ARTHRITIS & RHEUMATISM
Vol. 64, No. 12, December 2012, pp 4094–4103
DOI 10.1002/art.37692

Provision of an Explanation for the Inefficacy of Immunotherapy in Sporadic Inclusion Body Myositis

Quantitative Assessment of Inflammation and β-Amyloid in the Muscle

Jana Zschüntzsch,[1] Joachim Voss,[2] Kim Creus,[3] Stephan Sehmisch,[1] Raghavan Raju,[4] Marinos C. Dalakas,[5] and Jens Schmidt[6]

Objective. **In sporadic inclusion body myositis (IBM), inflammation and accumulation of β-amyloid–associated molecules cause muscle fiber damage. We undertook this study to determine why intravenous immunoglobulin (IVIG) and prednisone are not effective in sporadic IBM despite their effectiveness in other inflammatory myopathies.**

Methods. **Relevant inflammatory and degeneration-associated markers were assessed by quantitative polymerase chain reaction and immunohistochemistry in repeated muscle biopsy specimens from patients with sporadic IBM treated in a controlled study with IVIG and prednisone (n = 5) or with prednisone alone (n = 5). Functional effects were assessed in a muscle cell culture model.**

Results. **In muscle biopsy specimens, messenger RNA (mRNA) expression of the proinflammatory chemokines CXCL9, CCL3, and CCL4 and of the cytokines interferon-γ (IFNγ), transforming growth factor β, interleukin-10 (IL-10), and IL-1β was significantly reduced after treatment in both groups. No consistent changes were observed for tumor necrosis factor α, IL-6, inducible costimulator (ICOS), its ligand ICOSL, and perforin. Messenger RNA expression of the degeneration-associated molecule ubiquitin and the heat-shock protein αB-crystallin was also reduced, but no changes were noted for amyloid precursor protein (APP) or desmin. By immunohistochemistry, a significant down-modulation of chemokines was observed, but not of inducible nitric oxide (NO) synthase, nitrotyrosine, IL-1β, APP, and ubiquitin; β-amyloid was reduced in 6 of 10 patients. Pronounced staining of IgG was observed in the muscle after treatment with IVIG, indicating penetration of infused IgG into the muscle and a possible local effect. In muscle cells exposed to IFNγ plus IL-1β, IgG and/or prednisone down-regulated mRNA expression of IL-1β 2.5-fold. Accumulation of β-amyloid, overexpression of αB-crystallin, and cell death were prevented. In contrast, NO-associated cell stress remained unchanged.**

Conclusion. **IVIG and prednisone reduce some inflammatory and degenerative molecules in muscle of patients with sporadic IBM and in vitro, but do not sufficiently suppress myotoxic and cell stress mediators such as NO. The data provide an explanation for the resistance of sporadic IBM to immunotherapy and identify markers that may help to design novel treatment strategies.**

Supported by DFG grants Schm 1669-1-1 and Schm 1669-2-1, an unrestricted research grant from Octapharma, and a fellowship from The Myositis Association to Dr. Schmidt.

[1]Jana Zschüntzsch, MD, Stephan Sehmisch, MD: University Medical Centre, Göttingen, Germany; [2]Joachim Voss, PhD, RN: University of Washington, Seattle; [3]Kim Creus, MSc: Ghent University Hospital, Ghent, Belgium; [4]Raghavan Raju, PhD: University of Alabama at Birmingham; [5]Marinos C. Dalakas, MD: National University of Athens School of Medicine, Athens, Greece; [6]Jens Schmidt, MD: University Medical Centre and Institute for Multiple Sclerosis Research and Hertie Foundation, Göttingen, Germany.

Dr. Dalakas has received consulting fees, speaking fees, and/or honoraria from Baxter, Novartis, Octapharma, and Grifols (less than $10,000 each) and Therapath (more than $10,000). Dr. Schmidt has received consulting fees, speaking fees, and/or honoraria from CSL Behring, Octapharma, and Biotest (less than $10,000 each) and unrestricted research grants from Octapharma and CSL Behring (more than $10,000 each).

Address correspondence to Marinos C. Dalakas, MD, Neuroimmunology Unit, National University of Athens School of Medicine, 75 Mikras Asias Street, 115 27 Athens, Greece (e-mail: mdalakas@med.uoa.gr); or to Jens Schmidt, MD, Department of Neurology, Department of Neuroimmunology, University of Göttingen, Waldweg 33, 37073 Göttingen, Germany (e-mail: j.schmidt@gmx.org).

Submitted for publication February 6, 2012; accepted in revised form August 28, 2012.

Sporadic inclusion body myositis (IBM) is an inflammatory myopathy that leads to severe disability (1,2). The skeletal muscle pathology includes accumulation of aberrant molecules that are also found in neurodegenerative disorders, most of all β-amyloid (3). Along with the degeneration, there is a profound inflammation with a strong expression in the muscle of various proinflammatory chemokines, including CCL2, CCL3, CCL4, and CXCL9, and cytokines, including interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), and transforming growth factor β (TGFβ) (1,4,5). The network of interactions between degenerative and inflammatory pathomechanisms is now emerging as a significant factor in sporadic IBM, based on recent data that a severe chronic inflammation with an enhanced expression of IL-1β within the muscle can lead to in situ accumulation of β-amyloid and other degenerative molecules (6,7). Furthermore, this chronic inflammation triggers a specific immune response with clonal expansion of T cells (8,9) in the muscle and a cytotoxic attack of myofibers by CD8+ T cells (7,10).

Despite evidence of a specific and chronic inflammation, which is likely relevant to the pathology of sporadic IBM, several treatment studies with immunosuppressive drugs such as prednisone, methotrexate, oxandrolone, cyclosporin A/tacrolimus, anti–T lymphocyte immunoglobulin, and alemtuzumab (11–15) or immunomodulatory agents such as intravenous immunoglobulin (IVIG) (16,17) or interferon (IFN) beta-1a (18,19) have failed to demonstrate a significant clinical benefit except for individual improvements in some patients. This is in contrast to the other 2 inflammatory myopathies, polymyositis (PM) and dermatomyositis (DM), which clearly respond to these therapies (20).

In an effort to better understand mechanisms of antiinflammatory treatment in patients with sporadic IBM, we quantitatively assessed the messenger RNA (mRNA) and protein expression of key molecules relevant to the β-amyloid–associated degeneration as well as inflammation in repeated skeletal muscle biopsy specimens from a previous clinical trial on sporadic IBM with IVIG and prednisone or prednisone alone (21). To elucidate further the functional implications of the induced changes in vivo, the effects of prednisone and human IgG were also assessed in a cell culture model of sporadic IBM.

PATIENTS AND METHODS

Patients and muscle biopsy specimens. We investigated the repeated muscle biopsy specimens of 10 patients with sporadic IBM treated with IVIG and prednisone (n = 5) or prednisone alone (n = 5) in a recent treatment trial (21). All patients were treated with oral prednisone, which, after 4 weeks at 60 mg/kg/day, was tapered to alternate-day treatment during the 3-month trial before another tapered discontinuation. Half of the patients received monthly infusions of 2 gm/kg IVIG for 3 months. Before treatment and upon completion of the trial, open biopsies were performed on the same biceps or quadriceps muscle.

