Increased CD40 Expression on Muscle Cells of Polymyositis and Dermatomyositis: Role of CD40-CD40 Ligand Interaction in IL-6, IL-8, IL-15, and Monocyte Chemoattractant Protein-1 Production

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Increased CD40 Expression on Muscle Cells of Polymyositis and Dermatomyositis: Role of CD40-CD40 Ligand Interaction in IL-6, IL-8, IL-15, and Monocyte Chemoattractant Protein-1 Production

Tomoko Sugiura, Yaoshi Kawaguchi,2 Masayoshi Harigai, Kae Takagi, Shuji Ohta, Chikako Fukasawa, Masako Hara, and Naoyuki Kamatani

In polymyositis (PM)/dermatomyositis (DM), T cells infiltrate the muscle tissues and interact with muscle cells via cell surface molecules. Recently, myoblasts have been reported to express CD40, but little is known about the role of CD40 in myoblasts. In the present study we examined the expression and involvement of CD40 and CD40 ligand (CD40L) in the interaction between muscle cells and T cells in PM/DM. Immunohistochemical staining revealed that CD40 was expressed on muscle cells in five of five PM and four of five DM patients, and that infiltrating mononuclear cells (MNCs) expressed CD40L in all cases of PM/DM. These CD40L-expressing MNCs were primarily CD4+ T cells. IFN-γ, which is known to induce CD40 expression on various types of cells, was also expressed on the MNCs in four of the PM and four of the DM patients. Although cultured human myoblasts (SkMC 2859) did not express CD40 constitutively, IFN-γ induced CD40 expression in a dose-dependent manner. To clarify the functional roles of CD40-mediated signals, the effects of a trimeric form of recombinant human CD40L on cytokine production were studied in SkMC 2859 that were prestimulated with IFN-γ to express CD40. Recombinant human CD40L markedly increased the production of IL-6, IL-8, IL-15, and monocyte chemoattractant protein-1 of SkMC 2859. The expression of these humoral factors in muscle cells of PM and DM was demonstrated by immunohistochemistry. These results suggest that interaction between T cells and muscle cells via the CD40-CD40L system contributes to the immunopathogenesis of PM/DM by augmenting inflammation via cytokine production by the muscle cells.


I diopathic inflammatory myopathies (IIMs)1 include polymyositis (PM), dermatomyositis (DM) and inclusion body myositis. Although the pathogenesis remains to be elucidated, interactions between infiltrating immunocompetent cells, especially T cells, and muscle cells are thought to be involved in the etiology of IIMs (1). Infiltrating T cells in IIMs are considered to be in their activated state because a vast majority of them are positive for HLA-DR Ag (2) and LFA-1 (3) and have a memory phenotype (4). In PM, infiltrating T cells express restricted TCRs with common CDR3 motifs (5, 6). In vitro, CD8+ T cells expanded from muscle tissues of IIMs show cytotoxicity against autologous myotubes (7), CD4+ T cells obtained from normal human PBMC proliferate and secrete IFN-γ in response to allogenic myoblasts upon costimulation with anti-CD28 Ab (8). These results suggest the importance of T cells in the pathogenesis of IIMs. On the other hand, muscle cells of IIMs constitutively express various cell surface molecules, such as CD54, CD106, MHC class I and II Ags, and apoptosis-related molecules (i.e., CD95) (3, 9–12). A recent study (13) showed that muscle cells in IIMs express BB-1, as do cultured myoblasts after stimulation with IFN-γ, TNF-α, IL-4, or CD40L transfectant. Because these molecules are not expressed on muscle cells of normal healthy controls and their counter-receptors are expressed on infiltrating T cells in IIMs, they might mediate interaction between T cells and muscle cells and provide signals for regulation of muscle cells in the disorders.

