Alteration of the Serotonergic Nervous System in Fatal Familial Insomnia

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Serotonergic Nervous System

Fatal familial insomnia (FFI) is a rare form of human transmissible spongiform encephalopathies (TSEs), or prion diseases. FFI is inherited by an autosomal dominant trait and is genetically linked to a point mutation at codon 178 of the prion protein (PRNP) gene, coupled with the methionine polymorphism at codon 129 of the mutant allele.1,2 Neuropathology differs from other human TSEs, with predominance of lesions in the thalamus, whereas involvement of the cortex is mild or absent.3,4 Patients with FFI display unique clinical features, including complex alterations of the sleep-wake cycle, attention deficit, sympathetic hyperactivity, attenuation of autonomic circadian and endocrine oscillations, and motor signs.5 On polysomnography, non–rapid eye movement (NREM) sleep is abolished, sleep spindles and K-complexes disappear early, and brief, often clustered, REM sleep episodes are associated with orexin behavior because of lack of physiological muscle atonia.6 Impairment of sleep and autonomic and endocrine functions in FFI has been related to selective damage of anterointernal and mediodorsal thalamic nuclei, the cingulate gyrus, and orbitofrontal gyrus, leading to interruption of thalamocortical limbic circuits involved in the control of the sleep-wake cycle.5–7 Ascending serotonergic projections arising in median raphe nuclei densely innervate the limbic thalami and related cortical regions. The brain serotonergic system is considered to play an integral role in the regulation of sleep homeostasis, autonomic functions, and control of locomotor activities.9 To investigate whether alterations of the serotonergic system are present in FFI, we immunohistochemically visualized and evaluated quantitatively serotonin-synthesizing neurons in the median raphe.

Materials and Methods

We used formal-fixed, paraffin-embedded brain tissue obtained at autopsy from 8 FFI affected subjects: 4 members of the Austrian FFI family,10 and 4 cases from 3 different, previously published kindreds.2,11 All cases were methionine homozygous at codon 129. Eight sex- and age-matched individuals without neurological disease served as controls (details summarized in the Table). Additionally, 4 sporadic and 2 familial subjects with Creutzfeldt-Jakob disease (1 reported previously12) were investigated as disease controls. The following brain regions were examined: upper pons, including the dorsal raphe nucleus (DRN) and superior central nucleus (SCN), and medulla oblongata with raphe obscurus nucleus (RON). In 2 FFI cases, only the medulla oblongata and in another only the pons could be examined.

Immunohistochemistry was performed on 4-μm-thick sections using a monoclonal antibody against tryptophan hydroxylase (PH8; Pharmingen, San Diego, CA; 1:500) to visualize serotonin (5-HT)-synthesizing neurons.13 Sections for tryptophan hydroxylase labeling were boiled 10 minutes in citrate-buffered saline (pH 6.0). As secondary antibody, we used a ChemMate detection kit (Dako, Glostrup, Denmark) as described previously.14 Immunostained sections were counterstained with cresyl violet.

Total numbers of neurons and numbers of tryptophan hydroxylase–immunoreactive (TH+) neurons were quantified on two consecutive sections across the upper pons and medulla with a 40-μm interval by counting cell bodies in six continuous fields per section with a 40× objective, including four fields next to the median line and two more lateral fields in SCN, and six fields next to the median line in RON. These fields covered most or all of the area of the nucleus. Only nucleated cells or cells with their complete soma visible were counted. Relative numbers of TH+ neurons were evaluated in PH8-stained sections by counting positive and negative neurons in the same fields. Mean values of 12 fields, respectively, were entered into statistical evaluation. Since sections of the pons from some brains contained only caudal parts of the DRN, quantitation was not done there.
The significance of the difference of mean regional cell numbers and the ratio of TH$^+$ to TH$^-$ neurons were tested using a Kruskal-Wallis one-way test followed by the exact version of a two-tailed Mann-Whitney U test. For all tests, $p \leq 0.05$ was considered as the level of significance.

### Results

#### Controls

Hematoxylin and eosin (H&E)-stained sections showed no histopathological changes. TH immunohistochemistry visualized cell bodies and axons of serotonergic neurons in raphe nuclei. Morphology of neurons was similar in all cases, appearing as either small or large cells with multipolar or fusiform-shaped perikarya (Fig 1). In SCN, 38.9% $\pm$ 4.8 SEM were TH$^+$ and 61.1% $\pm$ 4.8 SD were TH$^-$. In RON, 34.6% $\pm$ 3.1 SEM of the neurons were TH$^+$, whereas 65.4% $\pm$ 3.1 SD were TH$^-$ (Fig 2).

#### Creutzfeldt-Jakob Disease

H&E sections yielded mild to moderate gliosis and spongiform change in the tegmentum. Quantification of neurons showed no loss of total cell numbers and no changed ratio of TH$^+$ to TH$^-$ neurons in SCN and RON. In SCN, 42.4% $\pm$ 4.4 SEM ($p = 0.818$ vs controls) of neurons were TH$^+$ and 57.6% $\pm$ 4.4 SEM were TH$^-$. In RON, 40.5% $\pm$ 3.4 SEM of the neurons were TH$^+$ ($p = 0.366$ vs controls) and 59.5% $\pm$ 3.4 SEM were TH$^-$ (Fig 2).

### Discussion

The main clinical features of FFI—namely, loss of circadian sleep patterns, flattening of circadian and neuroendocrine rhythmicity, and unbalanced sympathetic hyperactivity—indicate a profound disturbance of control of homeostasis. The pathophysiological role of the thalamus has been emphasized based on the invariable and severe degeneration of anteroventral and mediodorsal thalamic nuclei during the disease, while other areas classically known to control autonomic functions, such as the hypothalamus and several regions of the brainstem, appear morphologically spared by the disease process.
The serotonergic system has been implicated in the regulation of the sleep-wake cycle and circadian rhythmicity. It has been demonstrated that serotonin suppresses REM sleep and ponto-geniculo-occipital waves by inhibition of cholinergic laterodorsal tegmental and pedunculo-pontine tegmental nuclei. Atonia of anti-gravity muscles is a fundamental feature of REM sleep. Neurophysiological studies in pontine-lesioned cats showed increased activity of 5-HT neurons during REM sleep associated with high muscle tone of anti-gravity muscles and overt behavior, resembling the REM sleep abnormalities in FFI. Moreover, experimental data document a sympathoexcitatory role of 5-HT, causing exaggerated cardiovascular responses.

In this study, we demonstrate increased numbers of TH neurons and a changed ratio of TH to TH neurons in the pons (SCN) and medulla (RON) of FFI patients compared with controls. These changes were consistently observed in FFI cases from 4 different families. There is no decrease in total cell numbers; thus, relative increase of TH neurons in FFI cannot be explained by loss of TH neurons. This observation...
more likely reflects a recruitment of TH$^+$ neurons from a pool of “silent” raphe neurons with the potential to synthesize serotonin or the ability to activate serotonin production. Because we have not observed similar changes in the serotonergic system of sporadic and familial CJD or cases with a selective ischemic thalamic lesion (unpublished data of our group), the changed ratio of TH$^+$ to TH$^-$ neurons is unlikely to occur secondary to the thalamic damage in FFI. The imbalance between TH$^+$/TH$^-$ neurons indicates a disturbance in the homeostasis of serotonin metabolism. These observations provide evidence for an enhanced serotonergic neurotransmission as possible functional substrate for some symptoms in FFI.

Several serotonin-related disorders show no detectable morphological abnormalities, such as mood disorders.$^{19}$ Investigation of the TH$^+/TH^-$ ratio in these disorders may provide deeper insight into the pathophysiology of these disorders and contribute to the formation of a rationale for adequate treatment.

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References

7. Benarroch EE, Stotz-Potter EH. Dysautonomia in fatal familial insomnia as an indicator of the potential role of the thalamus in autonomic control. Brain Pathol 1998;8:527–530
Inhibition of Cyclooxygenase-2 Protects Motor Neurons in an Organotypic Model of Amyotrophic Lateral Sclerosis

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The pathogenesis of motor neuron loss in amyotrophic lateral sclerosis (ALS) is thought to involve both glutamate-mediated excitotoxicity and oxidative damage due to the accumulation of free radicals and other toxic molecules. Cyclooxygenase-2 (COX-2) may play a key role in these processes by producing prostaglandins, which trigger astrocytic glutamate release, and by inducing free radical formation. We tested the effects of COX-2 inhibition in an organotypic spinal cord culture model of ALS. The COX-2 inhibitor (SC236) provided significant protection against loss of spinal motor neurons in this system, suggesting that it may be useful in the treatment of ALS.


There is increasing evidence that glutamate-mediated excitotoxicity plays an important role in the pathogenesis of amyotrophic lateral sclerosis (ALS). Glutamate released by presynaptic neurons normally stimulates motor neurons, and its synaptic actions are rapidly terminated by specific glutamate transporters on astrocytic and neuronal membranes surrounding the synapse. Accumulation of glutamate in the synaptic space can exert neurotoxic effects mediated by the entry of Ca\(^{2+}\) in motor neurons and the production of various toxic molecules. Impairment of glutamate uptake by the astrocytic transporters is thought to play an important role in the pathogenesis of ALS. It is now clear that astrocytes not only take up glutamate but that they also synthesize and release substantial amounts of glutamate. Astrocytic glutamate release may be stimulated by prostaglandins via a calcium-dependent pathway, and this triggers additional glutamate release from the astrocytes by a positive feedback mechanism. Prostaglandins are produced within the central nervous system by the enzymatic action of cyclooxygenase-2 (COX-2), which catalyzes the synthesis of prostaglandins from arachidonic acid. Inhibitors of COX can powerfully inhibit astrocytic glutamate release. Thus, COX-2 inhibitors might have a therapeutic effect in ALS by inhibiting astrocytic release of glutamate. Furthermore, the fact that COX-2 plays a pivotal role in inflammatory processes in the central nervous system has led us to anticipate that its inhibition could have additional therapeutic benefit in ALS.