Extraction of mRNA and reverse transcription–polymerase chain reaction (RT-PCR). RT-PCR was performed as previously described (6). Total RNA was extracted from muscle biopsy specimens using a commercial kit (RNeasy from Qiagen), following the supplier's instructions. Briefly, the tissue was homogenized in 350 μl lysis buffer with a plastic tissue grinder and pestle (Kontes Glass). RNA was eluted in 30 μl water and stored at −80°C. Complementary DNA (cDNA) synthesis was performed with SuperScript II reverse transcriptase (Invitrogen), following the supplier's instructions. The resulting cDNA was stored at –20°C. For amplification, 5 ng cDNA was used in a 20 μl ready-to-use master mix for quantitative (real-time) PCR (Eurogentec) using 6-FAM–labeled probes and specific primers (Applied Biosystems) for the following compounds: GAPDH (Hs99999905_m1), amyloid precursor protein (APP; Hs00169098_m1), TGFβ1 (Hs00171257_m), IL-1β (Hs00174097_m1), CCL3 (Hs00234142_m1), ubiquitin (Hs00430290_m1), CXCL9 (Hs00171065_m1), αB-crystallin (Hs00157107_m1), desmin (Hs00157258_m1), CCL4 (Hs00605740_g1), IFNγ (Hs00174143_m1), TNFα (Hs00174128_m1), IL-6 (Hs00174131_m1), IL-10 (Hs00174086_m1), inducible costimulator (ICOS; Hs00359999_m1), ICOSL (Hs00323621_m1), and perforin (Hs00169473_m1).

The reactions were run in duplicate on an Opticon 2 DNA engine (MJ Research/Applied Biosystems) following the standard cycle protocol and instructions provided by the supplier. Similar to a reliable assay control that was also used in our previous study (6), a reanalysis in triplicate reactions was performed for all targets with a difference in the duplicate expression of >1 C_t. The quantitative expression analysis of all targets remained within the linear part of the amplification by PCR. Target mRNA expression was quantified using the ΔC_t method in relation to the expression of GAPDH mRNA.

Immunohistochemistry. Sections (5 μm) of frozen muscle biopsy specimens were fixed in 4% paraformaldehyde at room temperature (for β-amyloid) or acetone at −20°C (for all other stainings) for 10 minutes. Nonspecific binding was reduced by 30 minutes of incubation with 5% bovine serum albumin (BSA) and 3% goat or chicken serum (all from Jackson ImmunoResearch) in Tris buffered saline (TBS) (0.05*M* Tris, pH 7.4, 0.15*M* saline). All primary and secondary reagents were diluted in 1% BSA in TBS. The following primary anti-human antibodies were used at various concentrations and incubation times: anti-APP (goat polyclonal, 1 hour at 10 μg/ml; R&D Systems), antiubiquitin (rabbit polyclonal, 30 minutes at 15 μg/ml; Dako), anti–IL-1β (rabbit polyclonal, 24 hours at 4°C at 10 μg/ml; Abcam), anti-CXCL9 (goat polyclonal, 24 hours at 4°C at 10 μg/ml; R&D Systems), anti-CCL3 (rabbit polyclonal, 30 minutes at 4 μg/ml; Abcam), anti–β-amyloid (mouse clone 6E10, 24 hours at 4°C at 10 μg/ml; Signet), anti–inducible nitric oxide synthase (anti-iNOS)

(rabbit polyclonal, AB5384, 1 hour at 1:500; Chemicon/Merck Millipore), antinitrotyrosine (rabbit polyclonal, 06-284, 2 hours at 1:200; Upstate/Merck Millipore), and anti-IgG (Texas Red–conjugated rabbit polyclonal, ABIN101567, 4 hours at 20 μg/ml; Antibodies-online).

Apart from the fluorochrome-conjugated primary anti-human IgG antibody, immunoreactivity was detected using Alexa Fluor 488– or Alexa Fluor 594–conjugated preadsorbed secondary goat or chicken antibodies against mouse, goat, or rabbit IgG (all from Molecular Probes/Invitrogen). To avoid cross-reactions in double labeling with primary goat antibodies, sections were blocked with chicken serum and the other secondary antibodies were preadsorbed with chicken serum as previously described (10). Negative controls were performed by omitting one of each primary antibody in every staining. Nuclear counterstaining was performed with DAPI (Molecular Probes/Invitrogen) at 1:50,000 for 1 minute, followed by mounting in Fluoromount-G (Electron Microscopy Sciences). Immunofluorescence microscopy and digital photography were performed on a Zeiss Axiophot microscope using a 20× objective, appropriate filters for green (488 nm), red (594 nm), and blue (350 nm) fluorescence, and a cooled CCD digital camera (Retiga 1300; QImaging) using the QCapture software (QImaging).

For quantitative assessment of immunohistochemical staining, photomicrographs that covered a cross-section of each biopsy specimen were taken by an investigator who was blinded to the source of the specimen (JS). Scion Image software was used for gray-scale analysis. Semiquantitative assessment of the staining of nitrotyrosine was performed by 2 observers (JZ and JS) who were blinded to the source of the specimen, according to a previously established grading method (6): 0 = no signal; 1 = low signal (<30% of the section); 2 = moderate signal (30–50% of the section); 3 = strong signal (>50% of the section).

Cell culture stimulation, treatment studies, and immunocytochemistry. As described previously (6), muscle cell progenitors (satellite cells) from diagnostic biopsy specimens from patients without myopathic changes were grown according to the following protocol. The muscle piece of 27 mm^3 was minced and washed in phosphate buffered saline and trypsinized. The fragments were seeded to a 25-cm^2 flask in Dulbecco's modified Eagle's medium with pyruvate, high glucose, and L-glutamine (Gibco Invitrogen), supplemented with 10% fetal calf serum (Cambrex Bioscience), penicillin, streptomycin (Gibco Invitrogen), and 0.5% chick embryo extract (Accurate). After 21 days, myoblasts were labeled with neural cell adhesion molecule (anti-CD56, mouse clone Eric-1; Neomarkers/Labvision), followed by magnetic bead–labeled secondary antibodies and subsequently separated by magnets (Dynal/Invitrogen). For further experiments, myoblasts were seeded either in 8-chamber slides (LabTek II; Nunc) or in 24-well plates (Nunc), and at 80% confluence, fusion was induced by serum deprivation (6). Well-differentiated myotubes, as revealed by immunocytochemical staining for the muscle marker desmin, were either kept as unstimulated controls in X-Vivo 15 medium (Cambrex Bioscience), exposed to the cytokines IFNγ (300 units/ml) and IL-1β (20 ng/ml) (both from R&D Systems) in serum-free X-Vivo 15 medium, or additionally treated with different concentrations of prednisone (10 μM or 100 μM; Jenapharm), human IgG (2 mg/ml or 10 mg/ml; Octapharma), or a combination of both for 72 hours.

