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Signal Transducer and Activator of Transcription 1 in Human Muscle

Implications in Inflammatory Myopathies

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Polymyositis (PM) and dermatomyositis (DM) are two major and distinct inflammatory myopathies. Cytokines, implicated in the immune process, have been recognized in the muscle tissue from PM and DM patients, but their functional* in situ *role has not been identified. We analyzed the expression of the signal transducer and activator of transcription 1 (STAT1), a molecule whose up-regulation indicates the interaction of cytokines, or growth factors, with their target receptors in muscle fibers and inflammatory infiltrates in PM and DM. An immunohistochemical analysis was performed using monoclonal antibodies to STAT1 in 57 muscle biopsies from 10 patients with DM, 10 with PM, and 37 controls. The profile of STAT1 up-regulation was also investigated in cultured muscle stimulated by interferon-γ, epidermal growth factor, platelet-derived growth factor, and interleukin-2, using semiquantitative polymerase chain reaction and Western blot. High STAT1 expression was observed in many perifascicular atrophic muscle fibers from DM patients in 10/10 biopsies. In contrast, only a few muscle fibers undergoing necrosis were STAT1 positive in 2/10 patients with PM and in 2/37 controls. STAT1 reactivity was noted in most cells of the infiltrates in DM, PM, and controls.* In vitro, *STAT1 was stimulated by interferon-γ but not by the other molecules studied. These results suggest that in DM, but not in PM, there is distinctive functional local cytokine activity able to increase STAT1 expression in muscle fibers. As interferon-γ specifically activates STAT1* in vitro, *this cytokine in conjunction with ischemia is probably involved in perifascicular muscle fiber pathology in DM. (Am J Pathol 1997, 151:81–88)

Polymyositis (PM) and dermatomyositis (DM) are distinctive inflammatory myopathies of unknown etiology that have in common the presence of muscle weakness and inflammation. Studies indicate that these two disorders involve different immunopathogenic mechanisms and targets.[1–3] Muscle injury in PM is presumably T-cell mediated and directed against unknown antigens expressed in the sarcolemma of muscle fibers, whereas in DM the predominant immune response is humoral, against unidentified antigens in the muscle microvasculature.

Cytokines probably play a major role in determining immune response in inflammatory myopathies. These soluble, short-lived molecules exert pleiotropic effects on a variety of target cells. They influence cell activation and modulate the expression of many genes.[4,5] Their *in situ* detection is used to establish the mechanism leading to and maintaining inflammation. In muscle, the local expression of different cytokines has been studied using various methods, such as immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR) amplification.[6–8] These studies have found poor correlation between cytokine profiles in affected muscles and the different types of inflammatory myopathies. Several factors could account for such lack of cor-

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relation. First, the study of cytokines is hampered by their low concentration and transient expression. Second, the level of cytokine mRNA does not always correlate with the level of secreted cytokine[9] and may not represent functional activity if the cytokine has been inactivated without altering its antigenicity.

To gain insight into the functional implications of cytokines in the pathogenesis of PM and DM while circumventing the above-mentioned difficulties, we focused our attention on the study of a new family of transcription factors that are specifically activated in response to cytokines and growth factors. These molecules, called signal transducers and activators of transcription (STATs),[10] are a family of latent cytoplasmic proteins that are activated by protein kinases through phosphorylation of tyrosine residues. Activated STAT proteins are able to form complexes and translocate to the nucleus, where they regulate the transcription of many genes.[11] One of the STAT proteins that have been characterized to date,[11,12] STAT1, was first described as involved in cell response to interferon (IFN)-α[13] and IFN-γ[14] and has recently been implicated in other activation pathways where it is assembled into multimeric complexes with other proteins.[15–18]

We hypothesized that STAT1 might be up-regulated locally in muscle in response to cytokines if they exert *in situ* activity, and we performed the present study to examine this possibility.

