On noxious desmin: functional effects of a novel heterozygous desmin insertion mutation on the extrasarcomeric desmin cytoskeleton and mitochondria

Rolf Schröder¹,∗,†, Bertrand Goudeau²,‡, Monique Casteras Simon², Dirk Fischer¹, Thomas Eggermann³, Christoph S. Clemen⁴, Zhenlin Li⁵, Jens Reimann¹, Zhigang Xue⁵, Sabine Rudnik-Schöneborn³, Klaus Zerres³, Peter F. M. van der Ven⁶, Dieter O. Fürst⁶, Wolfram S. Kunz⁷ and Patrick Vicart²

¹Department of Neurology, University Hospital Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany, ²Laboratoire Cytosquelette et Développement, Faculté de Médecine Pitié-Salpêtrière, Paris, France, ³Institute of Human Genetics, RWTH Aachen, 52057 Aachen, Germany, ⁴Center for Biochemistry I, Medical Faculty, University of Cologne, 50931 Cologne, Germany, ⁵Biologie Moléculaire de la Différenciation, Université Paris 7, France, ⁶Department of Cell Biology, University of Potsdam, 14471 Potsdam, Germany and ⁷Department of Epileptology, University Hospital Bonn, 53105 Bonn, Germany

Received November 15, 2002; Revised December 20, 2002; Accepted January 9, 2003

Recent studies in desmin (−/−) mice have shown that the targeted ablation of desmin leads to pathological changes of the extrasarcomeric intermediate filament cytoskeleton, as well as structural and functional abnormalities of mitochondria in striated muscle. Here, we report on a novel heterozygous single adenine insertion mutation (c.5141_5143insA) in a 40-year-old patient with a distal myopathy. The insertion mutation leads to a frameshift and a truncated desmin (K239fsX242). Using transfection studies in SW13 and BHK21 cells, we show that the K239fsX242 desmin mutant is incapable of forming a desmin intermediate filament network. Furthermore, it induces the collapse of a pre-existing desmin cytoskeleton, alters the subcellular distribution of mitochondria and leads to abnormal cytoplasmic protein aggregates reminiscent of desmin-immunoreactive granulofilamentous material seen in the ultrastructural analysis of the patient’s muscle. Analysis of mitochondrial function in isolated saponin-permeabilized skeletal muscle fibres from our patient showed decreased maximal rates of respiration with the NAD-dependent substrate combination glutamate and malate, as well as a higher amytal sensitivity of respiration, indicating an in vivo inhibition of complex I activity. Our findings suggest that the heterozygous K239fsX242 desmin insertion mutation has a dominant negative effect on the polymerization process of desmin intermediate filaments and affects not only the subcellular distribution, but also biochemical properties of mitochondria in diseased human skeletal muscle. As a consequence, the intermediate filament pathology-induced mitochondrial dysfunction may contribute to the degeneration/regeneration process leading to progressive muscle dysfunction in human desminopathies.

INTRODUCTION

Mutations of the human desmin gene on chromosome 2q35 cause a familial or sporadic form of skeletal myopathy frequently associated with cardiac abnormalities (OMIM no. 125660) (1–7). Desmin, the main intermediate filament (IF) protein in skeletal and cardiac muscle cells, is a structural component of the extrasarcomeric cytoskeleton which forms a three-dimensional scaffold around myofibrillar Z-discs, thereby interlinking neighbouring myofibrils and connecting the
myofibrillar apparatus to nuclei, the subsarcolemmal cytoskeleton and cytoplasmic organelles such as mitochondria (8–14).

A milestone in our current understanding of desmin function was the generation of desmin (−/−) mice by two laboratories independently (15,16). Although desmin (−/−) mice are viable and fertile, they develop progressive muscle weakness and dystrophic alterations in both cardiac and skeletal muscle. Since severe structural changes with disorganization and deranged alignment of myofibrils, as well as sarcolemmal disruption, were most prominent in highly used striated muscles, it was concluded that the lack of desmin results in an increased susceptibility of muscle fibres to physical strain during muscle contraction (14–16). Even in the absence of desmin, interconnecting filamentous structures were observed between neighbouring myofibrils and from the Z-discs of most peripheral myofibrils to the overlying sarcolemma (9). In normal striated muscle, the filamentous intermyofibrillar cytoskeleton is composed of a network of various components comprising the IF proteins desmin, synemin and paranemin, the molecular chaperone αB-crystallin, and the multifunctional cytoskeletal linker plectin (8,14,17–23). The latter protein has been attributed to play an essential role in the proper spacing, stabilization and subcellular attachment of intermediate filaments (20,24).