To evaluate the therapeutic effect of COX-2 inhibition, we used an in vitro model of glutamate excitotoxicity. Mammalian spinal cord slices are maintained in an organotypic culture system in which the motor neurons normally remain intact for more than 3 months. When astroglial transport of glutamate is inhibited by threo-hydroxyaspartate (THA), however, the spinal cord sections exhibit persistent elevation of glutamate levels and undergo gradual loss of motor neurons. This model system has been used in the preclinical evaluation of agents of potential interest for the treatment of ALS. In the present study, we used a selective COX-2 inhibitor (SC236; Monsanto/Searle, St Louis, MO), which is homologous to celecoxib, a drug currently in use for the treatment of arthritis. Our findings show that the addition of SC236 to THA-treated spinal cord cultures resulted in highly significant protection against loss of motor neurons.

Material and Methods

Organotypic spinal cord cultures were prepared from lumbar spinal cords of 8-day-old rat pups sectioned transversely into 350-μm slices and cultured on Millicell CM (Millipore Corp, Bedford, MA) semipermeable culture inserts at 37°C with 5% CO\(_2\) and 95% humidity. Under these conditions, more than 95% of cultures retain cellular organization, with survival of motor neurons in excess of 3 months. Culture media consisted of HEPES-buffered minimum essential medium (50%) with 25% heat-inactivated horse serum and 25% Hanks’ balanced salt solution supplemented with D-glucose (25.6 mg/ml) and glutamine (0.2 M). Seven days after culture preparation, THA was added to experimental cultures at 150 μM, which produces excitotoxic damage to motor neurons within 3 to 4 weeks. SC236 was added as indicated to achieve final concentrations from 0 to 100 μM. Parallel spinal cord cultures with no drugs added, THA alone, SC236 alone, and SC236 plus THA were run simultaneously. Experiments at each concentration of SC236 were repeated three to five times. A total of 25 to 47 cultures were used for each treatment. The medium, with THA and SC236 at indicated concentrations, was changed twice a week. The cultures were maintained for 4 weeks and pre-
pared as described below for quantification of motor neurons.

Motor neurons were identified by two immunocytochemical markers: SMI-32 stains nonphosphorylated neurofilaments, which are abundant in motor neuron cell bodies, and Islet-1 is a motor neuron–specific marker. Cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 minutes and then permeabilized with cold methanol or 0.1% Triton X-100 for SMI-32 and Islet-1 staining, respectively. Incubation with SMI-32 antibodies (1:8,000) or Islet-1 antibodies (1:100) was performed overnight at 4°C. After incubation with biotinylated horse anti-mouse antibodies and avidin-biotin complex reagents (Vector Labs, Burlingame, CA), color was developed with diaminobenzidine.

To quantify surviving motor neurons, whole-mount cultures immunostained by SMI-32 were used. The microscopist was unaware of the treatments used. Motor neurons were identified by three criteria: immunostaining with SMI-32, size greater than 25 μm, and localization to the ventral gray region of spinal cord (Fig 1). The motor neuron count using SMI-32 was verified by Islet-1 staining in some cases.

Results

Treatment with THA produces gradual motor neuron toxicity attributable to excessive extracellular glutamate. This mimics, in part, the loss of glutamate transport that occurs in sporadic ALS patients. We used a concentration of THA (150 μM) that produces motor neuron damage in 3 to 5 weeks.

Addition of THA alone resulted in a highly significant loss of motor neurons compared with control untreated cultures (see Figs 1 and 2). SC236 added to the THA-treated cultures potently and significantly protected motor neurons against excitotoxic damage at all concentrations tested (see Figs 1 and 2). The apparent lack of dose response is unexplained but could reflect a plateau effect. Nevertheless, this COX-2 inhibitor prevented the excitotoxic loss of motor neurons. We also noted a moderate decrease of motor neurons in cultures treated with SC236 alone.

Discussion

These experiments were undertaken to test the hypothesis that inhibition of COX-2 activity could prevent excitotoxic motor neuron loss in an ALS-like organotypic spinal cord culture model. This hypothesis was based on the key role of COX-2 in the production of prostaglandins and the demonstrated effects of prostaglandins in stimulating astroglial glutamate release and producing inflammatory changes as well as a variety of toxic molecules. Our findings show that COX-2 inhibition had a highly significant protective effect on motor neurons in this model system.

Different forms of COX exist in two isoforms: COX-1, which is constitutively expressed at extremely low levels in the nervous system but is not inducible, and COX-2, which is present throughout the central nervous system and is highly inducible. The different forms of COX catalyze the rate-limiting steps in the synthesis of prostaglandins. COX-2 is present in neurons and astrocytes as well as in macrophages and microglia. COX-2 has been demonstrated in the anterior horns of the spinal cord and specifically in motor neurons of rat spinal cord by immunocytochemistry. COX-2 is an inducible enzyme that increases in the brain after synaptic activity, seizures, or ischemia and in the spinal cord after trauma to the cord or peripheral noxious stimuli such as inflammatory arthritis. Proinflammatory cytokines, including interleukin-1β, upregulate the expression of COX-2 in astrocytes as well as in other cells. Of particular interest, interleukin-1β is markedly elevated in spinal cords of a mutant superoxide dismutase mouse model of ALS and plays a role in apoptotic neuronal cell death in that model.

It is likely that the effectiveness of COX-2 inhibition in preventing excitotoxic motor neuron death in our model system is due to the interruption of multiple processes, which can interact in a vicious cycle to produce progressive damage to motor neurons and astrocytes. One such mechanism is the prostaglandin-induced release of glutamate from astrocytes and secondarily from neurons, which induces excitotoxic damage. Excitotoxicity of the released glutamate is further enhanced because of the decreased buffering ca-
Capacity of astrocytes due to impairment of astrocytic glutamate transport, actual loss of astrocytes, and potentiation of excitotoxic effects of glutamate by overexpression of COX-2 in neurons. In ALS, any of the stimuli or inflammatory molecules noted above could initiate upregulation of COX-2. The process may be self-perpetuating, with damage and dysfunction of neurons and astrocytes spreading contiguously in widening areas as is commonly observed clinically in ALS.

COX-2 inhibitors also interrupt inflammatory processes that result in the production of free radicals and other toxic molecules. Inhibition of COX-2 has been shown to protect against the effects of global ischemia or reperfusion injury in the brain and trauma to the spinal cord. There is a recent report that a soluble aspirin analog may delay the onset of motor deficits in mutant superoxidase dismutase 1 mice.

The present results suggest that COX-2 inhibition could have therapeutic effects in ALS by altering the cascade of pathogenic processes that otherwise cause relentless progression of motor neuron damage. Inhibition of COX-2 could break the pathogenetic cycle by (1) preventing prostaglandin-mediated release of glutamate from astrocytes and (2) interrupting an important pathway that otherwise leads to the production of free radicals, toxic and proinflammatory molecules which damage neurons and glia and upregulate the activity of COX-2. The availability of COX-2 inhibitors that readily penetrate the central nervous system and are relatively nontoxic facilitates therapeutic trials in animal models of ALS.

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References


Down’s Syndrome Is Associated with Increased 8,12-iso-iPF2α-VI Levels: Evidence for Enhanced Lipid Peroxidation In Vivo

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Postmortem and in vitro studies have shown that oxidative stress plays a role in the pathogenesis of many of the clinical features of Down’s syndrome. The isoprostane 8,12-iso-iPF2α-VI is a specific marker of lipid peroxidation. We found elevated levels of this isoprostane in urine samples of subjects with Down’s syndrome compared with those of matched controls, which correlated with the duration of the disease. These results suggest that increased in vivo lipid peroxidation is a prominent component early in the course of Down’s syndrome.


Caused by the triplication of chromosome 21, Down’s syndrome (DS) is one of the most common human cytogenetic abnormalities. Those with DS suffer from a wide range of symptoms that are considered to occur through damage caused by overexpression of normal gene products encoded by extra gene copies present on this chromosome. Human copper/zinc-superoxide dismutase (SOD1) is one of these genes, and increased levels of this enzyme as a result of gene dosage have been shown in DS subjects. The increased gene dosage for SOD1 has been proposed to contribute to the precocious dementia of Alzheimer’s type and mental retardation that occurs as part of the syndrome. High

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SOD1 activity results in the accumulation of hydrogen peroxide, which leads to the formation of an excess of hydroxyl radicals, the most reactive oxygen species (ROS), through a Fenton-type reaction and to subsequent oxidative stress with loss of cellular function through damage to macromolecules.4

Despite the fact that some studies have associated DS with increased levels of markers of oxidative stress, there are shortcomings to these investigations. They have used markers that are not specific and also have used mostly tissue assays and in vitro cellular systems rather than changes in plasma or urine as potential biomarkers of oxidative stress in living patients with DS.5,6

F2-isoprostanes are isomers of the prostaglandin F2α produced by ROS attack on polyunsaturated fatty acids. They are chemically stable, sensitive, and specific quantitative biomarkers of lipid peroxidation in vitro and in vivo.7

Here, we measured levels of 8,12-iso-iPF2α-VI, one of the most abundant F2-isoprostanes in human beings,7,8 in the urine of young living subjects with DS and compared those levels with the levels in the urine of matched controls.

