Acute quadriplegic myopathy (AQM; also called “critical illness myopathy”) shows acute muscle wasting and weakness and is experienced by some patients with severe systemic illness, often associated with administration of corticosteroids and/or neuroblocking agents. Key aspects of AQM include muscle atrophy and myofilament loss. Although these features are shared with neurogenic atrophy, myogenic atrophy in AQM appears mechanistically distinct from neurogenic atrophy. Using muscle biopsies from AQM, neurogenic atrophy, and normal controls, we show that both myogenic and neurogenic atrophy share induction of myofiber-specific ubiquitin/proteosome pathways (e.g., atrogin-1). However, AQM patient muscle showed a specific strong induction of transforming growth factor (TGF)-β/MAPK pathways. Atrophic AQM myofibers showed coexpression of TGF-β receptors, p38 MAPK, c-jun, and c-myc, including phosphorylated active forms, and these same fibers showed apoptotic features. Our data suggest a model of AQM pathogenesis in which stress stimuli (sepsis, corticosteroids, pH imbalance, osmotic imbalance) converge on the TGF-β/MAPK pathway in myofibers. The acute stimulation of the TGF-β/MAPK pathway, coupled with the inactivity-induced atrogin-1/proteosome pathway, leads to the acute muscle loss seen in AQM patients.
To provide a more complete picture of the biochemical pathways activated in AQM, we defined a molecular fingerprint unique to myogenic atrophy (AQM) as opposed to neurogenic atrophy by genomewide microarray analysis of patient muscle biopsies. We show that unsupervised data analysis is easily able to differentiate myogenic atrophy from neurogenic atrophy via expression profiles, and that constitutive stimulation of the proapoptotic transforming growth factor (TGF)–β/MAPK pathway underlies myogenic atrophy. We also show that the muscle-specific Skp1-Cullin-F-box protein (SCF) ubiquitin ligase component, atrogin-1 (FBX32), is strongly induced in AQM, consistent with the significant loss of myofilaments in this disorder. We propose a model for AQM in which a combination of cellular stress, inactivity of muscle, and drug stimulation (corticosteroids) all converge on the TGF-β/MAPK and atrogin-1 pathways, leading to acute stimulation of apoptosis and myofilament loss.

Patients and Methods

Patients and Biochemical and Morphological Analysis of Muscle Biopsies

Diagnostic muscle biopsies were obtained, with informed consent, from five patients, affected by acute paraplegic myopathy (Table 1). Muscle biopsies from all patients were studied by standard histochemistry for morphological assessment, and for enzyme biochemistry for acid and neutral maltase. Disease control muscle biopsies were five patients with neurogenic disorders, including motor neuron disease, lower motor neuron disease, spinal muscle atrophy type III, and chronic axonal neuropathy. Seven normal control muscles were from patients with asymptomatic elevations of creatine kinase (hyperCKemia), with no evidence of histological changes on muscle biopsy. TUNEL, electron microscopy, and immunocytochemistry for apoptosis-related proteins caspase 1, caspase 3, caplain, and cathepsin B were done as previously described (see Table 1).

Expression Profiling

Expression profiling was done as we have described previously. In brief, RNA was extracted from each frozen muscle biopsy using TRIzol reagent (GIBCO BRL, Gaithersburg, MD). Seven milligrams of total RNA from each tissue sample (17 samples total) was processed accordingly to protocol (Affymetrix) to obtain fragmented cRNA that was hybridized on the chip.