Mutations in the human desmin, αB-crystallin and plectin gene all give rise to a progressive skeletal myopathy (1,3,24–27). This indicates that these essential members of the extrasarcomeric cytoskeleton have complementary (but not interchangeable) roles in the structural and functional maintenance of striated muscle fibres in response to physical stress. All three disorders, which are morphologically characterized by myofibrillar abnormalities, and abnormal cytoplasmic accumulation of desmin-immunoreactive material, share their structural myofibrillar and intermyofibrillar abnormalities with the large group of the so-called myofibrillar myopathies. These disorders comprise sporadic and familial neuromuscular conditions of considerable clinical and genetic heterogeneity. In a recently reported series of 53 patients with myofibrillar myopathy only three cases could be attributed to either desmin or αB-crystallin mutations (27), indicating that the vast majority of myofibrillar myopathies are due to so far unidentified gene defects. Accordingly, familial myopathies mapping to chromosomes 12q, 10q23 and 2q24–31 are not yet genetically defined (28,29). Furthermore, mutation analysis of the human genes encoding the IF proteins synemin, paranemin and syncoilin (a novel desmin-binding protein) will be a central issue in deciphering the complex genetic and pathophysiological background of myofibrillar myopathies (30,31).

Although the primary event in the pathogenesis of myopathies caused by desmin αB-crystallin and plectin mutations seems clearly related to structural and functional defects of the three-dimensional extrasarcomeric intermediate filament cytoskeleton, the exact molecular mechanisms leading to progressive muscle damage in these disorders are unclear. A possible explanation was indicated by recent studies in desmin (−/−) and plectin (−/−) mice, that suggested that perturbations of the desmin/plectin cytoskeleton are associated with an abnormal distribution of mitochondria and respiratory function abnormalities (13,14,32–34).

In the present study, we report the functional consequences of the first desmin insertion mutation in a patient and in cultured cells. We demonstrate that the K239fsX242 mutation leads to a dual phenotype in diseased skeletal muscle with focal derangements of the extrasarcomeric cytoskeleton characterized by prominent desmin, synemin, plectin and αB-crystallin–immunoreactive protein aggregates, whereas others areas within the same muscle fibre display a normal cross-striated distribution of these proteins. Using transfection studies in SW13 and BHK21 cells, we show that the K239fsX242 desmin mutant is incapable of forming a desmin intermediate filament network, induces a collapse of a pre-existing desmin cytoskeleton with consecutive changes in the subcellular distribution of mitochondria, and leads to the abnormal aggregation of cytoplasmic proteins. Changes in the subcellular distribution of mitochondria in BHK21 cells that express the desmin mutant are mirrored in the diseased skeletal muscle fibres of our patient, that showed an irregular succinate dehydrogenase (SDH) staining pattern in a large number of fibres. Furthermore, our analysis of mitochondrial function in isolated saponin-permeabilized skeletal muscle fibres from our patient provided evidence for an in vivo inhibition of complex I activity. Our results strongly indicate that primary intermediate filament pathology in human skeletal muscle induces mitochondrial abnormalities which may contribute to the vicious circle leading to progressive muscle dysfunction in human desminopathies and other forms of myofibrillar myopathies.

RESULTS

Clinical phenotype

A 40-year-old Caucasian male patient presented with a history of slowly progressive muscle weakness since the age of 18. Although muscle weakness was initially restricted to the distal anterior leg muscle compartment, weakness and atrophy gradually spread over the years to his arm and proximal leg muscles. In his mid-twenties, he first developed episodes of cardiac arrhythmia and at the age of 31 a defibrillator device was implanted because of episodes with malignant ventricular tachycardia. He has two healthy children but lost one child at the age of 9 years, who was in a persistent vegetative state for 8 years after a cardiac arrest of unknown cause. Otherwise the family history was negative for neuromuscular or cardiac disorders. In June 2001 neurological examination demonstrated generalized muscle weakness and wasting which was pronounced in distal muscle groups. Repeated laboratory testing revealed normal creatine kinase levels. Needle electromyography demonstrated positive sharp waves and fibrillation potentials as well as short-duration polyphasic motor unit potentials with decreased amplitudes in all muscles analysed.

Mutation analysis

Mutation analysis using direct nucleotide sequencing of PCR products amplified from all nine coding exons of the desmin gene revealed a novel heterozygous single adenine insertion mutation (c.5141_5143insA; GenBank ID 181539, accession M63391) in the very 3’ end of exon 3 (Fig. 1A and B). This mutation resides in a part of the gene that encodes in the evolutionary highly conserved 1B domain of the alpha-helical
coiled-coil rod domain of the desmin protein. It causes a frame shift and a premature termination signal located four codons downstream of the insertion site, thereby leading to a truncated desmin molecule with a predicted molecular weight of 27 kDa (K239fsX242; Swiss-Prot P17661). This mutation was not detected in 50 normal control chromosomes. We did not have the chance to perform a desmin mutation analysis in any other family members. However, the negative family history for cardiac or neuromuscular diseases might indicate that the detected monoallelic insertion is due to a de novo mutation in the reported patient.

