Plasminogen Activator Inhibitor Type 1 Up-Regulation Is Associated with Skeletal Muscle Atrophy and Associated Fibrosis

Jasmin Naderi,* Christian Bernreuther,[†]
Nicole Grabinski,[‡] Charles T. Putman,[§]
Birgit Henkel,* Gordon Bell,[§] Markus Glatzel,[†]
and Karim R. Sultan*

From the Institute of Biochemistry and Molecular Biology II, and I,[‡] and the Institute of Neuropathology,[†] University Medical Center Hamburg-Eppendorf, Hamburg, Germany; and the Exercise Biochemistry Laboratory,[§] Faculty of Physical Education and Recreation, University of Alberta, Edmonton, Alberta, Canada*

Muscle wasting remains a feature of many diseases and is counteracted by anabolic supplementation or exercise. Persisting atrophy-inducing conditions can be complicated by skeletal muscle fibrosis, which leads to functional impairment. Identification of early mechanisms that initiate atrophy-induced fibrosis may reveal novel targets for therapy or diagnosis. Therefore, we investigated changes in the expression of genes involved in extracellular matrix homeostasis during glucocorticoid-induced atrophy of myotubes and compared them with insulin-like growth factor-1-induced hypertrophy. Obtained results were verified in rat gastrocnemius muscle that was exposed to microgravity by space flight for 2 weeks. Myostatin and atrogen-1 mRNA levels reflected the magnitude of atrophy. Despite differential induction of these negative muscle mass regulators, no major changes in matrix metalloproteinases-2, -9, and -14 mRNAs or their physiological inhibitors could be detected in either atrophy model. In contrast, transcript levels of plasminogen activator inhibitor type 1 (PAI-1) was dramatically increased in atrophic myotubes and microgravity-exposed rat gastrocnemius muscle, while plasminogen activators remained unaltered. In contrast to atrophy, no increase in PAI-1 mRNA levels could be detected in rat hindlimb that was electrically stimulated for 21 days. Furthermore, a strong increase in PAI-1 mRNA levels was identified in skeletal muscle of patients with neurogenic muscle atrophy. Our study suggests that increased PAI-1 expression in atrophic skeletal muscle may lead to muscle fibrosis by reducing

plasmin generation. (*Am J Pathol* 2009, 175:763–771; DOI: 10.2353/ajpath.2009.081009)

Muscle wasting remains a major feature of many diseases. In transient conditions such as starvation, short-term immobilization, or after surgical interventions, atrophy can be reversed by anabolic supplementation or exercise. In contrast, in chronic conditions, such as HIV, in multimorbid patients, in chronic neurological disorders but also in healthy individuals exposed to prolonged immobilization or microgravity, loss of muscle mass is difficult to counteract. Furthermore, sarcopenia is an increasing health concern due to general aging of society.¹ Diagnosis of muscle wasting can be complicated by a number of mechanisms including fatty infiltration or fibrosis.²

Maintenance of muscle mass is determined by equilibrium between protein synthesis and degradation. Enhanced proteolysis can be initiated by the calpain system followed by enhanced activity of the proteasome system.³ The latter is executed by increase in expression of E3-ubiquitin ligases such as atrogen-1.⁴ Inhibition of skeletal muscle growth can also be regulated by the differentiation factor myostatin as identified in mice,⁵ cattle,⁶ and humans.⁷ Interestingly, recent studies have identified a fibrogenic property of myostatin as it stimulates proliferation of muscle fibroblasts and their production of extracellular matrix (ECM) proteins.⁸

Fibrosis as seen in sarcopenia seems to be a general complication of prolonged atrophy. This has been de-

Supported in part by research grants from the Natural Sciences and Engineering Council of Canada (to C.T.P.), the Alberta Heritage Foundation for Medical Research (to C.T.P.), and the Canadian Space Agency (to G.B.). Work in the laboratory of M.G. is supported by grant GL589/5-1 from the Deutsche Forschungsgemeinschaft.

J.N. and C.B. contributed equally to this work. M.G. and K.R.S. are joint senior authors.

Accepted for publication April 22, 2009.

