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The Fas/Fas Ligand System Is Involved in the Pathogenesis of Autoimmune Myocarditis in Rats

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The mechanisms responsible for myocardial injury and cell death in myocarditis are still unclear. We examined whether myocardial cell death occurs via apoptosis in myosin-induced autoimmune myocarditis in rats and whether the Fas/Fas ligand (FasL) system plays a role in this apoptosis. On days 14, 17, 21, and 35 after immunization with porcine heart myosin, some cardiomyocytes and infiltrating lymphocytes were found to be apoptotic on in situ terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay, but none was on day 60 and in control rats. Apoptotic indices peaked at day 17, and lagging of genomic DNA from the affected myocardium was observed on days 17 and 21 on agarose gel electrophoresis. The expression of Fas mRNA and protein was detected on days 17 and 21 in some cardiomyocytes and infiltrating lymphocytes by Northern blot analysis and immunohistochemistry, respectively. In addition, FasL was detected in some infiltrating lymphocytes on days 14, 17, and 21 by both in situ hybridization and immunostaining, and FasL-positive lymphocytes were mainly CD4+ cells. Some rats were injected with anti-Fas Ab (0.1 mg/kg) or anti-FasL Ab (0.1 mg/kg), and subsequently, inflammatory lesions exhibited less severe than did untreated rats with myocarditis. These findings suggest that cell death via apoptosis of cardiomyocytes and lymphocytes is one of the mechanisms of myocardial injury in autoimmune myocarditis, and that the Fas/FasL system might play a role in the induction of this apoptosis. The Journal of Immunology, 1998, 161: 4695–4701.

Programmed cell death is a selective process of physiologic cell destruction and plays an important role in the process of morphologic and functional maturation of a number of systems (1, 2). Programmed cell death usually occurs via apoptosis, which is visualized as condensation of the cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, and extensive degradation of chromosomal DNA into oligomers of about 180 bp (3). This DNA fragmentation is observed as DNA ladders on agarose gel electrophoresis, as a characteristic pattern of nucleic staining with nick end labeling in situ, and as a characteristic pattern of nuclear changes on electron microscopy. It has been demonstrated that programmed death can be induced by many pathologic conditions, including irradiation (1, 2), UV light exposure (1), anticancer drugs (4–6), viral infection (7–9), and hyperthermia (10).

Recent reports have indicated that programmed death of myocytes plays an important role during the postnatal development of the heart (11) as well as during ischemia reperfusion injury of the myocardium (12). Moreover, we demonstrated that under hypoxic conditions, the programmed death of cultured cardiomyocytes occurs via apoptosis, and obtained evidence suggesting that Fas Ag may play a role in this apoptotic process (13). It has since been reported that apoptosis plays a role in the damage to myocardial cells in acute myocardial infarction (14), the loss of myocytes in patients with end-stage cardiomyopathy and the progressive myocardial dysfunction associated with it (15), and the loss of myocardial cells in arrhythmogenic right ventricular dysplasia (16).

We recently reported that excess nitric oxide produced by inducible NO synthase appears to contribute to the progression of myocardial necrosis in myocarditis (17). However, the molecular mechanisms of myocardial injury and cell death in myocarditis remain unclear. We therefore prepared a rat model of autoimmune myocarditis useful for examination of the damage to myocardial cells, and investigated whether apoptotic cell death of cardiomyocytes and lymphocytes occurs in this model and whether the Fas/FasL system plays a role in this apoptosis.