Materials and Methods

Immunohistochemical Study

A total of 57 fresh-frozen muscle biopsy specimens were processed for immunohistochemistry from 10 patients fulfilling the criteria for DM, 10 patients with PM, and 37 controls including 10 patients with different muscle dystrophies (dystrophinopathies and limb girdle and facioscapulohumeral syndromes), 10 muscle biopsies from patients with neurogenic diseases (amyotrophic lateral sclerosis and chronic neuropathies), 10 morphologically normal muscles from patients undergoing orthopedic surgery, and 7 samples from patients who underwent limb amputation due to an acute or chronic ischemic process.

A series of five consecutive slides were immunostained with monoclonal antibodies to STAT1 (Transduction System, Lexington, KY) and to $CD8^+$, $CD4^+$ macrophages (Dako, Glostrup, Denmark) and an isotype-matched IgG antibody to STAT1 as a negative control. All specimens were also stained with hematoxylin and eosin (H&E) to detect muscle fiber necrosis and regeneration.

Four-micron sections were fixed in acetone, and after incubation with hydrogen peroxide and a blocking solution containing 1% bovine serum albumin, 10% normal human serum, and 3% normal horse serum, the slides were further incubated with the primary monoclonal antibody (10 μg/ml). A secondary biotinylated horse anti-mouse IgG antibody and avidin-biotin peroxidase complex (Dakopatts and Vector Laboratories, Burlingame, CA) were then applied. The reaction was developed with diaminobenzidine, and the section was counterstained with hematoxylin or methyl green.[19] The same procedure was used for cells in culture, before and after stimulation with several cytokines.

Double-label immunostaining was performed as previously described.[20] Briefly, after 30 minutes in blocking solution, the slides were incubated with anti-STAT1 monoclonal antibody for 1 hour and then with biotinylated horse anti-mouse IgG (5 μg/ml) (Vector) for 45 minutes. After rinsing with PBS and incubation with avidin-rhodamine (1 μg/ml; Vector), sections were incubated with an excess of goat anti-horse IgG (140 μg/ml; Jackson, West Grove, PA) for 1 hour to avoid cross-reactivity of the horse anti-mouse IgG with the next mouse monoclonal antibody. The slides were then incubated with a second blocking solution (2% bovine serum albumin, 10% normal human serum, and 3% normal donkey serum), followed by overnight incubation with the appropriate second monoclonal antibody to CD8, CD4, or macrophage antigens. After 15 minutes of washing and 30 minutes of incubation with donkey anti-mouse fluorescein-isothiocyanate-conjugated IgG (10 μg/ml), slides were mounted in a glycerol medium containing *p*-phenylenediamine (Sigma Chemical Co., St. Louis, MO) and examined using a Zeiss photomicroscope (Zeiss, Oberkochen, Germany) equipped with epifluorescence optics. Tissue sections were first viewed and photographed with fluorescein isothiocyanate and then with rhodamine, and finally a double exposure was made so that both markers could be visualized on the same cell. Negative controls were processed by substituting the primary antibodies for normal mouse serum and isotype-matched mouse IgG at the same concentration.

Muscle and Rhabdomyosarcoma Culture

Muscle biopsies from patients with myopathies were minced into small pieces and cultured in Eagle's minimal essential medium (Mediatech, Reston, VA) supplemented with 10% heat-inactivated fetal calf serum (MA Bioproducts, Springville, MD), 2% chick embryo extracts (GIBCO BRL, Bethesda, MD), 50

mmol/L glutamine, and gentamycin according to standard technique.[21] Cultures were refed twice a week and examined to assess the development of confluent myotubes.

The human rhabdomyosarcoma cell line TE671 (ATCC HT139) was grown in Dulbecco's minimal essential medium (Bio-Whittaker, Walkersville, MD) supplemented with penicillin/streptomycin, 2 mmol/L L-glutamine, and 10% fetal calf serum.

