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Roles of proinflammatory cytokines and the Fas/Fas ligand interaction in the pathogenesis of inflammatory myopathies

Masahiro Kondo,[1] Yohko Murakawa,[1] Nanae Harashima,[2] Shotai Kobayashi,[3] Shuhei Yamaguchi[4] and Mamoru Harada[2]

[1]*Department of Rheumatology, Shimane University Faculty of Medicine, Izumo, Shimane,* [2]*Department of Immunology, Shimane University Faculty of Medicine, Izumo, Shimane,* [3]*Shimane University Hospital, Izumo, Shimane, and* [4]*Department of Neurology, Shimane University Faculty of Medicine, Izumo, Shimane, Japan*

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Correspondence: M. Harada, MD, PhD, Department of Immunology, Shimane University Faculty of Medicine, Izumo, Shimane 693-8501, Japan.
Email: haramamo@med.shimane-u.ac.jp
Senior author: Mamoru Harada

Summary

Within the lesions of inflammatory myopathies, muscle fibres and invading mononuclear cells express Fas and Fas ligand (FasL), respectively. However, the roles of the Fas/FasL interaction in the pathogenesis of inflammatory myopathies are not fully understood. In the present study, we investigated the roles of proinflammatory cytokines and the Fas/FasL system in the pathogenesis of inflammatory myopathies. *In vitro* culturing of muscle cells with the proinflammatory cytokines interferon-γ, tumour necrosis factor-α, and interleukin (IL)-1β synergistically increased Fas expression, susceptibility to Fas-mediated apoptosis, and the expression of cytoplasmic caspases 8 and 3. In addition, culturing of muscle cells with activated $CD4^+$ T cells induced muscle cell apoptosis, which was partially inhibited by anti-FasL antibody. We also tested the possibility that T helper (Th) 17, which is an IL-17-producing helper T-cell subset that plays crucial roles in autoimmune and inflammatory responses, participates in the pathogenesis of inflammatory myopathies. Interestingly, *in vitro* culturing of dendritic cells with anti-Fas immunoglobulin M (IgM) or activated $CD4^+$ T cells induced the expression of mRNA for IL-23p19, but not for IL-12p35, in addition to proinflammatory cytokines. Furthermore, IL-23p19 and IL-17 mRNAs were detected in the majority of biopsy samples from patients with inflammatory myopathies. Taken together, these results suggest that proinflammatory cytokines enhance Fas-mediated apoptosis of muscle cells, and that the Fas/FasL interaction between invading dendritic cells and $CD4^+$ T cells induces local production of IL-23 and proinflammatory cytokines, which can promote the proliferation of Th17 cells and enhance Fas-mediated apoptosis of muscle cells, respectively.

Keywords: dendritic cells; Fas; inflammatory myopathy; interleukin-23; T helper 17

Introduction

The immunological response is a central event in the pathogenesis of inflammatory myopathies, which include polymyositis (PM) and dermatomyositis (DM).[1] Regarding the underlying mechanism, we have previously reported that Fas-mediated apoptosis of muscle fibres occurs in both PM and DM.[2] Immunohistochemical analysis has revealed that Fas-expressing muscle fibres are surrounded by FasL-expressing mononuclear cells, and

Abbreviations: DC, dendritic cell; DM, dermatomyositis; FasL, Fas ligand; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; PI, propidium iodide; PM, polymyositis; TNF, tumour necrosis factor; TUNEL, terminal deoxynucleotidyl transferase nick-end labelling.

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apoptotic muscle cells have been detected by the terminal deoxynucleotidyl transferase nick-end labelling (TUNEL) method in some patients. In addition, many invading $CD4^+$ T cells, as well as $CD8^+$ T cells, are detected in the inflamed tissues of PM, and Fas ligand (FasL) is preferentially expressed on $CD4^+$ T cells. Despite reports showing the expression of Fas and FasL,[3–5] it remains unclear whether Fas-mediated apoptosis occurs in inflamed muscle tissues,[6–8] and the roles that the Fas/FasL interaction plays in disease pathogenesis have not been fully elucidated.

Fas, which is a 45-kDa membrane protein that belongs to the tumour necrosis factor (TNF) receptor superfamily, is constitutively expressed in many cell types.[9] The ligand for Fas, FasL, is expressed on activated T cells and natural killer (NK) cells, as well as at some immune-privileged sites.[10–12] When cross-linked with FasL, Fas transduces an apoptotic signal. The Fas/FasL system is essential for homeostasis of the immune system, and its impairment leads to autoimmune disease.[13,14] Several reports suggest a stimulatory function for Fas-mediated signalling. Murine macrophages can be activated by FasL stimulation,[15] and Fas-mediated signalling can induce human dendritic cells (DCs) to produce proinflammatory cytokines.[16] These lines of evidence suggest the possibility that the Fas/FasL interaction in inflamed tissues of inflammatory myopathies results not only in muscle fibre apoptosis but also in the promotion of inflammatory responses.

In the present study, we investigated whether proinflammatory cytokines influence Fas-mediated apoptosis of muscle fibers, as they have been detected in the inflamed tissues of inflammatory myopathies.[17–20] We also tested the hypothesis that the Fas/FasL interaction in the lesions of inflammatory myopathies results in the activation of inflammatory responses, by focusing on interleukin (IL)-23 and T helper (Th) 17, which is a new helper T-cell subset that is responsible for autoimmune and inflammatory responses.[21,22] We reveal that proinflammatory cytokines enhance Fas-mediated apoptosis of muscle cells, and that the Fas/FasL interaction between invading DCs and $CD4^+$ T cells induces the local production of IL-23 and proinflammatory cytokines, which in turn promote the proliferation of Th17 cells and enhance Fas-mediated apoptosis of muscle cells, respectively.

Materials and methods

Cultured human skeletal muscle cells

Human skeletal muscle cells (Lonza, Walkerscille, MD) were cultured in gelatin-coated plastic dishes (Iwaki, Tokyo, Japan) with Dulbecco's modified Eagle's medium (Nissui Pharmaceuticals, Tokyo, Japan) that contained 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Corp., Carlsbad, CA), 10% heat-inactivated donor horse serum (ICN Biomedicals, Seattle, WA), 2 mM L-glutamine (Invitrogen Corp.), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen Corp.), and 1·25 μg/ml amphotericin B (Nacalai, Kyoto, Japan). The muscle cells used for the experiments were grown to confluence in a humidified 5% carbon dioxide atmosphere at 37°.