Materials and Methods

Induction of autoimmune myocarditis and experimental design

Autoimmune myocarditis was induced in male 7-wk-old Lewis rats, as previously described (18). In brief, 1 mg (0.1 ml) of porcine heart myosin (10 mg/ml) (Sigma, St. Louis, MO) was mixed with an equal volume of CFA (Sigma) and then injected into a footpad on days 1 and 7. On days 2 and 5, the animals were injected with 0.1 ml of Bordetella pertussis (Nacalai Tesque, Kyoto, Japan) dissolved in 0.9 ml of saline solution via the caudal vein. They were killed on days 14 (n = 10), 17 (n = 10), 21 (n = 10), 35 (n = 7), and 60 (n = 7), and formed day 14 (initial inflammation), day 17 (early inflammation), day 21 (climax of inflammation), day 35 (healing stage), and day 60 (healed stage). The other 20 rats were i.p. injected with anti-Fas Ab (anti-APO-1/FAS, SM1/23, mouse
Table I. Summary of the various treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Myosin</th>
<th>Anti-F anti-FasL</th>
<th>Isotype Control mAb</th>
<th>Anti-F anti-Fas</th>
<th>Isotype Control pAb</th>
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<tbody>
<tr>
<td>Day 14</td>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Day 14</td>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Day 17</td>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Day 21</td>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Day 21-M(–)</td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Day 21-F</td>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Day 21-F-C</td>
<td>5</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Day 21-F-L</td>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Day 21-F-L-C</td>
<td>5</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Day 60</td>
<td>7</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Rats were treated with myosin except group day 21-M(–).
* Anti-Fas or isotype control mAb to anti-Fas was administered on day 14.
* Anti-FasL, or isotype control pAb (polychromic rabbit Ab) to anti-FasL was administered on day 14. The day given in the group identity is the day of sacrifice.

Summary of the various treatment groups

IgG2b (day 21-F, n = 10) (Boehringer Ingelheim GmbH, Ingelheim, Germany) (0.1 mg/kg) (19–21), on day 14 (day 21-F, n = 10) or anti-FasL Ab (0.1 mg/kg) (anti-P, rabbit IgG) (day 21-F-L, n = 10) (22, 23). They were killed on day 21. Only saline solution was injected instead of myosin on days 1 and 7, and B. pertussis was injected on days 2 and 5, as a control compared with day 21. Animals were killed on day 21 (day 21-M(–), n = 5). Mouse IgG2b (C1998; Dainippon Pharmaceutical, Suita, Japan) (0.1 mg/kg) was injected instead of anti-Fas Ab (anti-APO-1/FAS) on day 21 (day 21-F-L, n = 5), as a control compared with day 21-F. Rabbit IgG (0.1 mg/kg) (InterCell Technologies, Hopewell, NJ) was injected instead of anti-FasL Ab on day 21 (day 21-F-L-C, n = 5) as a control compared with day 21-F-L. Animals were killed on day 21. The various treatment groups are summarized in Table I.

The hearts were removed and divided into four portions: 1) optimal cutting temperature (OCT) compound-fixed frozen specimens for terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay and immunohistochemistry; 2) Formalin-fixed specimens for histopathology and TUNEL assay; 3) specimens fixed in 4% paraformaldehyde and 0.5% glutaraldehyde for in situ hybridization; and 4) frozen specimens for DNA electrophoresis and Northern blot analysis, and their hearts were removed and fixed in Formalin for histopathology.

To examine the effect of anti-Fas or anti-FasL treatment on the organs other than heart of this model, we investigated pathologically systemic organs, such as brain, lung, aorta, liver, spleen, pancreas, stomach, intestine, colon, kidney, and adrenal gland. Each organ was removed and fixed in 10% buffered Formalin for histopathology.

Histopathology

Tissues of heart and other organs were fixed in 10% buffered Formalin and embedded in paraffin, were cut into sections, 3 μm thick, and stained with hematoxylin-eosin. The tissue preparations were then examined for degree of myocardial cellular damage and infiltration of inflammatory cells, or tissue injury.