Morphological analysis

Morphological analysis of a muscle biopsy taken from the left vastus lateralis muscle showed mild myopathic changes consisting of slightly increased endomysial connective tissue, increased fibre diameter variability and roundness of muscle fibres. Furthermore, haematoxylin and eosin and Gomori trichrome stains revealed multiple basophilic inclusions, which could be detected in the subsarcolemmal region and, to a lesser extent, in the cytoplasm of the vast majority of fibres (Fig. 2A and B). While multiple muscle fibres displayed areas of attenuated or even absent SDH-staining indicating a focal depletion of mitochondria, other fibres showed focal areas with increased SDH-staining indicating focal mitochondrial proliferation (Fig. 2C). Cytochrome c oxidase (COX)-negative fibres were present (<2% of all fibres; Fig. 2D), but no classical ragged-red fibres were observed.

Ultrastructural analysis demonstrated Z-disc streaming (data not shown), and the presence of intermyofibrillar and subsarcolemmal desmin-immunoreactive granulofilamentous protein aggregates (Fig. 2E and F). Furthermore, multiple fibres displayed areas with a focal clustering of mitochondria in areas with pathological protein aggregates (Fig. 2E), whereas other regions with granulofilamentous inclusion showed a reduction or even absence of mitochondria (data not shown). In areas with Z-band streaming, we noted a reduction of mitochondria in the vicinity of some but certainly not all of these myofibrillar lesions.

Indirect immunofluorescence analysis of isolated muscle fibres showed an intense labelling of these pathological aggregates with antibodies against desmin, synemin, αB-crystallin (Fig. 3) and, to a somewhat lesser extent, with antibodies against plectin and ubiquitin (data not shown). However, it is noteworthy that other areas within the same muscle fibres showed a normal cross-striated distribution of desmin, synemin, αB-crystallin and plectin. Furthermore, double immunolabelling with antibodies against desmin in conjunction with antibodies against fast-, slow- and fetal myosin demonstrated that the abnormal desmin inclusions were not specific for a certain fibre type, since inclusions were detected in type I, type II and regenerating fibres (data not shown).

Desmin protein expression

To study the consequences of the K239fsX242 desmin mutation on desmin protein expression, we performed one- and two-dimensional gel electrophoresis of total protein extracts from normal and diseased skeletal muscle in conjunction with western blotting. Both the polyclonal desmin antiserum (Fig. 4A), and the monoclonal desmin antibody (data not shown) showed a strong labelling of a single 53 kDa band in both normal and diseased muscle with no apparent differences in the intensity of the signals. However, no mutated
desmin protein with a predicted molecular weight of 27 kDa could be detected by using these two desmin antibodies. Two-dimensional gel electrophoresis and immunoblotting analysis of total protein extracts from normal human skeletal muscle detected desmin isoforms in a range of pH 5.4–5.9 (calculated pI is 5.2). In contrast, our analysis of diseased muscle showed a spectrum with a range of immunolabelled desmin isoforms between pH 5.4 and 5.8 (Fig. 4B), indicating the absence of the less acidic desmin variants. This shift was confirmed using IPG stripes with a linear pH range from 3 to 10 (data not shown).

**Transfection studies**

The functional consequences of the novel desmin insertion mutation were studied by means of transfection studies in SW13 cells that do not contain an endogenous intermediate filament cytoskeleton, and BHK21 cells that express desmin, vimentin and keratin intermediate filaments. Our western blot studies with two different desmin antibodies failed to detect the mutated 27 kDa desmin protein. To overcome these problems in the visualization of mutant desmin in our transfection studies, wild-type and K239fsX242 mutant human desmin cDNA were cloned into a myc-tag expression vector which allows the immunodetection of recombinant wild-type and mutant desmin protein by using an anti-myc tag antibody. As demonstrated in Figure 5A and B, transfection of SW13 cells with wild-type desmin cDNA carrying a N-terminal myc-tag resulted in the formation of a desmin filament network similar to that observed in cells transfected with wild-type desmin cDNA lacking the N-terminal myc-tag. These findings indicate...
Mitochondrial function

Mitochondrial function was studied in skeletal muscle homogenates and isolated muscle fibers from the reported patient. As shown in Table 1, biochemical analysis of respiratory chain enzyme activities in skeletal muscle homogenates showed normal levels of complex I, complex IV and citrate synthase, as well as normal complex I/citrate synthase and COX/citrate synthase ratios. However, our analysis of mitochondrial function in isolated saponin-permeabilized skeletal muscle fibers, which more closely represent the in vivo situation, showed decreased maximal rates of respiration with the NAD-dependent substrate combination glutamate and malate, and a higher amytal sensitivity of respiration (Table 2). This indicates an in vivo inhibition of complex I activity in skeletal muscle harbouring the K239fsX242 desmin insertion mutation.