CD40 is a 50-kDa type I cell surface molecule originally identified on B cells and some epithelial carcinomas (14, 15) that interacts with CD40L expressed on activated T cells (16, 17). In B cells, signals mediated by CD40-CD40L interaction induce B cell proliferation, differentiation, and Ig production (14–17) and also rescue B cells from apoptosis (18). Recent studies have shown that nonlymphoid cells such as fibroblasts (19–21), epithelial cells (22), and endothelial cells (23–25) also express CD40. In vitro, CD40 ligation results in up-regulation of several cell surface molecules and cytokine production in these cells. In the present study we demonstrate that 1) muscle cells of PM/DM express CD40 and muscle-infiltrating mononuclear cells (MNCs) express CD40L; 2) CD40 is induced on cultured myoblasts by IFN-γ stimulation; and 3) CD40 ligation increases IL-6, IL-8, IL-15, and monocyte chemoattractant protein-1 (MCP-1) production by myoblasts. The results are discussed in terms of the implication of the roles of CD40-CD40L interaction in the immunopathogenesis of PM/DM.

Materials and Methods

Patients

Muscle specimens were obtained from five patients with PM and five patients with DM. The patients’ profiles are summarized in Table I. All subjects fulfilled the diagnostic criteria of Bohan and Peter (26). None was
receiving an immunosuppressive treatment at the time of muscle biopsy. As normal controls, we chose three individuals who were suspected to have muscle disorders, but whose biopsyed muscle tissues showed normal histologic findings. The ages of the PM patients, DM patients, and normal controls were 57 ± 4.8, 56 ± 14.6, and 49 ± 7.8 years, respectively.

**Immunohistochemistry**

Four-micron thick sections were air-dried and fixed in 2% paraformaldehyde for 30 min at 4°C. Immunohistochemical staining was performed using a commercial kit (Vectorstain Universal Quick Kit, Vector, Burlingame, CA). Briefly, all sections were incubated with 2.5% normal horse serum for 30 min, followed by mAbs recognizing CD40 (Mab98, 10 μg/ml, mouse IgG1; Coulter, Hialeah, FL), CD40L (CD40 ligand) or control rabbit IgG1 for 30 min at 4°C, washed, and incubated with biotinylated anti-CD4 mAb (10 μg/ml; Dako, Glostrup, Denmark) for 30 min. Mouse IgG1 (Dako), goat IgG (Dako), and human IgG (Dako) were used as control Abs. After being washed with PBS, the sections were incubated for 10 min with horse biotinylated goat IgG (Dako) for 30 min, and visualized with phosphate substate (1.0 mg/ml of BioSource, Camarillo, CA) for 30 min, and visualized with phosphate substrate (Vector). Next, all sections were incubated first with 10 μg/ml of mouse IgG1 for 30 min to avoid nonspecific binding and then with biotinylated anti-CD4 mAb (10 μg/ml, mouse IgG1; Coulter), biotinylated anti-CD8 mAb (10 μg/ml, mouse IgG1; Coulter), or biotinylated control mouse IgG1 (Ancell, Bayport, MN). After washing, the sections were exposed to streptavidin/peroxidase-preformed complex for 5 min and then covered with diaminobenzidine tetrahydrochloride for 2 min. All sections were counterstained with hematoxylin.

Double staining was performed with biotinylated mouse mAb and non-labeled mouse mAb. Briefly, sections were fixed, blocked with 2.5% normal horse serum for 30 min, followed by mAbs recognizing CD40 (Mab98, 10 μg/ml, mouse IgG1; Coulter, Hialeah, FL), CD40L (CD40 ligand) or control rabbit IgG1 for 30 min at 4°C, washed, and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (10 μg/ml; BioSource, Camarillo, CA) for 30 min, and visualized with phosphate substrate (Vector). Next, all sections were incubated first with 10 μg/ml of mouse IgG1 for 30 min to avoid nonspecific binding and then with biotinylated anti-CD4 mAb (10 μg/ml, mouse IgG1; Coulter), biotinylated anti-CD8 mAb (10 μg/ml, mouse IgG1; Coulter), or biotinylated control mouse IgG1 (Ancell, Bayport, MN). After washing, the sections were exposed to streptavidin/peroxidase complexes and colored with diaminobenzidine. All sections were counterstained with Methyl Green (Dako).

