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

Shigeru Ishiyama, Michiaki Hiroe, Toshio Nishikawa, Takashi Shimojo, Shinji Abe, Hiroyuki Fujisaki, Hiroshi Ito, Katsutoshi Yamakawa, Nobuyuki Kobayashi, Takeshi Kasajima and Fumiaki Marumo

*J Immunol* 1998; 161:4695-4701; http://www.jimmunol.org/content/161/9/4695

---

**References**

This article cites 39 articles, 16 of which you can access for free at: http://www.jimmunol.org/content/161/9/4695.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
The Fas/Fas Ligand System Is Involved in the Pathogenesis of Autoimmune Myocarditis in Rats

Shigeru Ishiyama, Michiaki Hiroe, Tosio Nishikawa, Takeshi Shimojo, Shinji Abe, Hiroyuki Fujisaki, Hiroshi Ito, Katsutoshi Yamakawa, Nobuyuki Kobayashi, Takeshi Kasajima, and Fumiaki Marumo

The mechanisms responsible for myocardial injury and cell death in myocarditis are still unclear. We examined whether myocardial cell death occurs via apoptosis in myocardium-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 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 laddering 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), respectively. The other 20 rats were i.p. injected with anti-Fas Ab (anti-APO-1/FAS, SM1/23, mouse

Copyright © 1998 by The American Association of Immunologists

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan (076770765 and 08670828); by a grant from the Japan Private School Promotion Foundation; by a grant from the Japan Research Promotion Society for Cardiovascular Diseases; and by a Research Grant for Diseases from Ministry of Health and Welfare of Japan.

*Second Department of Internal Medicine, Tokyo Medical and Dental University, Tokyo, Japan; †Department of Pathology, Tokyo Women’s Medical University School of Medicine, Tokyo, Japan; and ‡Department of Internal Medicine, Matsuoka Hospital, Tosu, Japan, and Department of Clinical Pharmaceutics, Faculty of Pharmaceutical Sciences, Nagasaki University, Nagasaki, Japan

Received for publication March 25, 1998. Accepted for publication July 1, 1998.

3 Abbreviations used in this paper: FasL, Fas ligand; DAB, diaminobenzidine tetrahydrochloride; DDW, double-distilled water; OCT, optimal cutting temperature; RT, room temperature; TUNEL, in situ terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling.

5 Address correspondence and reprint requests to Dr. Michiaki Hiroe, Cardiology Division, Second Department of Internal Medicine, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113, Japan.
Table I. Summary of the various treatment groups

<table>
<thead>
<tr>
<th>Groupa</th>
<th>n</th>
<th>Myosin Anti-Fb</th>
<th>Isotype Control mAbb</th>
<th>Anti-F-Lc</th>
<th>Isotype Control pAbb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 14</td>
<td>10</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>
</tr>
<tr>
<td>Day 17</td>
<td>10</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>
</tr>
<tr>
<td>Day 21-M(−)</td>
<td>5</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>
</tr>
<tr>
<td>Day 21-F-C</td>
<td>5</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>
</tr>
<tr>
<td>Day 21-F-L-C</td>
<td>5</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 35</td>
<td>7</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 60</td>
<td>7</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a Rats were treated with myosin except group day 21-M(−).
b Anti-Fas or isotype control mAb to anti-Fas was administered on day 14.
c Anti-Fasl, or isotype control pAb (polychromat rabbit Ab) to anti-Fasl was administered on day 14. The day given in the group identity is the day of sacrifice.

The Fas/FasL LIGAND SYSTEM PLAYS AN IMPORTANT ROLE IN MYOCARDITIS

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-PS, 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). MoAb IgG2b (Genzyme, Danvers, Mass., Japan) (0.1 mg/kg) was injected instead of anti-Fasl Ab (anti-APO-1/FAS) on days 14 (day 21-F-C, n = 5), as a control compared with day 21-F. Rabbit IgG (0.1 mg/kg) (Cell Interce Technologies, Hopewell, NJ) was injected instead of anti-Fasl Ab on day 14 (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-biotin nick end-labeling (TUNEL) assay and immunohistochemistry; 2) formalin-fixed specimens for histopathology and TUNEL assay; 3) 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.