DISCUSSION

Morphological and biochemical consequences of the novel K239fsX242 desmin mutation

Over the past 4 years, an increasing number of genetically proven cases with myopathy cardiomyopathy caused by mutations in the desmin gene have been reported (1–7,35). Apart from a single case with a missense mutation residing in the desmin tail domain, all other pathogenic desmin gene alterations (missense mutations, n = 8; deletion mutation, n = 1; splice site mutations, n = 2) were found in the evolutionary highly conserved z-helical coiled-coil rod domain.

Mutation analysis in our patient with the clinical phenotype of a distal myopathy and malignant cardiac arrhythmias revealed a heterozygous single adenine insertion mutation (c.5141_5143insA) in exon 3 which encodes the C-terminal part of the z-helical 1B segment of the central rod domain (Fig. 1C). This, to our knowledge first, desmin insertion

that the N-terminal myc-tag does not interfere with the assembly and formation of desmin filaments. In contrast, immunodetection of myc in myc/K239fsX242 transfected SW13 cells showed that the mutant desmin is incapable of assembling a three-dimensional desmin intermediate filament cytoskeleton but leads to a speckled pattern of myc-immunoreactive cytoplasmic protein aggregates (Fig. 5C). In BHK21 cells, endogenous and myc-tagged labelled desmin networks give similar patterns (Fig. 5D–F), whereas transfection with myc/K239fsX242 disrupts the endogenous desmin cytoskeleton in BHK21 cells (Fig. 5G–I).

Upon transfection of these cells with myc/K239fsX242 aggregates of myc-immunoreactive material were observed 48h after transfection but the endogenous desmin cytoskeleton still had a normal appearance (not shown). Seventy-two hours following transfection, when expression levels of the mutant protein are significantly higher, the endogenous desmin cytoskeleton was completely disrupted, and endogenous wild-type desmin was found together with mutant desmin in aggregates (Fig. 5G–I). This indicates that the ratio of wild-type desmin to mutant desmin determines the effects of the expression of the mutated desmin. Furthermore, these experiments show that in cells with pre-existing desmin intermediate filaments the mutated desmin can induce a collapse of the desmin cytoskeleton.

To address the issue whether the disruption of the endogenous desmin cytoskeleton exerts an effect on the subcellular distribution of Mitochondria, transfected BHK21 cells were incubated with the mitotracker red fluorescent dye, which allows a specific labelling of mitochondria. In myc/wild-type desmin transfected BHK21 cells, mitochondria were localized throughout the cytoplasm with a somewhat denser concentration in the perinuclear area when compared to the cell periphery (Fig. 6A–C). In contrast, cells transfected with the myc/K239fsX242 desmin mutant showed a dramatic increased clustering of mitochondria in the perinuclear region where mitochondria seemed to be entrapped in the aggregates of the disrupted desmin filament cytoskeleton (Fig. 6D–F).
mutation leads to a frame shift and premature termination of translation only four codons downstream of the insertion site, thereby leading to a truncated desmin mutant lacking the last 227 amino acids (K239fsX242).

Standard histological and indirect immunofluorescence analysis of cross-sections from desmin myopathy muscle showed mild myopathic changes and multiple subsarcolemmal and cytoplasmic desmin-immunoreactive inclusions, which are the light microscopic equivalent of the desmin-immunoreactive granulofilamentous protein aggregates seen at the ultrastructural level. These pathological protein aggregates were also

<table>
<thead>
<tr>
<th></th>
<th>CS</th>
<th>CI</th>
<th>CI/CS</th>
<th>COX</th>
<th>COX/CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>10.51</td>
<td>1.32</td>
<td>0.13</td>
<td>6.00</td>
<td>0.57</td>
</tr>
<tr>
<td>Controls</td>
<td>12.2 ± 2.5</td>
<td>1.1 ± 0.4</td>
<td>0.1 ± 0.03</td>
<td>5.8 ± 0.9</td>
<td>0.48 ± 0.07</td>
</tr>
</tbody>
</table>