For immunocytochemistry, chamber slides were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) at room temperature or in acetone at –20°C. After thorough washing and blocking of nonspecific binding of proteins, the myotubes were incubated with the following primary anti-human antibodies: anti–NF-κB p65 (mouse monoclonal, 12H11, overnight at 1:20; Chemicon), anti–NF-κB p50 (rabbit polyclonal, H-119, overnight at 1:50; Santa Cruz Biotechnology), antinitrotyrosine (rabbit polyclonal, 06-284, 2 hours at 1:200), and anti–αB-crystallin (rabbit polyclonal, AHP-256, 1 hour at 1:1,000; Serotec). Immunoreactivity was detected using Alexa Fluor 594–conjugated preadsorbed secondary goat antibody against rabbit IgG. Staining in 1% thioflavin S (T1892-25G; Sigma) was performed at room temperature for 5 minutes to identify β-amyloid aggregates. Cell death was analyzed by propidium iodide (PI) staining. After thorough washing, the nucleus was counterstained with DAPI. After mounting in Mowiol (Carl Roth), the specimens were analyzed by immunofluorescence microscopy as described above. Alternatively, for quantitative (real-time) PCR analysis, RNA from myoblasts (rhabdomyosarcoma cell line CCL-136) was extracted as described above.

Statistical analysis. For statistical analysis, Student's *t*-test, analysis of variance with Tukey's post hoc test, and the nonparametric Wilcoxon signed rank test were used (GraphPad Prism software, version 4.0). *P* values less than 0.05 were considered significant.

RESULTS

Reduction of mRNA expression of inflammatory chemokines and cytokines in muscle from patients with sporadic IBM. As revealed by quantitative (real-time) PCR analysis, the mRNA expression of all major proinflammatory chemokines and cytokines was significantly down-regulated by IVIG and prednisone or prednisone alone (Figures 1A and B). The most striking reductions in mRNA expression were observed for the chemokines CXCL9, CCL3, and CCL4 (fold reduction given as mean ± SEM: 14.7 ± 5.5–fold, 6.2 ± 1.6–fold, and 7.3 ± 2.2–fold, respectively; all $P < 0.01$) and for the cytokine IFNγ (6.2 ± 1.2–fold; $P < 0.01$), for which the expression was reduced to a nondetectable level in 2 patients. We observed a similar reduction in mRNA expression of the fibrosis-associated cytokine TGFβ (3.1 ± 1.0–fold; $P < 0.01$), the amyloidogenic IL-1β (5.8 ± 2.5–fold; $P < 0.05$), and the potentially antiinflammatory IL-10 (3.3 ± 1.1–fold; $P < 0.05$). The cytokines TNFα and IL-6, the costimulatory molecule ICOSL and its receptor ICOS, and the cytotoxic effector molecule perforin were not detectable in some patients, and no consistent changes were observed (Figure 1B).

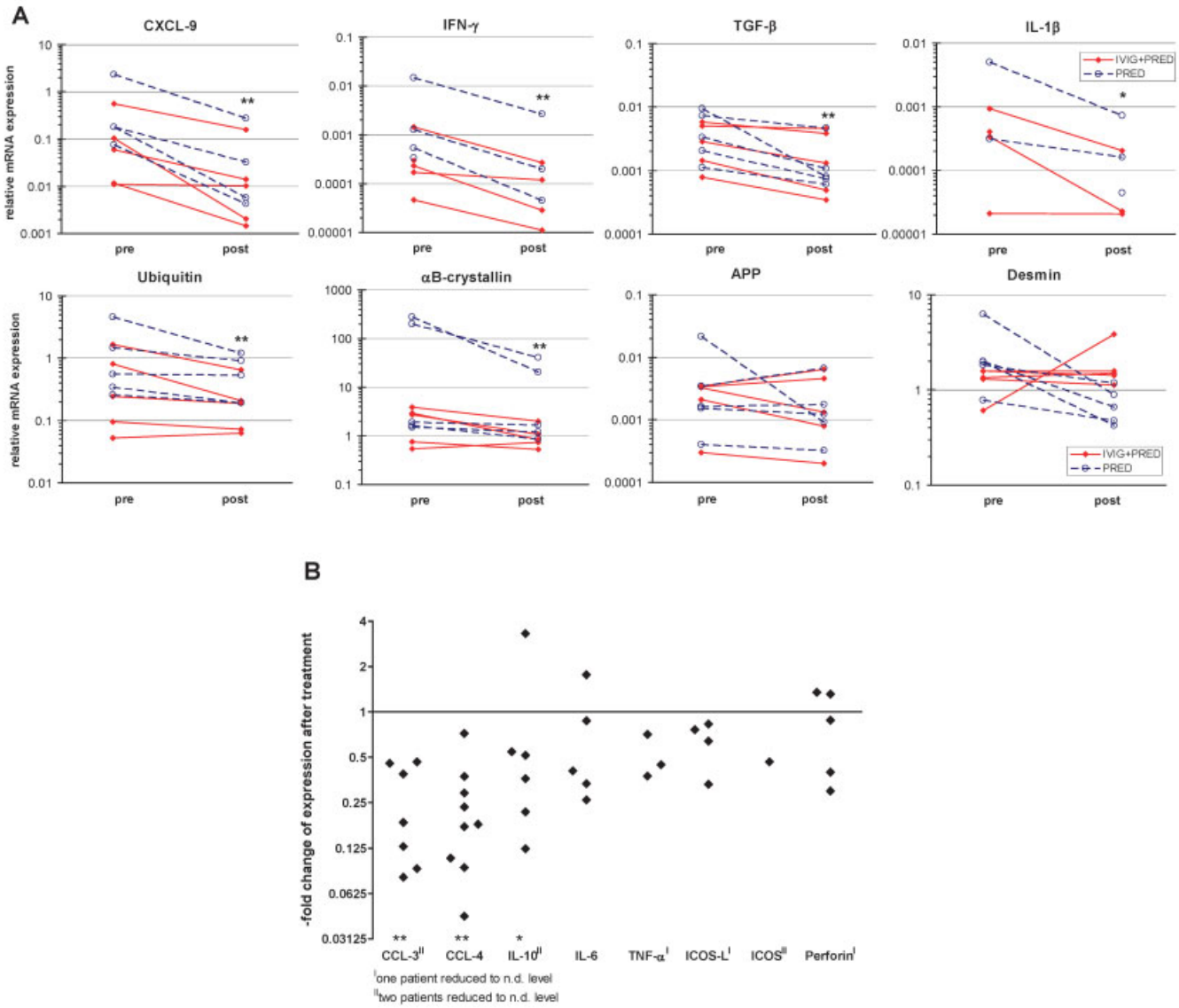

Figure 1. GAPDH-normalized mRNA expression of inflammatory and degenerative markers as assessed by quantitative (real-time) polymerase chain reaction in skeletal muscle from patients with sporadic inclusion body myositis (IBM) before (pre) and after (post) treatment with prednisone (pred) and intravenous immunoglobulin (IVIG) or prednisone alone. **A,** Significant reduction of proinflammatory mediators CXCL9, interferon-γ (IFNγ), transforming growth factor β (TGFβ), interleukin-1β (IL-1β), the tagging molecule ubiquitin, and the heat-shock protein αB-crystallin. No changes were observed in amyloid precursor protein (APP) (a marker for degeneration in sporadic IBM) or in desmin (a marker for degeneration/regeneration of muscle fibers). **B,** Same specimen as in **A** analyzed for mRNA expression of chemokines, cytokines, and costimulatory molecules. Data are presented as fold change in expression from before treatment to after treatment. A significant reduction was noted for CCL3, CCL4, and IL-10, but not for IL-6, tumor necrosis factor α (TNFα), inducible costimulator (ICOS), ICOSL, and perforin. $* = P < 0.05$; $** = P < 0.01$ versus before treatment. ND = not detectable. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

