of our functional investigation of Atm1 we found that the protein is essential for the biogenesis of cytosolic Fe/S proteins suggesting that the transporter mediates the mitochondrial export of components required for Fe/S cluster incorporation into cytosolic apoproteins. Nfs1, the orthologue of the bacterial cysteine desulphurase NifS, is involved in formation of both intra- and extra-mitochondrial Fe/S proteins. The protein initiates Fe/S cluster synthesis by producing elemental sulphur in the mitochondrial matrix. The central role of mitochondria in the synthesis of extra-mitochondrial Fe/S proteins is further evident from the requirement of a membrane potential for biogenesis. Our data demonstrate a hitherto unknown essential biosynthetic function of mitochondria in the generation of extra-mitochondrial Fe/S clusters. This raises the interesting question of whether mitochondrial iron homeostasis is regulated by cytosolic Fe/S cluster-containing proteins. We are currently testing whether frataxin also plays a role in cytosolic Fe/S protein biogenesis.

Biochemical consequences of frataxin deficiency

Anthony H. V. Schapira^{1,2}, Jane Bradley¹, Raffaele Lodi³, and J. Mark Cooper¹

Deletion of YFH1 in yeast results in impaired respiratory function, increased intramitochondrial iron and decreased mitochondrial DNA (mtDNA) levels. We have investigated mitochondrial respiratory chain function and oxidative phosphorylation capacity, aconitase activity, iron levels and mtDNA content in Friedreich's ataxia (FRDA) patients. We demonstrate: (1) ³¹P magnetic resonance spectroscopy (³¹pMRS) abnormalities in vivo in skeletal muscle from FRDA patients. The decline in Vmax correlated with the length of the patients' GAA repeat and therefore inversely with expected frataxin levels; (2) Abnormal ³¹pMRS in myocardium from FRDA patients; (3) Data on the results of treatment design to reverse these abnormalities will be presented; (4) Severe and highly significant defects in the activities of complexes I-III and aconitase in FRDA heart; (5) Iron accumulation in FRDA heart, liver and spleen; (6) Defects of complexes I-III and aconitase in FRDA skeletal muscle; (7) Mitochondrial DNA levels were not significantly decreased in tissues studied.

These results provide insight into the biochemical consequences of frataxin deficiency and the mechanisms that may be involved in FRDA pathogenesis. The pattern of mitochondrial defects suggests that oxidative damage is important. Abnormal mitochondrial iron metabolism may result in impaired Fe-S construction and the resulting impairment in respiratory chain function may cause increased free radical release and cell damage.

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Session IV

Frataxin & Pathogenesis: Lower Organisms

- Yeast
- *C. elegans*
- *Drosophila*
- Bacteria

Robert B. Wilson, Chair

Mitochondrial intermediate peptidase and the yeast frataxin homologue together maintain mitochondrial iron homeostasis in *Saccharomyces cerevisiae*

Steven S. Branda, Zhi-yong Yang, Anne Chew, Patrizia Cavadini, and **Grazia Isaya**

Friedreich's ataxia (FRDA) is a neurodegenerative disease typically caused by a deficiency of frataxin, a mitochondrial protein of unknown function. In *S. cerevisiae*, lack of the Yeast Frataxin Homologue (YFH1, gene; Yfh1p, polypeptide) results in mitochondrial iron accumulation, suggesting that frataxin is required for mitochondrial iron homeostasis and that FRDA results from oxidative damage secondary to mitochondrial iron overload. This hypothesis implies that the effects of frataxin deficiency could be influenced by other proteins involved in mitochondrial iron usage. We show that Yfh1p interacts functionally with the Yeast Mitochondrial Intermediate Peptidase (OCT1, gene; YMIP, polypeptide), a metalloprotease required for maturation of ferrochelatase and other iron-utilizing proteins.

YMIP is activated by ferrous iron *in vitro*, and loss of YMIP activity leads to mitochondrial iron depletion, suggesting that YMIP is part of a feedback loop in which iron stimulates maturation of YMIP substrates and this in turn promotes mitochondrial iron uptake. Accordingly, YMIP is active and promotes mitochondrial iron accumulation in a mutant lacking Yfh1p (*yfh1*), while genetic inactivation of YMIP in this mutant (*yfh1 oct1*) leads to a two-fold reduction in mitochondrial iron levels. Moreover, overexpression of Yfh1p restores mitochondrial iron homeostasis and YMIP activity in a conditional *oct1*ts mutant, but does not affect iron levels in a mutant completely lacking YMIP (*oct1*). Thus, we propose that Yfh1p maintains mitochondrial iron homeostasis both directly, by promoting iron export, and indirectly, by regulating iron levels and therefore YMIP activity, which promotes mitochondrial iron uptake. This suggests that human MIP may contribute to the functional effects of frataxin deficiency and the clinical manifestations of FRDA.