Figure 5. Transfection studies in SW13 (A–C) and BHK21 cells (D–I). (A) Immunodetection of desmin 48 h after transfection with wild-type human desmin cDNA in SW13 cells (mab-D33). Note the formation of a desmin cytoskeleton. (B) Immunodetection of myc 48 h after transfection with wild-type human desmin cDNA carrying an N-terminal myc-tag in SW13 cells (anti-myc antibody). Note the formation of a normal three-dimensional desmin cytoskeleton in SW13 cells. (C) Immunodetection of myc 48 h after transfection with K239fsX242 mutant desmin carrying an N-terminal myc-tag. Note that the desmin mutant is incapable of forming a desmin intermediate filament network but leads to abnormal myc-immunoreactive protein aggregates. (D) Immunodetection of the transfected and endogenous desmin network in BHK21 cells 72 h after the transfection with myc-tagged wild-type human desmin cDNA. (E) Immunodetection of myc in the same cells transfected with myc-tagged wild-type human desmin cDNA. (F) Visualization of endogenous and myc-labelled wild-type desmin BHK21 transfected cells. (G) Desmin immunolabelling reveals the disruption of the endogenous desmin cytoskeleton and the formation of pathological protein aggregates in BHK21 cells 72 h after transfection with the K239fsX242 desmin mutant. (H) Immunodetection of myc in pathological protein aggregates in the same cells. (I) Colocalization of endogenous desmin and myc-labelled mutant desmin in pathological protein aggregates in the same cells.

Table 1. Analysis of respiratory chain enzyme activities in skeletal muscle homogenates from diseased muscle (DM) showed normal values for CI (complex I), COX (cytochrome c oxidase) and CS (citrate synthase) as well as normal CI:CS and COX:CS ratios.
stained by antibodies against the intermediate filament synemin, the intermediate filament-associated protein plectin and the small heat shock protein βB-crystallin, all components of the extrasarcemeric intermediate filament system. Although pathological protein aggregates were a prominent feature in the majority of muscle fibres, analysis of isolated skeletal muscle fibres also showed widespread areas with a normal appearing desmin, synemin, plectin and βB-crystallin staining pattern, indicating that the structural organization of the extrasarcemeric cytoskeleton is disturbed in some but not all areas of diseased muscle fibres. This concept is further corroborated by a recently published paper on the cytoskeletal derangements in muscle harbouring the K239/X242 desmin insertion mutation. Using a two-sided t-test, we found a significant (P < 0.05) difference between diseased and normal control muscle fibres. The expression level and subcellular distribution of mutant desmin in diseased muscle remains elusive.

Table 2. Rates of respiration (four independent measurements) with the NAD-dependent substrate combination glutamate and malate, succinate, TMPD (N,N,N,N-tetra-methyl-paraphenyl-diamine) plus ascorbate. Note the decreased maximal rates of respiration with the NAD-dependent substrate combination glutamate and malate as well as a higher amytal sensitivity of respiration, indicating an in vivo inhibition of complex I activity in skeletal muscle containing the K239/X242 desmin insertion mutation. Using a two-sided t-test, we found a significant (P < 0.05) difference between diseased and normal control muscle fibres. Ci (amytal) = flux control coefficient with amytal; Ci (KCN) = flux control coefficient with KCN.

<table>
<thead>
<tr>
<th></th>
<th>Glutamate +</th>
<th>Succinate</th>
<th>TMPD + Ci</th>
<th>Ci (amytal)</th>
<th>Ci (KCN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>6.6 ± 1.3</td>
<td>10.1 ± 0.8</td>
<td>13.5</td>
<td>0.5 ± 0.15</td>
<td>0.33 ± 0.12</td>
</tr>
<tr>
<td>Controls</td>
<td>8.6 ± 1.9</td>
<td>9.2 ± 1.9</td>
<td>13.1 ± 4.0</td>
<td>0.1 ± 0.06</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>(n = 25)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The K239/X242 mutant desmin is incapable of forming a desmin intermediate filament network, induces a collapse of a pre-existing desmin cytoskeleton and leads to abnormal cytoplasmic protein aggregation

Analysis of myc/Des K239/X242 transfected SW13 showed that the mutant desmin is incapable of assembling a (de novo) filament network and leads to abnormal cytoplasmic myc-immunoreactive protein aggregates. In BHK21 cells, which contain an endogenous desmin intermediate filament network, transfection with myc/K239/X242 leads to a disruption of this pre-existing desmin cytoskeleton, and abnormal protein aggregation reminiscent of the desmin-containing aggregates in the patient's muscle. The fact that this effect in BHK21 cells was only observed in cells with a high level of mutated desmin, after 72 h of transfection, points to a dosage effect. These findings, which are in line with previous transfection studies using mutated desmin variants (2–6), indicate that the K239/X242 desmin mutant exerts a dominant negative effect on the processes that are required for maintaining a normal intermediate filament network formation, at least in cultured cells. The observed dosage effect has already been found in in vitro experiments that demonstrated that the phenotype of the desmin filamentous that are assembled from mixtures of wild-type and mutant desmin depends on the stoichiometry of both proteins (2). These data might explain the observed variations in ultrastructural findings in the muscle fibres of patients suffering from a desminopathy.