**Myoblast culture**

Myoblasts of human skeletal muscle (SkMC 2859) were obtained from BioWhittaker (Walkersville, MD) and maintained in serum-free culture medium (QBSF 51, Sigma, St. Louis, MO). SkMC 2859 were grown to confluence and detached from culture flasks or plates with 0.25% trypsin-EDTA (Life Technologies, Frederick, MD). SkMC 2859 were isolated by enzymatic digestion of ventral thigh tissue of a 22-wk-old black male barnyard chicken according to the manufacturer’s data sheet. This cell strain is positive for sarcomeric myosin, α-actinin (sarcomeric), and troponin-T.

**Flow cytometry**

At the end of culture, SkMC 2859 were detached from culture flasks by trypsinization. In all experiments, 1 x 10^6 cells were treated with human Ig to block nonspecific binding. Cells were then stained with anti-CD40 mAb or control mouse IgG1 for 30 min at 4°C, washed, and incubated with fluorescein-conjugated Fab1/2 goat anti-mouse IgG (Becton Dickinson, San Jose, CA) for 30 min at 4°C. The cells were washed and fixed with 2% paraformaldehyde, and the fluorescein intensity was measured by Cytoron Absolute (Ortho-Clinical Diagnostics, Tokyo, Japan).

**RT-PCR**

Total cellular RNA was isolated from SkMC 2859 with Trizol (Life Technologies, Gaithersburg, MD) according to the manufacturer’s protocol. Total RNA was reverse transcribed to cDNA using Superscript II RT (Life Technologies). For PCR, 2 μl of RT product was used in a total volume of 50 μl containing the following reagents: 1.5 mM MgCl2, 1.5 μM of each primer (1.50 mM KCl and 10 mM Tris-HCl, pH 8.3), 0.2 mM of each dNTPs, 1 U of AmpliTaq polymerase (Roche, Mannheim, Germany), and forward and reverse primers (20 μM each). The sequences of the primers were as follows: CD40 forward, 5'-ATGGTGTCGCTGCTCTGCTGAG-3'; CD40 reverse, 5'-CTCGGCGAGGCGTCACGATG-3'; IL-6 forward, 5'-ATGAAGCTCT TCTCCGCAAGCCGGC-3'; IL-6 reverse, 5'-GAAGAGCCCTCAGGCTG GTAAG-3'; IL-8 forward, 5'-ATGACCTGACGTTGCGTGCT-3'; IL-8 reverse, 5'-TCTCAGCCTCTTTAAAACCTCTC-3'; IL-15 forward, 5'-TTTGATGGAGGACGAT-3'; IL-15 reverse, 5'-AAGAGCTCATCTG ATCAAGAGG-3'; MCP-1 forward, 5'-TGTGGTGCCGTCTGGCTCATA-3'; MCP-1 reverse, 5'-GGTGGTCTTGGCAGGTTG-3'; β-actin forward, 5'- AAGAGGAGCTATCTACCCCT-3'; and β-actin reverse, 5'-TACATGGCT CGGTGTCGTA-3'. The PCR cycling conditions used were 95°C for 30 s (denaturing), 72°C for 30 s (annealing), and 72°C for 1 min (extension). The PCR products were electrophoresed in a 2% agarose gel and visualized with ethidium bromide.

**Preparation of cell extracts**

SkMC 2859 were detached by trypsinization, washed three times in PBS, and lysed in 100 μl of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% sodium deoxycholate, and 1% Nonidet P-40, containing 1 mM PMFS (Sigma) and 0.2 U/ml aprotinin (Sigma). The extracts were centrifuged at 5000 x g, and the supernatants were used for ELISA.