Analysis of the role of YFH1 in yeast iron homeostasis

Jerry Kaplan, Derek Radisky and Opal Chen

The yeast homologue of Frataxin, YFH1 encodes a mitochondrial protein that plays a critical role in mitochondrial and cellular iron homeostasis. Deletion of YFH1 (Delta yfh1) results in poor growth on glucose containing medium, loss of respiratory competence and excessive mitochondrial iron accumulation. The growth deficit and loss of mitochondrial respiratory activity is a consequence of increased mitochondrial iron accumulation. Conditions that limit mitochondrial iron accumulation, decreased medium iron or deficits in the yeast high affinity iron transport system, limit or prevent the loss of respiratory activity. Alternatively, high medium iron accelerates the loss of mitochondrial respiration in cells that show decreased Yfh1p content but not in normal cells. Pulse chase experiments demonstrate that the increase in mitochondrial iron seen in Delta yfh1 cells is the result of defect in a mitochondrial iron export system. Expression of YFH1 from a regulateable plasmid in cells containing a defective chromosomal copy of delta yfh1 leads to a loss of mitochondrial iron. This result indicates that Yfh1p affects mitochondrial iron egress, and that iron accumulated by defective mitochondria can be made bioavailable. Genetic screens have identified genes that affect mitochondrial iron metabolism and ameliorate the toxic affects of defects in YFH1. These studies indicate that accumulation of mitochondrial iron due to a defective egress pathway can be modified, and that the toxic affect of mitochondrial iron accumulation may be limited. (This work is supported by a grant from the National Institutes of Health, National Institute of Diabetes, Digestive and Kidney Disease, and a Center of Excellence in Hematology grant).

Isolation and characterization of the frataxin gene homologue in *Drosophila*

Joaquin Cañizares¹, José M . Blanca¹, Rosa de Frutos¹, **Francesco Palau**^{1,2}, and M. Dolores Moltó¹

Friedreich's ataxia is a neurodegenerative disorder caused by mutations in the FRDA gene. This gene encodes a 210 amino acids protein, frataxin, involved in the iron metabolism in mitochondria. Frataxin gene homology has been observed in mouse and in lower organisms such as *C. elegans*, *S. cerevisiae*, and *g -purple*, a gram-negative bacterial species. We present the characterization of the frataxin homologue gene in *Drosophila melanogaster*, called Dfh (*Drosophila* frataxin homologue).

Dfh is mapped on the chromosome X 8D region. The gene has two exons separated by an intron of 69 bp. The first exon has 340 bp and exon 2 has two alternative polyadenylation signals. In the promotor region there are two TATA boxes and two CAAT boxes. Dfh is expressed throughout the *Drosophila* development, from early embryo stages to adult with higher expression in the embryo 6 to 12 hours interval. Dfh protein is predicted to have 190 amino acids and a molecular weight of 21 Kd. Analysis of the secondary structure suggested that the protein has a β -sheet central region flanked by two α -helix regions. Analysis of the protein sequences by using the SignalP v1.1 program showed two amino-terminal peptide signals, being the first one a mitochondrial peptide signal. Blast2 analysis showed homology of Dfh frataxin with human, mouse, *C. elegans*, and *S. cerevisiae* frataxins. Dfh exon 2 encodes the highly conserved amino acid domain that is encoded by the exons 4 and 5a of the FRDA gene. We conclude that *Drosophila melanogaster* may be a model system to Friedreich's ataxia. This work has been funded by CICYT grants no. SAF96-0312 and SAF97-0082

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Knock-out of the *cyaY* gene in *Escherichia coli* does not affect cellular iron content and sensitivity to oxidants

Dong Sheng Li¹, Keiichi Ohshima¹, Sarn Jiralerspong¹, Michel W. Bojanowski², and Massimo Pandolfo¹

Frataxin shows homology with the CyaY proteins of γ -purple bacteria, whose function is unknown. We knocked out the CyaY gene in *E. coli* MM383 by homologous recombination and we generated an *E. coli* MM383 strain overexpressing CyaY.

Bacterial growth, iron content and survival after exposure to H₂O₂ did not differ among these strains, suggesting that, despite structural similarities, *cyaY* proteins in bacteria may have a different function from frataxin homologues in mitochondria.

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Requirement of either of a pair of iscU-related genes of *Saccharomyces cerevisiae* for cell viability

Robert B. Wilson¹, Padmini A. Menu¹, Huy Minh Le¹, Larisa Touloukhonova², Tomoka Ohnishi², and David M. Roof³

The proteins encoded by the iscU genes of bacteria have been hypothesized to be involved in the mobilization of iron for the formation or repair of iron-sulfur clusters. *Saccharomyces cerevisiae* contains two homologs of the iscU proteins, which have been designated ISU1p and ISU2p. Because of the demonstrated reductions in iron-sulfur-cluster-enzyme activities in Friedreich's Ataxia patients, and the presence of a structural and functional homolog of frataxin in yeast, we knocked out the ISU1 and ISU2 genes in *Saccharomyces cerevisiae* to determine the effects, if any, on iron-sulfur-cluster proteins and iron-sulfur-cluster formation or repair. Yeast lacking ISU1p or ISU2p have no obvious growth defect on normal media or media containing only non-fermentable carbon sources, and preliminary visible absorption spectra of mitochondria from these yeast are not significantly different from those of normal controls. We are currently testing the effects of media supplementation with iron, iron chelators, and oxidants. Genetic crosses between yeast lacking ISU1 and yeast lacking ISU2 demonstrate that yeast lacking both genes are inviable. A GFP fusion of ISU1p was used to localize ISU1p to mitochondria by fluorescence microscopy.