TUNEL assay and apoptotic index

TUNEL assay was performed using ApopTag Kit (Oncor, Gaithersburg, MD), as described by Ioth et al. (14) and Gavrieli et al. (23). In brief, paraffin sections were affixed to slides. After deparaffinization in xylene (3×, 10 min) and acetone (2×, 5 min), the sections were washed in double-distilled water (DDW), incubated with 20 mg/ml proteinase K for 15 min at room temperature (RT), and then washed four times in DDW for 2 min. After inactivation of endogenous peroxidase by covering sections with 2% hydrogen peroxide for 5 min at RT, sections were rinsed with DDW and immersed in TdT buffer (30 mmol/L Tris-HCl buffer, pH 7.2, 140 mmol/L sodium chloride, 1 mmol/L sodium citrate, 0.3 U/ml deoxynucleotidyl transferase, and biotinylated dUTP in TdT buffer was then added to cover the sections, followed by incubation in a humid atmosphere at 37°C for 60 min. The reaction was terminated by placing the slides in buffer containing 300 mmol/L sodium chloride and 30 mmol/L sodium citrate for 15 min at RT. The sections were rinsed in DDW, covered with a 2% aqueous solution of BSA, allowed to stand for 10 min at RT, rinsed in DDW, and immersed in PBS for 5 min. The sections were then covered with streptavidin peroxidase, incubated for 10 min at 37°C, washed in DDW, immersed for 5 min in PBS, and stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB) for approximately 10 min at RT. In addition, the TUNEL assay consisting of a treatment without TdT, but with biotinylated dUTP, was performed as a negative control.

The apoptic index (24) (the percentage of TUNEL-positive myocytes or lymphocytes) of inflammatory lesions was estimated by light microscopy at 400-fold magnification. A minimum of 5000 cells was counted for each animal. Counting was performed by two observers.

Agarose gel electrophoresis of DNA

DNA extraction and electrophoresis were performed in principle, as previously reported by Facchinetti et al. (25). Myocardial tissues from days 21-M(–), 14, 17, 21, and 35, and 60 were examined. Tissue specimens (50 mg each) were incubated in 700 ml of cytolytic buffer (50 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH 8.5), 100 mM NaCl, and 1% SDS containing 500 μM protease K (10 mg/ml) at 55°C overnight. Then, they were incubated at 37°C for 60 min with RNase (Boehringer Mannheim Biochemica, Mannheim, Germany). After that, 700 μl of chloroform was added to each sample, and samples were shaken gently for 20 min at RT. They were then centrifuged at 15,000 rpm and 4°C for 15 min. The supernatants were then centrifuged with 600 μl of ice-cold ethanol (100%) at 15,000 rpm and 4°C for 15 min. The pellets were rinsed with ice-cold ethanol (70%) and dried. The content of DNA in pellets was measured by absorptionmetry, and TE buffer was added to equalize DNA concentrations in all samples. Electrophoresis was performed at 50 V with 1.5% agarose gels. DNA was visualized with ethidium bromide.

Immunohistochemistry

OCT compound-fixed frozen specimens of myocardial tissues were cut into sections, 7 μm thick, and immunostained with anti-FasL Ab (FAS-L (N-20); Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Fas Ab (FAS (M-20); Santa Cruz Biotechnology) (26–30). To identify the subpopulation of anti-FasL Ab-positive lymphocytes, the sections were double stained with anti-FasL and anti-rat CD4 Abs or anti-FasL and anti-rat CD8 Abs, and with the anti-Fas and anti-FasL Abs. Immunostaining was performed as follows. Endogenous and exogenous peroxidase activities were blocked by placing tissue sections in 1% periodic acid at RT for 10 min and then washing them thoroughly with PBS. As primary Abs, anti-FasL Ab (1/1000 dilution) and anti-Fas Ab (1/500 dilution) were used, with incubation overnight at 4°C. As the secondary Ab, anti-rabbit IgG-conjugated peroxidase (Gout) (MBL, Nagoya, Japan) (1/300 dilution) was used, with incubation at RT for 60 min. They were examined under a light microscopy after development of color using DAB. For double staining, anti-Fas and FITC-conjugated anti-CD4 rat Abs (BMA Biomedicals, August, Switzerland), or anti-FasL and FITC-conjugated anti-CD8 rat Abs (BMA Biomedicals) were used as primary Abs at 1/500 and 1/500 dilutions, respectively, with incubation overnight at 4°C. The secondary Ab for anti-FasL Ab was stained with goat anti-rabbit IgG-conjugated peroxidase (MBL) (1/300 dilution) and examined under light and fluorescence microscopes after development of color using DAB.