Microarray (genechip) Quality Control, Data Scrubbing, and Statistical Analysis

We used stringent quality control methods as previously published and detailed on our Web site: (http://microarray.cnmresearch.org/pgaoutline-qcsofamples.asp). Three of the neurogenic atrophy subject biopsies did not meet our usual threshold for “present” calls. We attributed this to the severe atrophy and fibrosis shown by muscle of these patients. The scaling factors determinations were done using default Affymetrix algorithms (MAS 5) with a target intensity of chip sector fluorescence to 800. Both preamplification (s1) and postamplification with streptavidin/phycocerythrin (s2) scans were done, and the scans were compared by scatterplots and correlation coefficients. Saturated probe sets showing evidence of saturation of the PMT in s2 were eliminated with our custom Array Data Manipulation software. We have shown recently that use of Affymetrix MAS 5.0 signal intensity values, together with a “present call” noise filter achieves an excellent signal to noise balance for human muscle relative to other probe set analysis methods (RMA). Data analyses were limited to probe sets that showed one or more “present” (P “calls”) in the 17 genechip profiles in our complete data set. Experiment normalization was performed by normalizing gene chips as described. Normalized data then were compared for differential gene expression analysis between AQM patients and the two control groups. Genes that showed a Welch analysis of variance (ANOVA) t test with p value less than 0.05 between groups were retained for further analysis. Initial data analysis also included a fold change filter of greater than 1.5 (50% difference) increase or decrease relative to control groups (Affymetrix MAS 5.0). Although a p value of less than 0.05 alone would give many false-positives, the combination of present call filters, fold change thresholds, and p value thresholds, eliminates most false-positives that are obtained with only p values less than 0.05 (all confirmed by other methods). We also confirmed nearly all expression changes at the mRNA and/or protein level using independent techniques.

Table 1. Summary of Clinical, Electrophysiological, and Muscle Pathological Features of AQM patients

<table>
<thead>
<tr>
<th>Pt. No.</th>
<th>Age (yr)</th>
<th>ICU Systemic Illness</th>
<th>Surgery/Anesthesia</th>
<th>Corticosteroids</th>
<th>Atrophy/Weakness</th>
<th>EMG</th>
<th>CK</th>
<th>Myofiber Atrophy</th>
<th>Apoptotic Signs (% fibers)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>66</td>
<td>No COPD-diabetes</td>
<td>No</td>
<td>Yes</td>
<td>+++</td>
<td>1/10 n.v.</td>
<td>+++</td>
<td>Myopathy</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>Yes Sepsis-MD</td>
<td>Yes</td>
<td>Yes</td>
<td>+++</td>
<td>1/2 n.v.</td>
<td>+++</td>
<td>Myopathy</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>77</td>
<td>No Lymphoma-hyper cortisol</td>
<td>No</td>
<td>No</td>
<td>+++</td>
<td>1/5 n.v.</td>
<td>++</td>
<td>Myopathy</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>Yes Sepsis-ARDS-MODS</td>
<td>No</td>
<td>Yes</td>
<td>+++</td>
<td>n.v.</td>
<td>++</td>
<td>Myopathy</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>76</td>
<td>Yes Sepsis-MODS</td>
<td>Yes</td>
<td>Yes</td>
<td>+++</td>
<td>1/20 n.v.</td>
<td>++</td>
<td>Myopathy</td>
<td>40</td>
</tr>
</tbody>
</table>

ICU = intensive care unit; EMG = electromyogram; CK = creatine kinase; COPD = chronic obstructive pulmonary disease; n.v. = normal values; MD = myelodisplasia. ARDS = acute respiratory distress syndrome; MODS = multiorgan distress syndrome; AQM = acute quantitative myopathy.
Real-time Reverse Transcription Polymerase Chain Reaction

We studied gene expression in muscle from AQM patients and normal controls, by real-time polymerase chain reaction (PCR) for both validation purposes (gadd45 β, p21, c-myc, and c-jun) and to measure expression of genes not represented on the genechip (atrogin-1). Fluorophore-labeled LUX primers (forward) and their unlabeled counterparts (reverse) were provided by Invitrogen (La Jolla, CA). LUX primers were designed within Affymetrix probe sets sequences for each gene, and all primers were designed using the software called LUX Designer (Invitrogen, www.invitrogen.com/lux). We performed multiplex PCR combining in the same PCR mix each experimental gene with the housekeeping gene GAPDH.

For each sample, 20μl PCR contained 2μl cDNA (first diluted 1:10 after reverse transcription [RT]), 200nM of each gene-specific primer (two pairs for multiplex PCR) and 1 × Platinum Quantitative PCR SuperMix-UDG (Invitrogen), and 1 × ROX reference dye (500nM in SuperMix). PCR conditions were standard (Invitrogen, www.invitrogen.com/lux), and reactions were conducted in a 96-well spectrophotometric thermal cycler (ABI PRISM 7700 Sequence Detection System; Applied Biosystems, Foster City, CA). Fluorescence was monitored during every PCR cycle at the annealing or extension step and during the post-PCR temperature ramp. Fold changes were measured according to the manufacturer instructions (Invitrogen), and ANOVA t test was used for statistical analysis.