Modulation of mRNA expression of molecules associated with degeneration, cell stress, and regeneration in muscle from patients with sporadic IBM. Similar to the down-regulated proinflammatory molecules, quantitative PCR revealed reduced mRNA expression of ubiquitin, the tagging molecule for aberrant proteins, and αB-crystallin, a cell stress–associated heat-shock protein, after treatment with IVIG and prednisone or prednisone alone (1.9 ± 0.3–fold and 3.3 ± 1.2–fold, respectively; $P < 0.01$ for both) (Figure 1A). In con-

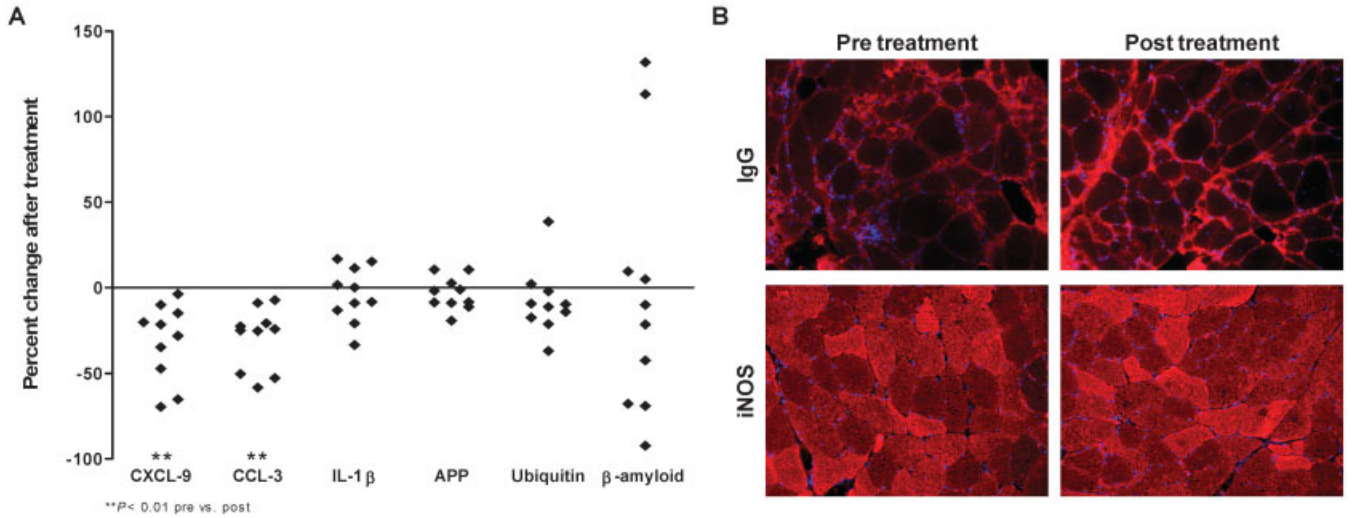

Figure 2. Immunohistochemical analysis of protein expression of proinflammatory and degeneration-associated molecules. **A,** Gray-scale quantification of photomicrographs, obtained by a blinded observer, of stained skeletal muscle biopsy specimens from all patients. Values are the mean change in staining intensity from before treatment to after treatment. Staining for CXCL9 and CCL3 revealed a significant reduction in protein expression after treatment. No significant changes were noted in the expression of IL-1β, APP, ubiquitin, or β-amyloid, or in the expression of inducible nitric oxide synthase (iNOS) (not shown). **B,** Immunohistochemical staining for IgG (Texas Red) or iNOS (Alexa Fluor 594; red) in representative biopsy specimens from patients with sporadic IBM before and after treatment with IVIG and prednisone. While IVIG and prednisone led to a marked increase in the staining signal for IgG, there was no change in the labeling for iNOS. Photomicrographs were obtained with a CCD camera using a 20× objective. See Figure 1 for other definitions.

trast, there was no consistent change in mRNA expression of APP, the most important degeneration-associated molecule in sporadic IBM. Furthermore, no significant change could be observed in mRNA expression of desmin, a marker for degeneration/regeneration of muscle fibers.

Modulation of protein expression of chemokines and β-amyloid–associated markers in muscle from patients with sporadic IBM. The protein expression of selected markers was analyzed by immunohistochemistry, followed by a computerized gray-scale analysis of photomicrographs. Consistent with reduced mRNA expression, the protein levels of CXCL9 and CCL3 were reduced (reductions of 31.5 ± 7.1% and 29.5 ± 5.7%, respectively; both $P < 0.01$) (Figure 2A). Despite changes at the mRNA level, the protein expression of IL-1β remained basically unchanged. Consistent with its unchanged mRNA level, APP did not show noticeably altered expression. The 2-fold reduction of ubiquitin mRNA upon treatment could not be observed at the protein level. Interestingly, some patients displayed a tremendous change in the staining intensity for β-amyloid, with a substantial down-regulation in most patients and an up-regulation in 2 patients (Figure 2A). Upon exclusion of these 2 outliers for the final analysis, there was a significant reduction in the gray-scale value of the immunosignal for β-amyloid (36.1 ± 13.3% reduction; $P < 0.05$).

NO-associated cell stress has recently been dem-

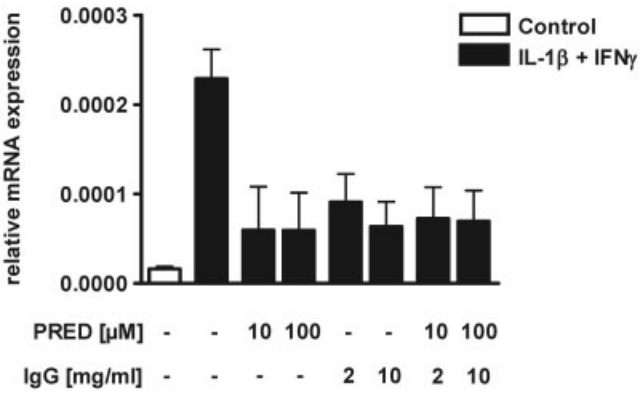

Figure 3. In vitro analysis of IL-1β mRNA expression in cultured human myoblasts. Myoblasts (rhabdomyosarcoma cell line CCL-136) were exposed to IL-1β (10 ng/ml) plus IFNγ (300 units/ml) for 72 hours. In addition, myoblasts were treated with different concentrations of prednisone (10 or 100 μM) or IgG (2 or 10 mg/ml) or both in combination. GAPDH-normalized expression of IL-1β was up-regulated upon exposure to IL-1β and IFNγ compared to controls. Addition of prednisone and/or IgG reduced cytokine-induced expression of IL-1β 2.5-fold. Values are the mean ± SEM of duplicates of 1 experiment representative of 4 experiments with similar results. See Figure 1 for definitions.