Antibodies

We used CH11 [immunoglobulin M (IgM); Beckman Coulter, Marseille, France] as the anti-Fas IgM monoclonal antibody (mAb) and MOPC 104 (Sigma-Aldrich, St Louis, MO) as the IgM-type control antibody. Anti-Fas mAb (AbD Serotec, Oxford, UK) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (H + L) antibody were used to detect the expression of Fas by cultured skeletal muscle cells. Anti-caspase 3 mAb (Transduction Laboratories, Lexington, KY) and anti-caspase 8 mAb (MBL, Nagoya, Japan) were used to detect caspase expression by western blotting. Polyclonal anti-caspase 8 (Mch5 p20; Santa Cruz Biotechnology, Santa Cruz, CA), anti-caspase 3 (CPP32; Santa Cruz Biotechnology), and control goat polyclonal IgG (Vector Laboratories, Burlingame, CA) were used for immunohistochemistry.

Cytokines

IL-1β (PeproTech, London, UK), interferon (IFN)-γ (donated by Shionogi Pharmaceuticals, Osaka, Japan), and TNF-α (donated by Asahikasei Industries, Osaka, Japan) were used to examine the effects of these cytokines on cultured muscle cells.

Preparation of purified $CD4^+$ T cells

Heparinized venous blood samples were collected from healthy volunteers and separated into peripheral blood mononuclear cells (PBMC) by Ficoll Hypaque density gradient centrifugation. The PBMC were cultured with 5 μg/ml phytohaemagglutinin (PHA) and 100 U/ml human recombinant IL-2 (PeproTech) for 3 days. Subsequently, PHA-activated $CD4^+$ T cells were isolated using the CD4-positive isolation kit (Dynal, Oslo, Norway). The purity of the obtained $CD4^+$ T-cell population exceeded 90%, as assessed by flow cytometry.

Detection of Fas expression

Cells were cultured in the presence or absence of cytokines and detached from culture dishes with phosphate-buffered saline (PBS; Nissui Pharmaceuticals) that contained 0·025% trypsin (Invitrogen Corp.) and 0·01%

ethylenediaminetetraacetic acid (EDTA; Invitrogen Corp.). After three washes with PBS that contained 1% heat-inactivated FBS and 0·1% sodium azide (Nacalai), the cells were incubated with phycoerythrin (PE)-conjugated anti-Fas mAb or isotype-matched control mAb for 40 min on ice. After washing, the cells were immediately assessed by flow cytometry.

Detection of apoptotic cells

Skeletal muscle cells were precultured with or without the indicated cytokines for 48 hr, and then incubated with anti-Fas IgM mAb (1 μg/ml) or a control IgM mAb for the indicated time. The cells were then detached from the culture dishes and washed as described above. Apoptotic muscle cells were stained with Annexin V and propidium iodide (PI) (Beckman Coulter) and analysed by flow cytometry.

Caspase detection by western blotting

Cultured human skeletal muscle cells were lysed in lysis buffer that contained 1% Nonidet P-40 (Nacalai), 0·3 M NaCl, 0·05 M Tris, 1 mM phenylmethylsulphonyl fluoride (PMSF; Wako Pure Chemical Industries, Osaka, Japan), 10 μg/ml leupeptin (Roche Diagnostics, Mannheim, Germany), and 10 μg/ml aprotinin (Roche Diagnostics). The lysates were centrifuged at 13 000 ***g*** for 15 min to remove insoluble material, and the protein concentrations of the extracts were determined by colorimetric bicinchoninic acid analysis (Pierce, Rockford, IL). One hundred and fifty micrograms of protein was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (9% for caspase 8 and 15% for caspase 3) under reducing conditions, and electroblotted onto nitrocellulose membranes. The membranes were treated sequentially with anti-caspase 8 mAb (1 μg/ml) or anti-caspase 3 mAb (0·25 μg/ml), alkaline phosphatase-conjugated anti-mouse IgG (1 : 1000; Bio-Rad Laboratories, Hercules, CA), and a substrate-containing buffer [0·165 mg/ml 5-bromo-4-chloro-3-iodolylphosphate-*p*-toluidine salt, 0·33 mg/ml nitroblue tetrazolium chloride, 0·1 M Tris (pH 9·5), 0·2 M NaCl and 0·1 M $MgCl_2$].

To examine the expression of the active form of caspase 3, protein aliquots (50 μg) were subjected to SDS-PAGE (12%), and western blotting was performed using anti-caspase 3 mAb (3G2; Cell Signaling Technology, Danvers, MA), which can detect both the full-length and cleaved forms of caspase 3, and the WesternBreeze Chemiluminescent Immunodetection System (Invitrogen Corp.). The expression value was determined by densitometry using IMAGEJ software (http://rsb.info.nih.gov/ij/index.html) and the ratio of the cleaved form to the full form of caspase 3 was calculated.

Patients

The study was approved by the Ethics Review Board of Shimane University Faculty of Medicine (study no. 373). All of the patients gave written informed consent for sample collection in accordance with institutional policy. Muscle biopsy specimens were taken from nine patients with PM, three patients with DM, and three control subjects with normal histological findings. The controls had no clinical evidence of muscle disease. Each diagnosis was confirmed clinically and with routine histological preparations of the muscle biopsy. All of the patients were designated as 'definite' according to the criteria of Bohan and Peter.[23]

Immunohistochemistry

Serial 3·0-mm-thick sections were air-dried and fixed in acetone for 10 min. The samples were incubated with normal rabbit serum (Vector Laboratories) for 30 min. The samples were then incubated with 10 μg/ml polyclonal antibody against caspase 8 (Mch5 p 20), caspase 3 (CPP32) or purified control goat IgG. After washing with PBS, the sections were incubated with biotinylated rabbit anti-goat IgG (Vector Laboratories) for 30 min. After extensive washing, all the sections were exposed to avidin–biotin–peroxidase complex (Vector Laboratories) for 30 min, covered with diaminobenzidine tetrahydrochloride for 2 min, and counterstained with haematoxylin.