From intermediate filament pathology to mitochondrial dysfunction: a relevant aspect in human desminopathies?

Varying degrees of mitochondrial dysfunction have been reported in a wide range of inflammatory as well as hereditary neuromuscular disorders (24,41–45). To date, the exact pathologic mechanisms leading to secondary mitochondrial dysfunction, as well as the functional relevance of a secondary ‘metabolic crisis’, in various muscle diseases is an unresolved issue (42). However, with respect to desminopathies there are good reasons to contemplate a more specific relationship between intermediate filament pathology and mitochondrial dysfunction. Several ultrastructural studies have documented a structural relationship between the intermediate filament cytoskeleton and mitochondrial dysfunction (13,46,47). Furthermore, recent studies in desmin (−/−) mice that showed the targeted ablation of desmin leads to alterations in the spatial distribution of mitochondria, and to structural and biochemical mitochondrial abnormalities in striated muscle (14,32–34).

To analyse putative effects of the K239/X242 desmin mutant on mitochondrial localization, we studied their distribution in...
Figure 6. Transfection studies in BHK21 cells with respect to the subcellular distribution of mitochondrias. (A) Immunodetection of myc 72 h after transfection of wild-type human desmin cDNA carrying an N-terminal myc-tag. (B) Visualization of mitochondria using the Mitotracker<sup>®</sup> fluorescent dye in the same cells. Mitochondria are labelled throughout the cytoplasm with a denser aggregation in the perinuclear region when compared with the cell periphery. (C) Dual visualization of mitochondria and myc-tagged wild-type human desmin. (D) Immunodetection of myc in pathological protein aggregates after transfection with the K239fsX242 desmin mutant. (E) Note the markedly altered subcellular distribution of mitochondria with clustering of mitochondria in the perinuclear region in response to the collapse of the endogenous desmin network in BHK21 cells transfected with the K239fsX242 desmin mutant. (F) Dual visualization of mitochondria and myc-tagged mutant desmin. Note the clustering of mitochondria in the perinuclear region as well as the entrapment of mitochondria in pathological protein aggregates.
transfected BHK21 cells using the Mitotracker® fluorescent dye. In myc/K239fsX242 transfected cells, we observed marked changes in the subcellular distribution with massive clustering of mitochondria in the perinuclear region that coincided with the disruption of the pre-existing desmin cytoskeleton and formation of pathological protein aggregates. These changes in the subcellular distribution of mitochondria are mirrored, at least in part, in the skeletal muscles from our patient. SDH staining, a histochemical marker for mitochondria, showed patchy enzyme activity in multiple muscle fibres with areas of attenuated or even absent SDH activity, indicating focal depletion of mitochondria. Interestingly, we also observed focal areas with increased activity, indicating focal mitochondrial proliferation or aggregation. In this context, it is important to note that the focal derangements of the desmin cytoskeleton due to the K239fsX242 desmin mutation are present in some but not all areas of individual muscle fibres, which in turn might explain focal differences in the subcellular distribution of mitochondria within a single muscle fibre.

Our biochemical analysis of respiratory chain enzyme activities in desmin myopathy skeletal muscle homogenates showed normal results, which rules out any gross abnormalities of cell respiration. However, analysis of mitochondrial function in isolated saponin-permeabilized skeletal muscle fibres, an optimal tool for the detection of subtle changes of oxidative phosphorylation that more closely represents the in vivo situation, showed decreased maximal rates of respiration with the NAD-dependent substrate combination glutamate and malate as well as a higher amytal sensitivity of respiration. Considering the observed normal enzyme activities in skeletal muscle homogenates, these findings suggest an in vivo inhibition of complex I activity in intact skeletal muscle fibres. In analogy to the findings in desmin (−/−) mice, these mild changes of oxidative phosphorylation in diseased muscle may be attributed to a reduced focal diffusion of mitochondrial substrates caused by the myofibrillar disorganization and/or pathological protein aggregates. We speculate that the focal depletion of mitochondria and/or focal disturbance in ATP production might further weaken the structural integrity and functional interplay of neighbouring myofibrils, and thus aggravate the focal myofibrillar changes caused by the lack of an intact intermyofibrillar cytoskeleton. Furthermore, mitochondrial abnormalities might result in the non-lysosomal degradation of pathological protein aggregates via the ATP-dependent 26S-proteasome pathway. These aspects will have to be addressed in future experimental studies.

Since mitochondrial abnormalities usually increase with age, mitochondrial dysfunction might progress undetected until an as yet undefined threshold is reached. At this point a ‘metabolic crisis’ is induced which contributes to progressive muscle dysfunction in primary desmin myopathies and other forms of myofibrillar myopathies.

