The Cardiac Fas (APO-1/CD95) Receptor/Fas Ligand System
Relation to Diastolic Wall Stress in Volume-Overload Hypertrophy
In Vivo and Activation of the Transcription Factor AP-1
in Cardiac Myocytes

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Background—Fas (APO-1/CD95) is a transmembrane receptor belonging to the tumor necrosis factor receptor superfamily. Cross-linking of Fas by Fas ligand (FasL), a tumor necrosis factor-α–related cytokine, promotes apoptosis and/or transcription factor activation in a highly cell-type–specific manner. The biological consequences of Fas activation in cardiomyocytes and the regulation of Fas and FasL abundance in the myocardium in vivo remain largely unknown.

Methods and Results—As shown by immunohistochemistry, Fas was expressed on the sarcolemma of cardiomyocytes in left ventricular tissue sections. Moreover, FasL was constitutively expressed in the myocardium and in isolated cardiomyocytes, as revealed by reverse transcription polymerase chain reaction and Western blotting. Left ventricular abundance of Fas but not FasL was upregulated in a rat model of compensated volume-overload hypertrophy and was closely related to diastolic but not systolic wall stress as determined by MRI. Cardiomyocyte apoptosis was not enhanced in volume-overload hypertrophy despite the increased expression of Fas and the presence of FasL in the myocardium. Moreover, injection of mice with an agonistic anti-Fas antibody promoted hepatocyte but not cardiomyocyte apoptosis in vivo. Stimulation of isolated cardiomyocytes with recombinant FasL promoted an activation of the transcription factor AP-1 as shown by electrophoretic mobility shift assays but did not induce cell death.

Conclusions—Fas and FasL are constitutively expressed in the myocardium and in cardiomyocytes. Myocardial expression of Fas is closely related to diastolic loading conditions in vivo. Signaling pathways emanating from Fas are coupled to an activation of the transcription factor AP-1 in cardiomyocytes. (Circulation. 2000;101:1172-1178.)

Key Words: receptors ♦ myocytes ♦ apoptosis ♦ hypertrophy

Cytokines including tumor necrosis factor-α (TNF-α) are emerging as potent regulators of cardiomyocyte hypertrophy and apoptosis and have been proposed to play an important role in the remodeling of the myocardium in response to chronic increases in hemodynamic load.1-2 Fas (APO-1/CD95) is a transmembrane receptor belonging to the TNF receptor superfamily.3-6 Activation of Fas requires cross-linking by Fas ligand (FasL), a TNF-α–related cytokine.7 Fas is expressed rather abundantly in the myocardium and in cardiomyocytes, which indicates that cardiomyocytes are potential targets for FasL–mediated effects.4,8,9 Indeed, a recent study has demonstrated that short-term exposure of cardiomyocytes to FasL can alter [Ca2+]i homeostasis.9 In many cell types, engagement of Fas by FasL is followed by caspase activation and apoptotic cell death.6 However, the response to Fas activation has been shown to be highly cell-type specific, with certain cells responding to Fas ligation with the induction of transcription factors and gene transcription rather than cell death.10-12 In this regard, the biological consequences of Fas activation in cardiomyocytes remain largely unknown. Overstretching of papillary muscles ex vivo has been shown to enhance cardiomyocyte Fas expression.13 Because Fas expression levels can determine cell susceptibility to FasL–mediated effects,6 it is crucial to understand whether more physiological levels of mechanical load that can be observed in cardiac hypertrophy in vivo are involved in the regulation of myocardial Fas expression.

In the present study, we investigated the regulation of Fas and FasL in the myocardium in relation to hemodynamic load in the in vivo setting, and we explored the functional
significance of Fas activation in cardiomyocytes. With the use of a combined in vivo and in vitro approach, we demonstrated that the abundance of Fas in the myocardium is closely related to diastolic loading conditions and that signaling pathways emanating from Fas are coupled to an activation of the transcription factor AP-1 in cardiomyocytes.

Methods

Immunohistochemistry

The expression pattern of Fas in left ventricular (LV) myocardium was determined by immunohistochemistry. Rabbit polyclonal IgG (1:400) directed against the intracellular domain of mouse/rat Fas was used as the primary antibody (FAS-M20, Santa Cruz). Secondary (mouse anti-rabbit) and tertiary (goat anti-mouse alkaline phosphatase) antibodies were obtained from Dako. Fas expression was visualized with Fast blue. The slides were counterstained with hematoxylin. Fas peptide (FAS-M20P, Santa Cruz) was used in control experiments.