Subjects and Methods
The participants were randomly recruited from the Children’s Hospital of the University of Chieti, Chieti, Italy. Patients were all karyotyped at birth. Each participating family with a DS child was asked to provide a urine sample. Control samples were from non-DS siblings of the patients or from healthy nonrelated children attending the clinic. The study was approved by the Ethics Committee of the Institutional Review Board of the hospital where it was conducted. Informed and signed consent was obtained from a parent or guardian of each participant. The urine samples were collected in plastic tubes early in the morning and brought to the clinic within 4 hours of collection. An aliquot (1 ml) was stored for creatinine determination. Immediately afterward, the samples were frozen in a coded fashion at −80°C and kept frozen until analysis.

8,12-iso-iPF2α-VI Assay
Samples were spiked with internal standard [4H2]-8,12-iso-iPF2α-VI, extracted, purified, and finally assayed by gas chromatography and mass spectrometry as previously described.7,8 The intra-assay and interassay variability was ±4% and ±5%, respectively. All assays were performed in a coded fashion.

Statistical Analysis
Comparisons between groups were performed by nonparametric one-way ANOVA (Kruskal-Wallis test) with the use of Dunn’s postest. Correlation was studied by linear regression analysis. Statistical significance was set at p < 0.05.

Results
Thirty-three subjects with DS and 33 control subjects participated in the study; their characteristics are shown in Table 1. No significant difference in age or gender was observed between controls and DS patients. Study participants stopped taking any vitamins or antioxidants 4 weeks before the study. Three patients were taking substitutive hormonal therapy (thyrotropin-stimulating hormone) for a mild form of hypothyroidism, which was well controlled at the time of the study (not shown).

A scatterplot diagram of urine 8,12-iso-iPF2α-VI levels for the 2 groups of patients is shown in Figure 1. Control subjects had a median value of 1.20, with a range of 0.25 to 2.5 ng/mg of creatinine. DS patients had significantly higher levels than controls, with a median of 1.97 and a range of 0.75 to 4.25 ng/mg of creatinine (p < 0.01). This significant difference persisted, excluding the patients taking thyrotropin-stimulating hormone and the ones with congenital heart disease (data not shown). Interestingly, 15 (45%) of the DS subjects had levels of 8,12-iso-iPF2α-VI higher than 2 ng/mg of creatinine (mean ± 2 SD higher than that of control subjects). Creatinine values between the 2 groups did not differ (p = 0.9). There was no difference between male and female DS subjects (p = 0.8). Next, we determined whether the increase in 8,12-iso-iPF2α-VI observed in DS subjects was correlated with the duration of the disease. A significant correlation was observed between those levels and the age of the DS subjects (r² = 0.35, p = 0.002) (Fig 2); however, the same was not found in controls (data not shown).

Discussion
In this article, we demonstrate that young DS patients have significantly higher urinary levels of 8,12-iso-iPF2α-VI, a sensitive and specific marker of lipid peroxidation, than age- and sex-matched controls. This finding supports the hypothesis that increased lipid peroxidation and oxidative stress occur in vivo and are important components early in the life of DS subjects. Furthermore, the increased levels significantly corre-

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lated with the age of the patients, indicating that they also correlate with the duration of the disease.

Although DS was described over 100 years ago, and the relation between trisomy 21 and Down’s phenotype has been known for more than three decades, little is still known about the mechanisms responsible for the clinical manifestation of the disease.9 Elevated levels of normal products encoded by genes residing in this chromosome are thought to alter various biochemical pathways and their homeostasis, giving rise to the specific pathologies affecting these subjects. The SOD-1 gene is located on this chromosome, and it is known that DS patients have elevated levels of this enzyme and activity.2 This can produce an excess in ROS and a subsequent imbalance in the antioxidant enzymatic pathways.3,4 Based on this observation, it is believed that the DS fetus is situated in an environment of increased oxidative stress early in the course of its life. Indeed, DS fetal brains have increased lipid per-oxidation, glycoxidation, and DNA oxidation products.5,10 In addition, the augmented SOD-1 activity, by increasing the amount of ROS, mediates degeneration and apoptosis of DS cortical neurons in vitro.11 Thus, cellular damage may begin accumulating in utero and continue during the life span of the individual with DS, contributing to the phenotypes associated with aging changes such as the Alzheimer’s type of neuronal pathology.12,13 Another possible mechanism that can underlie oxidative brain damage in DS is the presence on chromosome 21 of the gene that codifies for the amyloid precursor protein.14 This gene dosage effect would create an increased concentration of amyloid β (Aβ) in the brain of these patients. It is known that amyloid Aβ can directly produce ROS by binding directly to the receptor of advanced glycation end products15 and that accumulation of intracellular Aβ, similar to the case in Alzheimer’s disease patients, induces cellular oxidative stress.16 Interestingly, we and others have reported that F2-isoprostane formation is increased in Alzheimer’s disease patients.17,18

In the present study, by using a reliable, specific, and sensitive quantitative index, we provided evidence that lipid peroxidation is a prominent component early in the course of the syndrome in living patients with DS. The temporal increase in lipid peroxidation observed in DS subjects may reflect accumulation over time of these products, which could derive from overproduction and/or reduced clearance.

Quantification of this noninvasive specific marker of in vivo lipid peroxidation may be especially helpful in testing the hypothesis that antioxidant therapy could be efficacious in slowing down the evolution of this disease.

Fig 1. Urinary 8,12-iso-iPF2α-VI levels in Down’s syndrome (▲) and control subjects (■) (n = 33 in each group).

Fig 2. Linear regression analysis of urinary 8,12-iso-iPF2α-VI levels versus age in Down’s syndrome subjects (r² = 0.35, p = 0.002).

References
PABP2 Polyalanine Tract Expansion Causes Intranuclear Inclusions in Oculopharyngeal Muscular Dystrophy

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Oculopharyngeal muscular dystrophy (OPMD) is a hereditary disease that affects all skeletal muscles, especially those responsible for eyelid elevation and swallowing. OPMD may lead to severe dysphagia with malnutrition and aspiration pneumonia, which can be fatal.1,2 It has a worldwide distribution but is particularly frequent in French Canadians.1–3 The OPMD gene, which was mapped to chromosome 14q11.2-q134 and positioned cloned,5 is the human homologue of the bovine poly(A) binding protein type II (PABP2) gene.6 The 306–amino acid protein has a predicted molecular mass of 32.8 kd.6 PABP2 is a nuclear protein7 that is involved in the polyadenylation of mRNA.8,9 The normal PABP2 gene has a (GCG) 6 trinucleotide repeat coding for a polyalanine stretch at

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the 5’ end. The disease occurs when a small expansion of (GCG)$_{10}$ to (GCG)$_{18-13}$ occurs.\(^5\) Because there are three GCA and one GCG codon adjacent to the GCG tract, the normal PABP2 protein has a 10-alanine stretch whereas the mutant proteins have 12 to 17 alanines.

In 1980, Tomé and Fardeau described clusters of intermediate-sized tubular filaments in the nuclei of OPMD skeletal muscle, intranuclear inclusions, a feature that has since served as the pathological hallmark of this condition.\(^10\) It has been proposed that these nuclear filaments might be “nuclear toxic” and cause cell death.\(^2,5\) In vitro studies have shown that polyalanine polymers as small as 12 amino acids form β-sheet complexes,\(^11\) which are extremely insoluble.\(^12\) The presence of intranuclear inclusions in OPMD suggests that the expanded polyalanine tract of mutant PABP2 protein may play a direct role in disease pathogenesis by accumulating as insoluble aggregates. Interestingly, a similar pathogenetic model has been proposed for diseases caused by expanded polyglutamine encoding CAG tracts.

To determine whether expression of the mutated PABP2 protein alone is sufficient for formation of intranuclear inclusions, both normal and mutated (GCG-13 repeat) PABP2 fused to enhanced green fluorescent proteins was transfected into COS-7 cells. We show that mutated proteins, and not the normal protein, oligomerize and form aggregates leading to intranuclear inclusions in a time-dependent manner. Furthermore, we show that the aggregates contain the complete PABP2 protein. This model for OPMD will help to study the mechanisms of toxicity of mutant PABP2 protein in vitro.

Materials and Methods

Antisera

A 15-mer peptide corresponding to the N-terminus of human PABP2 protein (GRGSGPGRRRHLVPG) was used to raise antisera, termed anti-NterPABP2, using standard protocols.\(^13\) We also used antisera raised against bovine PABP2 (Dr Wahle, University of Giessen) and EGFP (Clontech, Palo Alto, CA).

Transfections

COS-7 cells were seeded at 1.5 × 10^5 cells per well in six-well plates containing sterile coverslips. In each experiment, 2.0 µg of plasmid DNA was transfected using lipofectamine (Gibco BRL) in OPTI-MEM, using the manufacturer’s protocol.

Immunocytochemistry

Cells grown on coverslips were fixed in 4% paraformaldehyde in purified buffered saline (PBS), mounted, permeabilized with 0.05% Triton-X, blocked with 10% normal goat serum for 1 hour, and incubated with primary antibody (1:1:000) at room temperature overnight. Cells were rinsed in PBS and incubated with biotinylated secondary antibody (1:500). Amplification was performed using the ABC kit (Vector; Burlingame, CA) and products visualized using the VIP kit (Vector).

Western Blotting

Cells from 100-mm plates transfected with various constructs were scraped, collected, and lysed in Laemmli sample buffer. Proteins were quantitated using the Bio-Rad (Hercules, CA) protein assay. One hundred micrograms of total protein from each sample were electrophoresed on a 10% polyacrylamide gel and transblotted to nitrocellulose membrane. Immunodetection was performed with immunopurified anti-NterPABP2 (1:1,000), anti-bovine PABP2 (1:1,000), and anti-GFP (1:10,000). Horseradish peroxidase–conjugated secondary antibodies were used (1:10,000) and visualized by chemiluminescence (Renaissance: NEN, Boston, MA).