Address reprint requests to Prof. Dr. Markus Glatzel, Institute of Neuropathology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. E-mail: m.glatzel@uke.de.

scribed in response to unloading hindlimb muscle in mice,⁹ in denervation-induced atrophy in rats¹⁰ and in humans.¹¹ Skeletal muscle fibrosis is also a well described pathological hallmark of chronic myopathies.¹² Excessive fibrosis is associated with decreased strength and elasticity of muscle and may inhibit the diffusion of nutrients to myofibers. In addition, fibrotic tissue forms a mechanical barrier that leads to incomplete skeletal muscle healing.¹³ Potential future therapies for myopathies, involving gene and myoblast transfer, may also be hampered by fibrosis. Thus, the identification of factors that regulate fibrosis is an important goal, as it may reveal new targets for the therapy of myopathies and may pave the way toward novel diagnostic procedures.

The excessive accumulation of ECM molecules can be counteracted by two major proteolytic systems represented by plasmin and the matrix metalloproteinases (MMPs). Current findings of skeletal muscle MMP have been reviewed.¹⁴ The most prominent changes include elevation of MMP-2 and MMP-9 activity in disuse-mediated atrophy. Local delivery of MMP-1 into the fibrotic injury zone enhanced regeneration and improved muscle healing.¹⁵ Plasmin is able to limit ECM accumulation directly or indirectly by activation of latent MMPs. Its expression is mainly regulated by the serine protease inhibitor, PAI-1, which inhibits both plasminogen activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) and thus reduces plasmin generation. This concept is supported by studies in human and animal models of organ fibrosis, which show that PAI-1 is up-regulated in glomerulosclerosis and liver, pulmonary, and cardiac fibrosis.^{16–19} The present study investigates early changes in ECM homeostasis related to fibrotic development in atrophying skeletal muscle. For this purpose we analyzed changes in gene expression in glucocorticoid- and microgravity-induced atrophic development. We identified elevation of PAI-1 expression in both models of atrophy. These changes were confirmed in skeletal muscle specimen of patients suffering from neurogenic atrophy. Our findings indicate that the switch toward fibrotic state in atrophying muscle may be due to PAI-1 up-regulation. Thus PAI-1 may constitute a novel target in diagnosing and monitoring muscular atrophy and atrophy-related fibrosis.

Materials and Methods

In Vitro Analysis of L6 Myotubes

Culture conditions of the rat myogenic cell line L6 were described in detail.²⁰ Myoblast fusion was achieved by Dulbecco's modified Eagle's medium with 2% adult horse serum. The medium was changed every 2 days, and differentiation was allowed to continue for up to 10 days before the experimentation period. Muscle fiber diameters were assessed using published methods.²¹ The experiments lasted from 24 to 72 hours under serum-free conditions with daily medium renewal. Dexamethasone was dissolved in dimethyl sulfoxide at a final concentration of 100 nmol/L (final concentration of dimethyl sulfoxide

was below 0.01%). Insulin-like growth factor-1 (IGF-1) (R&D Systems) was used at a concentration of 10 ng/ml. To exclude unspecific effects of the different vehicles, all conditions contained the same amount of dimethyl sulfoxide of 0.01%. Characterization of the differentiated myotubes has been published.²⁰

Microgravity-Induced Atrophy in Rodent Skeletal Muscle

Adult male Sprague-Dawley rats from the SLS-2 space shuttle mission were used. Rats exposed to 14 days of microgravity ($n = 4$) or ground-based controls ($n = 4$) were housed individually in identical research animal housing facility flight cages. Muscles used in this study were obtained through the NASA tissue-sharing program for the SLS-2 mission via the Canadian Space Agency. Details of the SLS-2 flight conditions and of other studies performed using these tissue are reported elsewhere.²² The muscles used in this study (medial gastrocnemius muscle) showed early signs of atrophic development such as slow-to-fast fiber transitions; no late signs of atrophy such as a reduction of the cross-sectional area could be observed.²²

Chronic Low-Frequency Stimulation (CLFS) Induced Transformation of Rat Skeletal Muscle

All animal procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and received ethical approval from the University of Alberta. Details of experimental procedures are described.²³ Briefly, adult male Wistar rats (Charles River Laboratories, Montreal, PQ, Canada) were individually housed under controlled environmental conditions. Four animals received 21 days of chronic low-frequency stimulation (CLFS: 10 Hz, impulse width 380 μ s, 12 hours) applied across the left common peroneal nerve. The contralateral right leg received no stimulation and served as an internal control. On completion of the stimulation period, animals were anesthetized and tibialis anterior and extensor digitorum longus muscles were excised from both hindlimbs and snap-frozen in a slightly stretched position in isopentane (-159°C). Muscles were stored in liquid nitrogen (-196°C). Immunohistochemistry-based fiber typing has been described in detail.²³ The monoclonal antibodies directed against adult myosin heavy chain (MHC) isoforms were harvested from hybridoma cell lines obtained from the American Type Culture Collection (Manassas, VA): BA-D5 (IgG, anti-MHC I), SC-71 (IgG, anti-MHC IIa), BF-F3 (IgM, anti-MHC IIb), BF-45 (IgG, anti-MHC-embryonic) and clone BF-35 (purified IgG, anti-MHC IIx). Their specificity has been described.²⁴