Culture Stimulation

Rhabdomyosarcoma cells and human muscle cells at myotube stage in culture were stimulated with human IFN-γ at a concentration of 100 U/ml (Boehringer Mannheim, Mannheim, Germany), epidermal growth factor (EGF) at a concentration of 20 U/ml (Collaborative Biomedical Products, Bedford, MA), platelet-derived growth factor (PDGF) at 20 ng/ml, and interleukin (IL)-2 at 100 ng/ml (Boehringer Mannheim). After 90 minutes, 24 hours, 2 days, and 6 days of incubation, cells were collected for RNA or protein extraction or were fixed and used for immunocytochemistry.

Western Blot Analysis

Cytoplasmic extracts were prepared by modification of the method of Schreiber et al.[22] Muscle and rhabdomyosarcoma cells in culture were washed three times in PBS and resuspended in buffer A (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA and 0.1 mmol/L EGTA, 1 mmol/L dithiothreitol) containing the following proteinase inhibitors: 1 mmol/L phenylmethylsulfonyl fluoride, 33 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A. After 10 minutes, an equal volume of buffer A containing 0.2% Nonidet P-40 was added and allowed to stand for 15 minutes. Nuclei were spun down, and the supernatant was collected, labeled as cytoplasmic extract, and stored at −80°C. The protein content of the cytoplasmic extract was determined by performing the Bio-Rad protein assay (Bio-Rad, Richmond, CA).

Equivalent quantities of proteins obtained as described above from unstimulated cells or from cells stimulated for 24 hours with IFN-γ, EGF, PDGF, and IL-2 were subjected to electrophoresis on a 7% SDS-polyacrylamide gel[23] and transferred to nitrocellulose.[24] Blots were blocked for 2 hours in 5% nonfat dry milk and incubated with mouse anti-STAT1 monoclonal antibody (dilution 1/100) and a mouse monoclonal antibody as a loading control (anti-desmin, Novocastra Laboratories; dilution 1/50) in 3% nonfat dry milk in PBS for 1 hour, washed three times with the same solution, and incubated with peroxidase-conjugated rabbit anti-mouse antibody diluted 1/1000 in 3% nonfat dry milk in PBS. After extensive washing, blots were developed using enhanced chemiluminescent reagent according to the manufacturer's instructions (Amersham International, Little Chalfont, UK).

Semiquantitative RT-PCR

We studied the expression of STAT1 mRNA in human muscle culture after treatment with IFN-γ, EGF, PDGF, and IL-2 and in untreated cells by means of semiquantitative RT-PCR. Total RNA was isolated from muscle cells in culture and TE671 cells using Ultraspec (Biotecx, Houston, TX). Two micrograms of RNA were reverse transcribed (1 hour at 42°C and 5 minutes at 94°C) using oligo dT (Promega, Madison WI), 75 mmol/L KCl, 50 mmol/L Tris/HCl, 3 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol, 500 mmol/L each dNTP, and 20 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda MD) in a final volume of 20 μl. The whole reverse transcription reaction was used as a template for PCR using the following primers: 5′CATCCTCGAGAGCTGTCTAG 3′ and 5′GTGCCAGGTACTGTCTGATT 3′ for STAT1. Primers for Abelson tyrosine kinase, 5′CAGCGGCCAGTAGCATCTGACTT 3′ and 5′ TGTGATTATAGCCTAAGACCCGGAG 3′ were used as the internal control, as synthesis of mRNA of Abelson tyrosine kinase is unaffected after treatment with the cytokines used in the study. Each reaction also included 2.5 U of *Taq* DNA polymerase, using buffer (BRL) and 500 mmol/L each dNTP to a final volume of 100 μl. To facilitate the detection of changes in the levels of STAT1 mRNA after the different treatments, 30, 25, and 20 cycles (15 seconds at 94°C, 15 seconds at 62°C, and 30 seconds at 72°C) of PCR were performed for each sample to check the exponential phase of the reactions. All PCR products were analyzed by 1.2% agarose gel electrophoresis with ethidium bromide staining.