Plasmid Construction

The longest PABP2 cDNA available (~950 bp) did not have the complete 5’ sequence. The 5’ fragment of the cDNA was removed and replaced with a genomic Radiation and Bsu36I fragment (complete 5’ end) of either the normal or mutant (13 GCGs) PABP2. The insert was digested with HindIII and AciI and cloned in-frame into pEGFP-N1 (Clontech) with the EGFP at the 3’ end of the PABP2 gene.

Results

Cellular Distribution of PABP2

Normal and expanded (GCG)$_{13}$ PABP2 cDNA fused in-frame to EGFP and driven by the cytomegalovirus promoter were transiently transfected into COS-7 cells and the protein detected as green fluorescence at regular time points (12, 24, 48, and 72 hours posttransfection). At 12 and 24 hours, in both constructs, there was diffuse nuclear fluorescence, consistent with previous studies of PABP2.\(^7,14\) At 48 and 72 hours post-transfection, normal PABP2-transfected cells continued to show diffuse nuclear fluorescence (Fig 1b). With mutant PABP2, however, the fluorescence was now exclusively observed as nuclear punctate patches (see Fig 1a). Transfection with pEGFP-N1 vector alone resulted in fluorescence throughout the cell.

Protein Expression

Expression of PABP2-EGFP in transiently transfected COS-7 cells was analyzed using anti-NterPABP2 and anti-EGFP antisera. Equal amounts of protein lysates from normal and mutant PABP2, as well as pEGFP-N1 or mock transfected cells (Fig 2, lanes 1–4) were analyzed. No difference in migration was observed between normal and mutant protein, although the latter had seven additional alanine residues. Figure 2A shows the presence of endogenous PABP2 protein migrating as a 50-kd band in all lanes and the expected 80-kd fusion protein in transfected cells. Similar results were obtained using immunopurified anti-NterPABP2 and bovine antibovine PABP2 (data not shown). The same blot was stripped and reprobed with polyclonal EGFP antibody.
Both normal and mutant PABP2-EGFP fusion proteins were detected as an 80-kd band (see Fig 2b, lanes 1 and 2), confirming our previous observations using antibodies against PABP2. Cells transfected with the vector alone (see Fig 2b, lane 3) detected a 30-kd band, the predicted size of EGFP protein. Mock transfected cells showed no bands using this antibody. Similar observations were made with cell extracts from 24, 48, 72, and 96 hours. At 96 hours and later, the fluorescence began to decrease as cells were detaching from the plate.

COS-7 cells transfected with normal or mutant constructs were immunostained at different time points using anti-NterPABP2. At 24 and 48 hours, both constructs showed heavy nuclear staining. At 72 hours, compact nuclear inclusions were observed only in cells transfected with the mutant PABP2 construct (Fig 3a), whereas cells transfected with the normal construct (see Fig 3b) still showed diffuse nuclear staining. Cells transfected with pEGFP-N1 vector alone (see Fig 3c) or mock transfected (see Fig 3d) were used as controls. Cells transfected with mutant construct showed one major intranuclear inclusion with a few cells also having two to three smaller inclusions. The inclusion pattern was consistent in all transfected cells. These data are also consistent with our previous observations, in which compact fluorescent nuclear patches were seen under fluorescent microscopy at 72 hours in the cells transfected with the mutant PABP2.

Discussion
Our data suggest that the expanded polyalanine tract from the PABP2 protein as seen in OPMD patients is sufficient to cause nuclear aggregates in cultured mam-

---

*Fig 1. Distribution of the normal and expanded PABP2-EGFP fusion protein in transfected COS-7 cells. Cells transfected with the constructs were observed for the expression of the fluorescent fusion protein at 72 hours posttransfection: (a) cells transfected with the mutated PABP2 cDNA; (b) cells transfected with normal PABP2 (both the normal and the expanded PABP2 cDNAs have EGFP in frame); and (c and d) cells transfected with pEGFP-N1 vector alone and mock transfected cells, respectively. (Magnification, ×100.)

*Fig 2. Western blot analysis of COS-7 cell extracts transfected with the normal and expanded PABP2 constructs. Equal amounts of cell extracts from COS-7 cells transfected with various constructs were immunodetected using immunopurified anti-PABP2 polyclonal antiserum or polyclonal EGFP antiserum. (A) Lane 1, lysates of COS-7 cells containing the expanded PABP2 construct; lane 2, lysates expressing the normal PABP2 construct; and lanes 3 and 4, cell lysates of COS-7 cells transfected with vector alone and mock transfected cells, respectively. (A) Cells probed with anti-PABP2 serum. (B) The same blot was stripped of the original antibody and reprobed with anti-EGFP serum.*
malian cells. We provide here an in vivo model for the formation of nuclear inclusions in OPMD.

A small increase in the polyalanine repeat from (GCG)₆ to (GCG)₁₃ causes aggregation in our system, suggesting that a threshold exists for polyalanine tract-induced formation of inclusions. It remains unclear how aggregation occurs, however. We may speculate that the expansion of the alanine tract beyond a critical number may lead to formation of degradation resistant oligopeptides that aggregate and subsequently recruit normal PABP2 protein. An alternate model would be that mutant PABP2 protein is abnormally folded, resists degradation, and aggregates as insoluble inclusions. The fact that normal PABP2 protein forms dimers and oligomers⁶ is consistent with either model. Further experiments are needed to differentiate between these two models.

Our results show that expression of the PABP2 cDNA bearing an extra seven GCG codons requires 2 to 3 days before formation of nuclear inclusions (see Fig 1a). This delay in inclusion formation may result from the time needed to obtain a critical quality of mutant protein. Alternatively, spontaneous conformational changes in the protein induced by the polyalanine tracts may take time to occur,¹⁵ eventually leading to protein-protein aggregation.

Immunostaining transfected cells using PABP2 antibodies raised against the N-terminus shows nuclear inclusions only with the mutant protein (see Fig 3a). In addition, inclusions also contain EGFP, which is attached to the C-terminus, suggesting that the inclusion contains intact PABP2 protein. These results are consistent with the identification of intact filamentous aggregates of PABP2 in muscle fibers from OPMD patients.¹⁶ We propose a model in which protein conformation is altered by the expanded polyalanine tract, leading to abnormal protein-protein interactions. Over time these proteins accumulate as insoluble nuclear aggregates, leading to premature cell death. While speculative, this model is supported by the fact that nuclear inclusions are also a common phenomenon in polyQ/CAG triplet disorders, which are also thought to be initiated by altered protein conformation¹⁷ and protein misfolding.¹⁸ These aggregates are resistant to degradation and may prevent ubiquitin-mediated proteolytic destruction by disrupting the proteasome machinery.¹⁹ The fact that we see a similar phenomenon in a polyA/GCG triplet disease suggests that the nucleus may be particularly susceptible to aggregate formation.

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We thank Dr Elmar Wahle for providing the bovine PABP2 antibody.

References

High Mortality in Nipah Encephalitis Is Associated with Presence of Virus in Cerebrospinal Fluid

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During the outbreak of Nipah virus encephalitis in Malaysia, stored cerebrospinal fluid (CSF) samples from 84 patients (27 fatal and 57 nonfatal cases) were cultured for the virus. The virus was isolated from 17 fatal cases and 1 nonfatal case. There were significant associations between CSF virus isolation and mortality as well as clinical features associated with poor prognosis. In addition, there was a positive linear correlation of CSF virus isolation with age. There was no significant association between CSF virus isolation and the character of the CSF, presence of Nipah-specific antibody in the serum or CSF, duration of illness before collection of samples, or sex or ethnicity of the patients. This study suggests that high viral replication in the central nervous system may be an important factor for high mortality.


A novel paramyxovirus, now named Nipah virus, was first isolated from the cerebrospinal fluid (CSF) of a patient from Sungei Nipah village in March 1999 and subsequently identified as the etiological agent responsible for the outbreak of a fatal encephalitis in Malaysia.1–4 The infection subsequently spread to neighboring Singapore, where it involved abattoir workers.5,6 This virus is antigenically related to, but distinct from, Hendra virus, which was first isolated in September 1994 during an outbreak of a severe respiratory illness that killed 14 horses and 2 humans in Queensland, Australia.7–12 The outbreak of Nipah virus encephalitis

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started in September 1998 and reached its peak in March 1999, with 268 human cases reported and 105 fatalities. The virus also caused a respiratory and central nervous system (CNS) illness in pigs, and most of the human cases occurred in individuals who had occupational exposure to infected pigs.\textsuperscript{1–3} The spread of the disease was due to the movement of infected pigs from farm to farm. The outbreak was controlled only after successful isolation and identification of the etiological agent followed by the culling of a million pigs in the infected areas.

Between February and June 1999, 110 patients suspected of Nipah encephalitis were admitted to the University of Malaya Medical Centre (UMMC), Kuala Lumpur.\textsuperscript{13} From the CSF of 18 patients, Nipah virus was initially isolated from 5 fatal cases but from none of the patients who survived. In this current study, Nipah virus isolation was further attempted on CSF samples to determine the possible relation between mortality and virus isolation.