Cytotoxicity assay using activated $CD4^+$ T cells and muscle cells

Skeletal muscle cells (1×10^5) were cultured with IL-1β (1 ng/ml), TNF-α (100 U/ml), and IFN-γ (100 U/ml) in 60-mm dishes (Asahi Glass, Tokyo, Japan) for 2 days. Then, PHA-activated $CD4^+$ T cells ($1{\cdot}5 \times 10^5$) were added with or without anti-FasL mAb (mouse IgG2a; BD Biosciences, Franklin Lakes, NJ) or control anti-Bcl-X_L mAb (mouse IgG2a; Trevigen, Gaithersburg, MD) at a dose of 5 μg/ml. After 24 hr of culture, whole cells were harvested and examined by flow cytometry using FITC-conjugated Annexin V and PE-conjugated anti-CD4 mAb (Beckman Coulter). $CD4^-$, Annexin V^+ cells were deemed to be apoptotic muscle cells.

Preparation of DCs and monocytes

To prepare immature DCs, adherent PBMC from a healthy donor were cultured in 24-well plates in the presence of IL-4 (500 U/ml) (PeproTech) and granulocyte–macrophage colony-stimulating factor (GM-CSF) (500 U/ml; PeproTech) for 6 days. To prepare monocytes, adherent PBMC from a healthy donor were harvested in EDTA/trypsin using a cell scraper.

Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA samples from DCs, monocytes, and biopsies of inflammatory myopathies were extracted using the TRIzol Reagent (Invitrogen Corp.). The RNA samples were treated with DNase I (amplification grade; Invitrogen Corp.) to remove residual genomic DNA. Two total RNA samples from normal human skeletal muscle were purchased from Clontech (Palo Alto, CA) and from BioChain (Hayward, CA). cDNAs were synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen Corp.) for RT-PCR and oligo$(dT)_{20}$ primer. One microgram of RNA was reverse-transcribed into cDNA. PCR amplification was carried out in 30 μl of PCR mixture that contained cDNA template aliquots, 0·2 mM dNTPs, 1·5 mM $MgCl_2$, 1 U Platinum Taq polymerase (Invitrogen Corp.), and 0·2 μM of the sense and antisense primers. The following primers (sense and antisense, respectively) were used: for *IL-23p19*, 5′-gttccccatatccagtgtgg-3′ and 5′-gaggcttggaatctgctgag-3′; for *IL-12p35*, 5′-gccctgtgccttagtagtat-3′ and 5′-gctcgtcactctgtcaatag-3′; for *IL-12/23p40*, 5′-atgtcgtagaattggattggtatccg-3′ and 5′-gtactgattgtcgtcagccaccagc-3′; for *IL-1β*, 5′-atggcagaagtacctaagctcgc-3′ and 5′-acacaaattgcatggtgaagtcagtt-3′; for *TNF-α*, 5′-atgagcactgaaagcatgatccgg-3′ and 5′-gcaatgatcccaaagtagacctgccc-3′; for *IFN-γ*, 5′-gcatcgttttgggttctcttggctgttactgc-3′ and 5′-ctccttttcgcttccctgttttagctgctgg-3′; for *IL-6*, 5′-atgaactccttctccacaagcgc-3′ and 5′-gaagagccctcaggctggactg-3′; for *IL-17*, 5′-gaaggcaggaatcacaatc-3′ and 5′-cccacggacaccagtatct-3′; and for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), 5′-acaacagcctcaagatcatcag-3′ and 5′-ggtccaccactgacacgttg-3′.

PCR was performed for the indicated number of cycles using the iCycler thermal cycler (Bio-Rad Laboratories). The PCR products were analysed on a 2% agarose gel.

Results

Effects of proinflammatory cytokines on Fas expression by cultured skeletal muscle cells

Proinflammatory cytokines, including IFN-γ, TNF-α and IL-1β, are present in the inflamed tissues of idiopathic inflammatory myopathies.[17–20] Therefore, we investigated the effects of these cytokines on Fas expression by skeletal

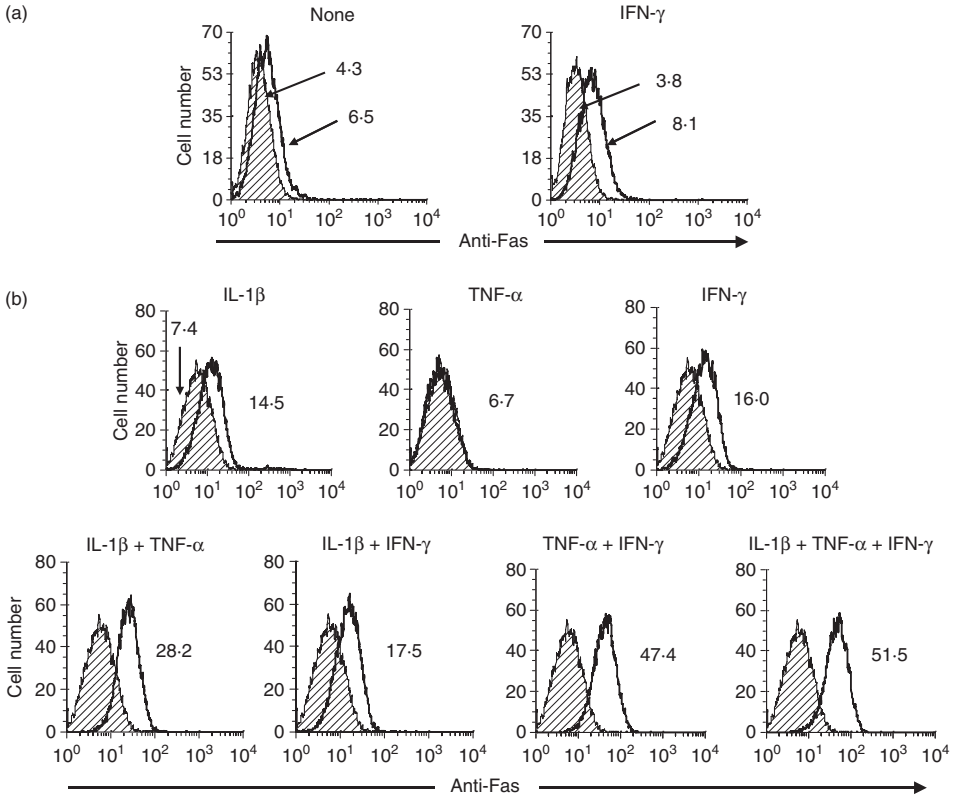

Figure 1. Effects of proinflammatory cytokines on Fas expression by cultured muscle cells. (a) The skeletal muscle cells were incubated with or without interferon (IFN)-γ (100 U/ml) for 48 hr, and then examined for Fas expression by flow cytometry. Controls that contained secondary antibody alone (oblique lines) are also shown. The number in parentheses is the mean fluorescence intensity (MFI). (b) Skeletal muscle cells were incubated with the indicated cytokines for 48 hr, and then examined for Fas expression by flow cytometry. The concentrations of interleukin (IL)-1β, tumour necrosis factor (TNF)-α, and interferon (IFN)-γ used were 1 ng/ml, 100 U/ml, and 100 U/ml, respectively. A control without any cytokine is indicated by the oblique line. The number in parentheses is the MFI.