For double staining, anti-Fas Ab (clone 13, mouse IgG1; MBL) and anti-FasL Ab (FAS-L (N-20), rabbit IgG, epitope corresponding to amino terminus of FAS-L, of rat origin; Santa Cruz Biotechnology) were used as primary Abs at 1/100 and 1/500 dilutions, respectively, with incubation overnight at 4°C. The secondary Ab for anti-FasL Ab was stained with goat anti-rabbit IgG-conjugated peroxidase (MBL) (1/300 dilution) and examined using light and fluorescence microscopes after development of color using DAB.

For double staining, and dye-conjugated mouse IgGs (DAKOPATTS Japan, Kyoto, Japan) (1/100) were used and left overnight at RT for 60 min. They were examined by light and fluorescence microscopy.

For identification of TUNEL assay-positive cells, the sections were double stained by TUNEL assay and an immunohistochemical method using anti-α-actinin (sarcomeric) mAb (Sigma) or FITC-conjugated anti-α actin mAb (Pharmingen, San Diego, CA). Immunostaining was performed as follows. After TUNEL assay, sections were incubated with the primary Abs, anti-α actinin mAb (1/100 dilution), or FITC-conjugated mouse anti-α actinin mAb (1/100 dilution), overnight at 4°C. Then the sections with anti-α-actinin (sarcomeric) Ab were incubated with the secondary Ab, anti-mouse Ig FITC (Dako, Glostrup, Denmark) (1/100 dilution), at RT for 4 h. Sections were then examined by light and fluorescence microscopy.

Northern blot analysis

Northern blot analysis of rat Fas mRNA was performed as previously described (24, 31). In brief, total RNA from rat heart tissues was isolated in guanidine thiocyanate and centrifuged through a 5.7 M CsCl cushion. RNA was then size fractionated through a 1.4% agarose gel in 0.7 M formalde-
M EDTA. 32 P-labeled cDNA probes were prepared by the random primer method. The membranes (Magnagraph Nylon; Micron Separations, Westborough, MA) were washed twice with 5× SSPE/10% SDS at room temperature, twice with 1× SSPE/0.1% SDS, and once with 0.1× SSPE/10% SDS at 60 for 15 min each time. Autoradiography was performed using Fuji RX film and an intensifying screen at -80. Radioactivity of the corresponding bands on Northern membranes was quantified using an image analyzing system (BAS 2000; Fuji Film, Tokyo, Japan). Results were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal. Statistical analyses were performed using quantitative data from four independent Northern blot experiments. The results shown are representative of four separate Northern blot analyses that yielded similar results.

In situ hybridization

A 520-bp fraction was excised from the noncoding region of rat FasL cDNA (33) to prepare an antisense probe and a sense probe labeled with digoxigenin. Tissue specimens were fixed in 4% paraformaldehyde with 0.5% glutaraldehyde and embedded in low melting point paraffin. After deparaffinization in xylene, sections were digested with 100 μg/ml proteinase K in 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA for 10 min at room temperature. The hybridization buffer contained 0.6 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, 10% dextran sulfate, 0.25% SDS, 200 μg/ml RNA. 1× Denhardt’s solution, 10 mM DTT, and 50% deionized formamide. A volume of 50 μl of hybridization buffer that contained either the antisense or sense probe was applied to each section. This was followed by incubation in a humid chamber with 2× SSC and 50% formamide for 16 h at 50°C. Sections were washed in 2× SSC and 50% formamide for 30 min at 50°C, followed by incubation with 20 mg/ml RNase A (Boehringer Mannheim) for 30 min at 37°C. After washing sections once in 2× SSC for 20 min and twice in 0.2× SSC for 20 min each at 42°C, the digoxigenin-labeled probe was detected using anti-digoxigenin-alkaline phosphatase, and visualized using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

Statistical analyses

Values are expressed as means ± SD. One-way ANOVA was used for statistical analysis, followed by Scheffe’s multiple comparison test. Findings of p < 0.05 were considered significant.