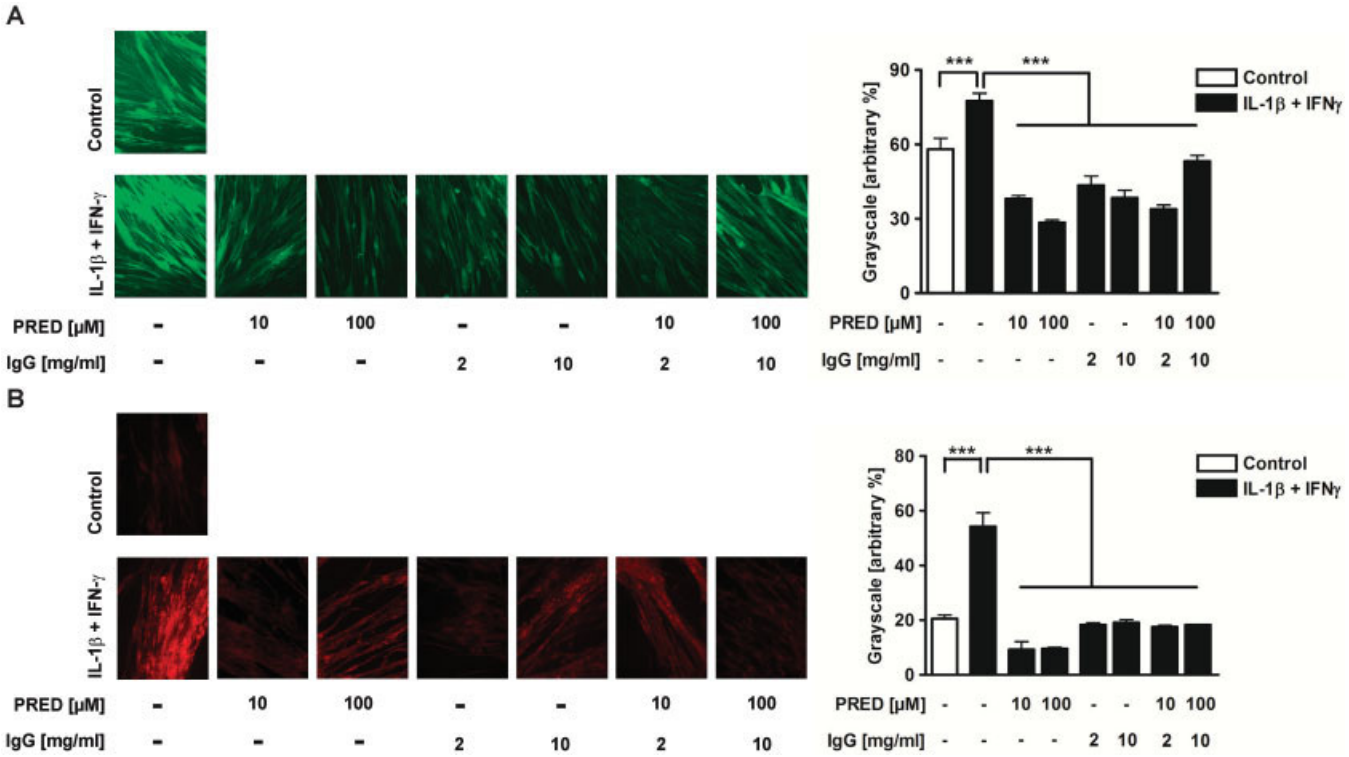

Figure 4. Immunocytochemical analysis of amyloid and cell death in cultured muscle cells. Primary human myotubes were exposed to IL-1β (10 ng/ml) plus IFNγ (300 units/ml) for 72 hours. In addition, myotubes were treated with different concentrations of prednisone (10 or 100 μM) or IgG (2 or 10 mg/ml) or both in combination. **A,** Thioflavin S staining revealed a significant increase of amyloid deposits compared to controls as reflected by photomicroscopy and subsequent gray-scale analysis. The cytokine-induced immunocytochemical signal was significantly diminished upon addition of prednisone or IgG. **B,** The degree of cell death was analyzed by staining for propidium iodide. The fraction of dead cells was significantly increased by IL-1β plus IFNγ, and this induction of cell death was abolished by prednisone and/or IgG. Values are the mean ± SEM of duplicates of 1 experiment representative of 3 experiments with similar results. *** = $P < 0.001$. Photomicrographs were obtained with a CCD camera using a 20× objective. See Figure 1 for definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

onstrated to be a crucial mechanism in muscle from patients with sporadic IBM (22). The level of iNOS, the main NO-producing enzyme, did not change significantly upon treatment (Figure 2B). Consistent with this, the nitration of tyrosine residues, an indicator of intracellular NO production, remained unchanged as revealed by immunohistochemical staining and semiquantitative assessment (1.86 ± 0.76 pretreatment versus 1.65 ± 0.74 posttreatment) (data not shown). Deposition of IgG in the muscle was evidenced by immunohistochemical labeling, which was particularly present around vessels in inflammatory areas and clearly augmented upon treatment with IVIG (Figure 2B).

Prednisone and/or IgG modulate cytokine-induced inflammatory responses in muscle cells. Functional effects of IgG and prednisone in skeletal muscle were addressed by using a recently established in vitro model (6). Consistent with the muscle biopsy results, mRNA expression for the amyloidogenic cytokine IL-1β was up-regulated upon exposure of muscle cells to IFNγ plus IL-1β, which was clearly diminished by prednisone and/or IgG (2.5-fold reduction) (Figure 3). In contrast, the level of the chemokine CXCL9 remained without consistent change upon addition of prednisone and/or IgG (data not shown).

Intracellular β-amyloid is augmented in skeletal muscle cells exposed to IL-1β plus IFNγ (6). As shown by thioflavin S staining and subsequent quantification by gray-scale analysis, the cytokine-induced accumulation of β-amyloid was significantly ameliorated by prednisone and/or IgG ($P < 0.001$) (Figure 4A). Subsequent induction of cell death as evidenced by staining for PI was abolished by prednisone and/or IgG ($P < 0.001$) (Figure 4B).