Immunoblotting

Protein extracts were recovered from the lower organic phase of TRIZOL after supernatant isolation for RNA extraction (GIBCO). Proteins then were quantified by the Bradford method, and 20μg of protein solubilized in Laemmli sample buffer supplemented with protease inhibitors (10mg of aprotinin per ml, 1mg of leupeptin per ml, 1mM phenylmethysulfonyl fluoride). Immunoblotting was done using standard methods with 4 to 12% and 10% SDS-PAGE gels. For immunoblotting, we used the following antibodies: goat polyclonal anti–TGF-β receptor I and II, goat polyclonal anti–TAK-1 (diluted 1:200); Santa Cruz Biotechnology, La Jolla, CA); rabbit polyclonal anti-pan-B (diluted: 1:1,000; Oncogene, Cambridge, MA); and rabbit polyclonal anti–R-Ras, anti–p38 MAPK, anti–phospho-p38 MAPK, anti–c-jun, anti–phospho-c-jun, anti–c-myc, anti–phospho-c-myc, anti–phospho-SMAD 1, 5, 8, anti–MKK3, anti–phospho-MKK3, anti–MKK4, anti–Ask1, anti–phospho-Ask1, mouse monoclonal anti–JNK, and anti–phospho-JNK (Cell Signaling, Beverly, MA). Immunocomplexes were visualized with ECL chemiluminescence (Amersham, Arlington Heights, IL).

TUNEL and Immunocytochemistry

The TUNEL technique was used for detection of nuclear DNA fragmentation in situ. Frozen muscle sections (10μm) from patients and control subjects were incubated under the same coverslip with TUNEL reaction mixture, and incorporated fluorescein-dUTP was detected by using alkaline phosphatase-conjugated antifluorescein antibodies according to the manufacturer’s instruction (In Situ Cell Death Detection Kit; Boehringer, Mannheim, Germany). Unfixed muscle sections adjacent to those analyzed by means of TUNEL were processed for immunocytochemistry accordingly to standard procedures. The same primary antibodies used for immunoblotting were used also for immunocytochemistry, and incubated overnight at 4°C. Sections were developed with peroxidase staining. The count of fibers positive by TUNEL and immunocytochemistry was performed in at least 100 muscle fibers per section.

Results

Clinical and Pathological Features of Acute Quadriplegic Myopathy and Controls

Muscle biopsies from five patients, each showing a clinical and histological pattern consistent with the diagnosis of AQM, were studied (see Table 1). Severe systemic illness was present in all patients, variably including sepsis, intensive care unit treatment, surgery, and corticosteroids administration. All patients had a moderate/severe muscle weakness and atrophy. Electromyography showed myopathic changes compatible with AQM. No clinical or electromyography evidence of nerve involvement was seen, including lack of spontaneous activity (see Table 1). Apoptotic signs, as shown by TUNEL and activated caspase 3 immunoreactivity were present in atrophic fibers in all patients (see Table 1), as we have previously described.18

Both neurogenic and normal control muscles were used to address the specificity of the changes in AQM muscles. These age-matched controls included seven biopsies from subjects with asymptomatic hyperCKemia (normal histopathology) and five biopsies from patients from neurogenic disorders (motor neuron disease, lower motor neuron disease, spinal muscle atrophy type III, and chronic axonal neuropathy; all showed neuropathic histopathology). A subset of neurogenic atrophic fibers showed also apoptotic features as previously reported.18

Unsupervised Hierarchical Clustering Accurately Diagnoses Acute Quadriplegic Myopathy from Neurogenic Atrophy

Each skeletal muscle biopsy was processed individually for expression profiling (five AQM patients, five neurogenic disorders, and seven normal muscles). We expression profiled approximately 12,000 transcripts using Affymetrix high-density oligonucleotide arrays Hu95 v2. Data were processed according to bioinformatic methods that we have previously shown provide good signal/noise ratios for human muscle biopsies.20 Normalizations included per chip (50th percentile) and per sample normalization (to the median of each gene). We used several types of data analysis: unsupervised and supervised hierarchical clustering; statistical analysis using analysis of variance (Welch ANOVA t test); nucleation of dysregulated transcripts using a 1.5-fold change cutoff be-
between the different groups of diseases (probe sets differing in 50% expression level); functional clustering with implementation of the GeneSpring gene query tool; and functional ontology visualizations using both GeneMapp,21 and David (http://apps1.niaid.nih.gov/david/upload.jsp) software and databases.