Results

Histopathology

On day 21, typical myocarditic findings, including extensive injury of cardiomyocytes along with marked infiltration by inflammatory cells, such as lymphocytes, macrophages, neutrophils, and giant cells, was observed in the myocardium (Fig. 1). On days 14 and 17, fibrosis associated with focal infiltration of inflammatory cells was observed, and on day 60, no inflammatory cells were present and inflammatory regions had been replaced by fibrosis. Neither infiltration by inflammatory cells nor myocardial injury was observed on day 21-M (data not shown). On day 21-F (Fig. 1B) and day 21-F-L (Fig. 1E), myocardial tissue exhibited only focal infiltration by inflammatory cells, and damage of myocytes was rare, while on day 21-F-C (Fig. 1C) and day 21-F-L-C (Fig. 1F), marked infiltration by inflammatory cells, including lymphocytes, macrophages, neutrophils, and giant cells, was also observed along with severe injury of cardiomyocytes, as on day 21. No inflammatory cell infiltration or tissue injury of each organ except heart was observed on days 21-F and 21-F-L (data not shown).

TUNEL assay

No positive staining of myocardial nuclei was observed on TUNEL assay on days 21-M (data not shown) and 60 (data not shown), while some of the nuclei of myocardial cells and lymphocytes stained positive on days 14 (Fig. 2b), 17 (Fig. 2, c and e), 21 (Fig. 2d), 35, 21-F-C (data not shown), and 21-F-L-C (data not shown), but rare on day 21-F (Fig. 2f) and day 21-F-L (Fig. 2g). On day 17, myocardial cells (Fig. 2, i and j) and lymphocytes (Fig. 2, k and l) exhibited a positive nuclear staining on TUNEL assay. No positive staining of the nuclei of myocardial cells and lymphocytes was observed on a TdT omitted control for the TUNEL assay (Fig. 2h).

Apoptotic index

The apoptotic indices of myocytes and lymphocytes were both highest on day 17 (Table II). Apoptotic indices in rats treated with anti-Fas Ab (day 21-F) or anti-FasL Ab (day 21-F-L) were significantly lower than those in rats nontreated with them, respectively (Table II).

Agarose gel electrophoresis of DNA

Laddering of nuclear DNA, which consisted of various bands of 180 bp, was confirmed for days 17 and 21. On days 21-M (data not shown), 14, 35, and 60, only the original DNA bands were observed (Fig. 3).

Immunohistochemistry

Immunohistochemical staining using anti-Fas Ab. On days 14, 17, and 21, the surfaces of myocardial cells stained positive for anti-Fas Ab. The cell membranes of some lymphocytes stained positive for anti-Fas Ab on days 17 and 21. Figure 4 shows some myocytes (Fig. 4A) stained positive for anti-Fas Ab on day 17, while on day 21-M (−) (Fig. 4B), no anti-Fas-positive myocytes were observed.

Immunohistochemical staining using anti-FasL Ab. On days 14, 17, 21, and 35, which exhibited inflammatory cell infiltration, some of the infiltrating lymphocytes stained positive for anti-FasL.
Figure 5A shows some lymphocytes stained positive for anti-FasL on day 17. The percentage of anti-FasL-positive lymphocytes was highest on day 17 (data not shown), while on days 21-M(−) and 60, no anti-FasL-positive lymphocytes were observed (data not shown).

**Double staining using anti-rat CD4 and anti-FasL Abs or anti-rat CD8 and anti-FasL Abs.** On days 14, 17, 21, and 35, lymphocytes stained positive for anti-FasL Ab were also positive mainly for anti-rat CD4 Ab and rarely for anti-rat CD8 Ab. Figure 6 discloses FasL-positive lymphocytes showing CD4-positive (Fig. 6, A and B) and CD8-positive (Fig. 6, C and D).

**Double staining using anti-Fas Ab and anti-FasL Ab.** On days 14, 17, 21, and 35, positive staining with anti-FasL Ab was observed in some of the Fas-positive lymphocytes (Fig. 6, E and F) and cardiomyocytes.