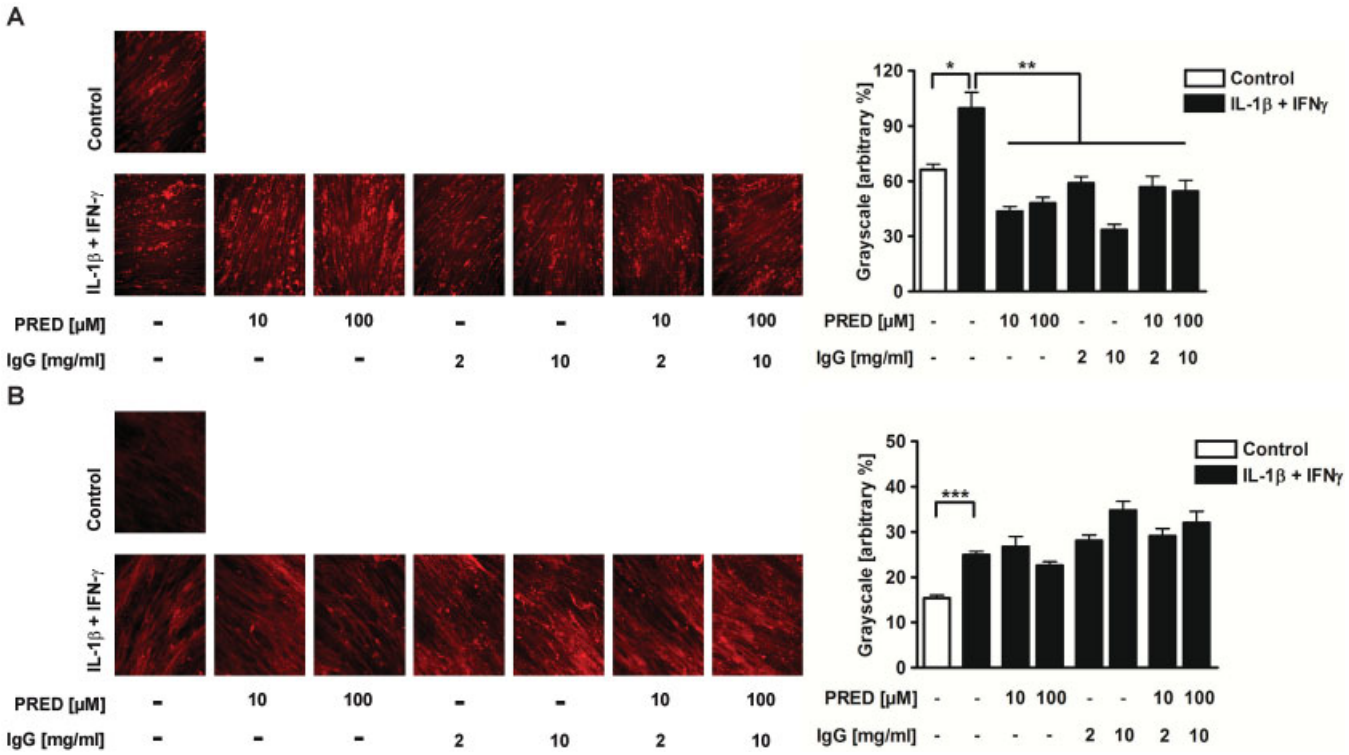

Figure 5. Immunocytochemical analysis of αB-crystallin and nitrotyrosine in cultured muscle cells. Primary human myotubes were exposed to IL-1β (10 ng/ml) plus IFNγ (300 units/ml) for 72 hours. In addition, myotubes were treated with different concentrations of prednisone (10 or 100 μM) or IgG (2 or 10 mg/ml) or both in combination. Heat-shock protein expression was detected by staining for αB-crystallin (**A**), and nitric oxide (NO)–associated cell stress was detected by staining for nitrotyrosine (**B**). IL-1β plus IFNγ significantly up-regulated αB-crystallin as well as nitrotyrosine. Prednisone and/or IgG did block up-regulation of αB-crystallin (**A**), but not intracellular generation of NO (**B**). Values are the mean ± SEM of duplicates of 1 experiment representative of 3 experiments with similar results. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. Photomicrographs were obtained with a CCD camera using a 20× objective. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

To address the underlying molecular changes of the protective effect of IgG/prednisone, cell stress mediators such as the transcription factor NF-κB were assessed. Both of its subunits, NF-κB p65 and NF-κB p50, remained unaltered upon exposure to prednisone and/or IgG (data not shown). Using immunocytochemistry and gray-scale analysis, the up-regulation of αB-crystallin upon exposure to IFNγ plus IL-1β was profoundly blocked by prednisone and/or IgG ($P < 0.01$) (Figure 5A).

No relevant synergy was observed between prednisone and IgG with regard to their effects on β-amyloid, cell death, and αB-crystallin. NO-associated cell stress has recently been shown to be a relevant cell stress mediator in a muscle cell culture model of sporadic IBM (22). However, prednisone and/or IgG did not prevent the cytokine-induced production of intracellular NO after a time interval of 72 hours, as evidenced by staining for nitrotyrosine (Figure 5B).

Collectively, these data demonstrate that in a cell culture model of sporadic IBM, modulation of inflammation by prednisone and/or IgG has a protective effect and reduces β-amyloid–associated degeneration, but fails to prevent production of NO.

DISCUSSION

IVIG alone or combined with prednisone is effective in DM and possibly in PM, and their efficacy is associated with reduction of relevant inflammatory molecules as demonstrated with repeated biopsy specimens. The same regimen, however, has been generally ineffective in sporadic IBM, in spite of the strong up-regulation of the same inflammatory molecules in this disease. We

reasoned that the inefficacy of IVIG/prednisone in patients with sporadic IBM might be related to their insufficient effect on key inflammatory and degenerative molecules, and that the myotoxicity of certain factors outweighs the down-modulatory capacity of IVIG/prednisone. Detailed deciphering of such inefficacy could provide valuable information as to which of the degenerative or inflammatory molecules are the most relevant in the pathogenesis of sporadic IBM. The study demonstrates that IVIG and prednisone or prednisone alone reduces certain proinflammatory chemokines and cytokines as well as ubiquitin and β-amyloid, but has no consistent effect on TNFα, IL-6, ICOSL, ICOS, or perforin and does not affect key mediators of myotoxicity such as iNOS, nitrotyrosine, and NF-κB.

The results are consistent with previously reported changes of down-regulation of inflammatory mediators as revealed by gene array in repeated biopsy specimens from patients with sporadic IBM after IVIG and prednisone treatment (23). The findings in the muscles of patients with sporadic IBM who did not improve after therapy are in sharp contrast to findings in DM patients who had improved significantly after IVIG (20); repeated biopsy specimens in DM displayed a clear reduction in mRNA expression of intercellular adhesion molecule, class I major histocompatibility complex, and TGFβ (24). In spite of the small number of studied specimens and the practical difficulties of obtaining repeated muscle biopsy specimens, the changes were robust and deemed sufficient for statistical analysis, which was similar to a previous study on DM (23).

The way in which IVIG exerts an immunomodulating effect is complex and includes inhibition of complement activation, up-regulation of inhibitory Fc receptors on macrophages, and modulatory effects on certain T and B cell functions, autoantibodies, cytokines, and chemokines (25–27). The detection of a pronounced signal for IgG upon treatment suggests that, apart from general immunomodulation, IVIG might exert local effects in muscle. However, its effectiveness may relate to the underlying immunopathology of a given disorder (28). In DM for example, in which complement activation is an early event, it intercepts the formation of the membranolytic attack complex and down-regulates downstream proinflammatory cytokines, chemokines, and key adhesion molecules (23,29). In sporadic IBM, however, in which CD8-mediated cytotoxicity is thought to be an important component of the pathology, IVIG had no effect on the expression of perforin, the main effector molecule. The down-regulatory effects of IVIG/prednisone on certain cytokines and chemokines, which may be important in certain diseases, seem to be clinically less relevant in sporadic IBM. In contrast, TNFα and IL-6, along with iNOS/nitrotyrosine, ICOS, and perforin, were not affected and thus may be among the most relevant immune molecules in sporadic IBM.