Results

Using the anti-STAT1 monoclonal antibody, we observed a distinctive immunostaining pattern in muscle fibers and inflammatory infiltrates whereas the isotype-matched control antibodies were negative at the same fields in consecutive slides. In all of the DM specimens studied (10/10), $STAT1^+$ muscle fibers

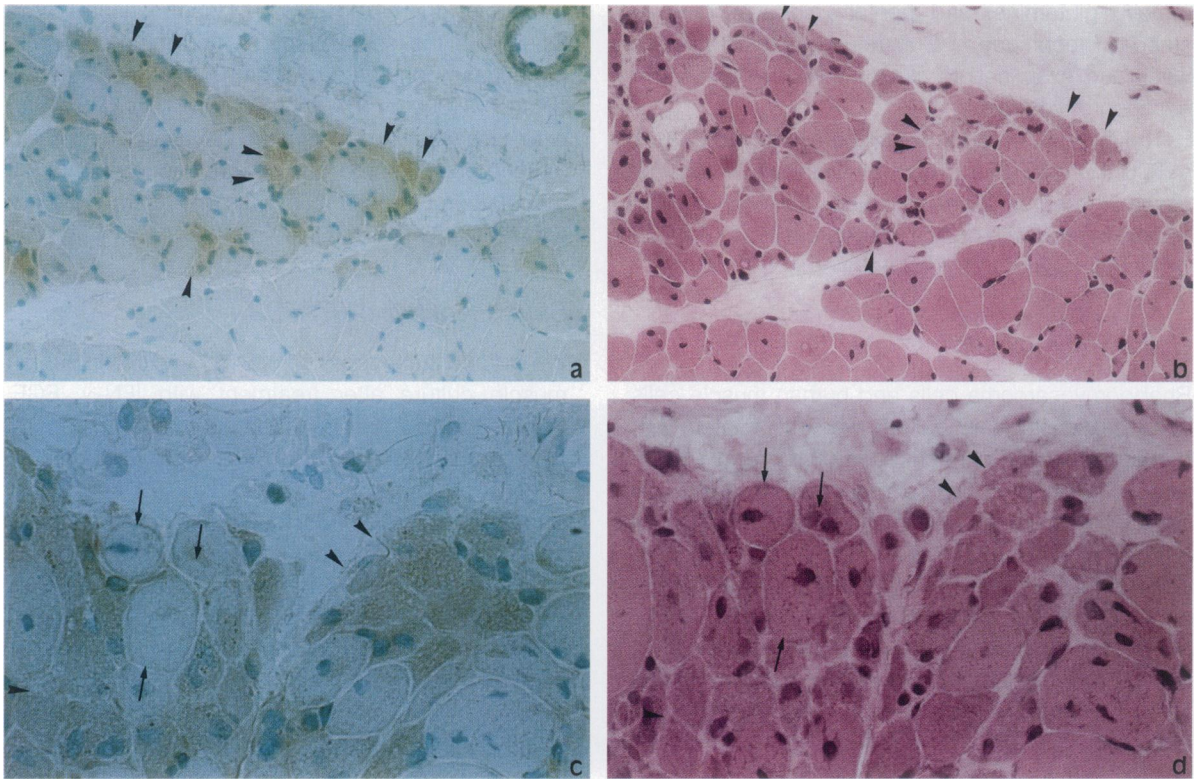

Figure 1. *Paired serial sections of fresh-frozen muscle biopsies from two different patients with DM stained with anti-STAT1 monoclonal antibody* (**a** *and* **c**) *and H&E* (**b** *and* **d**). *Degenerating fibers mainly located at the periphery of the fascicle are immunostained with STAT1* (**arrowheads**). *A few atrophic fibers are also STAT1$^+$ in the fascicle. Most regenerating fibers* (**arrows**) *are STAT1$^-$. Magnification, ×200* (**a** *and* **b**) *and ×400* (**c** *and* **d**).