Northern Blotting

Fas mRNA expression was quantified by Northern blotting with the use of a rat Fas cDNA probe obtained by polymerase chain reaction (PCR) cloning. Separate filters were hybridized to a rat atrial natriuretic factor probe. cDNA probes were [32P]-labeled by random priming. To control for loading and transfer efficiency, filters were hybridized to a [32P]-end-labeled oligonucleotide complementary to 18S rRNA.

Quantification of FasL mRNA Expression

FasL mRNA expression was quantified by standard calibrated reverse transcription (RT)-PCR. A 495-bp FasL cDNA fragment was amplified from LV total RNA and cloned into pCR2.1 (Invitrogen). Forward primer nt371 to 392, reverse primer nt844 to 865. The resulting pCR2.1-FasL(495) plasmid was then amplified by PCR with the use of the reverse primer and a mutagenic forward primer composed of nt371 to 392 fused to nt493 to 512, thereby producing a FasL cDNA fragment with a 100-bp deletion. The 395-bp fragment was cloned into pCR2.1, generating pCR2.1-FasL(395). Competitor rat FasL cRNA molecules were synthesized by in vitro transcription from pCR2.1-FasL(395) (T7-MEGASHortscript, Ambion).

LV total RNA (625 ng) was reverse-transcribed into cDNA along with increasing quantities of FasL competitor cRNA molecules (10^4 to 5 × 10^5) and subsequently amplified by PCR by use of the FasL forward and reverse primers (36 cycles: 1 minute at 94°C, 1 minute at 53°C, 1 minute at 72°C each). In no case was a PCR product obtained when reverse transcriptase was omitted from the reaction. The FasL target and FasL competitor RT-PCR products were separated by ethidium bromide agarose gel electrophoresis and analyzed by laser densitometry.

Western Blotting

FasL protein expression was analyzed by Western blotting, with a monoclonal mouse antibody generated against the extracellular domain of human FasL (Transduction Laboratories).

Volume-Overload Cardiac Hypertrophy Model

Aortic regurgitation was induced by cusp perforation in male Wistar-Kyoto rats (weight 250 to 300 g). Control animals were instrumented but did not undergo cusp perforation. Eight weeks after the procedure, LV end-diastolic and end-systolic dimensions were determined by MRI. After MRI data acquisition, an ultraminiature catheter pressure transducer (Millar) was inserted through the left carotid artery and advanced into the ascending aorta and LV cavity. Systolic blood pressure and LV end-diastolic pressure were recorded. On the basis of the hemodynamic data and the MRI-derived values for LV wall thickness and radius, in vivo end-diastolic and end-systolic wall stresses were calculated according to Laplace’s law. After completion of the hemodynamic analyses, the heart was quickly removed and rinsed in ice-cold saline. The LV and the right ventricular free wall were separated and weighed. The LV free wall was divided into halves and snap-frozen for later isolation of total RNA and genomic DNA or embedded in OCT medium for later preparation of cryosections.

DNA Agarose Gel Electrophoresis

To detect internucleosomal cleavage of genomic DNA, a hallmark of apoptotic cell death, DNA was isolated from LV tissue and subjected to ethidium bromide agarose gel electrophoresis (10 μg/sample).

In Situ Nick End-Labeling

Apoptotic nuclei were detected in LV myocardium by in situ terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-
conjugated dUTP nick end-labeling with the use of a commercially available kit (ApopTag Plus, Peroxidase, Oncor).22 LV cryostat sections (5 μm) were mounted on glass slides, fixed in 10% buffered formalin, and postfixed in ethanol:acetic acid (2:1) at −20°C. Sections were then treated with 8 μg/mL proteinase K for 5 minutes at room temperature. End-labeling was carried out according to the manufacturer’s instructions, and tissue sections were finally counterstained with hematoxylin and eosin to allow a discrimination between cardiac myocyte and nonmyocyte nuclei. Five tissue sections per animal were examined at ×400 magnification. Positive controls were prepared by treating selected slides with 0.5 μg/mL DNase I for 10 minutes at room temperature; dUTP labeling was never observed when TdT was omitted from the reaction.

Treatment of Mice With an Agonistic Anti-Fas Antibody

Five-week-old Balb/c mice were injected intraperitoneally with 10 μg of a monoclonal hamster anti-mouse Fas IgG (Jo2, Pharmingen).23 Control mice were treated with anti-trinitrophenol IgG (Pharmingen). After 24 hours, cryosections were prepared from the LV and liver and analyzed by in situ nick end-labeling.