Patient Samples

Twenty-one muscle biopsies obtained from vastus lateralis muscle of 37- to 75-year-old patients (mean, 55.9

Table 1. Primers Used for qRT-PCR

Target gene	Species	5'→3'	Primer sequences	GenBank accession no.
<i>Atrogin-1</i>	Rat	Forward	5'-TCACAGCTCACATCCCTGAG-3'	NM_133521
		Reverse	5'-GACTTGCCGACTCTCTGGAC-3'	
<i>Myostatin</i>	Rat	Forward	5'-AGAGAGAGGCGAATGTGGAA-3'	AF019624
		Reverse	5'-TCACTGCTGTCATCCCTCTG-3'	
<i>MMP-2</i>	Rat	Forward	5'-GATGGATACCCGTTTGATGG-3'	NM_031054
		Reverse	5'-CCATCAGCGTTCCCACTT-3'	
<i>MMP-9</i>	Rat	Forward	5'-CCACCGAGCTATCCACTCAT-3'	NM_031055
		Reverse	5'-GTCCGGTTTCAGCATGTTTT-3'	
<i>MMP-14</i>	Rat	Forward	5'-TTGGTGTGTGTCCAACCTA-3'	NM_031056
		Reverse	5'-GCTGCCAACTGGGTACTT-3'	
<i>TIMP-1</i>	Rat	Forward	5'-TCCCCAGAAATCATCGAGAC-3'	NM_053819
		Reverse	5'-TCAGATTATGCCAGGAACC-3'	
<i>TIMP-2</i>	Rat	Forward	5'-CAAGTTCTTTGCCTGCATCA-3'	U14526
		Reverse	5'-TCCAGGAAGGATGTCAAAG-3'	
<i>Col1a1</i>	Rat	Forward	5'-GTGCTAAAGGTGCCAATGGT-3'	NM_053304
		Reverse	5'-GGCTCCTCGTTTCTCTT-3'	
<i>Col4a1</i>	Rat	Forward	5'-TAAAGGTGATTCGGGCTTC-3'	NM_001135009
		Reverse	5'-GTCCAGGGGATCCTGGTATT-3'	
<i>P4Ha1</i>	Rat	Forward	5'-TTGGGCAAAGTGGCCTATAC-3'	X78949
		Reverse	5'-GGTCACCTGCTGGTACT-3'	
<i>PAI-1</i>	Rat	Forward	5'-TGGTGAACGCCCTCTATTTT-3'	M24067
		Reverse	5'-GAGGGGCACATCTTTTCAA-3'	
<i>uPA</i>	Rat	Forward	5'-TGAAGGTGGCAGTGAACCTG-3'	NM_013085
		Reverse	5'-CGGCCTTTGGTGTCTAGTATT-3'	
<i>tPA</i>	Rat	Forward	5'-GAGATGCTTCAATGGGGTA-3'	NM_013151
		Reverse	5'-TGCTCCATGTGCCTCTGTAG-3'	
<i>MLP</i>	Rat	Forward	5'-ATCAGAGAAGTGCCACGAT-3'	X81193
		Reverse	5'-GTAAGCCCTCCAAACCAAT-3'	
<i>GSα</i>	Rat	Forward	5'-TACCATCTCTGTGATCCTCTTC-3'	M17525
		Reverse	5'-GTGAGGGTAGCAGTAGTGAC-3'	
<i>PAI-1</i>	Human	Forward	5'-CTCTCTCTGCCCTCACCAAC-3'	NM_000602
		Reverse	5'-GTGGAGAGGCTCTTGGTCTG-3'	
<i>Myostatin</i>	Human	Forward	5'-TTCGTCTGGAACAGCTCCT-3'	NM_005259
		Reverse	5'-CATTTGGGTTTCCATCCAC-3'	
<i>TGF-β1</i>	Human	Forward	5'-CACGTGGAGCTGTACCAGAA-3'	NM_000660
		Reverse	5'-GAACCCGTTGATGTCCACTT-3'	
<i>Atrogin-1</i>	Human	Forward	5'-TCACAGCTCACATCCCTGAG-3'	NM_058229
		Reverse	5'-AGACTTGCCGACTCTTTGGA-3'	
<i>GAPDH</i>	Human	Forward	5'-GAGTCAACGGATTGTTCGT-3'	NM_002046
		Reverse	5'-TTGATTTTGGAGGGATCTCG-3'	