were found. The staining was sarcoplasmic and predominantly located on many small perifascicular muscle fibers (Figure 1, a and c). H&E staining performed on serial sections disclosed that most of these fibers were pale or presented rarefaction in the sarcoplasm, as corresponds to atrophic or damaged fiber, whereas only some of the regenerating fibers, basophilic with big nuclei, were also STAT1$^+$ (Figure 1, b and d). No myonuclear staining was observed. In contrast, most fibers in specimens from PM patients, including those surrounded or invaded by inflammatory infiltrates, were STAT1$^-$ (Figure 2). In 2/10 PM biopsies, STAT1 was detected only in a few muscle fibers showing necrosis on H&E staining. Of the 37 controls studied, all fibers were consistently STAT1$^-$ in normal muscles (10/10). In patients with dystrophies, most normal-looking muscle fibers as well as the hypertrophic or regenerating fibers were STAT1$^-$. Only a few scattered muscle fibers, all of which were necrotic on the H&E staining, were weakly stained in 2/10 specimens from one patient with Duchenne dystrophy and one patient with facioscapulohumeral syndrome. Inflammation was observed in the specimen in both cases. In patients with neurogenic diseases, normal-looking muscle fibers as well as angulated fibers were STAT1$^-$ (data not shown; 10/10). The muscle fibers from the seven ischemic biopsy specimens, studied because ischemia is thought to play an important role in perifascicular atrophy,[1–3] were all STAT1$^-$.

Most cells in the inflammatory infiltrates were STAT1$^+$, not only in PM and DM specimens but also in those from patients with dystrophies. Using double staining, STAT1 positivity was detected in CD8$^+$ and CD4$^+$ T cell subsets as well as in macrophages. There were no differences in the percentages of STAT1$^+$ cells found surrounding or invading muscle fibers and those found in the perimysial spaces or around vessels (Figure 2).

In culture, human myotubes showed very faint STAT1 cytoplasmic staining whereas controls were negative. The same results were obtained when the TE671 cell line was used (data not shown). Stimulation with IFN-γ resulted in the myonuclei becoming positive after 90 minutes, with strong sarcolemmal staining found after 24 hours (Figure 3). This STAT1 up-regulation persisted throughout the 6 days of the experiment. In contrast, no STAT1 immunostaining was evident in untreated cultures or after treatment with EGF, PDGF, or IL-2.

When immunoblots of muscle cultures were performed, a constitutive band of 91 kd corresponding