muscle cells. Fas expression was detected on the surfaces of muscle cells cultured without cytokines, and IFN-γ treatment (100 U/ml) increased the levels of surface Fas expression (Fig. 1a). IFN-γ up-regulated Fas expression in a time-dependent manner, with peak expression occurring after 48 hr (data not shown). We also examined the synergistic effects of IFN-γ, TNF-α and IL-1β on Fas expression by muscle cells in relation to Fas expression by untreated muscle cells (Fig. 1b). IL-1β increased Fas expression, whereas TNF-α alone did not. The combination of TNF-α and IFN-γ increased Fas expression synergistically, and the triple combination of IL-1β, TNF-α and IFN-γ produced the largest increase in Fas expression.

Effects of proinflammatory cytokines on Fas-mediated apoptosis

Several cytokines are produced within the lesions of inflammatory myopathies.[17–20] Therefore, we investigated the synergistic effects of proinflammatory cytokines on Fas-mediated apoptosis of muscle fibre cells (Fig. 2). The Fas signal was provided by anti-Fas IgM mAb, and apoptosis was evaluated by flow cytometry with Annexin V and PI staining. Although Annexin V^+ PI^+ cells are often called secondary necrotic cells and are probably derived from apoptotic cells that were not cleared before the membrane became damaged and unable to exclude PI, both the Annexin V^+ PI^- and Annexin V^+ PI^+ cells were deemed to be apoptotic in this assay. Although increased apoptosis was observed when the cells were precultured in the presence of TNF-α, the combination of the three cytokines resulted in the most efficient induction of Fas-mediated apoptosis of the muscle fibre cells (Fig. 2a). A representative result is shown in Fig. 2b. After 24 hr of culture with the anti-Fas IgM mAb, approximately 80% of the muscle cells that were precultured with the three cytokines were positive for Annexin V staining. These cells showed characteristic apoptotic changes, such as condensation of chromatin and formation of apoptotic bodies (Fig. 2c). In contrast, the muscle cells that were not treated with cytokines showed approximately 8% apoptosis, even after 24 hr of incubation.

Effects of proinflammatory cytokines on the expression of caspases 3 and 8

We investigated the effects of proinflammatory cytokines on the expression levels of caspases 3 and 8, which are essential for Fas-mediated apoptosis. The skeletal muscle cells were cultured for 48 hr with or without the indicated cytokines, and the expression levels of caspase 8 and caspase 3 were examined by western blotting (Fig. 3a). Caspase 8 was not detected in the unstimulated muscle cells, while IFN-γ alone significantly up-regulated the expression of caspase 8. TNF-α induced weak expression of caspase 8, whereas IL-1β did not. The expression of caspase 3 in unstimulated muscle cells was weak, and its expression was increased by either or both of TNF-α and IFN-γ. These results indicate that, among the three cytokines tested, IFN-γ is crucial for the up-regulation of caspase 8.

Although TNF-α alone could not augment the Fas expression of muscle cells (Fig. 1b), TNF-α alone increased the sensitivity of muscle cells to Fas-mediated apoptosis (Fig. 2a). To address the mechanism by which TNF-α influenced Fas-mediated apoptosis, we next examined the effect of TNF-α on the active form of caspase 3

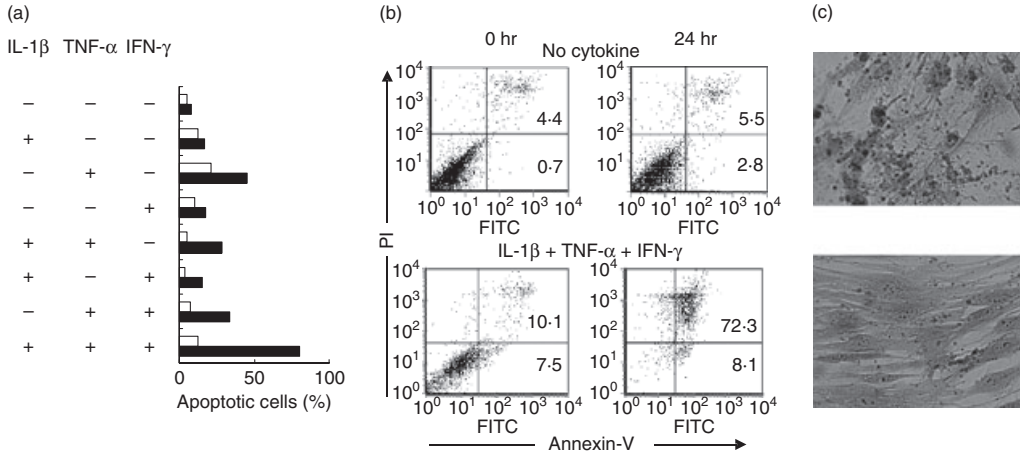

Figure 2. Effects of proinflammatory cytokines on the Fas-mediated apoptosis of cultured muscle cells. (a) Muscle cells that were pretreated with or without interleukin (IL)-1β (1 ng/ml), tumour necrosis factor (TNF)-α (100 U/ml) and/or interferon (IFN)-γ (100 U/ml) for 48 hr were cultured in the presence of anti-Fas immunoglobulin M (IgM) (closed bars) or a control antibody (open bars). Annexin V^+ cells were deemed to be apoptotic. (b) Muscle cells that were pretreated with or without the three cytokines for 48 hr were cultured in the presence of anti-Fas IgM and examined by flow cytometry. (c) Muscle cells that were pretreated with the three cytokines for 48 hr were cultured in the presence of anti-Fas IgM (upper image) or a control antibody (lower image). Haematoxylin and eosin staining was used (magnification ×400).