years, SD 9.5; 10 females, 11 males) were reviewed by two neuropathologists for confirmation of diagnoses (C.B. and M.G.). Ten biopsies with signs of neurogenic muscle atrophy and 11 biopsies showing no signs of neurogenic muscle atrophy or myopathy were chosen for further analysis. Processing and analysis of the specimen were performed in a double-blinded manner. The use of these anonymized specimens in research are in agreement with local ethical standards at the University Medical Center Hamburg-Eppendorf.

For histological analysis, muscle tissue was cut in 3- μ m paraffin or 7- μ m cryosections and standard techniques for hematoxylin and eosin and elastica van Gieson stainings were used. For immunohistochemistry, monoclonal antibodies to type 1 (1:200, M8421, Sigma, Deisenhofen, Germany) and type 2 (1:500, M4276, Sigma) myosin and polyclonal rabbit antibodies against collagen I (1:40, PS 047, Monosan, Uden, The Netherlands) were used according to published methods.²⁵ The percentages of atrophic fibers were determined in hematoxylin and eosin-stained serial cryosections by assessing more than 500 muscle fibers in at least eight visual fields (0.1 mm²) in each muscle biopsy. The extent of fibrosis in the

muscle tissue was assessed using a four-tiered scale in elastica van Gieson staining (no fibrosis = 0; mild fibrosis = 1; moderate fibrosis = 2; pronounced fibrosis = 3).

Analysis of Gene Expression by Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from cells and tissue using a TRIzol RNA extraction kit from Invitrogen (Carlsbad, CA) according to the manufacturer's protocol. RNA concentration was assessed spectrophotometrically and its integrity checked by electrophoresis in 1% agarose. Reverse transcription of total RNA (1 μ g) was performed using the reverse transcriptase SuperScript II (Invitrogen), random hexamer and oligo dT primers. Exon spanning primers for the investigated target genes were designed using the free software Primer3²⁶ and are listed in Table 1. cDNA levels of target genes were measured using real-time qRT-PCR in a LightCycler system (Roche Molecular Biochemicals). In general, PCR product size and the melting point analysis were performed for all

samples tested. Samples were run in duplicate, and all runs included a calibrator and a negative control. PCR efficiency of each value was taken into account using the LinRegPCR²⁷ program. Samples were omitted if PCR efficiency was deviating more than ± 0.05 . Target gene values were normalized to their corresponding GS α (GTP-binding protein, α stimulating subunit) in the case of L6 myotube and rat skeletal muscle. Human samples were normalized to their corresponding glyceraldehyde-3-phosphate dehydrogenase levels.

Statistical Analysis

Results for individual experiments were presented as means $\pm$ SD unless otherwise indicated. Student's *t*-test or in case of multiple groups one-way analysis of variance was used to assess the statistical significance with a *P* value of <0.05 or <0.001 .

Results

Up-Regulation of PAI-1 in Atrophic L6 Myotubes

The aim of the study was to investigate early changes in the expression of MMPs and the members of the plasmin-generating system in skeletal muscle atrophy. For this purpose, we compared atrophic and hypertrophic cultures of myotubes. These were obtained using the rat myogenic cell line L6. Myotube differentiation lasted 10 days and thereafter dexamethasone or IGF-1 was added under serum-free conditions for 48 hours to induce myotube atrophy or hypertrophy, respectively. Morphological changes, which were monitored and documented by phase contrast microscopy, confirmed the expected size changes (Figure 1, A and B).