Cardiomyocyte Culture

Neonatal rat ventricular cardiomyocytes and nonmyocytes were isolated from 1- to 3-day-old Sprague-Dawley rats.24,25 Myocytes were plated at a density of 5 × 10⁴ cells/cm² in gelatin-coated tissue culture plates. After overnight incubation in serum-containing medium, cardiomyocytes were switched to serum-free medium and stimulated with various agents. Cell survival was assessed by trypan blue exclusion under a phase contrast microscope. Nonmyocytes were enriched by differential plating for 1 hour in 10-cm tissue culture plates. Cells adhering to the culture dish were grown to confluence over a period of 2 to 3 days.25 Adult rat ventricular cardiomyocytes were isolated from male Sprague-Dawley rats (weight 380 to 420 g).26 Adult cardiomyocytes were plated in laminin-coated tissue culture plates (1 × 10⁶ cells/cm²). More than 98% of the cells displayed a rod-shaped morphology. Cells were stimulated in serum-free medium with various agents. Cell viability was determined by counting rod-shaped cells per field.27 Recombinant human soluble FasL expressed as an epitope tag fusion protein and a cross-linking mouse monoclonal IgG directed against the epitope tag were purchased from Upstate Biotechnology.

Electrophoretic Mobility Shift Assay

Neonatal rat ventricular cardiomyocytes were plated into 6-cm dishes and serum-starved for 24 hours before stimulation with recombinant FasL and cross-linking IgG. AP-1 and nuclear factor-κB DNA binding was detected by electrophoretic mobility shift assay.28–30 Supershift experiments were performed with rabbit polyclonal IgG directed against c-Jun. Rabbit polyclonal IgG directed against retinoblastoma protein was used as a control (both antibodies from Santa Cruz).

Statistical Analysis

Data are presented as mean ± SEM. The unpaired Student’s t test was used for intergroup comparisons. Linear regression analysis was performed to test for a correlation between 2 variables. A 2-tailed P value of <0.05 was considered to indicate statistical significance.

Results

Expression of Fas and FasL in Left Ventricular Myocardium

As shown by immunohistochemistry, Fas was localized primarily on the sarcolemma of cardiomyocytes (Figure 1B). No staining could be detected when the primary antibody was omitted from the reaction (Figure 1A). Furthermore, Fas staining was abolished by coincubation with the Fas peptide used for immunization (Figure 1C). FasL mRNA (reverse transcription–polymerase chain reaction) and protein (Western blotting) were readily detectable in the myocardium as well as in isolated cardiomyocytes and nonmyocytes (Figure 2, A and B).

Volume-Overload Hypertrophy Model

To analyze the abundance of Fas and FasL in the myocardium in relation to hemodynamic load in vivo, we used a rat model of volume-overload hypertrophy (Table 1). Left ventricular (LV) end-diastolic and end-systolic wall stresses were increased in volume-overloaded rats. As determined in post mortem examination, volume overload resulted in an increase

![Figure 2. Detection of FasL expression by RT-PCR and Western blotting. A, 495-bp FasL cDNA fragment was amplified from LV myocardium (lane 1), purified adult rat cardiomyocytes (lane 2), and neonatal rat ventricular nonmyocytes (lane 3) by a 36-cycle, non–standard-calibrated RT-PCR (625 ng of total RNA per reaction). On left, DNA ladder was loaded. By Western blotting, FasL could be detected as 32-kDa protein in LV myocardium (B, lane 1), purified adult rat cardiomyocytes (lane 2), and neonatal rat ventricular nonmyocytes (lane 3). Forty micrograms of protein was loaded per lane. On left, positions of molecular weight standards are shown.](Image 316x572 to 528x718)
in LV-to-body weight ratio and an upregulation of LV atrial natriuretic factor mRNA expression.

Regulation of Fas and FasL in Volume-Overload Hypertrophy

Chronic volume overload resulted in an upregulation of LV Fas mRNA expression (Figure 3A). LV abundance of Fas was closely related to end-diastolic (Figure 3B) but not end-systolic wall stress (not shown) in volume-overload hypertrophy. As shown by standard-calibrated RT-PCR, LV expression of FasL mRNA did not differ significantly between control and volume-overloaded animals (7.3 ± 1.2 vs 6.6 ± 1.0 × 10³ mRNA transcripts/625 ng RNA, respectively) and did not correlate with ventricular wall stress (not shown).