**ELISA**

To determine the concentrations of cytokines in the culture supernatants or cell extracts, a specific ELISA was developed for each cytokine using specific Abs. For IL-6, IL-15, and MCP-1, ELISA plates (Costar, Cambridge, MA) were preabsorbed with specific capture Abs for IL-6 (1 μg/ml, rat IgG; PharMingen, San Diego, CA), IL-15 (2 μg/ml, mouse IgG; R & D Systems) or MCP-1 (2 μg/ml, mouse IgG; PharMingen, San Diego, CA). After overnight incubation at 4°C, the plates were washed with PBS containing 0.05% Tween-20 and blocked with PBS containing 10% FCS for 2 h at room temperature. After more washing, serial dilutions of recombinant cytokines or samples were applied to the plates in duplicate. Reconstituent IL-6, IL-15, and MCP-1 were purchased from R&D Systems. After overnight incubation at 4°C, the plates were washed three times, then incubated with biotin-conjugated Abs for IL-6 (rat monoclonal, 0.5 μg/ml; PharMingen), IL-15 (mouse monoclonal, 200 ng/ml; R&D Systems), or MCP-1 (rabbit polyclonal, 1 μg/ml; PharMingen) for 45 min at room temperature. All plates were then incubated with avidin-peroxidase (10 ng/ml, Sigma) for 30 min at room temperature. An ABTS peroxidase substrate (1.0 mg/ml of BioSource, Tokyo, Japan) with a test wavelength of 405 nm (test wavelength) to 620 nm (reference wavelength). The absorbance was read at 405 nm (test wavelength) to 620 nm (reference wavelength). The sensitivity of the assay were 7.81, 7.81, and 31.3 pg/ml for IL-6, IL-15, and MCP-1, respectively.

For assessment of IL-8, ELISA plates were coated with anti-IL-8 mAb (1 μg/ml, mouse IgG; R&D Systems) overnight at 4°C. The plates were blocked with Tris-HCl containing 2% BSA (Sigma) for 2 h, and then hIL-8 (R&D Systems) or diluted samples were applied. After an overnight incubation at 4°C, the plates were incubated with polyclonal rabbit anti-IL-8 Ab (2 μg/ml; Endogen, Woburn, MA) for 2 h at room temperature. Plates were then incubated with a 1/2000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (BioSource) for 1 h and reacted with a phosphate substrate (1.0 mg/ml of p-nitrophenylphosphate; Sigma). Absorbance was read at 405 nm (test wavelength) to 490 nm (reference wave- length). The sensitivity of the IL-8 ELISA was 15.6 pg/ml.

**CD40 ligation on SkMC 2859**

Semiconfluent SkMC 2859 were stimulated with 100 U/ml of INF-γ (PharMingen) for 48 h to induce CD40 expression. Then, the plates were
laries and vessels were also stained by the anti-CD40 Ab (Fig. 1A). CD40L was expressed on infiltrating MNCs surrounding muscle cells in all cases of PM/DM, but not in the muscle fibers (Fig. 1B and Table II). In patient 10 the MNCs infiltrating muscle tissue showed relatively mild staining for CD40L, and the serum CK (creatine kinase) level at biopsy was low (Table I), which may explain the absence of CD40 expression on muscle fibers in this case. The percentages of CD40L-positive MNCs was 19.5 ± 15.4% in PM and 16.8 ± 12.4% in DM. IFN-γ is known to induce or up-regulate CD40 expression on various types of cells. As shown in Fig. 1C and Table II, IFN-γ was expressed on infiltrating MNCs in endomysial and perivascular areas in 8 of the 10 PM/DM patients. Serial section analysis showed that these IFN-γ-expressing MNCs localized close to CD40-positive muscle cells and CD40L-expressing MNCs (data not shown). The percentage of MNCs expressing IFN-γ was 6.9 ± 12.7% in PM and 10.2 ± 12.5% in DM. In normal individuals, CD40 was detected on some endomysial MNCs, but not on muscle cells. Neither CD40 nor IFN-γ was expressed in the control tissues. These CD40L and/or IFN-γ-expressing MNCs were localized predominantly in endomysial areas in both PM and DM, although a small number was also detected in perivascular areas in DM patients (data not shown).

Cell populations that express CD40L

Double staining was performed in two representative cases (patient 2 with PM and patient 6 with DM) to clarify which cell populations expressed CD40L (Fig. 2). At least 100 CD40L-positive MNCs were analyzed for each case. Of the CD40L-positive MNCs, 78.8 ± 9.68% (three different high power fields) were CD4+ T cells and 8.9 ± 3.5% were CD8+ T cells in patient 2 (PM). In patient 6 (DM), 85.2 ± 7.8% were CD4+ T cells and 5.3 ± 4.9% were CD8+ T cells.