**Patients and Methods**

**Patients**

Patients included in the study were defined to have Nipah virus encephalitis if they:

1. Had evidence of encephalitis:
   a. Clinical features of fever, headache, altered sensorium or focal neurological signs; or
   b. Abnormal CSF findings, more than 6 leukocytes/µL, or protein levels above 0.45 g/L; or
   c. Characteristic brain magnetic resonance scan findings
2. Came from known outbreak areas
3. Had direct or close contact with pigs or other infected animals

The clinical criteria of Nipah virus infection whenever possible was supported by the presence of positive cross-reacting antibodies against Hendra virus antigen in the blood or CSF. Microscopic, biochemical, and serological investigations were performed on all the CSF obtained. Serum and CSF samples were tested by IgM capture and enzyme-linked immunosorbent assay (ELISA) using culture cell slurry derived from Vero E6 cells infected with Hendra virus after treatment by gamma radiation. Detection of specific IgG was by indirect ELISA using cell lysate derived from Hendra-infected Vero E6 cells. Pooled serum samples derived from local healthy blood donors were used as the background negative control.

**Virus Isolation**

Virus isolation was carried out in a modified biosafety level 3 laboratory. CSF stored in a \(-20^\circ C\) freezer for 3 to 4 months was thawed and 100 µL of each sample was inoculated into each well of a 24-well culture plate preseeded with 1 ml of \(1 \times 10^5\) Vero cells (ATCC, CCL81) in Eagle’s minimal essential growth medium. The culture plate was sealed with adhesive tape, incubated at 37°C, and examined daily for the presence of characteristic syncytial formation of multinucleated giant cells. Infected cells were harvested, washed twice with sterile phosphate buffered saline, and centrifuged; the cells were fixed onto Teflon-coated slides with cold acetone and were ultraviolet irradiated. The identity of the virus was confirmed by indirect immunofluorescence using anti-Hendra hyperimmune mouse ascitic fluid as primary antibody and goat anti-mouse fluorescein isothiocyanate-conjugated IgG as secondary antibody.

**Statistical Analysis**

The \(\chi^2\) test and \(\chi^2\) for linear trend were used to analyze the association between the isolation rate of Nipah virus from CSF and the clinical outcome of the disease, the presence of certain clinical features related to poor prognosis, and the Nipah serological results. All significant results were based on \(p < 0.05\).

**Results**

Ninety-one patients were defined by the above criteria to have Nipah encephalitis. However, stored CSF samples for virus isolation were available from only 84 patients (27 fatal and 57 nonfatal cases). Nipah virus was isolated from the CSF of 17 fatal cases and 1 nonfatal case (Table 1). Positive virus isolation from CSF was significantly associated with mortality (\(\chi^2 = 37.21; p = 0.0000\)).

The mean age of the patients included in this study was 37.5 years (age range, 13–68 years). The positive isolation rate correlated with the increasing age of patients (\(\chi^2\) for linear trend = 6.96; \(p = 0.006\)). The isolation of the virus also significantly correlated with clinical features that indicate severe CNS involvement and poor prognosis. These include segmental myoclonus, altered conscious level (Glasgow Coma Scale score <15), the need for ventilatory support, tachycardia, and hypertension\textsuperscript{13} (see Table 1).

However, virus isolation was not significantly associated with sex, ethnicity, or a history of Japanese encephalitis vaccination (see Table 1). Positive virus isolation was also not associated with abnormal CSF biochemistry, cell count (Table 2), or duration of illness at the time of CSF collection (\(\chi^2\) for linear trend = 0.06; \(p = 0.861\)). The presence of anti-Nipah virus antibodies in the CSF or serum did not appear to influence the virus isolation (see Table 1).

**Discussion**

This study shows that positive virus isolation from the CSF of patients with Nipah encephalitis is associated with high mortality, a finding similar to Japanese encephalitis.\textsuperscript{14} Positive virus isolation also correlated with poor clinical prognostic features. The rate of positive viral isolation increased with age, and this was consistent with increased mortality risk in older patients. These findings differed from another study that showed that Nipah virus isolation from urine and...
throat secretions was not associated with poor clinical outcome or age.\textsuperscript{15}

One of the main pathological findings in Nipah encephalitis was disseminated microinfarction secondary to vasculitis-induced thrombosis of the cerebral blood vessels.\textsuperscript{4} MRI of the brain in acute, nonfatal cases also showed disseminated small, discrete high-signal lesions on T2-weighted and fluid attenuated inversion recov-

<table>
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<tr>
<th>Characteristic Features</th>
<th>No. Total</th>
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$^a\chi^2$ value: after Yates corrected value.

JE = Japanese encephalitis; CSF = cerebrospinal fluid.

Table 2. Cerebrospinal Fluid of Patients with Nipah Encephalitis

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<tr>
<th>No. of Patients</th>
<th>White Cell Count$^a$ (cells/$\mu$L)</th>
<th>Protein$^a$ (g/L)</th>
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$^a$Mean values are shown, with the range in parentheses.
ery (FLAIR) images, which seemed to correspond to the microinfarction seen in fatal cases, indicating vasculitis could be a key event. However, there has been no correlation between these lesions with depth of coma and outcome of patients.16 Clinically, there are characteristic features, such as segmental myoclonus, hypotonia, and areflexia, that indicate a predilection for certain groups of neurons, including those in the brainstem.13 Pathologically, meningitis is usually mild in Nipah encephalitis.4 This is consistent with the observation that 25% of the initial CSF was normal.13 There is also lack of correlation between virus isolation and abnormal CSF. Thus, viral isolation from the CSF probably does not reflect extent of viral replication in the cells lining the meninges. On the other hand, there is inflammation in the brain parenchyma adjacent to subarachnoid space (Wong KT, unpublished observation), which would allow virus in the parenchyma to reach the CSF. This study showed an association between CSF virus isolation and mortality. This support by direct neuronal invasion with high viral replication may be important in the pathogenesis of severe disease.

The presence of anti–Nipah virus antibodies in the CSF did not influence the isolation of the virus, in contrast to the study on virus isolation in urine and throat secretions in which positive viral isolation correlated with the presence of specific antibodies.15 This may suggest that humoral immune response in the CNS probably plays a minor role in the disease process and recovery. The appearance of antibodies to Nipah virus appears to be dependent on the viremic phase, which is associated with diffuse viral spread throughout the body, but is unrelated to the presence of virus in the CNS. The latter, however, is more important in determining the morbidity and mortality.

We thank Dr T. G. Ksiazek and the Centers for Disease Control in Atlanta for carrying out the serological testing of the Nipah virus and the supply of reagents for the continuation of serological testing.

References

Variant Alzheimer’s Disease with Spastic Paraparesis and Cotton Wool Plaques Is Caused by PS-1 Mutations that Lead to Exceptionally High Amyloid-β Concentrations

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We describe 3 new families affected by Alzheimer’s disease with spastic paraparesis. In affected individuals, including the earliest known patient with this clinical syndrome, neuropathological examination revealed large “cotton wool” plaques similar to those we have previously described in a Finnish family. In the families in which DNA was available, presenilin-1 mutations were observed. Transfection of cells with these mutant genes caused exceptionally large increases in secreted Aβ42 levels. Furthermore, brain tissue from individuals with this syndrome had very high amyloid-β concentrations. These findings define the molecular pathogenesis of an important subgroup of Alzheimer’s disease and have implications for the pathogenesis of the disease in general.


The molecular causes of two distinct diseases characterized by the combination of progressive dementia and spastic paraparesis have recently been defined: (1) a variant of Alzheimer’s disease (AD) with spastic paraparesis and large, nonneuritic “cotton wool” plaques caused by a variant of the PS1Δ9 mutation1,2 and (2) familial British dementia caused by a mutation in a novel gene, BRI.3,4 These discoveries led us to examine our sample archives to look for families with similar clinical syndromes and to subject these to neuropathological and molecular analysis. During this search, we identified a new branch of the familial British dementia kindred.3,4 (Houlden, 2000, unpublished data). We also identified a further 3 families in which the clinical features were reminiscent of those in the original Finnish family.1,2,5 Here, we report on our pathological examination of these new families and compared this with the original Finnish families, and we report our identification of presenilin-1 mutations within them. We also report analysis of the effects of these mutations on Aβ production.

Methods

The family material is summarized in Table 1.

Standard neuropathological examination was carried out on fixed specimens. Amyloid-β (Aβ) staining (Fig 1) of tissue sections was with the Dako anti-Aβ antibody (Dakopatts, CA), which does not discriminate between Aβ40 and Aβ42. Initially, analysis was carried out to define whether these samples had either of the two variants of the PS1Δ9 mutation.1,2,6 When this mutation was not found, the whole presenilin-1 gene was sequenced.7 Wild-type and Δ9 PS1 cDNA constructs (Invitrogen, Carlsbad, CA) were cloned as described previously.9 The mutation causing deletion of codons 83 and 84 (Del83M; see later) was introduced into the wild-type PS1 cDNA in pcDNA3 (Invitrogen) by site-directed mutagenesis using the Transformer mutagenesis system (Clontech, Palo Alto, CA). The P436Q mutation was introduced by polymerase chain reaction (PCR) mutation amplification of the PS1 gene using the Roche High Fidelity PCR kit (Roche, Indianapolis, IN).9 The amplified sequence was purified and cloned into the pGEM3 vector. All constructs were transfected into H4 glioma cells using Fugene transfection reagent (Roche).

H4 glioma cells were cultured in Optimem media with 1% penicillin/streptomycin and 10% fetal bovine serum (Gibco, Rockville, MD) with neomycin (800 mg/ml) to se-
lect for dellM and Δ9 PS1 expression or hygromycin (800 mg/ml) to select for P436Q PS1 expression. After 2 weeks under selection, cells were transfected with APP695 K/M670/1N/L cDNA in pAG3 overnight. The cells were incubated for 24 hours then were lysed in 200 ml of 1% triton in TBS with proteinase inhibitor complete cocktail (Roche).