Unsupervised hierarchical clustering based on standard distance metrics (GeneSpring) showed correct diagnosis of the three diagnostic groups (myogenic atrophy [AQM], neurogenic atrophy, normal controls) into three specific branches on the dendrogram (Fig 1). This analysis provides strong support for distinct molecular pathophysiological mechanisms for the two types of atrophy and also indicates that the biological variables were dominant over the sum of technical variables and interindividual noise.20

We then filtered data for dysregulated transcripts based on a significant Welch ANOVA \( t \) test (\( p < 0.05 \)) between AQM and neurogenic and AQM and normal controls, combined with a 1.5-fold change threshold. This resulted in 1,670 upregulated, and 709 downregulated transcripts (see http://microarray.cnmresearch.org/pgadatable.asp for the complete gene list). The AQM-specific transcripts then were filtered for functional ontologies and further analyzed and verified, as described below.

Strong Induction of Oxidative Stress Response and Protease/Proteasome Clusters in Acute Quadriplegic Myopathy

Visualization of known functional clusters showed consistent activation of oxidative stress, protease (including caspase 4 and 6), and ubiquitin pathways using “heat map” methods (see Fig 1). Some of these pathway members showed downregulation in neurogenic controls, consistent with distinct underlying molecular pathophysiology in the two types of atrophy. The ubiquitin-dependent proteolytic pathway was particularly strongly induced in myogenic atrophy (see Fig 1), including upregulation of several ubiquitin proteases and ubiquitin activating enzymes. There was no evident upregulation of ubiquitin ligases; however, it is known that many ubiquitin ligases are induced via activation of tissue specific F-box proteins of the SCF ligase complex.11 The muscle-specific F-box, atrogin-1 (FBx32),17 was not on the U95v2A microarrays used for our expression profiling studies. Therefore, we studied expression of the ubiquitin ligase atrogin-1, by quantitative real-time RT-PCR. We found atrogin-1 mRNA 10-fold increased in both AQM and neurogenic muscle relative to controls (Table 2). To our knowledge, this is the first data on atrogin-1 in humans in vivo, although our data are consistent with in vitro and animal models experiments showing atrogin-1 induced in muscle atrophy under diverse conditions and stimuli.

The Transforming Growth Factor–β/MAPK Signaling Cascade Is Specifically Activated in Acute Quadriplegic Myopathy Myogenic Atrophy

The GeneMapp and David databases were used for functional classification of dysregulated transcripts to identify potential signaling pathways specifically associated with myogenic atrophy. Both databases showed the induction of several members of the MAPK signaling cascade in atrophic AQM muscles. MAPK induction plays an important role in the regulation of cell growth, atrophy, mitosis, and apoptosis.22 This prompted us to further evaluate with both the expression profiling data set and RT-PCR the expression levels of all known members of this pathway and of possible related pathways, including TGF-β and RAS signaling cascades. We found the specific upregulation of TGF-β receptor II, TAK-1, ASK-1, RAS family members, SMADs (Smad 1, 3, and 4), JNKK2, JNK, MKK3/6, p38 MAPK, c-jun, junB, c-fos, c-myc, gadd-45β, and p21 cell cycle inhibitor. Fold changes and \( p \) values for each transcript are reported (Table 2). Transcription of the extracellular signal-repulsated kinase (ERK) branch of the large MAPK pathway was inhibited or not differentially regulated in AQM muscles (data not shown).

Real time RT-PCR confirmation was performed for a subset of the TGF-β/MAPK pathway members (p21, GADD45β, c-myc, c-jun; see Table 2). In all transcripts tested, the RT-PCR data were consistent with the microarray data, and all genes showed significant \( p \) values by both independent assay methods.