IFN-γ induced CD40 on SkMC 2859

RT-PCR revealed that unstimulated as well as IFN-γ-stimulated SkMC 2859 constitutively expressed CD40 mRNA (Fig. 3A). To investigate the cell surface expression of CD40, semiconfluent SkMC 2859 were stimulated with various concentrations of IFN-γ, TNF-α (R&D Systems) or IFN-γ plus TNF-α for 48 h, stained with anti-CD40 Ab or control mouse IgG1, and analyzed by flow

### Table II. Expression of CD40 on muscle fibers, and CD40L and IFN-γ in endomysial cells

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Patient No.</th>
<th>CD40-Positive Muscle Fibers/Total Fibers (%)</th>
<th>CD40L-Positive MNCs/Total MNCs (%)</th>
<th>IFN-γ-Positive MNCs/Total MNCs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM</td>
<td>1</td>
<td>23.9</td>
<td>44.3</td>
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<tr>
<td></td>
<td>2</td>
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<td>10.7</td>
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<td>1.1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.5</td>
<td>7.8</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.5</td>
<td>8.2</td>
<td>0</td>
</tr>
<tr>
<td>DM</td>
<td>6</td>
<td>39.1</td>
<td>31.4</td>
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<td>7</td>
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<td>22.3</td>
</tr>
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<td></td>
<td>8</td>
<td>13.2</td>
<td>10.0</td>
<td>2.1</td>
</tr>
<tr>
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<td>9</td>
<td>3.1</td>
<td>8.1</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
<td>5.3</td>
<td>0</td>
</tr>
<tr>
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<td></td>
<td>13</td>
<td>0</td>
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<td>0</td>
</tr>
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</table>

*At least 500 fibers and 300 MNCs were analyzed in each case.*

### Results

**Muscle cells expressed CD40, and infiltrating MNCs expressed CD40L and IFN-γ in PM/DM**

Immunohistochemistry revealed the sarcolemma of muscle cells in inflammatory foci to express CD40 in all the PM/DM patients except patient 10 (Fig. 1A and Table II). The percentage of fibers positive for CD40 was 11.6 ± 7.6% in PM and 15.1 ± 15.6% in DM (Table II). Infiltrating MNCs and endothelial cells of capillaries and venules were also stained by the anti-CD40 Ab (Fig. 1A). CD40L was expressed on infiltrating MNCs surrounding muscle cells in all cases of PM/DM, but not in the muscle fibers (Fig. 1B and Table II). In patient 10 the MNCs infiltrating muscle tissue showed relatively mild staining for CD40L, and the serum CK (creatine kinase) level at biopsy was low (Table I), which may explain the absence of CD40 expression on muscle fibers in this case. The percentages of CD40L-positive MNCs was 19.5 ± 15.4% in PM and 16.8 ± 12.4% in DM. IFN-γ is known to induce or up-regulate CD40 expression on various types of cells. As shown in Fig. 1C and Table II, IFN-γ was expressed on infiltrating MNCs in endomysial and perivascular areas in 8 of the 10 PM/DM patients. Serial section analysis showed that these IFN-γ-expressing MNCs localized close to CD40-positive muscle cells and CD40L-expressing MNCs (data not shown). The percentage of MNCs expressing IFN-γ was 6.9 ± 12.7% in PM and 10.2 ± 12.5% in DM. In normal individuals, CD40 was detected on some endomysial MNCs, but not on muscle cells. Neither CD40 nor IFN-γ was expressed in the control tissues. These CD40L and/or IFN-γ-expressing MNCs were localized predominantly in endomysial areas in both PM and DM, although a small number was also detected in perivascular areas in DM patients (data not shown).

**Statistical analysis**

Comparison of data, presented as the mean ± SEM, was performed using unpaired Student’s t test as indicated. $p < 0.05$ was considered significantly different.