Media samples were assayed for Aβ40, Aβ42, and secreted APP (sAPP). The Aβ40 and Aβ42 were captured using the Ban50 antibody (transfection studies) or BNT77 (brain amyloid studies) and detected (using BA27 HRP and BC05 HRP antibodies, respectively, each of which is specific to one of the two peptides) with peroxidase substrate/solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD). sAPP was assayed using a cELISA based around the 207 N terminal APP antibody (B. Greenberg). Aβ42 as a percentage of Aβ40 was then calculated.9

Results and Discussion

All of the families in which spastic paraparesis was a prominent feature, including that family (Family CO) in which this clinical feature was first described,10 shared the characteristic pathology of cotton wool plaques previously described in the family FINN2. This, therefore, represents a variant of AD. In all the cases in which DNA was available, presenilin-1 mutations were found: family FINN2 is the one in which we have previously described the PS1Δ9 mutation1,2,5; in family EB, an unusual and novel deletion of codons 83 and 84 removing isoleucine and methionine from exon 4 (the DelIM mutation); and in family D, the previously reported P436Q mutation.11 These findings, together with previous reports, are of considerable interest. First, it is clear that not all cases of the PS1Δ9 mutation necessarily have spastic paraparesis. Thus, in some cases with both the Finnish variant of the mutation and in others,1,5,6 dementia is the presenting feature. In some cases with dementia as the presenting feature, it seems as though the pathology is of more typical AD with more neuritic, rather than cotton wool, plaques,2,5,12 although this conclusion remains

<table>
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<th>Table 1. Clinical Summary</th>
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<tr>
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</tr>
<tr>
<td>EB (DelIM)</td>
</tr>
<tr>
<td>CO</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>

*Fig. Photomicrograph of serial sections from Family EB showing cotton wool plaques and amyloid angiopathy (arrow). (A, haematoxylin and eosin, ×200; B, Aβ immunohistochemistry, ×200).*

<table>
<thead>
<tr>
<th>Table 2. Shift in Aβ42/40 due to Spastic Paraparesis Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS1 Mutation</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>P436Q</td>
</tr>
<tr>
<td>Δ9</td>
</tr>
<tr>
<td>DelIM</td>
</tr>
<tr>
<td>M139V</td>
</tr>
<tr>
<td>PS1wt</td>
</tr>
</tbody>
</table>

The shift in Aβ42/Aβ40 due to spastic paraparesis mutations is significantly different to that shown by FAD M139V when analyzed by one-way ANOVA; *p < 0.001. In this system, the M139V mutation has had the largest effect on Aβ42/40 ratios of the following mutations (E280G, A285V, M146L, L286V [Golde, unpublished data]). All mutations were significantly different to PS1 wild-type values, as we and others have previously shown.
provisional until more extensive neuropathological investigations are performed.

These genetic and pathologic data suggest that the pathogenic route to disease can run down two distinct paths—either a standard AD route (dementia first, with neuritic plaques at end stage) or the unusual path we describe here (spastic paraparesis first with cotton wool plaques at end stage) (see Fig). This dichotomy suggests some form of threshold effect in the pathogenic pathways. Most presenilin mutations (and other causes of AD) fail to exceed this threshold, but some of these mutations have a different effect. We and others had previously noted that the PS1Δ9 mutation had a particularly large effect on Aβ42 production in transfected cells.8,13 This observation led us to investigate whether this is a more general effect of spastic paraparesis—causing mutations (Table 2). Examination of the data from transfection experiments revealed that all the spastic paraparesis-causing mutations have a larger effect on Aβ42 production than those mutations that lead to the more typical dementia phenotype, although the presenilin active site mutation, G384A, has a large effect on Aβ42 production without spastic paraparesis phenotype.14,15 This suggests that part of the pathogenesis of the spastic paraparesis variant relates to the amount of production of Aβ42, although it is likely that there are also other additional genetic and nongenetic factors. Examination of Aβ42 levels in the brain of individuals with this syndrome confirms that it is associated with particularly large quantities of Aβ (Table 3).

These data are provocative: a simple reading of the amyloid cascade hypothesis16 would be the prediction that those mutations that had the largest effects on Aβ42 production would be associated with the lowest age at onset of disease. This is not the case: rather, those mutations that have the largest effect on Aβ production are associated with a different clinical and pathological phenotype.

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**Table 3. Aβ Levels in Brains of Cases with Alzheimer’s Disease (AD)/Spastic Paraparesis Variant**

<table>
<thead>
<tr>
<th>Family</th>
<th>Mutation</th>
<th>Aβ40 (μg/g)</th>
<th>Aβ42(43) (μg/g)</th>
<th>Aβ42(43)/Aβ40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporadic AD* (n = 14)</td>
<td>DelIM</td>
<td>1.66</td>
<td>3.14</td>
<td>1.89</td>
</tr>
<tr>
<td>EB</td>
<td>56.18</td>
<td>35.34</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>20.05</td>
<td>35.21</td>
<td>1.76</td>
<td></td>
</tr>
</tbody>
</table>

*Data taken from Hosoda et al.17 Sporadic AD cases processed at the same time as the spastic paraparesis cases showed levels similar to these historical controls.

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**References**

Increased 8,12-iso-iPF$_{2\alpha}$-VI in Alzheimer’s Disease: Correlation of a Noninvasive Index of Lipid Peroxidation with Disease Severity

Domenico Praticò, MD,* Christopher M. Clark, MD,†‡§ Virginia M.-Y. Lee, PhD,‡ John Q. Trojanowski, MD, PhD,‡§ Joshua Rokach, PhD,∥ and Garret A. FitzGerald, MD*

The isoprostane 8,12-iso-iPF$_{2\alpha}$-VI is a sensitive and specific marker of in vivo lipid peroxidation. We found elevated levels in the urine, blood, and cerebrospinal fluid of patients with Alzheimer’s disease (AD) that correlated with measures of cognitive and functional impairment, established biomarkers of AD pathology (cerebrospinal fluid tau and amyloid) and the number of apolipoprotein E ε4 alleles. These results suggest that 8,12-iso-iPF$_{2\alpha}$-VI is a useful biomarker of oxidative damage in AD.


Alzheimer’s disease (AD) is a neurodegenerative disorder that impairs cognition and behavior.¹ Neuropathologically, the disease is characterized by the presence of senile plaques, neurofibrillary tangles, and neuron loss.² While there is argument about the “cause” of brain degeneration in AD, most agree that it is associated with an unusual form of inflammation that is accompanied by oxidative damage that may result, as the brain is rich in lipids, in widespread lipid peroxidation (LP).³ Isoprostanes are chemically stable prostaglandin isomers formed by free radical peroxidation of polyunsaturated fatty acids, which are stored in tissues, circulate in plasma, and are excreted in urine.⁴ These properties make them reliable quantitative in vivo LP biomarkers. We and others have shown that isoprostane levels in cerebrospinal fluid (CSF) obtained postmortem from the lateral ventricle of AD patients are higher than in control subjects.⁵,⁶ However, it is known that the pathological changes in AD begin years before a clinical diagnosis can be made. It is during this preclinical phase that therapies to slow, halt, or reverse the pathological process will be most effective. Thus, there is a compelling need to identify, in this phase, AD patients with increased LP by using biomarkers that can be acquired noninvasively.

We undertook this study to address the question whether living patients with AD have enhanced isoprostane generation in CSF, blood, and urine compared with matched healthy individuals, and whether their levels correlate with the severity of cognitive impairments and other biomarkers of AD brain degeneration.

Patients and Methods

Patients

This study was reviewed and approved by the Institutional Review Board of the University of Pennsylvania. Subjects were recruited from the university’s Alzheimer’s Disease Center Memory Disorders Clinic (MDC) between May 1998 and September 1999. Informed consent was obtained from all participants and their caregivers. The clinical diagnosis of probable or possible AD was based on the National Institute of Neurological and Communicative Diseases and Stroke-Alzheimer’s Disease and Related Disorders Association criteria.⁷ As part of their routine cognitive assessment, all patients received the Consortium to Establish a Registry for Alzheimer’s Disease psychometric battery, which assesses memory, language, and constructional praxis.⁸ The Dementia Severity Rating Scale (DSRS) and Mini-Mental State Examination (MMSE) assessments were performed to evaluate the clinical severity of the disease.⁹,¹⁰

Extensive laboratory and clinical examinations were performed, including magnetic resonance imaging, to exclude other causes of dementia. Subjects were excluded if they had an acute infectious or inflammatory disease, hepatic chronic disease, alcoholism, or cancer, or if they were treated with vitamins, since they all affect F$_{2\alpha}$-isoprostane biosynthesis.⁴ Thirty-five consecutive patients were enrolled; urine and blood samples were obtained from all of them. Two weeks later, 14 patients and 10 control subjects agreed to have a lumbar puncture for CSF and a second urine sample collection. There were no significant clinical or demographic differences between the patients who did or did not undergo lumbar puncture. Age- and sex-matched control subjects were recruited from a cohort of cognitively normal individuals followed by the Alzheimer’s Disease Center and from cognitively normal spouses of AD patients attending the MDC.

Isoprostane Analysis

Samples were analyzed by gas chromatography/mass spectrometry, as previously described.⁴,¹¹ The intraassay and in-
terassay variability for urine, plasma, and CSF, is ±4% and ±5%, ±5.5% and ±4%, ±5.0% and ±4.5%, respectively. Blood, anticoagulated with EDTA, was centrifuged to obtain plasma; CSF, collected visually free of blood contamination, was sedimented at 1,500 rpm; both were stored at −80°C. All assays were performed without knowledge of the clinical diagnosis of the patient.