Activation of the Transforming Growth Factor–β/MAPK Pathway Is Seen at the Protein Level

The majority of activation of the TGF-β/MAPK pathway occurs at the protein level (phosphorylation, localization, stability), and mRNA studies are a relatively insensitive means of assessing the status of the entire pathway. For this reason, we performed immunoblotting for many members of the TGF-β/MAPK cascade in protein extracts from all AQM muscle biopsies and compared them with the neurogenic and normal controls. We measured both total and phosphorylated (phospho) forms for the following proteins: ASK-1, SMADs, JNKKs, JNKs, MKK3/6, p38 MAPK, c-jun, c-fos, c-myc. Protein levels also were assessed for r-RAS, TGF-β receptors I and II, TAK-1, junB (Fig 2A). Our immunoblot data were consistent with a wide activation of the TGF-β/MAPK pathway, with increases of both total and phosphorylated protein forms in AQM atrophic muscles. These data were also in agreement with the transcriptional upregulation seen by both microarray and RT-PCR. Fold changes and \( p \) values from quantitated immunoblots were calculated for TGF-β receptor II, ASK-1, p38 MAPK, phosphorylated p38 MAPK, c-myc, phosphorylated c-myc, SMADs, and junB (see Fig 2B); all demonstrated highly significant activation.
specific for AQM myogenic atrophy, including the ratio of phosphorylated versus total p38-MAPK. We conclude that the TGF-β/MAPK cascade is coordinately activated at both at the transcriptional and protein level, including activated phosphorylated states of specific signaling proteins, in myogenic atrophy in AQM.
Table 2. TGF-β MAPK Signaling Cascade mRNA Expression Data Show Strong Upregulation Specific to Myogenic Atrophy

<table>
<thead>
<tr>
<th>Genbank No.</th>
<th>Gene Name</th>
<th>AQM vs Control</th>
<th></th>
<th>AQM vs Neurogenic Atrophy</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fold Change</td>
<td>p</td>
<td>Percentage of Positive Fibers*</td>
<td>Fold Change</td>
</tr>
<tr>
<td>D50683</td>
<td>TGFbeta type II receptor mRNA Affymetrix</td>
<td>3 &lt;0.01</td>
<td>2.4</td>
<td>90 ± 5(AQM) 20 ± 5(Neur)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>V00568</td>
<td>MYC</td>
<td>mRNA Affymetrix</td>
<td>9 &lt;0.01</td>
<td>82 ± 3(AQM) 12 ± 4(Neur)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AF078077</td>
<td>GADD45beta</td>
<td>mRNA Affymetrix</td>
<td>4.3 &lt;0.05</td>
<td>86 ± 5(AQM) 8 ± 2(Neur)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>U59423</td>
<td>SMAD1</td>
<td>mRNA Affymetrix</td>
<td>2.1 &lt;0.01</td>
<td>88 ± 4(AQM) 3 ± 1(Neur)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>U59912</td>
<td>SMAD1</td>
<td>mRNA Affymetrix</td>
<td>1.9 &lt;0.01</td>
<td>65 ± 10(AQM) 3 ± 1(Neur)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>X89750</td>
<td>SMAD2</td>
<td>mRNA Affymetrix</td>
<td>4.1 &lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U59913</td>
<td>SMAD5</td>
<td>mRNA Affymetrix</td>
<td>2.8 &lt;0.01</td>
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<td></td>
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<tr>
<td>L35263</td>
<td>P38 MAPK</td>
<td>mRNA Affymetrix</td>
<td>1.9 &lt;0.01</td>
<td>95 ± 5(AQM) 4 ± 1(Neur)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>M14949</td>
<td>R-RAS</td>
<td>mRNA Affymetrix</td>
<td>3 &lt;0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF043938</td>
<td>M-RAS R-RAS3</td>
<td>mRNA Affymetrix</td>
<td>1.6 &lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>J04111</td>
<td>JUN</td>
<td>mRNA Affymetrix</td>
<td>3.3 &lt;0.01</td>
<td>72 ± 4(AQM) 3 ± 1(Neur)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>U03106</td>
<td>CDKN1 P21</td>
<td>mRNA Affymetrix</td>
<td>6 &lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U52522</td>
<td>POR1</td>
<td>mRNA Affymetrix</td>
<td>5.4 &lt;0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Refers to immunohistochemistry experiments.