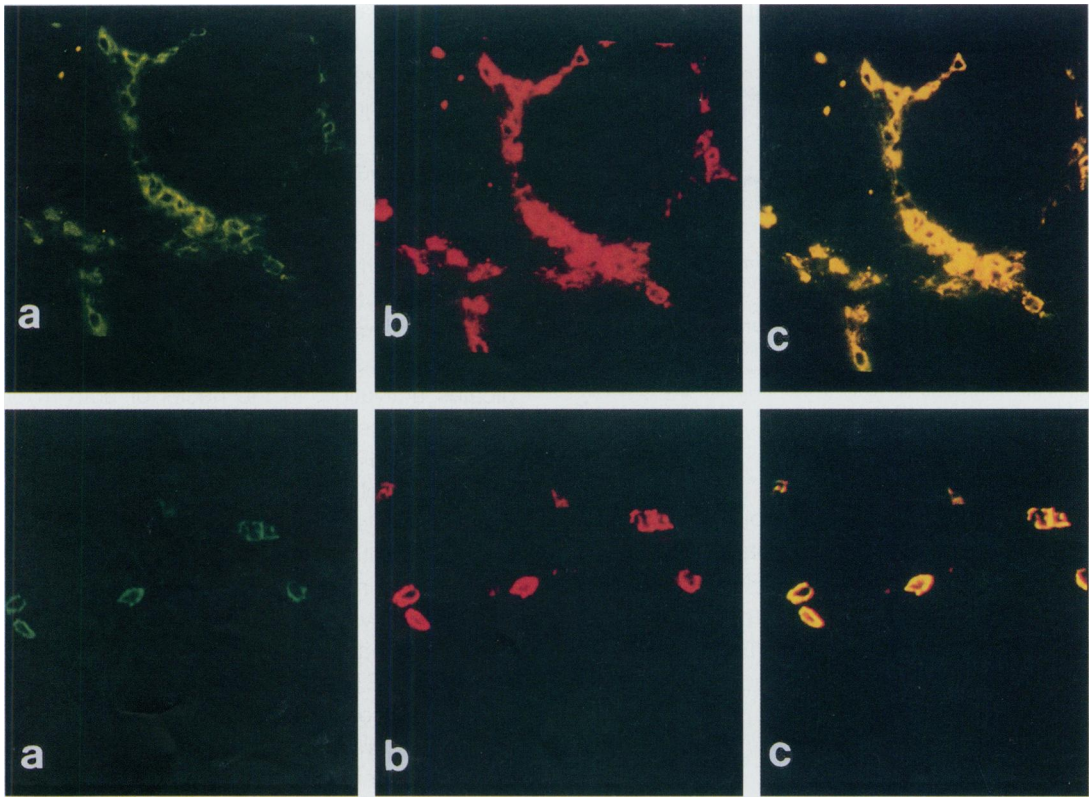

Figure 2. *STAT1-expressing cells in the infiltrates. Double staining with antibodies to STAT1 and CD8$^+$ T cells in transverse frozen sections of muscle biopsy from a patient with PM* (top) *and a patient with a facioscapulohumeral dystrophy* (bottom). *CD8$^+$ cells are visualized by fluorescein-conjugated IgG* (a *and STAT1$^+$ staining by avidin-rhodamine* (b). *Double exposure of* a *and* b, *seen as yellow due to overlap of the green and red signal* (c), *reveals that all of the CD8$^+$ T cells also express STAT1 in both the PM and the dystrophy muscle specimens. Note that the muscle fibers are STAT1$^-$. Original magnification, ×400.*

to STAT1 was detected in all samples. After 24 hours of stimulation with different cytokines, a clear increase in the intensity of the band corresponding to STAT1 was observed in the lane loaded with the culture stimulated with recombinant human IFN-γ, whereas unstimulated cultures and those treated with EGF, PDGF, and IL-2 remained at baseline levels (Figure 4). A band of 53 kd corresponding to desmin illustrated similar loading in all samples.

To further confirm the immunohistochemical results and corroborate that the anti-STAT1 antibodies used were not reacting with any other cross-reactive protein other than STAT1, we studied its mRNA expression in muscle in culture and in TE671 cells after treatment with IFN-γ, EGF, PDGF, or IL-2 and untreated cells by means of semiquantitative RT-PCR. Two bands were obtained in all cases, one of 240 bp corresponding to STAT1 and the other of 218 bp corresponding to Abelson tyrosine kinase. In unstimulated cells, indicated in Figure 5 as controls, the ratio between the two bands was 1:1 at 20 and 25 cycles, whereas in IFN-γ-treated cells, there was a

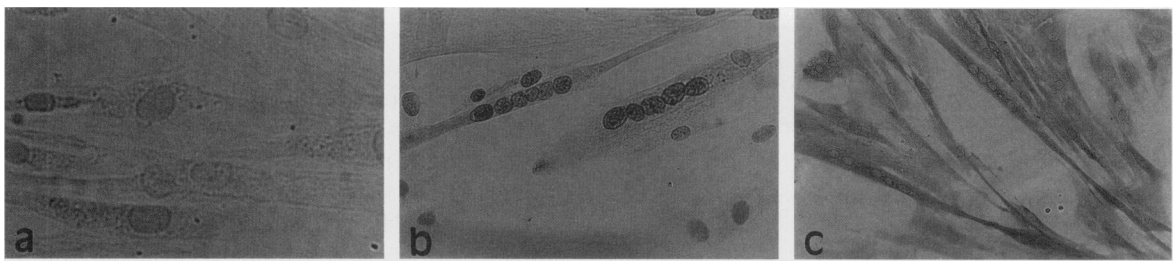

Figure 3. *Human muscle in tissue culture immunostained with antibodies to STAT1 using an immunoperoxidase technique.* a: *The myotubes show faint STAT1 staining before treatment.* b: *At 90 minutes after IFN-γ treatment, strong nuclear staining, indicating translocation into the nucleus, is noted.* c: *Diffuse cytoplasm staining is demonstrated after a longer exposure to IFN-γ (24 hours).*