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**FIGURE 2.** TUNEL assay. No positive staining of myocardial nuclei is observed on day 21-M(−) (a, original magnification, ×200). Some myocardial cells and lymphocytes exhibit positive nuclear staining on days 14 (b, original magnification, ×200), 17 (c, original magnification, ×200), and 21 (d, original magnification, ×200). Some of the nuclei of myocardial cells (arrows) and lymphocytes (arrowheads) stained positive on day 17 (e, original magnification, ×400), but rare on day 21-F (f) or 21-F-L (g) (f and g, original magnification, ×200). No positive staining of the nuclei of myocardial cells and lymphocytes was observed on a negative control for the TUNEL assay (h) (h, original magnification, ×200). i and j, Some anti-α actinin Ab-positive myocytes (j) are TUNEL assay positive (i) (original magnification, ×400) (white arrows) on day 17. k and l, Some FITC anti-rat CD3 Ab-positive lymphocytes (l) are TUNEL assay-positive lymphocytes (k) (original magnification, ×400) (white arrows) on day 17.

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### Table II. Apoptosis indices of myocytes and lymphocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>Myocytes (%)</th>
<th>Lymphocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 14</td>
<td>0.022 ± 0.059</td>
<td>0.98 ± 0.33</td>
</tr>
<tr>
<td>Day 17</td>
<td>0.99 ± 0.48*</td>
<td>1.67 ± 0.29#</td>
</tr>
<tr>
<td>Day 21</td>
<td>0.53 ± 0.71</td>
<td>0.61 ± 0.18</td>
</tr>
<tr>
<td>Day 21-M(−)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Day 21-F</td>
<td>0.012 ± 0.022**</td>
<td>0.12 ± 0.17###</td>
</tr>
<tr>
<td>Day 21-F-C</td>
<td>0.58 ± 0.23</td>
<td>0.72 ± 0.25</td>
</tr>
<tr>
<td>Day 21-F-L</td>
<td>0.029 ± 0.051***</td>
<td>0.23 ± 0.31###</td>
</tr>
<tr>
<td>Day 21-F-L-C</td>
<td>0.48 ± 0.33</td>
<td>0.62 ± 0.42</td>
</tr>
<tr>
<td>Day 35</td>
<td>0.009 ± 0.001</td>
<td>0.003 ± 0.002</td>
</tr>
<tr>
<td>Day 60</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Apoptotic indices are as follows: for myocytes: *p < 0.01 vs day 21-M(−), 14, 21, 35, and 60; **p < 0.001 vs day 21-F-C; ***p < 0.001 vs day 21-F-L-C; for lymphocytes: *p < 0.01 vs day 21-M(−), 14, 21, 35, and 60; **p < 0.001 vs day 21-F-C; ###p < 0.001 vs day 21-F-L-C.
Northern blot analysis

Northern blot analysis of total RNA from rat heart tissues with cDNA-encoding rat Fas revealed a distinct band corresponding to the size of rat Fas mRNA. The levels of expression of Fas mRNA were higher on days 17 and 21 than on days 21-M(2) and 14 (Fig. 7).

In situ hybridization

Some of the infiltrating lymphocytes were strongly positive for FasL mRNA. On day 17, some of the infiltrating lymphocytes exhibited positive reactivity for FasL cDNA antisense probe (Fig. 5B), but negative reactivity for FasL cDNA sense probe (Fig. 5C).

Discussion

The present study demonstrated for the first time that apoptotic death of cardiomyocytes and infiltrating T lymphocytes occurs in rat hearts with myosin-induced autoimmune myocarditis, suggesting that apoptosis plays a role in the pathogenesis of myocardial injury in myocarditis. The present study also demonstrated that myocardial inflammation was significantly less pronounced and that the number of TUNEL assay-positive cells in inflammatory lesions was significantly smaller in rats with myocarditis treated with anti-Fas Ab or with anti-FasL Ab than in those not treated with it. The occurrence of apoptosis of injured myocardial cells and T lymphocytes in animals with experimental myocarditis was detected on days 14, 17, 21, and 35 after immunization. The numbers of apoptotic myocardial cells and lymphocytes peaked on day

FIGURE 3. Agarose gel electrophoresis of DNA. M: marker. On days 17 (17) and 21 (21), laddering of nuclear DNA, which consisted of various bands of 180 to 200 bp, is shown. But there is no laddering formation on days 21-M(−) (C), 14 (14), 35 (35), and 60 (60); only the original DNA bands are observed.