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Session V

Frataxin & Pathogenesis: Mammalian Cells

- Human controls and patients
- Cells from animal models
- Transfected cells

Massimo Pandolfo, Chair

FRDA cells are sensitive to oxidative stress which is rescued by chelators of iron and calcium and apoptosis inhibitors

Alice Wong, Joy Yang, Patrizia Cavadini, Cinzia Gellera, Bo Lonnerdal, Franco Taroni² and **Gino Cortopassi**¹

The expansion of an intronic GAA repeat in Friedreich's ataxia (FRDA) reduces expression of frataxin, a novel mitochondrial protein. Disruption of the frataxin homolog in yeast results in increased cellular and mitochondrial iron, respiration deficiency, and sensitivity to oxidants. These data support the hypothesis that FRDA is a disease of oxidative stress. To determine if the FRDA mutations confer cellular sensitivity to oxidants, five fibroblastoid cell lines from FRDA patients homozygous for the GAA expansion and five control cell lines were exposed to oxidants. Cell viability was determined by the trypan blue exclusion assay. Each of the five FRDA cell lines was significantly more sensitive to hydrogen peroxide and iron than the control cell lines. Depletion of iron by desferoxamine, and depletion of calcium by BAPTA-AM provided significant rescue of cells from hydrogen peroxide-induced death. However mitochondrial iron levels were not statistically significantly different in mitochondria from fibroblasts of FRDA patients vs. controls. Exposure of cells to caspase inhibitors provided significant and preferential rescue of FRDA cells from death induced by hydrogen peroxide. Thus data are consistent with the notion that FRDA cells are sensitive to oxidative stress, and this sensitivity is dependent on iron, Ca⁺⁺ and caspase activity.

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Mitochondrial iron levels are higher in fibroblasts from people with Friedreich ataxia

Martin B. Delatycki¹, James Camakaris², Hilary Brooks², Tracy Evans-Whipp¹, David R. Thorburn¹, Robert Williamson¹, and Susan M. Forrest¹

Yeast deleted for the gene homologous to the human FRDA gene exhibit various features which indicate that Friedreich ataxia (FRDA) is due to mitochondrial iron overload with secondary mitochondrial dysfunction. In addition to mitochondrial iron overload, this yeast mutant called *Dyfh* has the additional features of absent mitochondrial DNA (*rho0*), marked reduction in the rate of mitochondrial respiration and increased sensitivity to oxidant stress. It is difficult to obtain samples of the tissues affected in FRDA including heart, dorsal root ganglia and spinal cord. Therefore we chose to investigate cell lines from people with FRDA. We found that mitochondrial iron levels in FRDA fibroblasts were significantly higher than in control fibroblasts when this was compared to the protein content. Mitochondrial iron levels compared to cell number and citrate synthase level approached but did not reach significance. FRDA fibroblast mitochondrial levels were about one and a half times higher than in controls. Copper levels were assessed as a control heavy metal, and levels were virtually identical in FRDA and control fibroblast mitochondria. By contrast, mitochondrial iron levels in lymphoblasts did not differ significantly in FRDA cell lines compared to controls. Mitochondrial iron levels were about five times higher in fibroblasts than lymphoblasts suggesting that mitochondrial iron metabolism may differ between these two cell types. We examined the ratio of mitochondrial to genomic DNA in FRDA and control fibroblasts with increasing passage number. We found no difference in this ratio in FRDA fibroblasts compared to controls. Finally we exposed patient and control fibroblasts to increasing concentrations of ferric iron (ferric ammonium citrate and FeCl₃). We did not find a consistent pattern of increased sensitivity in either FRDA or control cells. In conclusion, we have found evidence that the observations in *Dyfh* are very likely to underlie FRDA but that the mitochondrial pathology is much more subtle.

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Evidence for mitochondrial iron overload in patients with Friedreich ataxia

Julie C. Smith¹, James P. Kushner¹, Mark Bromberg², Elizabeth Hammond³, William H. Barry⁴, Massimo Pandolfo⁵, and Jerry Kaplan³

The underlying disease pathophysiology in patients with Friedreich Ataxia (FA) has long been elusive to investigators and physicians. Yeast strains with a deletion in Yfh1, an orthologous homologue of frataxin, show abnormal respiratory function (petite formation), and accumulation of iron in the mitochondria. In both yeast and human, the protein frataxin has been localized to the mitochondria. In yeast, frataxin regulates iron export from mitochondria. Although the frataxin-iron link is easily proven in yeast, the evidence thus far in humans has been lacking. Through the General Clinical Research Center at the University of Utah Medical Center in Salt Lake City, Utah, we are conducting a NIH funded clinical trial evaluating iron chelation therapy as treatment for Friedreich Ataxia. The primary objective of this trial is to determine if chelation therapy using desferrioxamine can deplete iron overload within affected tissues in patients with genotypically proven FA. Seven patients have been enrolled thus far. All patients are >18 years of age and have genotypically proven FA (450-1037 repeats). Upon enrollment, baseline measurements of affected tissues were conducted, including a complete cardiac work-up consisting of an EKG, echocardiography, and right heart catheterization with endomyocardial biopsy. In addition, muscle biopsies, bone marrow aspirates and biopsies, and a full neurologic evaluation were conducted (EMG, QST, ataxia scale, electroretinogram, visual evoked potentials). The planned duration of treatment is one year, at which point the above parameters will be reassessed. Definitive evidence of cardiac iron overload was seen in all study patients as evidenced by histologic examination of endomyocardial biopsy. Electron microscopic findings revealed accumulation of electron dense particles consistent with iron in mitochondria. Further evidence supporting mitochondrial iron overload in humans was determined by measurement of human cardiac mitochondrial (autopsy specimens) iron by inductively coupled plasma elemental analysis. (This work is supported through a Center of Excellence in Hematology Grant from the National Institutes of Health, NIDDK)

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Increased serum transferrin receptor concentrations in patients with Friedreich's ataxia