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 Itoh 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 specimens with 2% H2O2 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 cacodylate, 1 mmol/L cobalt chloride) containing 0.3 U/ml deoxynucleotidyltransferase, 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 300 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 apoptotic 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, 35, and 60 were examined. Tissue specimens (50 mg each) were incubated in 700 ml of cytosolic buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1% SDS containing 100 μM Na2EDTA containing 100 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-Fasl Ab (FAS (M-20); Santa Cruz Biotechnology, Santa Cruz, CA) (26–30). To identify the subpopulation of anti-Fasl Ab-positive lymphocytes, the sections were double stained with anti-Fasl and anti-CD4 Abs or anti-Fasl and anti-CD8 Abs, and with the anti-Fasl 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-Fasl 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 1 h. For double staining, anti-Fasl 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. For the secondary Ab, 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-Fasl 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. For the secondary Ab, 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-Fasl 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. For the secondary Ab, goat anti-rabbit IgG-conjugated peroxidase (Mbl) (1/300 dilution) and examined under light and fluorescence microscopes after development of color using DAB.

Histology

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-rat CD3 Ab (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-rat CD3 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) (1/300 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 formaldehyde and 20 mM morpholinopropanesulfonic acid/5 mM sodium acetate/1

Downloaded from http://www.jimmunol.org/ by guest on April 20, 2017
mM EDTA. Northern blot hybridization was performed with hybridization buffer containing 50% formamide, 5× Denhardt’s solution, 100 ng/ml salmon sperm DNA, and 5× SSPE (0.75 M NaCl/0.05 M NaH₂PO₄/0.005 M EDTA). ³²P-labeled cDNA probes were prepared by the random primer method (32). The membranes (Magnagraph Nylon; Micron Separations, Westborough, MA) were washed twice with 5× SSPE/10% SDS at room temperature, twice with 1× SSPE/0.5% SDS, and once with 0.1× SSPE/10% SDS at 65 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) signals. 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 protease K in 10 mM Tris-Cl (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-Cl, 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, were observed in the myocardium (Fig. 1, A and D), while on day 21-M(−), no myocardial injury was observed on day 21 (original magnification, ×200). B and E. Only focal infiltration by inflammatory cells is observed in the rats treated with anti-Fas Ab (anti-APO-1/FAS) (day 21-F, B) or anti-Fasl Ab (day 21-F-L, E) (original magnification, ×200). C and F. Marked infiltration by inflammatory cells, including lymphocytes, macrophages, neutrophils, and giant cells, and extensive injury of cardiomyocytes are observed in the rats treated with normal mouse IgG2b (day 21-F-C, C) or normal rabbit IgG (day 21-F-L-C, F) as in day 21 (original magnification, ×200). A, B, C, D, E, and F are stained with hematoxylin-eosin.

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(−), 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 lymphocytes (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.

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(–) 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...
target cells, i.e., cardiomyocytes, was mainly CD4^+ lymphocytes, which were mainly CD4^+ but rarely CD8^+.

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 cytolyis 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-I (CD95) 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 mimic 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^-.

17, and were decreased on day 21, when inflammatory cell infiltration was most prominent and necrotic cardiomyocytes were found scattered in inflammatory lesions. In addition, these findings suggested 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.

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.

Acknowledgments

We thank Dr. Shigekazu Nagata, Department of Genetics, Osaka University School of Medicine, for editorial advice.

References

11. Kajstura, J., M. Mansukhani, W. Cheng, K. Reiss, S. Krajewski, J. C. Reed, F. Quaini, E. F. Sonnenblick, and P. Anversa. 1995. Programmed cell death and apoptosis of cardiomyocytes and the deletion of infiltrating 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^-.