Dexamethasone-induced atrophic myotubes revealed a significant increase in mRNA levels of atrogen-1 (twofold increase, $P < 0.01$) (Figure 1C), whereas increases of myostatin mRNA could not be detected in the analyzed L6 myotubes (data not shown). In contrast to the atrogen-1 induction, no changes in the mRNA levels of MMP-2, MMP-9, MMP-14, tissue inhibitor of metalloproteinase (TIMP)-1 or TIMP-2 could be observed. IGF-1-induced hypertrophy was accompanied by a significant decrease (0.3-fold reduction, $P < 0.02$) of atrogen-1 mRNA levels (Figure 1C). In contrast to the unaltered MMP levels in atrophic myotubes, hypertrophy was accompanied by significant increases in MMP-9 mRNA (twofold increase, $P < 0.02$) and MMP-14 mRNA (1.4-fold increase, $P < 0.02$).

Interestingly, PAI-1 gene expression in atrophic myotubes revealed a strong increase of its mRNA levels following 24 hours (fivefold increase, $P < 0.01$) of dexamethasone treatment (Figure 2). This increase was also stable at 48 hours after treatment (fourfold increase, $P < 0.001$). In contrast to glucocorticoids, treatment with IGF-1 did not alter the mRNA levels of PAI-1.

Figure 1. Changes of ECM homeostasis in atrophying and hypertrophying L6 myotubes. **A:** Phase-contrast microscopy of L6 myotubes treated with dexamethasone (100 nmol/L) or IGF-1 (10 ng/ml) under serum-free conditions for 48 hours (scale bar = 20 μm). **B:** Quantification of myotube diameters in glucocorticoid-induced atrophy (black bar), IGF-1-induced hypertrophy (gray bar), and untreated control L6 myotubes (white bar). Values represent the average obtained from three independent experiments and are expressed as means $\pm$ SEM. * $P < 0.05$ versus untreated controls. **C:** qRT-PCR-based analysis of atrophy-related atrogen-1 expression and of the major members of MMPs (MMP-2, -9, and -14 and their inhibitors (TIMP-1 and -2) in L6 myotubes treated for 48 hours with dexamethasone (black bars) or IGF-1 (gray bars). Target gene mRNA values were normalized to GS α and compared with untreated controls (white bars). Values represent the average obtained from three to four independent experiments, each analyzed in duplicate and expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.001$ versus untreated controls.

Up-Regulation of PAI-1 in Microgravity-Induced Muscular Atrophy

Next, we analyzed changes in the expression of MMPs and the members of the plasmin-generating system in microgravity-induced atrophic development. We chose the microgravity model as a noninvasive *in vivo* system, which is glucocorticoid-independent.²⁸ Gene expression

Figure 2. Induction of PAI-1 mRNA in glucocorticoid-treated L6 myotubes. L6 myotubes were treated with dexamethasone (100 nmol/L) or IGF-1 (10 ng/ml) under serum-free conditions for 24 hours or 48 hours (black bars). Normalized PAI-1 values were compared with corresponding controls (white bars). Values represent the average obtained from three to four independent experiments, each analyzed in duplicate and expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.001$ versus untreated controls.

Figure 3. Changes of ECM homeostasis in microgravity-induced atrophy of rat hindlimb muscle. Microgravity-mediated atrophy (black bars) was analyzed in rat medial gastrocnemius muscles exposed to 14 days of space flight and compared with ground-based controls (white bars) by qRT-PCR. Target gene mRNA values were normalized to $GS\alpha$. **A:** Comparison of mRNA changes of the atrophy-related genes atrogen-1 and myostatin. **B:** Analysis of the plasmin regulating system composed of PAI-1, uPA, and tPA. **C:** Analysis of two collagen transcripts (Col1a1 and Col4a1) and the major collagen modifying enzyme P4H. All values represent the average obtained from four rats and are expressed as mean $\pm$ SD. * $P < 0.05$ versus corresponding controls.

analyses of the medial gastrocnemius muscle of rats exposed to space flight-induced microgravity for 2 weeks showed elevated levels of atrogen-1 mRNA, which did not reach statistical significance (1.7-fold increase, $P = 0.06$), whereas myostatin gene expression was significantly enhanced (2.5-fold increase, $P < 0.01$) (Figure 3A). PAI-1 mRNA levels in skeletal muscle were increased (threefold increase, $P < 0.04$) (Figure 3B). To assess whether expression of the plasminogen activators uPA and tPA were also altered, we determined their relative mRNA levels and observed no changes after 12 weeks of microgravity. Further changes of ECM regulating components were only detected for MMP-9 mRNA, which was significantly decreased (0.5-fold reduction, $P < 0.05$, data not shown). None of the other investigated MMPs or TIMPs (MMP-2, MMP-14, TIMP-1, or TIMP-2) were altered significantly at the transcript level (data not shown). To evaluate changes occurring in ECM production, we examined expression of procollagen type I, α -1 (Col1a1), procollagen type IV, α -1 (Col4a1), and the en-