TGF = transforming growth factor; AQM = acute quadriplegic myopathy; PCR = polymerase chain reaction; NS = not significant.
Specific Key Members of Transforming Growth Factor–β/MAPK Cascade Are Expressed in Atrophic Myofibers with Apoptotic Features in Acute Quadriplegic Myopathy

We have shown previously the presence of apoptotic features (activated caspases 3 and TUNEL-positive fibers) in atrophic myofibers in a subset of patients in AQM. To extend these previous findings, we localized several members of the TGF-β/MAPK cascade in cryosections of patient muscle biopsies by using immunohistochemistry. We found TGF-β, TGF-β receptor, p-ASK1, p38-MAPK, GADD45 β, c-jun, and c-myc all strongly expressed specifically in atrophic myofibers with apoptotic features (TUNEL-positive) and rare normotrophic fibers in AQM muscle (Fig 3, 4). TGF-β receptor showed both membrane and cytoplasmic staining, and p38-MAPK showed diffuse cytoplasm localization, whereas c-jun showed characteristic nuclear localization (see Fig 3). On serial sections, TUNEL-positive picnotic nuclei in atrophic fibers strongly immunoreacted for this transcription factor (see Fig 4). No or very faint staining was observed in control muscles and in neurogenic muscle fibers. Quantitation of the percentage of atrophic fibers (myogenic and neurogenic biopsies), immunoreactive for TGF-β II receptor, phosphorylated c-myc, GADD45 β, SMAD (1, 5, 8), P38, RAS, and phosphorylated ASK-1
was also performed. In all cases, the percentage of atrophic fibers immunopositive for TGF-β/MAPK members was significantly higher in myogenic (AQM) compared with neurogenic atrophy (see Table 2, boldface).

Discussion

Comparative Genomics of Myogenic and Neurogenic Atrophy

We present protein and mRNA data in AQM myogenic atrophy, neurogenic atrophy, and normal controls that shows activation of TGF-β/MAPK signaling cascade specifically in muscle in myogenic atrophy in AQM patients. Neurogenic atrophy and myogenic atrophy shared similar strong upregulation of the muscle-specific ubiquitin ligase atrogin-1 (FBx32), consistent with the myofiber atrophy seen in both conditions. The AQM-specific induction of the TGF-β/MAPK pathway was demonstrated at both the transcriptional and protein level, including protein phosphorylation consistent with constitutive activation of this signaling cascade. We also showed colocalization of TGF-β/MAPK pathway members with apoptotic atrophic myofibers in patient muscle, including TGF-β receptor II, ASK1, p38 MAPK, c-myc, and c-jun.

Transforming Growth Factor–β/MAPK Signaling Cascade: Mechanisms of Activation and Effectors

Atrophic myofibers in AQM patients showed strong coordinated upregulation of TGF-β MAPK signaling cascade, Ras family members, and cell cycle inhibitors (Table 2, Fig 5). Oxidative and osmolar stress, pH imbalance, and cytokine release all are documented triggers for the TGF-β/MAPK signaling cascade in diverse

Fig 3. Immunohistochemistry shows localization of transforming growth factor (TGF)-β MAPK cascade in atrophic fibers in acute quadriplegic myopathy (AQM). Shown is specific localization in atrophic fibers in AQM (A, C, E, G, I, M) and neurogenic muscles (B, D, F, H, L, N) of TGF-β receptor (A, B), SMADs (C, D), p38 (E, F), Ras (G, H), p-c-Myc (I, L), and gadd45 beta (M, N). Control muscles immunostained with TGF-β receptor (P), SMADs (Q), Ras (R), and p-c-Myc (S), show no signal. Intense immunostaining is present only in AQM atrophic muscles for all proteins. Several myofibers are positive for TGF-β receptor also in neurogenic atrophy (B). Original magnification, ×250
Fig 4. TUNEL shows colocalization with members of MAPK cascade in atrophic fibers in acute quadriplegic myopathy (AQM). Shown are TUNEL-positive fibers (B, F) that, respectively, co-localize with p38 (A, arrows) and c-jun (F, arrows show some representative fibers) in atrophic fibers in AQM. Shown are also control sections processed for p38 (C), c-jun (G), and TUNEL (D, H) that do not show TUNEL or p38 positivity and express faint nuclear staining for c-jun. Original magnification, ×250.
cell types. 22–28 Both the TGF-β and Ras pathways converge on the MAPK pathway, likely leading to constitutive activation, and the resulting proteolysis and apoptosis that are histological features of AQM. The association of AQM with oxidative and osmolar stress has been seen clinically; however, our data provide molecular confirmation of this via our observed upregulation of anti–oxidative stress enzymes HO-1, MnSOD, and GPX (see Fig 2), and these changes were not shared with neurogenic atrophy.