**CSF Tau, CSF Aβ_{1–40}, and Aβ_{1–42}**

Tau protein levels were measured by sandwich ELISA using the Innotest hTAU-Antigen kit (Innogenetics, Belgium). Aβ_{1–40} and Aβ_{1–42} levels were measured by a previously well-characterized sandwich ELISA. The Aβ and tau sandwich ELISA have detection limits of less than 1 femtomole of synthetic Aβ and less than 75 pg/ml of tau per sample. Both assays were performed in duplicate, and the variation between samples in the duplicate assay was less than 10% for each. They were performed without knowledge of the clinical diagnosis of the patient.

**Apolipoprotein E Genotype**

DNA was extracted from peripheral leukocytes and apolipoprotein E (ApoE) genotyping was performed as previously described, without knowledge of the patient’s clinical diagnosis.

**Statistical Analysis**

Comparisons among groups were performed by nonparametric one-way analysis of variance (Kruskall-Wallis test) with the use of Dunn’s posttest. Correlation was studied by linear regression analysis. Statistical significance was set at p < 0.05.

**Results**

Table 1 presents the characteristics of the AD patients and controls studied here. ApoE ε2, ε3, and ε4 allele distribution in the AD patients was as follows: ε2/ε3, n = 3; ε3/ε3, n = 8; ε4/ε3, n = 12; ε4/ε4, n = 12. No significant difference among groups was observed for plasma cholesterol and triglyceride levels (data not shown). Patients with probable or possible AD had urinary 8,12-iso-iPF_{2α}-VI levels significantly greater than controls (4.93 ± 0.42, 4.18 ± 0.56 vs 1.77 ± 0.17 ng/mg creatinine, respectively) (p < 0.0001, for both) (Fig, top). A similar pattern was observed in plasma (0.68 ± 0.05, 0.67 ± 0.08 vs 0.18 ± 0.01 ng/ml, respectively; p < 0.0001) (see Fig center). Subsequently, CSF was obtained from 10 patients with probable AD, 4 with possible AD, and 10 normal controls (see Fig, bottom). At this time, a second urine sample was collected, and the urinary 8,12-iso-iPF_{2α}-VI levels measured in these samples did not differ significantly from the ones initially obtained from the same subjects (not shown). In both groups of AD patients CSF 8,12-iso-iPF_{2α}-VI levels were significantly (p < 0.0001) higher than in controls (see Fig, bottom; Table 2). A direct correlation was observed between CSF and urinary levels of 8,12-iso-iPF_{2α}-VI and between CSF and plasma 8,12-iso-iPF_{2α}-VI. The coefficient of correlation (r^2) for each was 0.55 and 0.64 (both p < 0.001), respectively. AD patients had impaired cognitive function as shown by the MMSE and DSRS assessments (see Table 1). A direct correlation was observed between DSRS and CSF 8,12-iso-iPF_{2α}-VI levels (r^2 = 0.22, p = 0.02), whereas an inverse correlation was obtained between MMSE scores and CSF 8,12-iso-iPF_{2α}-VI levels (r^2 = −0.15, p = 0.04). CSF tau protein was elevated, while the percentage ratio between CSF Aβ_{1–40} and Aβ_{1–42} was lower in AD patients than in matched controls (see Table 2). CSF tau protein and CSF 8,12-iso-iPF_{2α}-VI levels were directly correlated (r^2 = 0.43, p < 0.0001), whereas 8,12-iso-iPF_{2α}-VI levels were inversely correlated with the percentage of CSF Aβ_{1–42} (r^2 = −0.32, p = 0.04). Finally, we investigated the relationship between the number of apoE ε4 alleles and the levels of 8,12-iso-iPF_{2α}-VI in CSF. Subjects with two ε4 alleles had significantly higher CSF 8,12-iso-iPF_{2α}-VI levels than those with one or no ε4 alleles (74 ± 7.4 [p = 0.04, versus both], 49 ± 9, 50 ± 5.7, pg/ml, respectively).

**Discussion**

In this study, we show for the first time that compared with matched controls, patients with a clinical diagnosis of AD have increased CSF, plasma, and urinary levels of 8,12-iso-iPF_{2α}-VI, a reliable marker of in vivo LP. The finding that urinary and circulating levels of this specific isoprostane significantly correlate with the levels in CSF of AD patients adds further credence to the hypothesis that LP occurs early in the course of this dementing disorder, thereby implicating it as contributor to brain degeneration in AD. Significantly, it is
plausible to infer that the assessment of 8,12-iso-iPF$_{2\alpha}$-VI in the urine may provide a convenient, non-invasive and reliable approach to study LP and oxidative damage in AD since both have been implicated mechanistically in its pathogenesis.$^{3,15}$

Another important finding we report here is the correlation between the levels of CSF 8,12-iso-iPF$_{2\alpha}$-VI and the severity of the dementia in AD patients as measured by the MMSE and DSRS scales. Further, since it has been demonstrated that CSF tau increases and Aβ$_{1-42}$ decreases with progression of the disease in AD patients,$^{16,17}$ it is notable that we observed a direct correlation of CSF 8,12-iso-iPF$_{2\alpha}$-VI levels and CSF tau and an inverse correlation of these levels with CSF Aβ$_{1-42}$ in AD patients. Thus, elevations in the level of 8,12-iso-iPF$_{2\alpha}$-VI not only reflect an increase in central nervous system oxidative stress but also correlate with the progression of the disease.

The risk of development of late-onset AD has been linked to the polymorphism of the ApoE gene, in particular the ε4 allele.$^{18}$ We found that subjects homozygous for ε4 allele had higher CSF 8,12-iso-iPF$_{2\alpha}$-VI levels than those with one or no ε4 alleles. Thus, this suggests that apoE ε4 allele could influence the response of the brain to oxidant injury in AD.$^{19}$

Because the neurological changes of AD are thought to begin insidiously well before a clinical diagnosis can be made, it would be attractive to be able to identify AD patients with increased LP by using reliable assays to noninvasively measure biomarkers of LP. Although the sample size in our study was small and follow-up studies of larger cohorts are needed, we observed no overlap between CSF and plasma 8,12-iso-iPF$_{2\alpha}$-VI levels of AD patients and controls. This strongly suggests that quantification of this specific isoprostane may be useful in early detection of AD. Finally, its correlation with clinical severity and other biomarkers of the disease could enable this marker to be exploited to monitor the response to antioxidant therapies in AD.

This work was supported by grants from the American Federation for Aging Research (P99143), the National Institutes of Health (HL 61364, M01RR00040 and AG10124), and a Pilot Grant from the ADC at the University of Pennsylvania. Dr Lee is the John M. Fig. Urinary (top), plasma (center), and cerebrospinal fluid (CSF) (bottom) 8,12-iso-iPF$_{2\alpha}$-VI levels in patients with probable (pro) or possible (pos) Alzheimer’s disease (AD) and age- and sex-matched controls (Con).

Table 2. Demographics, Cerebrospinal Fluid (CSF) 8,12-iso-iPF$_{2\alpha}$-VI, CSF Tau Protein, and CSF Aβ$_{1-42}$ Percentage Levels in Alzheimer’s Disease (AD) Patients and Controls

<table>
<thead>
<tr>
<th></th>
<th>AD (n = 14)</th>
<th>Controls (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (yr)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SE)</td>
<td>74 (1.3)</td>
<td>74 (2)</td>
</tr>
<tr>
<td>Range</td>
<td>64–82</td>
<td>60–82</td>
</tr>
<tr>
<td>Ratio F/M</td>
<td>11/3</td>
<td>7/3</td>
</tr>
<tr>
<td><strong>8,12-iso-iPF$_{2\alpha}$-VI (pg/ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SE)</td>
<td>66$^a$ (4.6)</td>
<td>25 (3.3)</td>
</tr>
<tr>
<td>Range</td>
<td>43–105</td>
<td>6–38</td>
</tr>
<tr>
<td><strong>CSF tau (pg/ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SE)</td>
<td>770$^b$ (70)</td>
<td>320 (65)</td>
</tr>
<tr>
<td>Range</td>
<td>300–1,500</td>
<td>170–460</td>
</tr>
<tr>
<td><strong>CSF Aβ$_{1-42}$ (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SE)</td>
<td>5.3$^b$ (0.7)</td>
<td>8.0 (0.8)</td>
</tr>
<tr>
<td>Range</td>
<td>2.4–7.8</td>
<td>5.4–16.7</td>
</tr>
</tbody>
</table>

Results are expressed as mean, standard error (SE), and range.

$^a$$p < 0.0001.$

$^b$$p < 0.01.$

Brief Communication: Praticò et al: Lipid Peroxidation in Alzheimer’s Disease 811
Oculopharyngeal muscular dystrophy (OPMD) is an autosomal dominant myopathy that presents in the fourth to sixth decade and is characterized by ptosis, dysphagia, and dysphonia often followed by limitation of eye movements, proximal limb weakness, and facial weakness\(^1\) (reviewed in Proceedings of the First International Symposium on OPMD\(^2\)). Pathologically, skeletal muscle from OPMD patients is not markedly abnormal, although occasional myofibers contain “rimmed vacuoles” in the sarcoplasm that appear to be apolipoprotein E type 4 and the risk of Alzheimer’s disease in late onset families. Science 1993;261:921–923


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**Intranuclear Inclusions in Oculopharyngeal Muscular Dystrophy Contain Poly(A) Binding Protein 2**

Mark W. Becher, MD,⁺ Joyce A. Kotzuk, BS,⁺ Larry E. Davis, MD,†‖ and David G. Bear, PhD‡§

Intranuclear inclusions are one of the ultrastructural hallmarks of oculopharyngeal muscular dystrophy (OPMD), a disorder caused by small polyalanine (GCG) expansions in the gene that codes for a ubiquitous nuclear protein called poly(A) binding protein 2 (PABP2). We studied OPMD skeletal muscle and found that 1.0 to 10.0% of myocyte nuclei contained discreet PABP2 immunoreactive intranuclear inclusions, providing the first direct evidence of the relation between the proposed gene for OPMD and the pathology of OPMD.