It is also conceivable that the IVIG-induced down-regulation and inhibition of certain myotoxic cytokines such as IL-1β might not have been sufficient during a 3-month treatment period. In particular, the direct effects of IL-1β on muscle fibers themselves were not sufficiently blocked in order to prevent the autoamplificatory process between inflammation and accumulation of β-amyloid (6). Duration and dosing of therapy may also account for the previously reported differences of the degree of inflammation noted after therapies (23,24,30). Therefore, the mere lack of clinical efficacy of antiinflammatory treatment does not exclude a significant contribution of inflammation to the pathology of sporadic IBM. Moreover, the significant reduction of some of the inflammatory mediators such as the chemokines CXCL9, CCL3, and CCL4 may account for a limited degree of clinical improvement, which is at least temporarily observed in individual patients.

On the other hand, it is possible that the degenerative component of sporadic IBM pathology is a more relevant driving force (3). In general, this could imply that immunomodulatory/immunosuppressive treatment cannot exert beneficial effects. However, patients with Duchenne's muscular dystrophy, a hereditary degenerative myopathy, can be clinically stabilized by glucocorticoid therapy. Therefore, the assumption that sporadic IBM could be a primary degenerative disorder does not per se exclude the possibility of beneficial effects of immunotherapy. Moreover, IVIG has shown some favorable effects in Alzheimer's disease (see below), suggesting that, in patients with sporadic IBM, this treatment could just as well help to ameliorate the degenerative pathology around β-amyloid in skeletal muscle.

Sporadic IBM is a complex disorder in which inflammation coexists with degeneration, which predominantly involves amyloid-related molecules (31). Inflammatory mediators can trigger cell stress–associated mechanisms as well as degenerative pathways including accumulation of β-amyloid (6,32,33). Therefore, we sought to examine if the inefficacy of IVIG was in part related to aggregation-associated molecules that are unaffected by this drug. To address an effect on the underlying autoamplificatory mechanisms of inflammation/degeneration in skeletal muscle, we used a recently established cell culture model (6) in which the combina-

tion of the proinflammatory cytokines IL-1β and IFNγ induces an accumulation of β-amyloid in association with up-regulation of cell stress–associated molecules such as αB-crystallin (32). We found that IgG and prednisone efficiently down-regulated the inflammatory response in muscle cells, but there was no synergism between the agents. Further, the accumulation of β-amyloid as well as induction of cell death was also prevented by IgG, which is consistent with a previous observation in neuronal cells (34) and the evidence of a clinical benefit in patients with Alzheimer's disease (see above) (35). However, the amount of intracellular NO stress remained unchanged.

Inducible NOS is up-regulated under inflammatory conditions in the muscle and may act via various pathways including mitochondrial respiration and induction of cell death (36). NO induces the accumulation of β-amyloid in human myoblasts in response to cytokine stimulation and thus has recently been identified as a key molecule in the network of pathomechanisms in sporadic IBM (22). Our current results solidify the finding that NO is an important and early mediator in sporadic IBM pathology and demonstrate the inability of prednisone and/or IgG to prevent nitrosylation or oxidation in skeletal muscle, which may explain their inefficacy in sporadic IBM. Apart from targeting antidegenerative molecules like β-secretase, it may be worth studying molecules that have been shown to be part of the muscle cell stress response, such as NO/iNOS, or giving renewed attention to blockade of IL-1β (6,22,37).

The lack of modulation of APP and the reduction of accumulation of β-amyloid in many but not all patients is consistent with previous studies that showed no change of the rate of congophilic inclusions (38) or even an increased number of vacuolated and β-amyloid–positive fibers (30). The different changes at the mRNA level compared to the protein level as seen for the expression of IL-1β (see above) may be due to posttranscriptional/posttranslational changes. Moreover, even under steady-state levels of protein expression of APP, different quantities of β-amyloid may be generated, since slight changes in the expression of APP-cleaving enzymes such as β-secretase may result in major alterations of the generation of β-amyloid (39,40). Thus, blockade of β-secretase (β-site APP–cleaving enzyme 1) may be an alternate treatment strategy (37).

Taken together, our data suggest that IVIG and prednisone effectively reduce some inflammatory mediators in the muscles of patients with sporadic IBM, which may explain some of the temporary clinical benefits observed in some patients. However, the treatment does not consistently reduce the molecules associated with cytotoxic T cells or the β-amyloid pathway. Particularly the inability to suppress inflammatory cell stress mediators such as NO may explain why the autoamplificatory network between inflammation and degeneration is not halted and the treatment lacks clinical efficacy in most patients. The data may help to better design and evaluate novel treatment strategies for sporadic IBM.

ACKNOWLEDGMENTS

We thank Fatima Agdas and Antje Ulrich for excellent technical assistance and Konstanze Kleinschnitz for help with some experiments.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Dalakas and Schmidt had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Zschüntzsch, Raju, Dalakas, Schmidt.

Acquisition of data. Zschüntzsch, Voss, Creus, Sehmisch, Dalakas, Schmidt.

Analysis and interpretation of data. Zschüntzsch, Voss, Creus, Dalakas, Schmidt.

ROLE OF THE STUDY SPONSOR

Octapharma had no role in the study design, data collection, data analysis, data interpretation, writing of the manuscript, agreement to submit the manuscript for publication, or approval of the content of the submitted manuscript. Publication of the manuscript was not contingent upon the approval of Octapharma.

REFERENCES

1. Dalakas MC. Inflammatory, immune, and viral aspects of inclusion-body myositis. Neurology 2006;66:S33–8.
2. Needham M, Mastaglia FL. Inclusion body myositis: current pathogenetic concepts and diagnostic and therapeutic approaches. Lancet Neurol 2007;6:620–31.
3. Askanas V, Engel WK. Inclusion-body myositis: a myodegenerative conformational disorder associated with Aβ, protein misfolding, and proteasome inhibition. Neurology 2006;66:S39–48.
4. De Paepe B, Creus KK, De Bleecker JL. Chemokines in idiopathic inflammatory myopathies. Front Biosci 2008;13:2548–77.
5. Raju R, Vasconcelos O, Granger R, Dalakas MC. Expression of IFN-γ-inducible chemokines in inclusion body myositis. J Neuroimmunol 2003;141:125–31.
6. Schmidt J, Barthel K, Wrede A, Salajegheh M, Bahr M, Dalakas MC. Interrelation of inflammation and APP in sIBM: IL-1β induces accumulation of β-amyloid in skeletal muscle. Brain 2008; 131:1228–40.
7. Wiendl H, Hohlfeld R, Kieseier BC. Immunobiology of muscle: advances in understanding an immunological microenvironment. Trends Immunol 2005;26:373–80.
8. Salajegheh M, Rakocevic G, Raju R, Shatunov A, Goldfarb LG, Dalakas MC. T cell receptor profiling in muscle and blood lymphocytes in sporadic inclusion body myositis. Neurology 2007; 69:1672–9.