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cytometry. Without stimulation, SkMC 2859 expressed marginal levels of CD40 (Fig. 3B). IFN-γ induced CD40 expression in a dose-dependent manner (Fig. 3B). The effect of IFN-γ became apparent at 12 h after stimulation and reached a plateau at 24 h (data not shown). TNF-α alone only weakly induced the expression of CD40, but it synergistically enhanced CD40 expression on SkMC 2859 in combination with IFN-γ (Fig. 3B). Neither IL-1α, IL-1β, IL-4, IL-6, nor GM-CSF induced CD40 expression on SkMC 2859 (data not shown).

Recombinant CD40L enhanced production of cytokines by IFN-γ-treated SkMC 2859

We next analyzed the function of CD40 on SkMC 2859. IFN-γ-treated SkMC 2859 were incubated with rhCD40L as described in Materials and Methods. Forty-eight hours after CD40 ligation, the culture supernatants and cell extracts were collected, and the concentrations of cytokines were measured by ELISA. As shown in Fig. 4A, unstimulated SkMC 2859 constitutively secreted low levels of IL-6, IL-15, and MCP-1, but not IL-8. Recombinant hCD40L showed marginal effects on the production of IL-6, IL-8, IL-15, and MCP-1 by untreated SkMC 2859. IFN-γ alone augmented IL-6, IL-8, IL-15, and MCP-1 production. The effects of IFN-γ on the production of these cytokines were dose dependent (data not shown). Ligation of CD40 further increased the production of these cytokines by IFN-γ-treated SkMC 2859 in a dose-dependent manner (Fig. 4A). It was interesting that >50% of IL-15 was detected in the cell extracts regardless of the pretreatment with IFN-γ. We also measured IL-1α, IL-1β, IL-10, GM-CSF, and TNF-α in the same culture supernatants, but their concentrations were below the sensitivity of the ELISA (data not shown). mRNAs of IL-6, IL-8, IL-15, and MCP-1 were detected by RT-PCR not only in IFN-γ plus rhCD40L-stimulated SkMC 2859 (Fig. 4B), but also in unstimulated and IFN-γ-stimulated SkMC 2859 (data not shown).
Expression of IL-6, IL-8, IL-15, and MCP-1 in PM/DM

Immunohistochemical staining for IL-6, IL-8, IL-15, or MCP-1 was performed in four representative patients (patients 1, 2, 6, and 7) and three normal individuals. Some of the muscle cells were stained with anti-IL-6 mAb or anti-MCP-1 mAb in the four patients with PM/DM, but not in the controls (Fig. 5, A, C, F, and H). Infiltrating MNCs also expressed IL-6 and MCP-1 in the patients. The cytoplasm of muscle cells was strongly stained with anti-IL-15 mAb in the four patients, although that of normal controls was only marginally stained (Fig. 5, B and G). Anti-IL-8 Ab stained muscle cells in neither patients nor controls (data not shown).

The effects of rhCD40L on cytokine production by SkMC 2859 were specifically inhibited by anti-human CD40L Ab

To assess the specificity of the effect of rhCD40L, IFN-γ-treated SkMC 2859 were stimulated with 0.2 mg/ml of rhCD40L in the presence of anti-human CD40L Ab (TRAP1) or control mouse IgG1. After 48 h, culture supernatants and cell extracts were collected, and the concentrations of cytokines were measured. As shown in Fig. 6, TRAP1 inhibited rhCD40L-stimulated IL-6 production in a dose-dependent manner, while control IgG1 showed no effect. TRAP1 (10 μg/ml) almost completely abrogated the effects of rhCD40L. Similar results were obtained with IL-8, IL-15, and MCP-1 (data not shown).

Discussion

In the present study we demonstrated that CD40 was constitutively expressed on muscle cells in the inflammatory foci of PM/DM and that CD40L-positive T cells infiltrated the endomysium and perivascular sites. Ligation of CD40 on SkMC 2859 augmented the production of inflammatory cytokines in vitro. These observations provide evidence of the importance of CD40-CD40L interaction in the immunopathogenesis of idiopathic inflammatory myopathies, especially PM/DM.