Cardiomyocyte Apoptosis in Volume-Overload Hypertrophy

We next investigated whether enhanced Fas expression levels in volume-overload hypertrophy were associated with an increase in apoptotic cell death in the myocardium. However, no evidence of DNA fragmentation was found in control hearts and in hearts with volume-overload hypertrophy (not shown). Because the DNA-laddering technique is rather insensitive, we used in situ nick end-labeling to detect apoptotic cardiomyocyte nuclei in LV myocardium: There was no significant difference in the prevalence of dUTP-labeled cardiomyocyte nuclei between control and volume-overloaded rats (Figure 4).

Effect of Fas Activation on Cardiomyocyte Viability

To assess whether Fas activation can trigger cardiomyocyte death in vivo, mice were injected with an agonistic anti-Fas antibody. In mice treated with control antibody, dUTP-labeled cells were very rare in the LV and the liver (Figure 5, A and C). Injection of anti-Fas induced severe liver damage resulting from extensive hepatocyte apoptosis (Figure 5D) but did not promote cardiomyocyte apoptosis in LV myocardium (Figure 5B). Conceivably, the lack of apoptosis in the heart after injection of anti-Fas may reflect a different efficiency of penetration of the antibody into cardiac versus hepatic tissues. To address this possibility, cardiomyocytes were isolated from neonatal and adult rats and exposed to recombinant soluble FasL in vitro for up to 48 hours. Even in the presence of cross-linking antibodies, however, FasL did not promote cell death in cultured cardiomyocytes (Table 2).

Effect of FasL on Activation of AP-1 and Nuclear Factor-κB

To investigate whether FasL can trigger transcription factor activation in cardiomyocytes, DNA-binding activities of AP-1 and nuclear factor (NF)-κB were analyzed by elec-
phoretic mobility shift assay. Stimulation of neonatal rat ventricular myocytes with recombinant FasL and cross-linking antibodies induced AP-1 DNA-binding activity (Figure 6) but did not enhance NF-κB DNA binding (not shown). The specificity of AP-1 DNA binding was supported by competition experiments with 100-fold molar excess of unlabeled AP-1. As expected, unlabeled NF-κB did not compete for AP-1 DNA binding. The AP-1/DNA complex was supershifted in part by an antibody directed against c-Jun but not by a control antibody directed against the unrelated retinoblastoma protein (Figure 6).

**TABLE 2. Effect of Recombinant FasL on Cardiomyocyte Viability**

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<th>24 h Cell Viability</th>
<th>48 h Cell Viability</th>
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<tr>
<td><strong>Adult CM</strong></td>
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<tr>
<td>Control</td>
<td>100±15</td>
<td>72±11</td>
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<tr>
<td>IgG</td>
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<td>68±9</td>
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<tr>
<td>FasL</td>
<td>97±9</td>
<td>75±8</td>
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<td>IgG+FasL</td>
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<td>68±6</td>
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<tr>
<td><strong>Neonatal CM</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>100±8</td>
<td>91±9</td>
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<td>IgG</td>
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Ventricular cardiomyocytes (CM) were isolated from adult or neonatal rats and cultured in triplicate in 24-well plates. Cells were stimulated with serum-free medium alone (control), cross-linking IgG (1.5 μg/mL), recombinant FasL (50 ng/mL), or cross-linking IgG and recombinant FasL combined. Cell viability was determined after 24 and 48 hours by counting rod-shaped cells adhering to the laminin substrate (adult CM) or by trypan blue exclusion (neonatal CM). Ten fields per well were evaluated in a blinded fashion. Control values at 24 hours were set at 100%. Values are mean±SEM from 3 independent experiments.

The specificity of AP-1 DNA binding was supported by competition experiments with 100-fold molar excess of unlabeled AP-1. As expected, unlabeled NF-κB did not compete for AP-1 DNA binding. The AP-1/DNA complex was supershifted in part by an antibody directed against c-Jun but not by a control antibody directed against the unrelated retinoblastoma protein (Figure 6).

**Discussion**

Cytokines, including TNF-α and members of the interleukin-6 cytokine family, have been recognized as potent regulators of cardiomyocyte growth and apoptosis. Fas is a member of the TNF receptor superfamily of structurally related cytokine receptors. In patients with heart failure, circulating levels of soluble Fas and FasL are increased, which suggests a potential role of Fas in this setting. Although Fas promotes caspase activation and apoptosis in susceptible cell types, Fas can mediate biological functions unrelated to cell death, such as transcription factor activation and the induction of cell growth and differentiation. In the present study, we investigated the regulation of Fas and FasL in the myocardium in relation to hemodynamic load in vivo and explored the functional significance of Fas activation in cardiomyocytes.