Becher MW, Kotzuk JA, Davis LE, Bear DG.


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Oculopharyngeal muscular dystrophy (OPMD) is an autosomal dominant myopathy that presents in the fourth to sixth decade and is characterized by ptosis, dysphagia, and dysphonia often followed by limitation of eye movements, proximal limb weakness, and facial weakness\(^1\) (reviewed in Proceedings of the First International Symposium on OPMD\(^2\)). Pathologically, skeletal muscle from OPMD patients is not markedly abnormal, although occasional myofibers contain “rimmed vacuoles” in the sarcoplasm that appear to be apolipoprotein E type 4 and the risk of Alzheimer’s disease in late onset families. Science 1993;261:921–923


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References

autophagocytic structures. Many years ago, it was found by ultrastructural studies that some OPMD skeletal muscle nuclei contain intranuclear inclusions of unknown significance. These inclusions are reportedly found in less than 10% of myocyte nuclei from affected individuals and, at the ultrastructural level, are composed of collections of tubulofilamentous structures (8.5 nm external diameter and 3.0 nm internal diameter) that displace the chromatin to the periphery of the nucleus. The nature of these tubulofilaments is unknown, although some inclusions have been shown to exhibit immunoreactivity to ubiquitin antibodies.

OPMD was linked to chromosome 14q11.2-q13 in 1995. Three years later, Brais and colleagues found that a polyalanine (GCG) repeat in the N-terminus of the poly(A) binding protein 2 (PABP2) gene in this region was expanded to (GCG)_{8-13} in a large series of OPMD pedigrees. PABP2 is a ubiquitous 33-kd nuclear protein with an acidic region near the amino terminus, a basic carboxy terminus, and a single RNA-binding domain near the center.

Thus, OPMD is a member of the growing number of disorders that have a very restricted neurological phenotype and are caused by expansion of a trinucleotide repeat sequence. Nearly all of the previously characterized triplet repeat disorders have been shown to develop intranuclear proteinaceous aggregates composed, at least in part, of the mutant gene product (reviewed in Kaytor and Warren), which are a novel morphological abnormality previously unheard of in any known cellular mechanism of disease. The significance of these intranuclear aggregates remains unclear. It is not known whether they are detrimental to cellular function, are protective in some manner, or represent a reactive process of sequestering abnormal proteins in an intranuclear compartment, perhaps as part of a dynamic process of protein degradation. In this study, we sought to determine whether nuclei of skeletal muscle from patients with OPMD contained the proposed gene product, PABP2, in an aggregate formation.

Subjects and Methods

Subjects
Archival skeletal muscle biopsy tissues from 4 unrelated adult patients with the clinical manifestations of OPMD from the Rio Grande Valley region of New Mexico were studied (UNM Human Research Review Committee approval #00-228). Samples were obtained from the deltoid or lower extremity muscles. Control skeletal muscle was obtained from archival surgical pathology adult cases without neurological symptoms.

Tissue Preparation and PABP2 Immunocytochemistry
Fresh skeletal muscle was fixed in 10% neutral-buffered formalin at the time of the original biopsy and embedded in paraffin. Immunocytochemistry was performed using a rabbit polyclonal antibody to PABP2 (gift of Dr Elmar Wahi, University of Halle, Germany) that was further purified on a protein A sepharose column (Pharmacia, Piscataway, NJ) and standard immunocytochemical methods with microwave antigen retrieval. Control samples were identically prepared and sectioned. Briefly, 4-μm deparaffinized sections were blocked with 3% H_{2}O_{2} in methanol for 15 minutes, microwave for 5 minutes in citrate buffer at pH 6.0, subjected to primary antibody at 1:1,000 in Tris-buffered saline (TBS) with 0.5% normal goat serum overnight at room temperature, and labeled with avidin-biotin complex reagents (Vector, Burlingame, CA) and 3,3'-diaminobenzidine chromagen. Sections treated with TBS or pooled rabbit IgG in place of the primary antibody served as technique controls. After the chromagen reaction, all sections were briefly stained in hematoxylin to provide a nuclear counterstain. Hematoxylin and eosin (H&E)–stained sections were present on each case to review the diagnostic light microscopic findings.

Results
None of the 4 OPMD cases had markedly abnormal skeletal muscle on H&E stain. Rare atrophic myocytes and occasional rimmed vacuoles were present. Myocyte nuclei had intact nuclear membranes by light microscopy and lacked chromatin or intranuclear abnormalities. Samples from control subjects were normal. Immunoreactivity to the PABP2 antibody was restricted to the nucleus in all cases. Strongly immunoreactive round intranuclear inclusions were found in all 4 OPMD cases (Fig) and none of the control subjects. Inclusion-bearing myocyte nuclei ranged from 1.0–10.0% (Table). Most nuclei with inclusions contained a single aggregate, although up to three distinct inclusions were identified in single nuclei (see Fig, B). Inclusions were approximately 2 to 3 μm in greatest dimension and myocyte nuclei were 8 to 10 μm in diameter. Given that 4-μm paraffin sections were studied, we evaluated approximately 40% of each nucleus. Therefore, it could be calculated that the actual number of inclusion-bearing myocyte nuclei could be up to 2.5 times higher than observed (ie, up to 25%). No inclusions were identified in control subject myocyte nuclei. Fine particulate and diffuse nuclear staining with PABP2 was identified in many myocyte nuclei in both OPMD cases and control subjects. Technique control slides (buffer or rabbit IgG as primary antibody) were blank (see Fig). Intranuclear inclusions in OPMD cases were distinct from fine particulate or diffuse nuclear staining and there was no predilection toward inclusions being present in nuclei with or without additional PABP2 immunoreactivity. The diffuse nuclear immunoreactivity in control subjects is consistent with what has been shown by others in mammalian nuclei.
Discussion

These studies show that the intranuclear inclusions of the first polyalanine repeat disorder to be identified, OPMD, which were previously best seen by electron microscopy, do indeed contain PABP2, the predicted gene product that harbors a triplet repeat expansion in affected individuals. It is possible that PABP2 antibodies will have diagnostic utility for the evaluation of muscle biopsies at the light microscopic level from suspected OPMD patients in the future.

With the exception of the androgen receptor of spino-bulbar muscular atrophy and the α subunit of a Purkinje cell–specific calcium channel in spinocerebellar ataxia type 6, the protein products of the majority of the triplet repeat disorder genes are of unknown function. PABP2 is a well-characterized nuclear protein that binds to newly created poly(A) tails after at least 10 adenine residues are in place, and it is thought that additional PABP2 molecules assemble as the poly(A) tail elongates. PABP2 has two known regulatory functions in polyadenylation. In conjunction with cleavage and polyadenylation specificity factor (CPSF), PABP2 enhances the processivity and rate of poly(A) tail elongation by poly(A) polymerase. PABP2 is also required to terminate the synthesis of the poly(A) tail after the polymerization of 250 to 300 adenine residues. Recently, Keller and colleagues have shown that PABP2-poly(A) complexes can form 7-nm linear filaments, as well as 21-nm oligomeric particles that included 200 to 300 adenine residues. Thus, the normal aggregation of PABP2 monomers may play a role in both elongation and termination of poly(A) synthesis. The PABP2 filament diameter is similar to the dimensions of the filaments aggregated within OPMD intranuclear inclusion bodies. PABP2 is found predominately in the nucleus, although it may shuttle between the nucleus and the cytoplasm. PABP2 also likely has a role in protein trafficking within the nucleus or to nuclear pores. These functions may be critical to our understanding of the pathogenesis of disease in OPMD.

Compared with other triplet repeat disorders in which pathogenic expansions are in the tens to hundreds of codons, the expansion in OPMD is remarkably small. How the small polyadenine expansion in PABP2 causes disease is unknown. Perhaps the intrinsic aggregation properties of PABP2 are altered enough to significantly increase its normal tendency to form oligomeric complexes.

The mechanism by which trinucleotide repeat expansion mutations in PABP2 lead to pathogenesis in OPMD is unknown. In addition, the relation between protein aggregation and the etiology of the triplet repeat disorders remains an enigma. Secondary pathology in these disorders needs to be examined. For example, structural and genomic mitochondrial abnormalities...
have been identified in OPMD, yet their significance and relationship to cellular dysfunction, age-related changes, or mutant gene products are unknown. Several hypotheses remain to be explored, such as the factors that impart cell specificity, proteins associated with gene product proteins, mitochondrial dysfunction, ubiquitin/proteasome/molecular chaperone abnormalities, the mechanism of protein aggregation, and the recently developed concept of perturbations in “protein clearance.” Given the large body of knowledge about the protein product of the OPMD gene and the evidence contained in this communication linking the proposed gene product to the pathology of OPMD for the first time, we expect that the study of OPMD and PABP2 will lead to a better understanding of the mechanism of intranuclear protein aggregation in this and other triplet repeat disorders in the near future.

We thank Elmar Wahle for the generous gift of PABP2 antiserum. This work was supported in part by grants from NIH NINDS (M.W.B.), National Science Foundation (D.G.B.), and Muscular Dystrophy Association (D.G.B.).

References