Recent studies have shown that CD40 is expressed on a broad range of cells and has various physiological roles, although it was initially thought to be expressed only on B cells and have limited functions (27). The cross-linking of CD40 on monocytes results in cytokine production (28) and nitric oxide synthesis (29). Cells of nonlymphoid origin, including fibroblasts, endothelial cells, epithelial cells, and myoblasts, also express CD40 molecules (19–25). Ligation of CD40 on fibroblasts enhances the expression of CD54 and CD106 (19), the production of IL-6 and IL-8 (21), and the proliferation of the cells (19). In endothelial cells, ligation of CD40 also up-regulates the expression of CD54, CD106, and CD68 (E-selectin) (24, 25) and increases the production of IL-8 and MCP-1 (25). These reports suggest that CD40-CD40L interaction induces chemotaxis and leukocyte adhesion to inflammatory sites in vivo. There are also reports that CD40-CD40L interaction...
is involved in chronic inflammatory diseases such as rheumatoid arthritis. Macrophage-like synovial cells in the lining region strongly express CD40, and the infiltrating T cells express CD40L (30–32). Ligation of CD40 on synovial cells in primary culture senhances the production of TNF-α (32) and also induces the proliferation of synovial fibroblasts (30). Thus, CD40-CD40L interaction should be important for the initiation and perpetuation of chronic inflammatory disorders.

Because the function of CD40 on myoblasts remains unknown in PM/DM, we set out to investigate the effects of CD40 ligation on cytokine production by cultured normal myoblasts. For CD40 engagement, we chose a trimeric form of CD40L-leucine zipper fusion protein. This rhCD40L has been demonstrated to have biological functions equivalent to those of its membrane-bound forms (33). In our study the production of IL-6, IL-8, IL-15, and MCP-1 was enhanced by CD40 ligation. We performed CD40 ligation using myoblasts of adult human isolated from muscle tissues in the manner described in Materials and Methods. Intracellular and extracellular IL-6 were measured by ELISA. TRAP1 (10 μg/ml) completely inhibited the effect of rhCD40L on IL-6 production. Control IgG1 had no effect. Similar results were obtained for IL-8, IL-15, and MCP-1 (data not shown). The results are expressed as the mean ± SE (n = 3). *, p < 0.05; **, p < 0.01.

IL-8 and MCP-1 are cytokines that mainly induce chemotaxis of neutrophils and monocytes, respectively (34, 35). Recent studies have revealed that these chemokines induce the expression of integrins such as LFA-1 on circulating leukocytes (36, 37), which results in leukocyte adhesion to the vascular wall, extravasation, and infiltration of inflammatory foci. Specific Abs against IL-8 or MCP-1 have been shown to inhibit leukocyte infiltration in vivo (38, 39). According to our data, it is plausible that the T cells infiltrating inflammatory foci in PM/DM stimulate the production of these chemokines by myoblasts via CD40-CD40L interaction, which further induces infiltration by MNCs around target muscle cells. This positive feedback loop may be relevant to the progression and perpetuation of inflammation in PM/DM.

Another remarkable observation was that CD40 ligation induces the production of IL-15 by myoblasts. IL-15 is a 14- to 15-kDa molecule and a member of the 4α-helix bundle cytokine family, with biological functions similar to those of IL-2 despite the absence of significant amino acid sequence homology. IL-15 and IL-2 share multiple biological functions, including stimulation of the proliferation and activation of T cells and NK cells, induction of cytotoxic effector cells, T cell chemoattraction and proliferation, and Ig synthesis by B cells (40–42). In contrast to IL-2, IL-15 mRNA is expressed in various nonlymphoid tissues and cells, such as placenta, skeletal muscle, kidney, lung, heart, fibroblasts, and monocytes, but not in resting or activated T cells (40). IL-15 also possesses biological functions not shared by IL-2. On muscle cells, IL-15 is reported to exert its effect as an anabolic cytokine (43) and to promote differentiation (44). Although IL-15 mRNA is widely.
expressed, it has been difficult to demonstrate IL-15 at the protein level in supernatants of various kinds of cell culture (45). We established a sensitive ELISA system for IL-15 and proved for the first time that myoblasts secrete this molecule. It is of interest that, in contrast to IL-6, IL-8, and MCP-1, >50% of IL-15 remained intracellularly. As shown in Fig. 5, the cytoplasm of muscle cells was strongly stained by anti-IL-15 mAb, but cytoplasmic staining was marginal in normal muscle cells; this suggests the presence of intracellular IL-15 in vivo in PM/DM. Increased production of IL-15 by muscle cells due to CD40 ligation might contribute to further T cell migration and activation, as discussed for IL-8. Further studies should attempt to clarify the molecular structure and biological function of intracellular IL-15.