9. Muntzing K, Lindberg C, Moslemi AR, Oldfors A. Inclusion body myositis: clonal expansions of muscle-infiltrating T cells persist over time. Scand J Immunol 2003;58:195–200.
10. Schmidt J, Rakocevic G, Raju R, Dalakas MC. Upregulated inducible co-stimulator (ICOS) and ICOS-ligand in inclusion body myositis muscle: significance for $CD8^+$ T cell cytotoxicity. Brain 2004;127:1182–90.
11. Dalakas MC, Rakocevic G, Schmidt J, Salajegheh M, McElroy B, Harris-Love MO, et al. Effect of alemtuzumab (CAMPATH 1-H) in patients with inclusion-body myositis. Brain 2009;132:1536–44.
12. Badrising UA, Maat-Schieman ML, Ferrari MD, Zwinderman AH, Wessels JA, Breedveld FC, et al. Comparison of weakness progression in inclusion body myositis during treatment with methotrexate or placebo. Ann Neurol 2002;51:369–72.
13. Rutkove SB, Parker RA, Nardin RA, Connolly CE, Felice KJ, Raynor EM. A pilot randomized trial of oxandrolone in inclusion body myositis. Neurology 2002;58:1081–7.
14. Quartuccio L, De Marchi G, Scott CA, Ferraccioli G, Beltrami CA, De Vita S. Treatment of inclusion body myositis with cyclosporin-A or tacrolimus: successful long-term management in patients with earlier active disease and concomitant autoimmune features. Clin Exp Rheumatol 2007;25:246–51.
15. Lindberg C, Trysberg E, Tarkowski A, Oldfors A. Anti-T-lymphocyte globulin treatment in inclusion body myositis: a randomized pilot study. Neurology 2003;61:260–2.
16. Dalakas MC, Sonies B, Dambrosia J, Sekul E, Cupler E, Sivakumar K. Treatment of inclusion-body myositis with IVIg: a double-blind, placebo-controlled study. Neurology 1997;48:712–6.
17. Amato AA, Barohn RJ, Jackson CE, Pappert EJ, Sahenk Z, Kissel JT. Inclusion body myositis: treatment with intravenous immunoglobulin. Neurology 1994;44:1516–8.
18. Muscle Study Group. Randomized pilot trial of betaINF1a (Avonex) in patients with inclusion body myositis [published erratum appears in Neurology 2002;58:334]. Neurology 2001;57: 1566–70.
19. Muscle Study Group. Randomized pilot trial of high-dose betaINF-1a in patients with inclusion body myositis. Neurology 2004;63:718–20.
20. Dalakas MC. Therapeutic approaches in patients with inflammatory myopathies. Semin Neurol 2003;23:199–206.
21. Dalakas MC, Koffman B, Fujii M, Spector S, Sivakumar K, Cupler E. A controlled study of intravenous immunoglobulin combined with prednisone in the treatment of IBM. Neurology 2001;56: 323–7.
22. Schmidt J, Barthel K, Zschuntzsch J, Muth IE, Swindle EJ, Hombach A, et al. Nitric oxide stress in sporadic inclusion body myositis muscle fibres: inhibition of inducible nitric oxide synthase prevents interleukin-1β-induced accumulation of β-amyloid and cell death. Brain 2012;135:1102–14.
23. Raju R, Dalakas MC. Gene expression profile in the muscles of patients with inflammatory myopathies: effect of therapy with IVIg and biological validation of clinically relevant genes. Brain 2005;128:1887–96.
24. Amemiya K, Semino-Mora C, Granger RP, Dalakas MC. Down-regulation of TGF-β1 mRNA and protein in the muscles of patients with inflammatory myopathies after treatment with high-dose intravenous immunoglobulin. Clin Immunol 2000;94:99–104.
25. Dalakas MC. Intravenous immunoglobulin in autoimmune neuromuscular diseases. JAMA 2004;291:2367–75.
26. Gold R, Stangel M, Dalakas MC. Drug insight: the use of intravenous immunoglobulin in neurology—therapeutic considerations and practical issues. Nat Clin Pract Neurol 2007;3:36–44.
27. Quick A, Tandan R. Mechanisms of action of intravenous immunoglobulin in inflammatory muscle disease. Curr Rheumatol Rep 2011;13:192–8.
28. Hughes RA, Dalakas MC, Cornblath DR, Latov N, Weksler ME, Relkin N. Clinical applications of intravenous immunoglobulins in neurology. Clin Exp Immunol 2009;158 Suppl 1:34–42.
29. Basta M, Dalakas MC. High-dose intravenous immunoglobulin exerts its beneficial effect in patients with dermatomyositis by blocking endomysial deposition of activated complement fragments. J Clin Invest 1994;94:1729–35.
30. Barohn RJ, Amato AA, Sahenk Z, Kissel JT, Mendell JR. Inclusion body myositis: explanation for poor response to immunosuppressive therapy. Neurology 1995;45:1302–4.
31. Shtifman A, Ward CW, Laver DR, Bannister ML, Lopez JR, Kitazawa M, et al. Amyloid-β protein impairs Ca^{2+} release and contractility in skeletal muscle. Neurobiol Aging 2010;31:2080–90.
32. Muth IE, Barthel K, Bahr M, Dalakas MC, Schmidt J. Proinflammatory cell stress in sporadic inclusion body myositis muscle: overexpression of αB-crystallin is associated with amyloid precursor protein and accumulation of β-amyloid. J Neurol Neurosurg Psychiatry 2009;80:1344–9.
33. Nagaraju K, Casciola-Rosen L, Lundberg I, Rawat R, Cutting S, Thapliyal R, et al. Activation of the endoplasmic reticulum stress response in autoimmune myositis: potential role in muscle fiber damage and dysfunction. Arthritis Rheum 2005;52:1824–35.
34. Magga J, Puli L, Pihlaja R, Kanninen K, Neulamaa S, Malm T, et al. Human intravenous immunoglobulin provides protection against Aβ toxicity by multiple mechanisms in a mouse model of Alzheimer's disease. J Neuroinflammation 2010;7:90.
35. Relkin NR, Szabo P, Adamiak B, Burgut T, Monthe C, Lent RW, et al. 18-month study of intravenous immunoglobulin for treatment of mild Alzheimer disease. Neurobiol Aging 2009;30:1728–36.
36. Brown GC, Borutaite V. Nitric oxide inhibition of mitochondrial respiration and its role in cell death. Free Radic Biol Med 2002; 33:1440–50.
37. Schmidt J, Dalakas MC. Inclusion-body myositis in the elderly: an update. Aging Health 2010;6:687–94.
38. Pruitt JN, Showalter CJ, Engel AG. Sporadic inclusion body myositis: counts of different types of abnormal fibers. Ann Neurol 1996;39:139–43.
39. Li Y, Zhou W, Tong Y, He G, Song W. Control of APP processing and Aβ generation level by BACE1 enzymatic activity and transcription. FASEB J 2006;20:285–92.
40. Querfurth HW, LaFerla FM. Alzheimer's disease [published erratum appears in N Engl J Med 2011;364:588]. N Engl J Med 2010;362:329–44.