Robert B. Wilson¹, David Lynch², David Brooks³, and Kenneth. H. Fischbeck²

Studies of the Yeast Frataxin Homolog, YFH1p, suggest that Friedreich's Ataxia may be a disease of abnormal cellular iron distribution. Yeast lacking YFH1p accumulate mitochondrial iron at the expense of cytosolic iron, and express high-affinity iron-uptake proteins even in iron-replete media. If the parallel between Friedreich's Ataxia and the yeast model system holds, then affected cells in Friedreich's Ataxia patients might be expected to behave in a similar fashion to yeast lacking YFH1p. The primary high-affinity iron-uptake protein in humans is the transferrin receptor (TfR), which is expressed on the cell surface, but can also be measured in the serum. We measured serum TfR in 12 patients with Friedreich's Ataxia, and found a statistically significant increase in the mean serum TfR concentration relative to that of normal controls. Our data support the hypothesis that Friedreich's Ataxia is a disease of abnormal cellular iron distribution, and may provide a quantitative means to follow disease progression and response to therapy

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Session VI

Frataxin & Pathogenesis: Animal models

- Knockout mice
- Transgenic mice

Michel Koenig, Chair

Targeted disruption of iron regulatory protein 2 leads to progressive neurodegeneration in mice

Tracey A. Rouault, Tim LaVaute, Kazu Iwai, Sophia Smith, Georgina Miller, Nancy Tresser, Paul Love, and Alex Grinberg

Mammalian cells contain two iron-sensing proteins known as iron regulatory proteins (IRPs). An RNA binding form of each protein accumulates in iron-depleted cells, but the mechanism of accumulation differs. The equilibrium between a form of IRP I that contains an iron-sulfur cluster and apoprotein generally reflects cellular iron concentrations. IRP2 is subject to iron-dependent oxidation, ubiquitination, and proteasomal degradation in iron-replete cells. The functions of these two proteins are redundant in most cells, and major regulatory targets such as the transferrin receptor and ferritin are normally regulated by changes in iron status when only one of the two proteins is present. However, some specific cell types in the intestinal mucosa and the central nervous system require IRP2 for appropriate regulation of iron metabolism. Animals that lack IRP2 develop a progressive neurodegenerative disease that is caused by excess deposition of iron within selected cell populations. Cytosolic iron accumulation appears to have a causal role in neuronal degeneration.

Towards a mouse model of Friedreich ataxia

Keiichi Ohshima¹, Julie C. Smith², Carlos Miranda¹, Manuela Santos¹,
Laura Montermini¹, Jerry Kaplan², and **Massimo Pandolfo**¹

Friedreich ataxia is the consequence of a deficiency of frataxin, mostly caused by GAA triplet repeat expansions which reduce but do not completely suppress frataxin gene expression. Individuals with frataxin point mutations, some of which are null mutations, are always compound heterozygotes for the point mutation and a triplet repeat expansion. Therefore, most individuals with Friedreich ataxia still make some normal frataxin, sometimes along with a mutated abnormal protein (cases with missense mutations) which may also retain some activity. This contrasts with knock-out models, which are completely devoid of frataxin. While yeast frataxin homolog knock-outs are viable, though severely impaired in mitochondrial function, an attempt to knock out the gene in the mouse, performed in Strasbourg by Dr. Koenig's group, resulted in early embryonic lethality (M. Koenig, personal communication and International Congress on Neuromuscular Diseases, Adelaide 1998). Therefore, in order to generate a mouse model that is viable and more closely resembles the human disease, it is necessary to reduce the amount of frataxin but leave some residual amount of the protein. We are using the following approaches to generate such a model: 1. Knock-in of a GAA repeat of about 250 triplets in the first intron of the mouse gene. 2. A conditional knock-out, in which exon 2 of the mouse frataxin gene is flanked by loxP sites and excised either by crossing with a Cre recombinase transgenic mouse or by infection with a viral vector carrying the Cre gene. In both cases the knock out should occur only in specific cells at a specific time in the animal's life. 3. Generation of a transgenic mouse expressing a mutant human frataxin (G130V), to be crossed with frataxin knock-out heterozygotes. Crosses should generate mice that only make mutated human frataxin, which should preserve some residual activity, as suggested by the mild phenotype of patients with the G130V mutation. 4. Generation of transgenic mice expressing an anti-frataxin ribozyme.

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Stability of GAA*TTC triplet repeats in transgenic mice

Keiichi Ohshima and Massimo Pandolfo

We generated transgenic mice carrying about 250 GAA*TTC triplets. The repeats were cloned in both orientations within the intron of a reporter gene, using a pSPL3-derived construct. DNA constructs were injected into the oocyte pronuclei of fertilized C57Bl6/C3H female mice. Pups were evaluated for the presence of the transgene by Southern blot and PCR analysis on DNA extracted from tail blood samples. Seven transgenic lines were obtained for the TTC orientation. Three transgenic lines were obtained for the GAA orientation.

We determined repeat sizes by PCR and by Southern blot analysis in the founders and in F1 and F2 mice. Most lines harbor multiple copies of the transgene, in some of which the GAA*TTC triplet repeat underwent size changes (mostly deletions) at the time of insertion into the mouse genome. However, repeats subsequently remained fully stable during meiotic transmission, with no detectable increases or decreases in size, regardless of orientation.

We conclude that GAA*TTC triplet repeats containing up to 250 triplets are stably transmitted in the mouse, at least in the context of the DNA construct we used to generate transgenic animals. In humans, repeats of similar length contained in the first intron of the frataxin gene show moderate meiotic instability.