In conclusion, we report for the first time a desmin insertion mutation. This mutation leads to a distal myopathy associated with malignant cardiac arrhythmias. The K239fsX242 desmin mutation has a dominant negative effect on the polymerization of desmin intermediate filaments, which leads to focal disruptions of the extrasarcromeric intermediate filament cytoskeleton and pathological protein aggregates in diseased human muscle fibres.

The occurrence only focal pathological changes might be explained by focal differences in the wild-type:mutant desmin ratio. The focal changes of the desmin cytoskeleton seem to induce focal myofibrillar disarray and abnormalities in the subcellular distribution and biochemical properties of mitochondria. Our transfection experiments provided evidence that at least the mitochondrial abnormalities are a direct cause of the expression of truncated desmin.

MATERIALS AND METHODS

Mutation analysis

Genomic DNA of the patient was isolated from peripheral lymphocytes by standard procedures. The complete coding region and intron–exon boundaries of the desmin gene were screened for variations by direct sequencing of PCR products. Information on primers and PCR conditions can be obtained from the authors upon request. PCR was carried out in a 25 μl volume, containing 80 ng genomic DNA, 50 pmol of each primer, 50 mM KCl, 10 mM Tris–HCl pH 8.3, 1.5 mM MgCl2, 0.01% gelatin, 200 μM of each dNTP, and 1 U Taq polymerase (Gibco BRL). Sequencing of PCR products was carried out using the BigDyeTermination-Cycle Sequencing System (Applied Biosystem), the sequencing products were analyzed on an ABI377 DNA Sequencer (Applied Biosystems).

Muscle biopsy

A diagnostic biopsy was taken from the left vastus lateralis muscle of the patient. Normal control muscle was obtained from a patient who underwent muscle biopsy for diagnosis of neuromuscular symptoms but was ultimately deemed to be normal by means of combined clinical, electrophysiological and histological criteria.

Morphological analysis

Single fibre preparation from normal human skeletal muscle was performed as described previously (48). Cryostat sections (9 μm) of snap-frozen, unfixed muscle were stained for haematoxylin and eosin, oil red O, periodic acid-Schiff, Gomori trichrome, nicotin adenine dinucleotide tetrazoli-um-H reductase, myofibrillar adenosine triphosphatase at pH 4.2, 4.6 and 9.4, cytochrome c oxidase, and SDH. These experiments were performed by standard procedures.

Antibodies

The following primary antibodies were used in this study: (1) mouse monoclonal anti-desmin antibody D33 (Dako, Germany); (2) rabbit polyclonal anti-desmin antiserum PDE 2203 (Euro-Diagnostica, The Netherlands); (3) rabbit polyclonal anti-αB-crystallin antiserum (Chemicon, USA); (4) P2 guinea pig serum, an antisem directed against the carboxy-terminal repeat domain 6 of human plectin (12); (5) rabbit polyclonal anti-synemin antisem (23). (6) mouse monoclonal anti-fast myosin heavy chain antibody (Sigma, Germany); (7) mouse monoclonal anti-slow myosin heavy chain antibody (Sigma, Germany); (8) mouse monoclonal anti-fetal myosin

...
heavy chain antibody (Sigma, Germany); (9) mouse monoclonal anti-ubiquitin antibody (Sigma, Germany); and (10) rabbit polyclonal anti-myc antibody (Sigma, Germany). Isotype-specific secondary antibodies conjugated with fluorescein isothiocyanate, Cy2, or Texas Red were applied according to the recommendations of the manufacturers (Southern Biotechnology Associates, USA; Jackson Immunoresearch Laboratories, USA; Molecular Probes, USA).

Indirect immunofluorescence

Indirect immunofluorescence analysis of human skeletal muscle was performed as described previously (20,49) and pictures were acquired digitally with a Nikon E800 microscope (Nikon, Düsseldorf, Germany), and a Zeiss Axiophot microscope (Zeiss, Germany).

Concerning functional studies, transfected cells were washed in PBS and fixed for 5 min in methanol–acetone (7:3 vol:vol) at 4°C, 48 and 72 h after transfection. Following washing steps in PBS, cells were processed for indirect immunofluorescence. Coverslips were mounted in Mowiol and pictures of transfected cells were acquired digitally with an Olympus BX60 microscope (Olympus, Germany).

Immunoelectron microscopy

Desmin immunogold electron microscopy of skeletal muscle was performed as described previously (24). The primary antibody against desmin was diluted 1:20. A secondary anti-rabbit antibody coupled to 10 nm colloidal gold particles was purchased from Amersham Pharmacia (Freiburg, Germany).