**Fas and FasL Are Expressed in Myocardium and in Cardiomyocytes**

As shown by immunohistochemistry in our study, Fas was localized on the sarcolemma of cardiomyocytes, supporting...
Regulation of Fas and FasL in Volume-Overload Hypertrophy

It has previously been shown that overstretching induces Fas expression in isolated papillary muscles.13 To study the regulation of myocardial Fas and FasL abundance in relation to more physiological levels of mechanical load in vivo, we used a rat model of volume-overload hypertrophy. As shown by MRI, volume-overload hypertrophy was characterized by LV chamber dilation but no change in wall thickness. The changes in LV geometry combined with the increases in LV end-diastolic pressure and systolic blood pressure translated into a significant elevation of LV diastolic and systolic wall stress. The increase in LV mass was associated with enhanced atrial natriuretic factor expression levels, confirming hypertrophy of the cardiomyocyte compartment. Right ventricular–body weight ratio was unchanged and mean arterial pressure was preserved, which indicates a compensated stage of LV hypertrophy (data not shown). LV expression of Fas was upregulated in relation to diastolic but not systolic wall stress in volume-overload hypertrophy. FasL, by contrast, was not induced, which indicates distinct regulatory mechanisms. Although a causal relation cannot be inferred from these data, the close relation between Fas expression and diastolic wall stress suggests that Fas expression levels in the myocardium are modulated by loading conditions in vivo.

Functional Significance of Fas Activation in Cardiomyocytes

In the present study, recombinant soluble FasL did not promote cell death in isolated cardiomyocytes. Like TNF-α, FasL is synthesized as a membrane-bound protein that can be converted by proteolytic cleavage into a soluble form that is then released into the circulation.35 Although soluble FasL has been shown to induce apoptosis in susceptible cell types,36,37 it has been reported that membrane-bound FasL is more active as compared with soluble FasL and that the proapoptotic activity of soluble FasL can be restored by cross-linking antibodies.38 As shown in the present study, however, cross-linking of soluble FasL did not enhance its cytotoxic activity in cultured cardiomyocytes. Moreover, supernatants from a murine neuroblastoma cell line stably transfected with a murine FasL expression vector did not promote cardiomyocyte death in vitro (data not shown), although FasL is released from these cells in vesicles, that is, in a membrane-bound, unprocessed form.39,40 In agreement with our in vitro results, an agonistic anti-Fas antibody induced hepatocyte apoptosis but did not promote cardiomyocyte apoptosis in vivo. Taken together, it appears that the signaling cascade coupling Fas to the induction of cell death is inhibited at some crucial point(s) in isolated cardiomyocytes and in normal myocardium. However, a general resistance of cardiomyocytes to Fas-mediated apoptosis cannot be inferred from these data. In the present study, increased Fas expression levels were not associated with enhanced cardiomyocyte apoptosis in rats with compensated volume-overload hypertrophy. By contrast, increased levels of Fas are accompanied by pronounced increases in cardiomyocyte apoptosis in overstretched and in ischemic myocardium.13,40 It is conceivable, therefore, that cardiomyocytes may become susceptible to Fas-mediated cell death under certain pathophysiological conditions.

As shown by EMSA in the present study, Fas-dependent signaling pathways are coupled to the activation of the transcription factor AP-1 in isolated cardiomyocytes. By contrast, Fas ligation did not result in NF-κB activation. AP-1 is a transcriptional activator composed of Jun and Fos gene family members.41 Indeed, supershift experiments indicated that c-Jun is an integral part of the AP-1/DNA complex induced by FasL in cardiomyocytes. FasL has been shown to activate AP-1 in other cell types as well, and in some cases, stimulation of AP-1 appears to be independent from the induction of apoptosis.10,42 In cardiomyocytes, AP-1 has been implicated in the transcriptional regulation of several genes associated with a hypertrophic response.43,44 Our data therefore raise the intriguing possibility that the Fas receptor can modulate gene expression through AP-1 in cardiomyocytes. In this regard, a preliminary study has recently demonstrated that cardiac-specific overexpression of FasL promotes cardiomyocyte hypertrophy in transgenic mice.45

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