Friedreich ataxia mouse models

Mireille Cossée, Helene Puccio, Hana Koutnikova, Victoria Campuzano,
Kenneth Fischbeck, Jean-Louis Mandel and **Michel Koenig**

Friedreich ataxia is caused by the loss of function of frataxin, a mitochondrial protein conserved through evolution. Yeast knock-out models, histological and biochemical data from heart biopsies or autopsies from patients suggest that frataxin may play a role in mitochondrial iron transport or in iron-sulfur proteins assembly. Affected human tissues are rarely available to test this hypothesis. We generated a mouse model by inactivation of the frataxin gene homologue. The gene disruption was obtained by deletion of exon 4, which causes a frame-shift. The genotype of surviving offsprings from heterozygote intercrosses was either wild-type or heterozygous; no mouse was homozygous for the deleted allele. We are analysing embryos to investigate the stage of embryonic lethality and the mechanism of degeneration, with a particular emphasis on iron accumulation. To circumvent embryonic lethality, we are generating a conditional knock-out model, based on the Cre-lox system in which exon 4 will be deleted under temporal and tissue specific control, and a knock-in mouse model by insertion of the H180R missense mutation found in a typical Friedreich ataxia patient (H183R). These models will allow to investigate the mechanism of the disease and to test anti-oxidant therapies.

Development of mouse models for Friedreich Ataxia

Kate Elliott, Lachlan McDonald, Tim Holloway, Kerry Fowler, Sophie Gazeas, Sue Forrest, Robert Williamson, and **Panos Ioannou**

The identification of frataxin as a mitochondrial protein involved in iron homeostasis indicates that Friedreich's ataxia is due to mitochondrial dysfunction as a result of mitochondrial iron overload. These findings and the elucidation of the molecular defects underlying Friedreich's ataxia have given impetus to efforts to establish accurate animal models for this disease.

In our group we have concentrated initially our efforts in creating a mouse model for the G130V mutation in exon 4 of the FRDA locus. In patients transheterozygous for this mutation and the GAA expansion within intron I, there appears to be a milder phenotype. It has thus been argued that the G130V mutation may provide a reasonable model for Friedreich ataxia in homozygous form. A mouse FRDA BAC clone was initially identified and the G127V mutation (equivalent to the human G130V mutation) was introduced into a 10kb subfragment carrying exon 4, together with a loxPflanked neomycin cassette in intron 4. Following standard procedures, we have been able to introduce the mutation in mouse embryonic stem cells and to produce mice carrying the mutation in heterozygous form. Work is in progress to produce mice homozygous for this mutation with and without the neo cassette, as well as to cross our mice with mice carrying a knockout mutation of the FRDA locus (M. Koenig et al.). It is hoped that these mouse models will reproduce at least some of the biochemical and phenotypic features of Friedreich ataxia and may thus provide useful models for developing treatment protocols aimed at preserving or restoring mitochondrial function.

Since about 98% of FRDA chromosomes carry the GAA expansion, we are also interested to explore gene therapy and pharmacological approaches which are aimed towards overcoming the impact of the GAA expansion on the expression of the frataxin locus. The strategies adopted by our group towards these objectives will be outlined at the meeting.

Session VII

Patient & Clinical Studies

- Genotype/phenotype correlations
- Heart disease
- Diabetes

Alexis Brice, Chair

Molecular and clinical characterization of Friedreich's ataxia compound heterozygote patients

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Rafael Vazquez Manrique¹, Juan J. Vílchez², Miguel Urtasun⁴, Miguel Lucas⁵,
Guillermo Navarro⁶, Guillermo Izquierdo⁶, and **Francesco Palau**¹

Friedreich's ataxia is caused by mutations in the FRDA gene that encodes frataxin, a mitochondrial protein. Most patients are homozygous for the expansion of a GAA triplet repeat within the FRDA gene, but a few patients show compound heterozygosity for a point mutation and the GAA-repeat expansion.

We analyzed DNA samples from a cohort of 241 patients with autosomal recessive or isolated spinocerebellar ataxia for the GAA triplet expansion. Patients heterozygous for the GAA expansion were screened for point mutations within the FRDA coding region. Molecular analyses included the single strand conformation polymorphism analysis (SSCP), direct sequencing to detect point mutations, and linkage analysis. Seven compound heterozygotes patients were identified. In four patients a point mutation that predicts a truncated frataxin was detected (two frameshift mutations, one nonsense mutation, and one 3' splicing site mutation). Three of them associated classic early onset Friedreich's ataxia with an expanded GAA allele greater than 800 repeats. The other patient associated late onset disease at the age of 29 years with a 350 GAA repeat expansion. In two patients, manifesting classical phenotype, no changes were observed by SSCP analysis. Linkage analysis in a family with two children affected with an ataxic syndrome, one of them showing heterozygosity for the GAA expansion, confirmed no linkage to the FRDA locus. We conclude that most point mutations in compound heterozygote Friedreich's ataxia patients are null mutations. Clinical phenotype seems to be related to the GAA repeat number in the expanded allele. Complete molecular definition in these patients is required for clinical diagnosis. This work is funded by the CICYT grant SAF97-0082 and Fundació "la Caixa" grant 97-134

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Point mutations and haplotype analysis in the FRDA gene

Susan M. Forrest¹, Martin B. Delatycki¹, Melanie Knight¹, Damien Paris¹, Leone Yeung², Najir Nassif², Garth Nicholson², Michel Koenig³, Mireille Cossée³, and Robert Williamson¹

Most cases of Friedreich ataxia (FRDA) are due to expansions of a GAA trinucleotide repeat sequence in the gene FRDA coding for frataxin. However, between 1-5% of mutations are single base changes in the sequence of the FRDA gene, causing missense, nonsense or splicing mutations. We describe three new mutations, IVS4nt2 (T to G), R165C and L182F, that occur in patients in association with GAA expansions. The splicing mutation is associated with a typical phenotype. Interestingly, R165C and L182F which occur in exon 5a at the carboxy terminal of the protein, result in a milder disease course. Therefore we propose that splicing, nonsense or initiation codon mutations (which cause a complete absence of functional frataxin) are associated with a severe phenotype. Missense mutations, even in highly evolutionarily conserved amino acids, may cause a mild or severe phenotype and the phenotype cannot be predicted from the position of the amino acid in the protein.