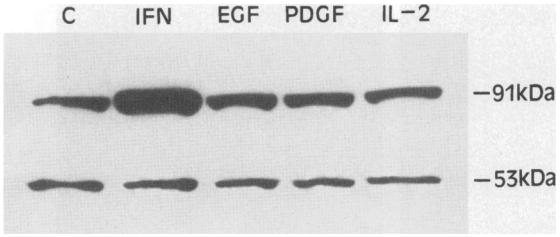

Figure 4. *Cytoplasmic extracts of TE671 were fractionated in a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. STAT1 protein (91-kd band) was detected using an anti-STAT1 antibody and visualized by chemiluminescence.* Lane 1, *untreated cells (CTRL);* lanes 2 *to* 5, *cells treated (24 hours) with IFN-γ, EGF, PDGF, and IL-2, respectively.* Lane 2 *shows the up-regulation of STAT1 after IFN-γ stimulation. Baseline levels of STAT1 detection are found in* lanes 3, 4, *and* 5 *stimulated by the other cytokines. The loading control is illustrated by a 53-kd band, which corresponds to desmin.*

predominance of the band corresponding to STAT1 after 20 and 25 cycles (exponential phase of the reaction; Figure 5), indicating that IFN-γ was able to increase the level of STAT1 mRNA in muscle cells in agreement with the immunohistochemical and Western blot studies.

Discussion

This is the first study to examine the STAT1 expression patterns in muscle tissue from patients with PM and DM and to study the activation of STAT1 in cultured muscle. Studies in other tissues have shown that STAT1 is up-regulated after IFN-γ stimulation, and it therefore appears to be a powerful marker of cytokine effect in the target cells.[25] Using immunohistochemistry, Western blot, and PCR, we have been able to demonstrate different patterns of STAT1 up-regulation. *In vivo*, there was an increase in STAT1 expression in DM, mainly in perifascicular atrophic muscle fibers, whereas most muscle fibers, including those invaded by $CD8^+$ T lymphocytes

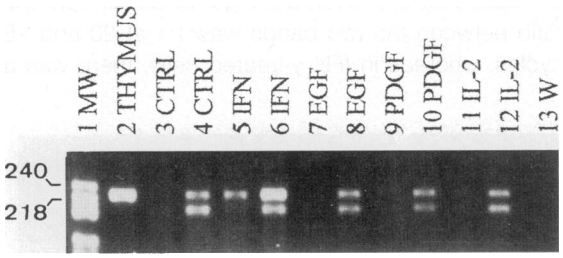

Figure 5. *Semiquantitative PCR amplification products obtained from RNA of muscle tissue in culture.* Lanes 3 *and* 4, *unstimulated cells;* lanes 5 *to* 12, *stimulated cells (24 hours) as indicated.* Lanes 3, 5, 7, 9, *and* 11, *20 cycles;* lanes 4, 6, 8, 10, *and* 12, *25 cycles.* Lane 2 *is a standard PCR control showing the expression of STAT1 mRNA in thymus after 30 cycles of amplification. The bands amplified correspond to STAT1 (240 bp) and Abelson tyrosine kinase (218 bp). Specific STAT1 amplification at 20 cycles (exponential phase of the reaction) is seen only after stimulation with IFN-γ* (lane 5).

and macrophages, were $STAT1^-$ in PM and controls. *In vitro*, IFN-γ, but none of the other cytokines examined, selectively stimulated STAT1 activation and transcription in myotubes and rhabdomyosarcoma cells.