The upstream triggers, downstream regulation, and cell-specific regulation of the TGF-β/MAPK pathway is complex; yet, it is recognized that this regulation is critical in determining the biological response of many cell types, toward either proliferation or atrophy/apoptosis. 21–23,25,26 A few members of this cascade appear to be key in influencing this biological outcome, namely, p38 MAPK, JNKs, c-myc, and c-jun. These specific pathway members have been shown to promote cell atrophy and death rather than mitosis, particularly in postmitotic tissues, including skeletal muscle. 29–38 Importantly, the MAPK and Ras pathways have been shown to be necessary for muscle proteolysis in Caenorhabditis elegans, 39 and TGF-β–mediated MAPK activation was shown to antagonize muscle hypertrophy and the insulin-like growth factor–1 pathway, through activation of proapoptotic insulin-like growth factor receptor binding proteins. 40

Taken together, our data and data from the literature allow us to propose a model for muscle cell atrophy in AQM in which activation of TGF-β receptors, exacerbated by stress response and corticosteroid/Ras pathways, 28,41 begins constitutive intracellular signaling of the MAPK cascade in specific myofibers leading to muscle atrophy with apoptotic features (see Fig 3, 4). Consistent with this model, we show that many of the pathway components are in the hyperphosphorylated, activated state, and that these pathway components colocalize with apoptotic cells in patient muscle biopsies.

To briefly describe the roles of some of the proteins we found specifically activated in AQM, the most important TGF-β signal transducers are SMADs and...
showing muscle atrophy, including cancer cachexia and "cade might also show efficacy in diverse conditions. We would anticipate that modulation of this signaling cascade toward growth as opposed to atrophy. An-Therefore, upregulation of these members might shift the cascade toward growth as opposed to atrophy. Another therapeutic target resides in the inhibition of key players of the proatrophic pathway, including SMADs, p38 MAPK, JNKs, ASK-1, and cell cycle inhibitor p21. JNK inhibitors will soon be in clinical trials in p38 MAPK, JNKs, ASK-1, and cell cycle inhibitor p21. JNK inhibitors will soon be in clinical trials in AQM. Importantly, our data prove that neurotrophic signals are known for their progrowth and proregenera-

TAK-1 that after phosphorylation translocate to the nucleus and promote transcription of downstream targets (c-jun, junD, and c-myc), and also phosphorylate downstream MAPK members (see Fig 5). Transcription factors c-jun, junD, and c-myc can both promote apoptosis and induce TGF-β transcription providing a positive feedback to the entire pathway. They can be activated at the transcriptional (activated by SMADs) and protein level (phosphorylated by p38MAPK, JNKs). Ras and Rho family members are typically triggered by TGF-β signaling, whereupon they mediate actin reorganization, possibly participating in cytoskeleton reshaping. SMADs activation can also promote transcription of p21 and gadd45 β, both of which induce cell growth arrest and interact with ASK-1. ASK-1 and other MAPK pathway members promote apoptosis and proteolysis.42–45

In summary, this signaling cascade likely leads to muscle actin-cytoskeleton reorganization, cell atrophy, apoptosis, and proteolysis, all distinguishing features of AQM muscle. Importantly, our data prove that neurogenic atrophy and myogenic atrophy (AQM) share the ubiquitin ligase pathway, but only AQM activates the TGF-β/MAPK pathway. Note also that the TGF-β/MAPK cascade is not dysregulated in forms of inflammatory myopathies associated with necrotic features, including dermatomyositis, polymyositis, and inclusion body myopathy.46,47

Transforming Growth Factor–β/MAPK Cascade: Possible Therapeutic Targets

The novel pathophysiological cascades defined here for AQM suggests new targets for potential therapies inhibiting muscle atrophy. As shown in Figure 5, expression of the ERK branch of MAPK cascade is either inhibited or unchanged in AQM. ERKs family members are known for their progrowth and proregeneration potential in diverse conditions and cell types. Therefore, upregulation of these members might shift the cascade toward growth as opposed to atrophy. Another therapeutic target resides in the inhibition of key players of the proatrophic pathway, including SMADs, p38 MAPK, JNKs, ASK-1, and cell cycle inhibitor p21. JNK inhibitors will soon be in clinical trials in cancer and rheumatoid arthritis.22 Importantly, we would anticipate that modulation of this signaling cascade might also show efficacy in diverse conditions showing muscle atrophy, including cancer cachexia and acquired immune deficiency syndrome.

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