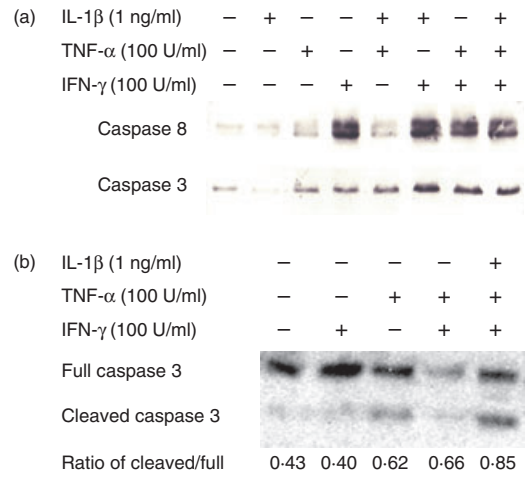

Figure 3. Effects of proinflammatory cytokines on the induction of caspases 3 and 8 in cultured muscle cells. (a) The proteins were extracted from muscle cells that were pretreated with the three cytokines for 48 hr. Protein aliquots (150 μg) were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (9% for caspase 8 and 15% for caspase 3) under reducing conditions, and were analysed by western blotting. The molecular masses of caspase 8 and caspase 3 were 55 and 54 kDa, respectively. (b) The proteins were extracted from muscle cells that were pretreated with or without the cytokines for 48 hr. Protein aliquots (50 μg) were subjected to SDS-PAGE (12%), and were analysed by western blotting using anti-caspase 3 monoclonal antibody which can detect both the full-length and the cleaved forms of caspase 3. The number represents the ratio of the cleaved form to the full form of caspase 3.

in muscle cells. The muscle cells were cultured for 48 hr with or without the indicated cytokines, and the expression level of the cleaved form of caspase 3 was examined by western blotting (Fig. 3b). Cleaved caspase 3 was faintly detected in the unstimulated or IFN-γ-stimulated muscle cells, while TNF-α alone significantly up-regulated the expression of cleaved caspase 3. The expression of cleaved caspase 3 was increased most effectively when the muscle cells were cultured with the triple combination of IL-1β, TNF-α and IFN-γ. These results indicate that TNF-α alone activates the caspase pathway.

Expression of caspases 3 and 8 in biopsy samples from patients with inflammatory myopathies

We next examined the expression of caspase 8 and caspase 3 in biopsy samples from inflammatory myopathies; a representative result is shown in Fig. 4. In a biopsy sample from a PM patient, the expression of caspase 8 was up-regulated in some atrophic muscle fibres (Fig. 4a), as compared with normal muscle (Fig. 4b). The expression of caspase 3 was strong in some of the muscle fibres of the inflamed muscle tissues (Fig. 4c), as compared with a normal control (Fig. 4d). The proportion of caspase 3-positive muscle fibres ranged from 1·6 to 8·6%. Longitudinal

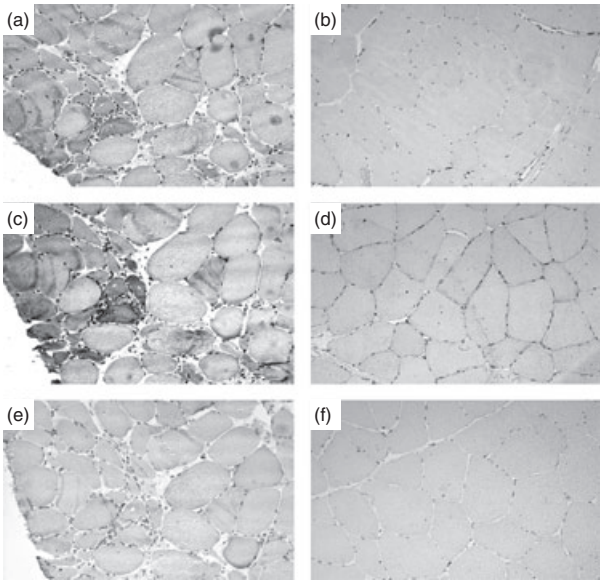

Figure 4. Immunohistochemical analysis of caspases 3 and 8 in muscle tissues. Biopsy samples from a polymyositis (PM) patient (left) and a normal control (right) were stained with anti-caspase 8 (a and b), anti-caspase 3 (c and d) or a control antibody (e and f). Magnification × 200.

sections revealed that caspase 3 overexpression occurred throughout the muscle fibre (data not shown). The control antibody did not stain muscle fibres from either the PM patient or the normal control (Fig. 4e,f). Similar findings were observed for five out of nine PM patients and all three DM patients (data not shown). These data suggest that caspase 8 and caspase 3 are induced in the inflamed lesions of some PM and DM patients.

Induction of Fas-mediated apoptosis of cytokine-stimulated muscle cells by activated CD4$^+$ T cells

Previously, we showed that most of the FasL-expressing cells in the inflammatory foci of PM and DM are CD4$^+$ T cells.[2] Therefore, in the present study, we investigated whether FasL-expressing, activated CD4$^+$ T cells could induce Fas-mediated apoptosis of cytokine-stimulated muscle fibre cells. Activated CD4$^+$ T cells were prepared from PBMC that were cultured with PHA and IL-2 (100 U/ml) for 3 days. Cytokine-stimulated muscle cells were cultured for 24 hr in the presence of activated CD4$^+$ T cells with either the anti-FasL mAb or a control mAb. Thereafter, whole cells were harvested, and the Annexin V expression of the CD4$^-$ subset was examined for apoptotic muscle cells. Co-culturing with activated CD4$^+$ T cells increased the percentage of apoptotic muscle cells (Fig. 5). In addition, the activated CD4$^+$ T-cell-mediated apoptosis of muscle cells was partially blocked by the addition of anti-FasL mAb, but not by the addition of the