FIGURE 4. Immunohistochemical staining for Fas Ag myocardial cells shows positive staining for anti-Fas Ab on day 17 (arrowheads) (A, original magnification, ×400). On day 21-M(−), the surfaces of myocardial cells stain negative for anti-Fas Ab (B, original magnification, ×400).

FIGURE 5. Immunohistochemical staining for FasL and in situ hybridization for FasL mRNA. On day 17, some of the infiltrating lymphocytes are positive for FasL (A, original magnification, ×400) (arrowheads). In particular, on day 17, the cytoplasm of some of the infiltrating lymphocytes shows positive reactivity for the rat FasL cDNA antisense probe (B, original magnification, ×200) (arrows), and negative reactivity for the rat FasL cDNA sense probe (C, original magnification, ×200).

FIGURE 6. Double staining using anti-FasL Ab/anti-rat CD4, anti-FasL Ab/anti-rat CD8, or anti-FasL Ab/anti-FasL Ab. Upper panels, Lymphocytes (arrows) that stain positive for both the anti-FasL Ab (A) and the anti-rat CD4 Ab (B) on day 17. Middle panels, Lymphocytes (arrows) that stain positive for both the anti-FasL Ab (C) and the anti-rat CD8 Ab (D) on day 17. Lower panels, On day 17, lymphocytes (arrows) that stain positive for the anti-FasL Ab (E) and the anti-Fas Ab (F), and cardiomyocytes (arrowheads) that stain positive reactivity for anti-Fas Ab (F). The left and right panels are two views of the same slide, the left adjusted to visualize peroxidase staining, and the right adjusted to show fluorescein staining. A, B, C, D, E, and F, original magnification, ×400.
target cells, i.e., cardiomyocytes, was mainly CD4+ lymphocytes, which were mainly CD4+ expressed distinctly in regions with myocarditis in some infiltrating T cells, in rats. The Fas/Apo-1 (CD95) molecule is a type I cell surface receptor belonging to the nerve growth factor receptor/TNF-receptor family and is expressed in a variety of tissues, including the thymus, liver, lung, ovary, and heart of mice (40). Fas mediates apoptosis when cross-linked with anti-Fas Ab or FasL (41). FasL is a type II protein and one of the TNF-related cytokines, which are found in membrane-bound or soluble form, and is expressed in activated T cells, testis, and lens (42).

Lymphocyte-mediated cytotoxicity is one of the defense mechanisms against viral infection and cancers, and plays significant roles in autoimmune disease (34), allograft rejection (35), and tumor surveillance (36). Recently, two distinct pathways, one perforin based, the other Fas based, accounting for the cellular injury and cell death induced by lymphocytes, have been defined at the molecular level (28, 37). Perforin-mediated cytolysis by CD8+ cytotoxic T cells and NK cells is one of the mechanisms involved in viral myocarditis (38) and allograft rejection (35), and leads to myocardial necrosis. On the other hand, the Fas/FasL system is a key molecular regulator of apoptosis and is normally involved in the deletion of autoreactive T cells in the immune system and the elimination of activated T cells after response to foreign Ags (39). The Fas/Apo-1 (CDD5) molecule is a type I cell surface receptor belonging to the nerve growth factor receptor/TNF-α receptor family and is expressed in a variety of tissues, including the thymus, liver, lung, ovary, and heart of mice (40). Fas mediates apoptosis when cross-linked with anti-Fas Ab or Fasl (41). FasL is a type II protein and one of the TNF-related cytokines, which are found in membrane-bound or soluble form, and is expressed in activated T cells, testis, and lens (42).