Figure 4. PAI-1 gene expression in fast-to-slow transforming skeletal muscle. **A–D:** MHC shifts in transforming rat tibialis anterior muscle. Representative photomicrographs of tibialis anterior sections immunohistochemically stained for adult MHC isoforms. Unstimulated control (**A** and **C**) and 21-day stimulated muscles (**B** and **D**) were stained to identify type I (**A** and **B**) and type IIA (**C** and **D**) fibers. Fast-to-slow fiber type transitions are shown by increases of type I and IIA fibers. **E:** Analysis of PAI-1 and MLP mRNA in 21-day stimulated extensor digitorum longus muscle (black bars) of the same hindlimbs as described in **A**. Target gene mRNA values were normalized to $GS\alpha$ and compared with the contralateral control extensor digitorum longus muscle (white bars). All values represent the average obtained from four rats and are expressed as mean $\pm$ SD. * $P < 0.05$ versus corresponding controls.

zyme proline 4-hydroxylase, α -1 (P4Ha1), which regulates collagen formation. Figure 3C shows the results, and again no significant changes of the mRNA levels were detectable.

Unaltered PAI-1 Expression during Chronic Low-Frequency Stimulation

To exclude non-atrophy-induced PAI-1 up-regulation, we determined PAI-1 mRNA levels in CLFS-treated skeletal muscle. CLFS-induced transformation of the fast extensor digitorum longus muscle and tibialis anterior encompasses the exchange of a multitude of myofibrillar structures from fast to slow isoforms.²⁹ These continuously stimulated fibers are exposed to a permanent loading resembling physiological stress not related to atrophy or fibrosis. This could be confirmed by unaltered mRNA levels of atrogen-1 and myostatin (data not shown). Figure 4, A–D, demonstrates the fiber type shifts occurring in 21-day stimulated rat tibialis anterior muscle. Fast-to-slow fiber type transitions are shown by increases of type I and IIA fibers. Unstimulated control (Figure 4, A and C) versus 21-day stimulated muscles (Figure 4, B and D) were

Table 2. Quantification of Fiber Type Transitions in 21-Day Stimulated Tibialis Anterior Muscles

Leg	% MHC-I	% MHC-IIA	% MHC-IIID(X)	% MHC-IIB
Right control	7.9 ± 1.5	31.2 ± 3.6	18.0 ± 2.0	43.2 ± 2.5
Left stimulated	11.7 ± 1.6*	90.5 ± 1.2*	4.4 ± 1.7*	41.3 ± 2.7

Data are the percentage of fibers expressing an MHC isoform. Because hybrid fibers are included in all analyses, the control adds to 100% but the stimulated adds to 148%, indicating at least 48% of the transitioning fibers expressed two or more MHC isoforms. Immunohistochemical staining data (mean ± SEM). Sample size $n = 8$.

*Different from contralateral control, $P < 0.05$.

immunostained to identify type I (MHCI) (Figure 4, A and B) and type IIA (MHCIIa) (Figure 4, C and D) fibers. Quantification of the immunohistochemical analysis is presented in Table 2 and confirms CLFS-induced fast-to-slow transitions. Since CLFS-induced transformation is associated with early and persisting induction of muscle LIM protein (MLP),³⁰ we used also MLP induction as a marker for ongoing transformation. As expected, levels of MLP were significantly elevated (2.7-fold increase, $P < 0.01$), while PAI-1 gene expression was not altered in transforming skeletal muscle (Figure 4E).

Up-Regulation of PAI-1 in Atrophic Human Skeletal Muscle

We investigated PAI-1 expression in skeletal muscle biopsies of patients with neurogenic atrophy (four patients with motor neuron disease, six patients with polyneuropathy) and age and sex matched control patients that were diagnosed clinically by auxiliary tests and by histological examination of muscle biopsies (for demographic data see Table 3). Skeletal muscle from patients with neurogenic atrophy showed a significantly higher percentage of atrophic fibers when compared with control patients (36.1%, ± 29.6 for neurogenic atrophy; 0.2%, ± 0.2 for controls, $P < 0.001$). Neurogenic atrophy was always accompanied by a variable yet significant muscle fibrosis, whereas we could not observe signs of fibrosis in

controls (0.9 ± 1.0 for neurogenic atrophy; 0%, ± 0 for controls; Table 3 and Figure 5). Interestingly, both atrogen-1 and myostatin mRNA levels were not changed in this chronic model of atrophy as compared with the control group (Figure 6A). The differences in PAI-1 mRNA levels were statistically significant (sevenfold increase, $P < 0.001$) with a vast up-regulation of PAI-1 mRNA in patients with neurogenic atrophy when compared with control patients (Figure 6B). Fibrosis was also confirmed at the molecular level by a significant mRNA increase (fourfold increase, $P < 0.01$) of the profibrotic acting growth factor transforming growth factor- β 1 (Figure 6B).