Of all point mutations, three appear to be relatively common. I154F is so far exclusively found in Southern Italy. MII has been found in three unrelated families and appears from haplotype analysis to have arisen from a common founder. G130V has been found in patients from around the world. We examined the haplotype background of the mutation in four G130V families. We have found that three of the four have a very similar haplotype whilst the fourth is different. This suggests that the G130V mutation may not always be due to a common founder mutation.

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What indications for a molecular test of Friedreich ataxia?

Massimo Pandolfo

We reviewed clinical records for more than 450 individuals with recessive or sporadic ataxia tested for Friedreich ataxia in our laboratory. About half of them had a confirmed molecular diagnosis, with homozygosity for expanded GAA repeats. Six patients were heterozygous for an expanded repeat and a frataxin point mutation. Linkage to the FRDA locus was excluded by haplotype analysis in some negative individuals for GAA expansions. The frequency of some signs and symptoms differs between positive and negative cases, but no clinical finding or combination exclusively characterizes positive cases. Therefore, different genetic entities determine phenotype that overlaps with FRDA. However, presence of the “classical” features of FRDA, as defined in Harding’s diagnostic criteria, is highly predictive of a positive test, while the presence of cortical cerebellar atrophy, demonstrated by MRI, appears a good predictor of a negative test.

In conclusion, a molecular test for FRDA is indicated for patients with progressive ataxia, no MRI evidence of cortical cerebellar atrophy, who present as sporadic cases or have affected sibs but no familial history compatible with a dominant ataxia.

Genetic relationships between Friedreich ataxia and Diabetes mellitus

Michael Ristow^{1,2}, Eleni Giannakidou², Judith Hebinck³, Ludger Schoels⁴,
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Friedreich Ataxia is frequently associated with diabetes mellitus. While the metabolic basis is unexplained, diabetes may be caused by insulin resistance due to frataxin deficiency, or oxidative damage of insulin-secreting beta-cells in the pancreas. While previous studies have repeatedly shown normal or even increased insulin secretion patterns, some evidence supports the occurrence of peripheral insulin resistance in FRDA-patients. To further elucidate this question, we evaluated eleven individuals heterozygous for the GAA-repeat expansion, thus asymptomatic carriers of the FRDA-genotype (as typically found in parents and siblings of FRDA-patients), and compared them to matched, non-expanded control subjects. All individuals underwent oral glucose tolerance testing (oGTT) and quantification of insulin resistance based on a parallel infusion of insulin, glucose and somatostatin, the latter one to suppress endogenous insulin secretion. One of the heterozygously affected individuals was shown to be diabetic based on the results of the oGTT. Using the somatostatin-based test, a significantly higher degree of insulin resistance ($p=0.001$) was found in every single heterozygously affected individual compared with the control subjects. Furthermore, a significant correlation between the degree of insulin resistance and the length of the GAA-tract was found ($r=0.69$). Secondly, we studied a potential association between the occurrence of type 2 diabetes (NIDDM) and intermediate expansions of the GAA-tract (i.e. number of repeats below 30, thus not conferring to the ataxia phenotype). Based on a PCR-amplification of DNA isolated from peripheral blood in diabetic individuals and matched, non-diabetic control persons, two different populations (Germans and US-Americans) were evaluated. Intermediate expansions (10 to 36 repeats), which are longer than normal but not sufficient for the appearance of the ataxia phenotype, were found in 24.7% and 27.3% of these two NIDDMz cohorts as compared with 7.6% and 6.3% of the matched control subjects (both $p < 0.001$). The odds ratios, indicating the increase of risk to develop type 2 diabetes, were 3.36 (95% confidence interval, 1.72-6.55) for the German group and 4.01 (2.08-7.74) for the US group. Therefore, we conclude that the frataxin GAA repeat polymorphism is associated with type 2 diabetes in the specific populations evaluated.

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Skeletal muscle abnormalities in Friedreich's ataxia patients demonstrated by Near-Infrared Spectroscopy

Robert B. Wilson¹, David R. Lynch², Kenneth H. Fischbeck², William J. Bank², and Britton Chance³

Recent studies indicate that mitochondrial dysfunction, secondary to mitochondrial iron accumulation and oxidant damage, underlies the pathophysiology of Friedreich's Ataxia. To test the hypothesis that the skeletal muscle weakness associated with Friedreich's Ataxia is at least in part intrinsic to muscle and secondary to mitochondrial dysfunction, and to test methodologies for objective, quantitative measurement of disease progression and response to therapy, we performed Near-Infrared Spectroscopy (NIRS) on Friedreich's Ataxia patients. NIRS is a non-invasive optical technique that uses the differential absorption properties of the oxy- and deoxy- forms of hemoglobin and myoglobin to assess kinetic changes in skeletal muscle oxygenation during exercise. Low-resolution imaging using 16-point data (interpolated to 64 points) allows spatial heterogeneity of deoxygenation to be visualized in 2 dimensions. Normal individuals deoxygenate skeletal muscle during exercise, reflecting an efficient extraction of oxygen by respiring mitochondria which exceeds the rate of oxygen delivery. In contrast, patients with known mitochondrial defects paradoxically oxygenate skeletal muscle during exercise, reflecting an inability to utilize increased oxygen delivery due to impaired mitochondrial metabolism. NIRS of the gastrocnemius muscle of Friedreich's Ataxia patients during a treadmill walking exercise demonstrated a regional heterogeneity across the muscle, with some areas deoxygenating and others oxygenating. Regions of paradoxical oxygenation were larger in more severely affected patients. Our results demonstrate that the skeletal muscle weakness associated with Friedreich's Ataxia is at least in part intrinsic to muscle, and that NIRS may be useful in following disease progression and response to therapy in ambulatory patients with Friedreich's Ataxia.