The positive STAT1 staining obtained in the perifascicular fibers in DM indicates that this transduction pathway is activated by specific molecules on the sarcolemma of the muscle fibers. According to our results, cytokines released by inflammatory cells are probably responsible for triggering the response. In fact, we observed STAT1 translocation, which indicates activation, to the nuclei of both myotubes and TE671 cells after 90 minutes of stimulation of cultures with IFN-γ, followed by a marked increase in the amount of STAT1 in cytoplasm after 24 hours. This up-regulation of the protein persisted after a single stimulation throughout the 6 days of the study. Sustained increase was observed whether immunohistochemistry, Western blot, or PCR was used. That IFN-γ could have a major role was highlighted by the absence of STAT1 up-regulation in muscle *in vitro* after stimulation with either EGF or PDGF, which are known to activate this protein in other tissues.[17,18] An absence of up-regulation could indicate either a lack of specific receptors for these factors in cultured muscle or their inability to activate this STAT1 transduction pathway. A significant correlation between IFNs and STAT1 has been clarified by studying targeted disruption of the STAT1 gene in a mice model.[26,27] Although STAT1 is activated in most cells by different cytokines,[28,29] the knockout mice model indicates that STAT1 is indispensable for the IFN pathway but may not be necessary for other signaling systems *in vivo*.

Previous authors consider that muscle fiber atrophy and degeneration at the periphery of the fascicles in DM is due to microvascular depletion.[1–3] In fact, immunohistochemical studies have shown immunocomplexes and the deposit of C5b-9 complement membrane attack complex in the endomysial capillaries. Consequently, the marked reduction of the number of capillaries would lead to impaired perfusion and ischemia. Therefore, we studied ischemia as a possible STAT1 up-regulating factor in muscle fibers. In contrast with the staining seen in DM, however, none of the seven ischemic muscles showed evidence of STAT1 increase. This result would indicate that ischemia is not an up-regulating factor for STAT1 and suggests that factors that act to increase STAT1 in conjunction with ischemia are likely to be involved in perifascicular atrophy. Although humoral effector mechanisms predominate, a feature of DM is the presence of inflammation,

mainly perivascular and perimysial. These infiltrates are predominantly $CD8^+$ T cells and macrophages. Our results are consistent with a hypothesis that these lymphocytes may play a role in triggering the STAT1 perifascicular response through local secretion of cytokines such as IFN-γ.

Our observation that none of the hypertrophic, atrophic, or regenerating muscle fibers in the control biopsies express STAT1 indicates that the up-regulation of this molecule is unrelated to muscle development, maturation, or regeneration. The perifascicular muscle fibers in DM biopsies are very active in the expression of molecules, such as neural cell adhesion molecule and others,[19] that are activated by these processes. In our study, in addition to the muscle fibers from DM patients, some fibers undergoing necrosis in 2/10 samples from PM patients and from 2/10 patients with dystrophies showed some degree of staining. Although we do not know the functional significance of this increase in STAT1 in muscle fiber physiology, such up-regulation may in fact be associated with muscle fiber degeneration .

STAT1 is apparently not activated in muscle fibers from patients with PM. This striking finding suggests that the inflammatory mediators in DM and PM are different. The infiltrates of activated mononuclear cells, which are clearly more important in PM than in DM, express high levels of STAT1. It is to be expected that many of the $CD4^+$ and $CD8^+$ T cells would be secreting IFN-γ, which in turn would activate STAT1 in muscle fibers. Nevertheless, activated T cells and monocytes also secrete other cytokines that could trigger additional signaling pathways and interfere with the activation of STAT1.[30,31] Several studies have shown an increase of such cytokines as IL-4, transforming growth factor-β1, granulocyte/macrophage colony-stimulating factor, tumor necrosis factor-γ, or IFN-γ or their mRNA in both PM and DM specimens, although a consistent profile has not been found.[7,8] Therefore, the absence of STAT1 activation in PM, despite large mononuclear infiltrates, could be explained by a particular network of cytokines produced in this disorder.

By studying STAT1 as a downstream effector of cytokine activation, we have been able to show its differential up-regulation in DM and its specific *in vitro* stimulation by IFN-γ. With further study of the STAT family by this approach, we should also be able to unravel the cytokine-mediated immune processes involved in inflammatory myopathies and to elucidate whether or not the induction of specific genes by this transcription factor contributes to secondary autodestructive damage of the muscle fibers.

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