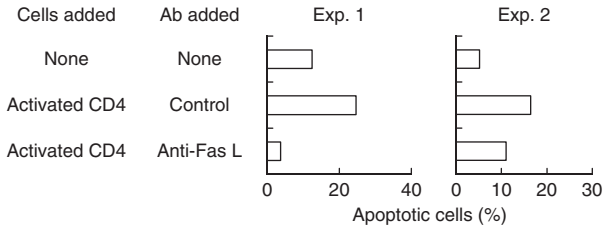

Figure 5. Fas-mediated apoptosis of muscle fibre cells by activated $CD4^+$ T cells. Muscle cells were pretreated with interleukin (IL)-1β (1 ng/ml), tumour necrosis factor (TNF)-α (100 U/ml), and interferon (IFN)-γ (100 U/ml) for 48 hr. Then, phytohaemagglutinin (PHA)-activated purified $CD4^+$ T cells were added and cultured for an additional 24 hr in the presence of anti-FasL monoclonal antibody (mAb) or a control mAb. Subsequently, whole cells were harvested and the percentages of apoptotic cells were determined by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated Annexin V and phycoerythrin (PE)-conjugated CD4 mAb. Annexin V^+, $CD4^-$ cells were deemed to be apoptotic muscle cells. The data from two separate experiments are shown.

control antibody. These results suggest that FasL-expressing, activated $CD4^+$ T cells induce Fas-mediated apoptosis of inflamed muscle cells.

Fas-mediated production of proinflammatory cytokines by DCs

Although Fas-mediated signalling is usually pro-apoptotic, the Fas molecules can also provide human DCs with stimulatory signals.[16] Therefore, we investigated the effect of Fas-mediated signalling on the production of proinflammatory cytokines by DCs, focusing on IL-23, which is a key cytokine in autoimmune and inflammatory responses.[21,22] Immature DCs and monocytes were prepared from the PBMC of a healthy donor. Human DCs and monocytes are positive for Fas molecules, as reported previously.[16,24] The DCs and monocytes were cultured with anti-Fas IgM or a control IgM, and the levels of IL-23p19 mRNA were examined by RT-PCR (Fig. 6a). As IL-23 is a member of the IL-12 cytokine family and both IL-23 and IL-12 share IL-12/23p40,[25] we examined the expression levels of IL-23p19, IL-12p35 and IL-12/23p40 mRNA. Interestingly, anti-Fas IgM induced IL-23p19 mRNA, but not IL-12p35 mRNA, in DCs. Anti-Fas IgM had no effect on IL-23p19 mRNA and decreased IL-12p35 mRNA expression in monocytes. Anti-Fas IgM enhanced the expression of IL-12/23p40 mRNA in the DCs, but appeared to decrease the levels of mRNA expression in monocytes. We also examined the expression of mRNA for other proinflammatory cytokines in Fas-stimulated DCs. We found that anti-Fas IgM increased the expression of mRNA for IL-1β, TNF-α and IL-6, but not for IFN-γ (Fig. 6b). These results indicate that Fas-mediated signalling stimulates DCs, resulting in the production of IL-23, as well as IL-1β, TNF-α and IL-6. We sought direct evidence that activated $CD4^+$ T cells could induce IL-23 and other proinflammatory cytokines in DCs via the Fas/FasL interaction (Fig. 6c). The *in vitro* culture of DCs with activated $CD4^+$ T cells increased levels of mRNA for IL-23p19, IL-1β, TNF-α and IL-6. The increased mRNA expression of these cytokines, as well as that of IL-12/23p40, was inhibited by the addition of anti-FasL mAb. These results indicate that activated $CD4^+$ T cells induce IL-23 and other proinflammatory cytokines in DCs via the Fas/FasL interaction.

Expression of IL-23 and IL-17 mRNAs in biopsy samples of inflammatory myopathies

We investigated whether the mRNAs for IL-23 and IL-17 were present in the biopsy samples of inflammatory myopathies (Fig. 6d). Tissues from five PM patients and one DM patient were used in this examination. The expression of IL-23p19 mRNA was detected in all the samples, although the expression levels varied. The expression of IL-12p35 mRNA correlated with that of IL-23p19 mRNA. We also examined the mRNA expression of IL-17, as IL-17-producing helper T cells (so-called Th17 cells) are thought to play an important role in autoimmune responses.[21,22] IL-17 mRNA was detected in four out of five PM patients, but not in the DM patient. We also investigated whether the IL-23p19, IL-12/23p40 and IL-17 mRNAs were present in human normal muscle tissues (Fig. 6e). In contrast to the control PBMC, these tissues showed faint expression of IL-12p35 mRNA but lacked expression of IL-23p19, IL-12/23p40 and IL-17 mRNA. Although cytokine messages were detected in the control PBMC, they were not activated by any stimulus. We postulate that these cytokine messages are expressed constitutively in a fraction of PBMC isolated from conventional healthy donors, and we ascribed this result to the difference in frequencies of cytokine-producing cells between PBMC and muscle tissues.

Discussion

Many studies have described the expression of Fas and FasL in the lesions of inflammatory myopathies.[3–5] Fas expression is induced on the muscle fibres in several muscle disorders, including inflammatory myopathies, muscular