Discussion

Muscle atrophy is a major feature of many diseases such as neoplasm, neurological disorders and para- or tetraplegia. In addition, muscle atrophy is a physiological reaction to prolonged immobilization or microgravity during space flight. Prolonged atrophy can lead to skeletal muscle fibrosis. This in turn will impede recovery and muscle function; thus understanding processes involved in atrophy induced muscle fibrosis is essential in diagnosing and monitoring these conditions to devise novel strategies preventing fibrosis. We have investigated early changes in ECM homeostasis in atrophying skeletal muscle, focusing on profibrotic processes by *in vitro* and *in vivo* experiments and correlated these data to human muscle specimen.

Table 3. Demographic Characteristics of Patients

	Age	Average age (yr)	Sex	Muscle-related disease	Atrophic fibers (%)	Fibrosis (0–3)
Neurogenic atrophy	51	57.5 ± 8	F	Motoneuron disease	36.1 ± 29.6	0.9 ± 1.0
	59		F	Motoneuron disease		
	67		M	Motoneuron disease		
	75		M	Motoneuron disease		
	51		F	Polyneuropathy		
	57		F	Polyneuropathy		
	60		F	Polyneuropathy		
	46		M	Polyneuropathy		
	54		M	Polyneuropathy		
	62		M	Polyneuropathy		
Control	39	51.2 ± 10	F	None	0.2 ± 0.2	0
	50		F	None		
	57		F	None		
	62		F	None		
	71		F	None		
	37		M	None		
	41		M	None		
	44		M	None		
	45		M	None		
	56		M	None		
	61		M	None

Figure 5. Fibrosis in neurogenic muscular atrophy. Morphology of atrophic skeletal muscle. Hematoxylin and eosin staining (**upper row**), immunohistochemical staining for type II myosin (**second row**), van Gieson staining (third row), and immunohistochemical staining for collagen type I (**bottom row**) showing groups of atrophic muscle fibers in patient samples with neurogenic atrophy (**left three columns**), whereas no such changes could be observed in controls (**right column**). The elastica van Gieson staining and the collagen immunostaining confirm variable grades of fibrosis in atrophying skeletal muscle. Scale bar = 100 μ m.

In accordance with previous reports, Glucocorticoid-mediated atrophy and IGF-1-mediated hypertrophy of L6 myotubes were accompanied by changes of atrogen-1.³¹ The twofold increase during catabolic reaction was sim-

ilar to that seen in dexamethasone-treated C2C12 myotubes,²⁰ whereas IGF-1 could only induce a slight reduction. On the other hand, IGF-1 led to an early and sustained induction of MMP-9, which has been described before in conjunction with myoblast migration.³² This context seems to be unlikely in our myotube cultures, as treatment started after the completion of myoblast fusion. Nevertheless, it cannot be excluded that remaining non-fused myoblasts are stimulated by IGF-1 to migrate. MMP-14 was also induced by IGF-1 but to a much lower degree. Whether this increase is also due to cell migration remains open, but regarding the ECM balance, both increases of MMP-9 and MMP-14 would favor enhanced degradation of ECM components and therefore an antifibrotic environment. Changes of MMP-2 or the inhibitors TIMP-1 and TIMP-2 could not be observed in either process.