The autoimmune myocarditis that can be induced by injecting rodents with porcine myosin is thought to mimick inflammatory disease of the heart (43). This myocarditis is characterized by massive infiltration of lymphocytes into the myocardium with myocardial necrosis induced by CTL specific for myosin protein, and is CD4+ T cell dependent (43). In the present study, we found that affected myocardium was infiltrated predominantly by CD4+ T cells and to lesser extent by CD8+ T cells, suggesting that lysis of target cells, i.e., cardiomyocytes, was mainly CD4+ T lymphocyte dependent. Dhein et al. (44) have demonstrated recently that TCR-triggered activation-induced death of peripheral T cells is mediated via Fas/FasL interaction. Following TCR stimulation, T cells express Fas and FasL, and FasL can cause autoimmune suicide of sensitive Fas-positive T cells in a cis or trans CD4+ and CD8+ manner (33). We therefore assumed that the loss of some cardiomyocytes and the deletion of infiltrating cytotoxic lymphocytes in the affected myocardium in this model resulted from apoptotic cell death through the Fas/FasL pathway. We found that expression of Fas Ag was increased significantly in some cardiomyocytes and lymphocytes, which were mostly CD4+ and CD8+ T cells, in rats with myocarditis, but not in control rats. Additionally, Fasl was expressed distinctly in regions with myocarditis in some infiltrating lymphocytes, which were mainly CD4+ but rarely CD8+.

We therefore assumed that apoptosis of cardiomyocytes precedes necrosis in this model of myocarditis. These data suggest that Fas/Fasl system might be involved in the pathogenesis of autoimmune myocarditis in rats. Lymphocyte-mediated cytotoxicity is one of the defense mechanisms against viral infection and cancers, and plays significant roles in autoimmune disease (34), allograft rejection (35), and tumor surveillance (36). Recently, two distinct pathways, one perforin based, the other Fas based, accounting for the cellular injury and cell death induced by lymphocytes, have been defined at the molecular level (28, 37). Perforin-mediated cytolysis by CD8+ cytotoxic T cells and NK cells is one of the mechanisms involved in viral myocarditis (38) and allograft rejection (35), and leads to myocardial necrosis. On the other hand, the Fas/FasL system is a key molecular regulator of apoptosis and is normally involved in the deletion of autoreactive T cells in the immune system and the elimination of activated T cells after response to foreign Ags (39). The Fas/Apo-1 (CD95) molecule is a type I cell surface receptor belonging to the nerve growth factor receptor/TNF-receptor family and is expressed in a variety of tissues, including the thymus, liver, lung, ovary, and heart of mice (40). Fas mediates apoptosis when cross-linked with anti-Fas Ab or FasL (41). FasL is a type II protein and one of the TNF-related cytokines, which are found in membrane-bound or soluble form, and is expressed in activated T cells, testis, and lens (42).

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Furthermore, we found that the anti-Fas Ab or anti-Fasl Ab inhibited the development of myocardial damage/cell death in this myocarditic model. These findings suggest that the Fasl expressed on activated T lymphocytes may bind to Fas Ag on cardiomyocytes, the level of which has been increased by proinflammatory cytokines, leading to apoptosis of these cells, and that suicide of infiltrating T lymphocytes may be induced in cis or trans fashion through the Fas system as inflammation wanes. Thus, impairment of the myocardium in myocarditis may be the result of the following cycle: initial focal myocarditic inflammatory reaction, followed by further infiltration of inflammatory cells induced by the resulting myocardial cell destruction. Some of the CD4+ and CD8+ positive T cells among the infiltrating inflammatory cells expressed Fasl protein. T cells expressing Fasl protein may contact cardiomyocytes expressing Fas protein, resulting in apoptosis of the latter cells. It remains to be determined whether CD4+ and CD8+ T cells can directly lyse cardiomyocytes via Fas-mediated pathways.

In conclusion, we have obtained evidence suggesting that apoptosis of cardiomyocytes and infiltrating lymphocytes may be one of the mechanisms of myocardial injury and cell death in experimental autoimmune myocarditis. Our findings also suggest that the Fas/Fasl system may play a role in apoptotic cell death. Apoptosis of cardiomyocytes and lymphocytes may be an essential part of the process of repair in autoimmune myocarditis. These molecular systems deserve thorough clinical investigation, and it may be possible to develop new therapeutic agents based on T cell-mediated cytotoxicity.

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References