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Genotype/phenotype relationship in Friedreich's ataxia

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We present the clinical and molecular data in 149 patients with Friedreich's ataxia originating mainly from Southern Italy. One hundred and thirty-nine patients were homozygous for the GAA expansion and 10 were compound heterozygotes for the expansion and different mutations in the X25 gene.

Mean onset age was 15.3 ± 8.0 years and mean disease duration was 14.9 ± 9.7 years in the homozygous patients. The mean GAA expansion on the smaller allele was 667 ± 230 triplets and it was significantly correlated with several clinical features. Patients with variant phenotypes (late onset or retained tendon reflexes) carried shorter expansions. A larger expansion was associated with lower limb areflexia, pes cavus and cardiomyopathy. Longer disease duration was associated with dysarthria, weakness, decreased vibration sense and diabetes. Both GAA expansion and disease duration influenced the occurrence of Babinski sign.

Concerning the neurophysiological features, only GAA expansion determined amplitude of sensory action potential at wrist and medial malleolus and the percentage of myelinated fibers in sural nerve biopsies. Central somatosensory, motor, visual and auditory pathways were influenced by either GAA expansion or disease duration or both. The multivariate analysis showed no effect of the expansion on the larger allele.

Ten patients from six families were compound heterozygotes. Three families carried a missense mutation within the exon 4 (I154F), two families two different missense mutations in exon 5a (W173G, R165P) and one family a truncating mutation (482+3delA). The mean onset age was 9.1 ± 6.1 years and mean disease duration was 14.4 ± 8.0 years. The mean GAA expansion was 829 ± 129 triplets. The heterozygous patients differed from homozygotes for an earlier age at onset and lower occurrence of dysarthria. Two patients carrying the R165P mutation had an atypical phenotype.

A modifying effect of variable-sized GAA repeats on clinical features in FRDA patients within one “pseudo-dominant” genealogy

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Irina A. Ivanova-Smolenskay**

We examined a large consanguineous family of Turkmen ethnic origin in which five patients from two generations were affected by Friedreich’s ataxia (FRDA). The parents of four affected siblings were first cousins. The “pseudo-dominant” inheritance of FRDA in this family is accounted for by consanguineous marriage of a FRDA patient (carrying alleles with 250 and 700 GAA repeats) to a heterozygote carrying a common ancestral mutated allele (700 repeats). Two distinct phenotypes of the disease co-segregated within this one genealogy. Two brothers from the lower generation exhibited “classical” FRDA with onset of symptoms before 10 years and a severe, relentlessly progressive course. On the other hand, three patients from the same family (two sisters from the lower generation and their farther) had a completely different “benign” phenotype (“late onset Friedreich’s ataxia”, or LOFA) with the onset at 26, 45 and 48 years and very slow progression over decades. All the patients developed moderate cardiomyopathy confirmed by the ECG study. The DNA analysis showed that the patients with “classical” FRDA had the only detectable expanded band (homozygosity for the longer 700 GAA repeats-containing allele). The sisters with LOFA had two different expanded alleles, the shorter one of paternal origin containing 250 GAA repeats and the longer one of maternal origin containing 700 GAA repeats. Taking into account a common environmental and genetic background in the present family, one may conclude that clinical variability of FRDA in our patients is caused predominantly by a modifying effect of one of the two (shorter or longer) expanded alleles inherited from the father. Our observation demonstrates very purely on a unique “clinical model” the significance of the alleles of different sizes for the phenotypic expression of the disease, implying the critical role of more “preserved” allele for maintaining a normal function of frataxin.

Session VIII

Therapeutic Approaches

- Antioxidants
- Iron chelation

Alessandro Filla, Chair

Towards a prevention of cardiomyopathy by idebenone in Friedreich ataxia

Pierre Rustin, Agnes Rötig, and Arnold Munnich

Friedreich Ataxia (FRDA) is a frequent autosomal recessive condition causing spinocerebellar degeneration and hypertrophic cardiomyopathy, and resulting from the deficiency of frataxin, a protein involved in regulation of mitochondrial iron content. We have recently reported a combined deficiency of a Krebs cycle enzyme, aconitase, and three mitochondrial respiratory chain complexes in endomyocardial biopsies of FRDA patients. All four enzymes share iron-sulfur cluster containing proteins (ISP) that are damaged by iron overload via generation of oxygen free radicals. We have devised an in vitro system using human heart homogenates both to elucidate the mechanism of iron-induced injury and to test the protective effects of various substances. Under our experimental conditions, reduced iron mimicked the damages observed in heart biopsies of FRDA patients. Ascorbate, which reduced iron and desferrioxamine, increased iron-induced injury in vitro. Conversely, reduced idebenone efficiently protected mitochondrial enzymes from iron-induced damages. Accordingly, three FRDA patients with hypertrophic cardiomyopathy were given idebenone orally (5 mg/kg/d). After 4-9 months, ultrasound evidence of drug efficiency was provided by the reduction of left ventricle hypertrophy in the three patients (mass index reduction: 21, 30, 32%, respectively). Our in vitro data suggest that both iron chelators and antioxidant drugs likely to reduce iron are potentially harmful in FRDA. Conversely, our preliminary in vivo data suggest that idebenone protects heart muscle from iron-induced injury in FRDA. Ongoing nation-wide clinical trial in process in France will hopefully confirm these preliminary observations.