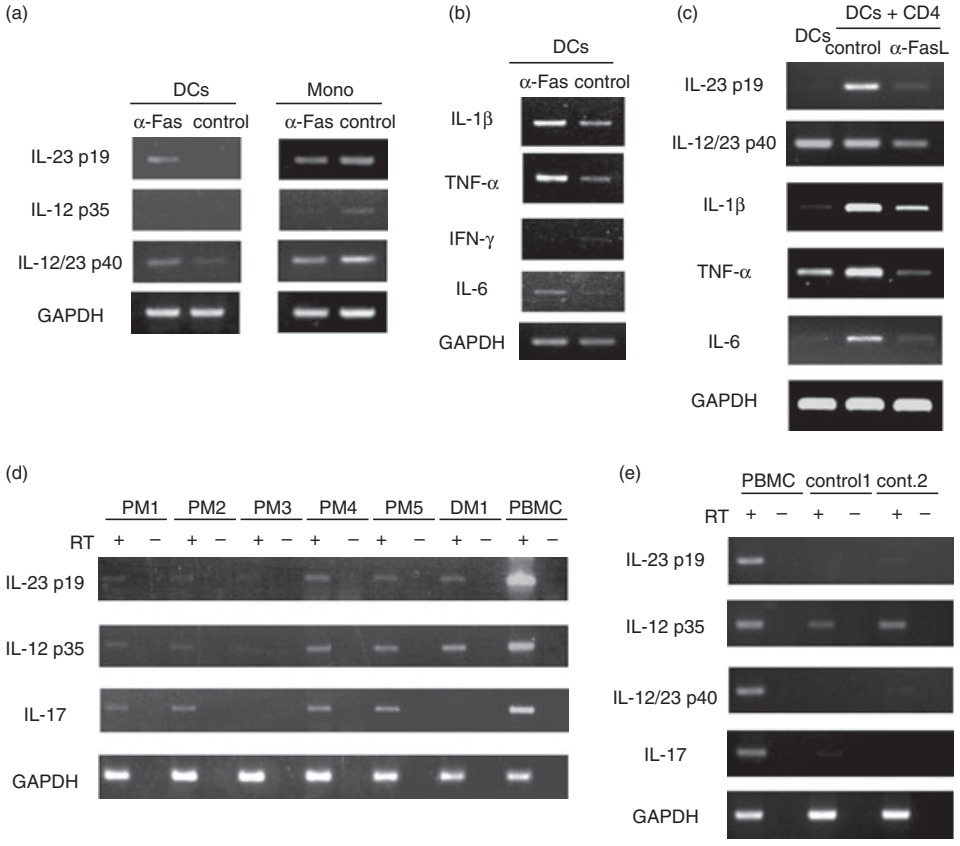

Figure 6. Fas-mediated induction of the mRNA for proinflammatory cytokines in dendritic cells (DCs) and the expression of interleukin (IL)-23 and IL-17 mRNA in inflamed muscle tissues from patients. (a) Immature DCs and adherent monocytes (Mono) were examined for the expression of IL-23p19, IL-12p35 and IL-12/23p40 mRNA after *in vitro* culture with anti-Fas immunoglobulin M (IgM) or control IgM for 20 hr. The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was examined as a control. Reverse transcription–polymerase chain reaction (RT-PCR) was performed for 35 cycles. (b) Immature DCs were examined for the expression of IL-1β, tumour necrosis factor (TNF)-α, interferon (IFN)-γ, and IL-6 mRNA after *in vitro* culture with anti-Fas IgM or control IgM for 20 hr. GAPDH mRNA expression was examined as a control. RT-PCR was performed for 33 cycles for IL-1β, IFN-γ and IL-6, and for 30 cycles for TNF-α and GAPDH. (c) Immature DCs were cultured with phytohaemagglutinin (PHA)-activated purified $CD4^+$ T cells in the presence of anti-FasL monoclonal antibody (mAb) or control IgG for 20 hr. Then, the immature DCs were examined for the expression of IL-23p19, IL-12/23p40, IL-1β, TNF-α and IL-6 mRNA after depletion of the $CD4^+$ T cells. RT-PCR was performed for 30 cycles for IL-23p19 and IL-6, and for 35 cycles for IL-12p40, IL-1β, TNF-α and GAPDH. (d) Tissue samples from five polymyositis (PM) patients and one dermatomyositis (DM) patient were examined for the expression of IL-23p19, IL-12p35 and IL-17 mRNA. Peripheral blood mononuclear cells (PBMC) were used as a control sample. The expression of GAPDH mRNA was examined as a control. RT-PCR was performed for 30 cycles for IL-23p19 and GAPDH, and for 35 cycles for IL-12p35 and IL-17. cDNA was transcribed from RNA in the presence (+) or absence (−) of reverse transcriptase (RT). (e) PBMC and two normal muscle tissues were examined for the expression of IL-23p19, IL-12p35, IL-12/23p40 and IL-17 mRNA. The expression of GAPDH mRNA was examined as a control. RT-PCR was performed for 30 cycles for IL-23p19 and GAPDH, and for 35 cycles for IL-12p35, IL-12/23p40 and IL-17. cDNA was transcribed from RNA in the presence (+) or absence (−) of RT.

dystrophy, and neurogenic disorders.[2,3,6,26] FasL expression has been detected on invading mononuclear cells, mainly $CD4^+$ T cells.[2,7] However, whether or not the Fas/FasL interaction in the lesions results in muscle cell apoptosis has remained a subject of controversy. Some groups have concluded that, although FasL-expressing mononuclear cells come into contact with Fas-positive muscle fibres, the Fas/FasL interaction does not induce muscle apoptosis, as a consequence of the up-regulated expression of anti-apoptotic proteins, such as bcl-2, inhibitors of apoptosis proteins (IAP)-like protein, and Fas-associated death domain-like IL-1beta-converting enzyme-inhibitory protein (FLIP).[3,4,27] In contrast, we have reported previously that the Fas/FasL interaction between inflamed muscle fibres in the inflammatory myopathies and the surrounding FasL-expressing mononuclear cells results in muscle apoptosis, based on the results of the TUNEL method and DNA fragmentation assay.[2] The proportion of TUNEL-positive muscle fibres is relatively small, probably because phagocytes promptly clear apoptotic muscle cells *in vivo*. In any case, the roles of the Fas/FasL interaction in the pathogenesis of inflammatory myopathies have not been fully elucidated to date.

Proinflammatory cytokines accumulate in the muscle tissues of inflammatory myopathies, and are proposed to play an important role in disease pathogenesis.[17–20] Therefore, we examined the effects of proinflammatory cytokines on Fas-mediated apoptosis of muscle fibre cells. We found that IFN-γ enhanced Fas expression on cultured muscle cells (Fig. 1a). These results are consistent with observations of cells from gliomas,[28] breast cancers,[29] and hepatomas,[30] and of normal epithelial cells.[31] IL-1β increased Fas expression, whereas TNF-α alone did not. The combination of TNF-α and IFN-γ increased Fas expression synergistically, and the triple combination of IL-1β, TNF-α and IFN-γ produced the largest increase in Fas expression. Furthermore, we found that cross-linking Fas with anti-Fas IgM induced the apoptosis of muscle cells that were pretreated with proinflammatory cytokines (Fig. 2). In addition, FasL-expressing, activated $CD4^+$ T cells induced the apoptosis of cytokine-pretreated muscle cells in a FasL-dependent manner (Fig. 5). These results suggest that muscle cells undergo apoptosis in inflammatory foci *in vivo* at sites of invasion of FasL-expressing T cells.