Atrophying L6 myotubes showed no changes in the expression of all measured MMP/TIMP components. In contrast to these findings, atrophic myotubes displayed an early and sustained increase of PAI-1. This increase would lead to reduced plasmin formation, decreasing MMP activation, and therefore augmenting the accumulation of matrix components. This development would shift the ECM homeostasis toward a fibrotic condition. IGF-1-induced hypertrophy was not accompanied by a decrease in PAI-1 levels, indicating that PAI-1 is not involved in the mechanism leading to atrophy but rather a consequence of atrophy. The fact that we could observe PAI-1 changes in myotube cultures indicates that PAI-1 up-regulation is not due to altered expression of PAI-1 in infiltrating immune cells or non-muscle tissue as seen in de-regenerating skeletal muscle¹⁴ or in myocardial infarction.¹⁸

Figure 6. PAI-1 induction in atrophying human skeletal muscle. qRT-PCR analysis of biopsies obtained from vastus lateralis muscle of patients as described in Table 3. **A:** Comparison of mRNA changes of myostatin and atrogen-1. **B:** Analysis of PAI-1 and transforming growth factor- β 1 mRNA. Target gene values were normalized to their corresponding glyceraldehyde-3-phosphate dehydrogenase value compared with control (white bar) and are expressed as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.001$ versus corresponding controls.

Regulation of PAI-1 by corticosteroids³³ is described. To clarify the role of PAI-1 in atrophy, we looked for an atrophy model that is less corticosteroid-dependent. Therefore we analyzed microgravity-induced atrophy in hindlimb muscle of the rat at a stage devoid of degenerative changes yet displaying early signs of atrophic development as the associated slow-to-fast fiber type transitions. Analyzed muscle showed myosin heavy chain shifts, affecting the more atrophy-susceptible fast type IID/X fibers.²² Since microgravity can directly induce atrophy independently of corticosteroids plasma levels,²⁸ the possibility that elevated glucocorticoid levels are responsible for microgravity-induced atrophy is unlikely. Furthermore, atrogen-1 and myostatin are both regulated by glucocorticoids,^{20,34} yet only myostatin and not atrogen-1 was elevated in microgravity-induced alterations. To our knowledge, this is the first study comparing myostatin and atrogen-1 changes in multiple atrophy models using qRT-PCR.

As with atrophying L6 myotubes, microgravity-induced atrophy did not result in major changes of MMPs and ECM components, and the elevation of PAI-1 was again the major change seen in atrophy altered ECM homeostasis. The unchanged levels of both plasminogen activators uPA and tPA suggest a functional change of the plasmin system toward reduced plasmin generation.

It remains open to which magnitude stress factors are involved in PAI-1 increase. Therefore we analyzed transforming rat hindlimb muscle induced by continuous electrical stimulation. Changes of structural proteins due to the fast-to-slow transformation could be verified by MHC shifts and induction of MLP.^{23,30} In contrast to these changes, PAI-1 gene expression was not altered. These findings suggest that the elevated levels of PAI-1 mRNA found in glucocorticoid-treated myotubes and microgravity-exposed muscles are indeed related to atrophy.

Finally, we confirmed PAI-1 up-regulation in muscle specimen of patients with neurogenic muscular atrophy and signs of muscle fibrosis. Known atrophy regulating factors such as myostatin and atrogen-1 were unaltered in their mRNA levels. It remains to be seen whether other factors are involved in modulating neurogenic muscular atrophy. All investigated muscle biopsy samples showed fibrosis, confirming the observation that chronic atrophy is complicated by fibrotic development. We could observe PAI-1 up-regulation in early stages of microgravity induced atrophy as well as in early and late stages of polyneuropathy or motor neuron disease-induced atrophy. This speaks in favor of a crucial role of PAI-1 in muscle atrophy. The molecular details of these mechanisms are targets of ongoing research projects.

The fact that robust PAI-1 up-regulation could be observed in patients with neurogenic muscle atrophy underscores the biological relevance of PAI-1. Since neurogenic muscle atrophy goes hand in hand with fibrosis in atrophied muscle, the connection between PAI-1 and a disturbed ECM homeostasis holds true in the human situation. PAI-1 is a secreted glycoprotein readily detected in blood, and monitoring PAI-1 levels in blood may offer the chance to monitor muscle atrophy. Given the

fact that there is a genuine need for biomarkers able to predict or track muscle atrophy or muscle wasting, plasma PAI-1 changes could supplement recent diagnostic approaches.³⁵ PAI-1 plasma levels can be influenced by other diseases such as sepsis, diabetes, and cardiovascular diseases.³⁶ Since these diseases present with distinct clinical symptoms, we do not believe that this lowers diagnostic relevance of PAI-1 up-regulation as a marker for skeletal muscle atrophy. Besides the diagnostic implications of our findings, our study offers new insights into the pathophysiology of atrophy-induced muscle fibrosis, possibly opening novel therapeutic approaches to this condition.

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