Evidence for mitochondrial iron overload in patients with Friedreich Ataxia

Julie C. Smith¹, James P. Kushner¹, Mark Bromberg², Elizabeth Hammond³, William H. Barry⁴, Massimo Pandolfo⁵, and Jerry Kaplan³

The underlying disease pathophysiology in patients with Friedreich Ataxia (FA) has long been elusive to investigators and physicians. Yeast strains with a deletion in *Yfh1*, an orthologous homologue of frataxin, show abnormal respiratory function (petite formation), and accumulation of iron in the mitochondria. In both yeast and human, the protein frataxin has been localized to the mitochondria. In yeast, frataxin regulates iron export from mitochondria. Although the frataxin-iron link is easily proven in yeast, the evidence thus far in humans has been lacking. Through the General Clinical Research Center at the University of Utah Medical Center in Salt Lake City, Utah, we are conducting a NIH funded clinical trial evaluating iron chelation therapy as treatment for Friedreich Ataxia. The primary objective of this trial is to determine if chelation therapy using desferrioxamine can deplete iron overload within affected tissues in patients with genotypically proven FA. Seven patients have been enrolled thus far. All patients are >18 years of age and have genotypically proven FA (450-1037 repeats). Upon enrollment, baseline measurements of affected tissues were conducted, including a complete cardiac work-up consisting of an EKG, echocardiography, and right heart catheterization with endomyocardial biopsy. In addition, muscle biopsies, bone marrow aspirates and biopsies, and a full neurologic evaluation were conducted (EMG, QST, ataxia scale, electroretinogram, visual evoked potentials). The planned duration of treatment is one year, at which point the above parameters will be reassessed. Definitive evidence of cardiac iron overload was seen in all study patients as evidenced by histologic examination of endomyocardial biopsy. Electron microscopic findings revealed accumulation of electron dense particles consistent with iron in mitochondria. Further evidence supporting mitochondrial iron overload in humans was determined by measurement of human cardiac mitochondrial (autopsy specimens) iron by inductively coupled plasma elemental analysis. (This work is supported through a Center of Excellence in Hematology Grant from the National Institutes of Health, NIDDK)

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Biochemical consequences of frataxin deficiency

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Deletion of YFH1 in yeast results in impaired respiratory function, increased intramitochondrial iron and decreased mitochondrial DNA (mtDNA) levels. We have investigated mitochondrial respiratory chain function and oxidative phosphorylation capacity, aconitase activity, iron levels and mtDNA content in Friedreich's ataxia (FRDA) patients. We demonstrate: (1) ³¹P magnetic resonance spectroscopy (³¹pMRS) abnormalities in vivo in skeletal muscle from FRDA patients. The decline in Vmax correlated with the length of the patients' GAA repeat and therefore inversely with expected frataxin levels; (2) Abnormal ³¹pMRS in myocardium from FRDA patients; (3) Data on the results of treatment design to reverse these abnormalities will be presented; (4) Severe and highly significant defects in the activities of complexes I-III and aconitase in FRDA heart; (5) Iron accumulation in FRDA heart, liver and spleen; (6) Defects of complexes I-III and aconitase in FRDA skeletal muscle; (7) Mitochondrial DNA levels were not significantly decreased in tissues studied.

These results provide insight into the biochemical consequences of frataxin deficiency and the mechanisms that may be involved in FRDA pathogenesis. The pattern of mitochondrial defects suggests that oxidative damage is important. Abnormal mitochondrial iron metabolism may result in impaired Fe-S construction and the resulting impairment in respiratory chain function may cause increased free radical release and cell damage.

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Clinical Aspects of Friedreich's Ataxia: Considerations for Treatment Trials

George R. Wilmot and Sue Gronka

The recent advances in our understanding of the molecular pathogenesis of Friedreich's ataxia has generated a great deal of interest in therapeutic trials. However, FRDA may be a difficult disease to study for therapeutic intervention given its relatively low prevalence, protracted clinical course and phenotypic variability. As a precursor to definitive treatment trials, outcome measurement techniques such as clinical rating scales, physiologic measurements, and biological markers should be validated and used to assess the natural history of the disease. As a step in that direction, we have begun collecting clinical data on the FRDA patients at Emory using a preliminary ataxia rating scale. Continued data collection with a greater number of patients should help to further define the natural history of FRDA and in turn will facilitate therapeutic trials.

Neuroprotective Effects of CoQ10 and Creatine

M. Flint Beal

The electron transport gene component CoQ10 or ubiquinone is a potent antioxidant which protects against glutamate toxicity in vitro. It produces dose-dependent protection against striatal lesions produced by ATP depletions. It also protects against both MPTP and 3-nitropropionic acid neurotoxicity and it extends survival in the transgenic mouse model of ALS. In HD patients, oral administration results in significant decreases in occipital cortex lactate concentrations which reversed following withdrawal of therapy. CoQ10 exerts additive effects with N-methyl-D-aspartate antagonists which has led to a proposal to test CoQ10 and an NMDA antagonist both alone and in combination in the treatment of HD. Another approach we recently examined, is to administer creatine to increase phosphocreatine levels and, thereby, buffer against energy depletion. Oral administration protects against striatal lesions produced by both malonate and 3-nitropropionic acid. It protects against phosphocreatine and ATP depletions as well as increases in 3-nitrotyrosine complex concentrations following administration of 3-nitropropionic acid. It also shows neuroprotective effects against MPTP toxicity and it significantly extended survival and exerted neuroprotective effects in a transgenic mouse model of ALS. These findings suggest that agents which improve bioenergetics may have significant neuroprotective effects in neurodegenerative diseases.