Recent studies have revealed an IL-17-producing $CD4^+$ T-cell subpopulation, termed Th17, which is distinct from Th1 and Th2 cells.[21,22] IL-17, which is a proinflammatory cytokine, is produced mainly by activated T cells, enhances T-cell priming, and stimulates many types of cells to produce multiple proinflammatory cytokines. The IL-23/Th17 axis is considered to play a central role in a variety of autoimmune and inflammatory diseases.[21,22] IL-23, which is an IL-12 family cytokine that consists of the p19 and p40 subunits, shares the p40 subunit with IL-12.[25] Current evidence suggests that IL-23 is responsible for the differentiation and expansion of Th17 cells from naïve $CD4^+$ T cells. With regard to DM and PM, Page *et al.*[32] have detected IL-17-expressing T cells and DCs in inflamed muscle tissues. Intriguingly, Caproni *et al.*[33] have reported granulocyte infiltration of the cutaneous lesions of DM, which suggests local production of IL-17, as this cytokine can attract granulocytes. However, several reports have indicated that Fas-mediated signalling induces IL-23 production in murine DCs,[15] and that Fas engagement induces the maturation of human DCs, resulting in the release of proinflammatory cytokines.[16] However, there have been no reports on whether Fas-mediated stimulation can induce IL-23 production in human DCs. In the present study, we show that Fas stimulation of immature human DCs induces IL-23p19 mRNA, but not IL-12p35 mRNA, as well as other proinflammatory cytokines, including IL-1β, TNF-α and IL-6 (Fig. 6). These results reveal new roles for the Fas/FasL interaction in the pathogenesis of inflammatory myopathies. Thus, besides Fas-mediated apoptosis of muscle cells, invading FasL-expressing T cells cause infiltrating DCs to produce IL-23 and other proinflammatory cytokines, thereby helping the Th17 cells to proliferate and increasing Fas expression on inflamed muscle cells. Interestingly, a recent study of the intestines of patients with Crohn's disease has revealed a helper T-cell subset that can produce both IFN-γ and IL-17.[34] These helper T cells have the ability to promote potent autoimmune and inflammatory responses *in vivo*. Further analysis of this cell type is needed to understand fully the pathogenesis of inflammatory myopathies.

It is important to speculate about the IFN-γ-producing cells in the myopathic lesions. Since $CD4^+$ T or $CD8^+$ T cells have been detected in the myopathic lesions,[2,35] they must be the major IFN-γ-expressing cells. Although NK and NKT cells produce IFN-γ, there is no report of their presence in myopathic lesions. Although macrophages and DCs have been detected in myopathic lesions,[32] we do not believe that they are the sources of IFN-γ.

Although the immunological response is a central event in the pathogenesis of inflammatory myopathies,[1] the pathogenetic mechanisms underlying tissue injury are strikingly different between PM and DM. In DM, antibody-mediated humoral immunity is implicated, because activated B cells and $CD4^+$ T cells infiltrate the perivascular area and C5b-9 complement membrane attack complexes are deposited in the microvascular walls. Therefore, muscle injury in DM is thought to result from ischemic change secondary to microvascular damage as a result of the deposition of immune complexes in the vascular walls.[35–37] In PM, numerous $CD8^+$ T cells invade the endomysial area and surround the muscle fibres, and they are thought to destroy muscle tissue directly.[35,37] However, we have previously reported that FasL-expressing $CD4^+$ T cells are present in the inflamed tissues of not only DM but also PM patients.[2] We assume that the $CD4^+$ T-cell response participates to some degree in the pathogenesis of PM.

Although the untreated muscle fibre cells were positive for surface expression of Fas, they were relatively resistant to Fas-mediated apoptosis. For the underlying mechanisms, we propose two possibilities: (i) the resistance of muscle fibre cells to Fas-mediated apoptosis is attributable to anti-apoptotic intracellular proteins, such as bcl-2, IAP-like protein, and FLIP, which are expressed in inflammatory myopathies;[3,4,27] or (ii) resistance can be ascribed to intracellular caspases that are essential for Fas-mediated apoptosis. The clustering of the Fas cytoplasmic domain generates an apoptotic signal via the death domain,[38] and Fas ligation by FasL activates caspases.[39] Caspase 8 links Fas receptor signalling to downstream caspases, such as caspase 3.[40] We tested this possibility by focusing on caspases 3 and 8 (Fig. 3). The expression in untreated muscle cells of caspase 8 was nil and that of caspase 3 was weak, whereas IFN-γ significantly increased the expression of caspase 8. Either IFN-γ or TNF-α augmented the expression of caspase 3. IL-1β did

not increase the expression of caspase 8 or caspase 3. The combination of three cytokines showed a synergistic effect on Fas-mediated apoptosis of muscle cells. In addition, we observed that caspase 8 and caspase 3 were over-expressed in biopsy samples from patients with PM or DM (Fig. 4, and M. Kondo, Y. Murakawa, N. Harashima, M. Harada, unpublished data). Because shorter-lived cells express caspase 3 more strongly than longer-lived cells,[41] the increased expression of caspase 3 in inflamed tissues seems to be related to muscle cell apoptosis. Considering these findings, we propose that Fas/FasL-mediated apoptosis plays a role in the pathogenesis of inflammatory myopathies.

Despite the comparable levels of caspase 8 and caspase 3 expression noted for treatment with IFN-γ alone and treatment with a combination of IFN-γ and TNF-α or IL-1β, the percentages of Fas-mediated apoptotic myocytes were markedly different. As shown in Fig. 1, surface Fas expression was apparent on untreated or IFN-γ-treated myocytes, but they were relatively resistant to Fas-mediated apoptosis (Fig. 2). TNF-α and IL-1β seem to facilitate the Fas-mediated apoptosis of myocytes. As shown in Fig. 1b, TNF-α and IL-1β increased IFN-γ-induced Fas expression. Interestingly, TNF-α alone did not increase Fas expression by myocytes, although this cytokine enhanced myocyte sensitivity to Fas-mediated apoptosis. As shown in Fig. 3b, TNF-α alone was able to induce the active form of caspase 3. This suggests that TNF-α alone can activate the caspase pathway that is shared with Fas-mediated apoptosis. In addition, as shown in Fig. 1b, TNF-α can augment the Fas expression on myocytes when combined with IL-1β and/or IFN-γ. In summary, TNF-α seems to enhance Fas-mediated apoptosis of myocytes through two different mechanisms: direct activation of the caspase pathway and augmentation of Fas expression in combination with other proinflammatory cytokines.

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Disclosure

The authors have declared no financial or commercial conflict of interest.

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