CD40-CD40L interaction also induced IL-6 production by IFN-γ-stimulated SkMC 2859. IL-6 has multiple biological functions, such as B cell differentiation and T cell activation (46). IL-6 production by muscle cells in PM/DM may activate T cells at inflammatory foci and contribute to autoantibody production in these disorders.

Because CD40 ligation induces CD80 and CD86 expression on macrophages (27), we examined its effect on the expression of these molecules on SkMC 2859. Recombinant hCD40L did not induce the expression of either cell surface CD80 or CD86, although it did induce the expression of CD80 mRNA in SkMC 2859 (data not shown). Behrens et al. used anti-CD40 mAb (EA-5) or CD40L-transfected mouse fibroblasts for CD40 ligation (13). CD40L transfectant induced weak expression of CD80 and strong expression of BB-1 in myoblasts, although EA-5 stimulated BB-1 expression alone. It is possible that CD40-mediated signals induce CD80 expression in myoblasts in vitro, but not in vivo, because we cannot find CD80-expressing muscle cells in IMs (13) (our unpublished observation).

Anti-human CD40 Ab inhibited rhCD40L-stimulated cytokine production in SkMC 2859 (Fig. 6). Thus, blocking of the CD40-CD40L interaction, which is reported to ameliorate several autoimmune disorders such as collagen-induced arthritis (47) and lupus-like nephritis in animal models (48, 49), may also have therapeutic value in PM/DM.

Regarding the inducers of CD40 on muscle cells in PM/DM, IFN-γ would be a prime candidate. We demonstrated that infiltrating MNCs expressed IFN-γ in eight of the 10 PM/DM patients, and in all these cases, muscle cells expressed CD40. Expression of IFN-γ in infiltrating cells was also shown by other investigators using immunohistochemistry and RT-PCR (50–52), consistent with our data. Furthermore, IFN-γ induced CD40 expression on cultured SkMC 2859 in a dose-dependent manner (Fig. 3B), as reported for B cells, endothelial cells, macrophages, fibroblasts, and synovial cells of RA by other investigators (27). These results support the hypothesis that IFN-γ is an inducer of CD40 on muscle cells in PM/DM. Another candidate for a CD40 inducer in PM/DM is TNF-α, although its effect is not as marked as that of IFN-γ (Fig. 3B). Expression of TNF-α has also been reported in PM/DM (53). Because TNF-α in combination with IFN-γ had a synergistic effect on CD40 expression on SkMC 2859 in vitro (Fig. 3B), the two molecules, IFN-γ and TNF-α, may synergistically stimulate CD40 expression on muscle cells in vivo.

CD40L is transiently expressed on activated T cells. The T cells that express CD40L are predominantly CD4+ T cells, with some CD8+ T cells also expressing the molecule in vitro (54). This agrees with our immunohistochemical data, wherein most of CD40L-positive cells are CD4+ T cells and some populations are CD8+ T cells in both PM and DM. Thus, CD40L-expressing CD4+ T cells are in their activated state and play a central role in the CD40-CD40L interaction in inflammatory lesions of PM/DM.

In conclusion, our results indicate that cross-talk between the inflammatory cytokines and the CD40-CD40L system contributes to T cell recruitment and activation in PM/DM and to perpetuation of the inflammatory process. Blocking of CD40-CD40L interaction may have therapeutic value in PM/DM.

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