Gel electrophoresis and western blotting

For one-dimensional gel electrophoresis, preparation of total protein extracts, SDS–PAGE using 12% polyacrylamide gels, protein transfer and visualization of proteins on membranes were carried out as described previously (12).

Two-dimensional gel electrophoresis was performed using an adaptation of the protocols described by Goerg et al. (50) and Rabilloud et al. (51) and the Amersham Pharmacia Biotech protocol. For protein extraction, 4 mg of each muscle specimen were cut at 0°C. Lysed in 200 µl lysis buffer [7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, 2% IPG-buffer, 2% DTT, 1 mM PMSF, complete mini protease inhibitor (Roche), bromophenol blue], homogenized and centrifuged at 16 000 g. For the first dimension 18 cm IPG-strips (pH 4–7 linear, Amersham) were rehydrated (7 M urea, 2 M thiourea, 2% CHAPS, 2% IPG-buffer, 13 mM DTT, bromophenol blue) and samples of the supernatants were loaded using cups placed at the anodic side. Isoelectric focusing was performed at 18°C (Multiphor II horizontal, Amersham) using the following voltage gradient (power supply EPS 3501 XL, Amersham): linear on 400 V in 1 min, 400 V for 4 h, linear on 3500 V in 5 h, 3500 V for 13 h. After IEF, stripes were equilibrated for 15 min each in buffer 1 (6 M urea, 50 mM Tris–HCl, 2% SDS, 30% glycerin, 1% DTT, bromophenol blue, pH 8.8) and buffer 2 (6 M urea, 50 mM Tris–HCl, 2% SDS, 30% glycerin, 4% iodacetamide, bromophenol blue, pH 6.8), SDS–PAGE was carried out using 12% polyacrylamide gels according to Laemmli (52).

Immunodetection of desmin using the polyclonal antiserum was performed with a peroxidase-coupled secondary antibody (1:30 000) and the ‘Supersignal’ enhanced chemiluminescence system (Pierce, Rockford, IL, USA).

Site-directed mutagenesis

The mutated human desmin cDNA which includes the A insertion was obtained using a mutated oligonucleotide for site-directed mutagenesis into a pcDNA3 vector (Invitrogen, Germany) containing the human desmin cDNA (pcDNA3/Des) (53). The following synthetic oligonucleotides were used: DESForward, 5'-CCACGCACCAACGAGAAGGTGG-3'; DESReverse, 5'-CGGATATCCTTAGGGCCGCAATG-3'; INSF, 5'-GATCGCGTCTCTTAAGAAAACTGCTGA-3'; INSAR, 5'-TCATGCACCTTTTAAAGGAAACGCGATC-3'. The A insertion is underlined. The wild-type and mutant cDNA in plasmid pcDNA3 were excised and cloned into a Myc-tag expression vector (54) to generate the myc/Des and myc/K239fsX242 constructs. Sequencing of cloned DNAs was performed using the DyePrimer Sequencing apparatus according to the manufacturer's instructions (Perkin-Elmer Cetus, USA) on an automated DNA sequencer.

Cell culture and DNA transfection

The expression vectors were transfected into SW13 and BHK21 cell lines. The SW13 (vim-) cells do not express desmin, vimentin and keratin, which makes them an optimal model system for intermediate filament expression studies in cultured cells. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% fetal bovine serum and penicillin/streptomycin (Gibco). One day before transfection, cultured cells were trypsinized and plated on glass coverslips placed in a culture dish. Transfection was carried out using Fugene 6 (Roche, Germany) according to the instructions of the manufacturer. Expression vectors pcDNA3/Des, myc/Des or myc/K239fsX242 (1.5 µg) were added to each dish and the cells were incubated overnight.

Mitochondrial function

Measurements of respiratory chain enzyme activities of NADH [CoQ1 oxidoreductase (complex I), cytochrome c oxidase, citrate synthase] in skeletal muscle homogenates as well as analysis of mitochondrial function in isolated saponin-permeabilized skeletal muscle fibres were performed as described previously (20,48,55).

ACKNOWLEDGEMENTS

The excellent technical assistance of Mrs K. Kappes-Horn, Mrs M. Altenschmidt Mering and Mrs K. Tolksdorf is gratefully acknowledged. Furthermore, we thank Doctor Fernando Rodrigues-Lima for the generous gift of the myc-tag plasmid and helpful discussions. This project is part of the German network on muscular dystrophies (MD-NET, research project R12, 01GM0302) funded by the German ministry of education and research (BMBF, Bonn, Germany). Part of this work was supported by the Association Française contre les Maladies Musculaires.
Myopathies. This article is dedicated to Professor H.H. Goebel on the occasion of his 65th birthday.

REFERENCES


