Published in final edited form as:

J Mol Cell Cardiol. 2006 June ; 40(6): 810–820.

Targeted cardiac expression of soluble Fas prevents the development of heart failure in mice with cardiac-specific expression of MCP-1[★]

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Abstract

Monocyte chemoattractant protein-1 (MCP-1) plays a crucial role in initiating coronary heart disease by recruiting monocytes/macrophages to the vessel wall. Transgenic mice with cardiac-specific expression of MCP-1 manifest cardiac inflammation and develop heart failure. The pathways mediating the detrimental effects of MCP-1 expression have not been defined. We postulate that the Fas ligand (FasL) derived from the infiltrating mononuclear cells causes death of cardiac cells resulting in the development of heart failure. Here, we tested this hypothesis by determining whether inhibition of FasL function through cardiac-specific expression of soluble Fas (sFas) would rescue the MCP-1 transgenic mice from developing heart failure. We generated mice with cardiac-specific expression of sFas and double homozygous transgenic mice that express both MCP-1 and sFas. Cardiac-specific expression of sFas in MCP mice, in fact, inhibited apoptosis of infiltrating mononuclear cells, normalized circulating C-reactive protein (CRP) levels, and prevented macrophage activation as well as production of proinflammatory cytokines, tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6 in the hearts. sFas expression resulted in restoration of cardiac structure, preservation of cardiac function, and a significant prolongation of survival of MCP mice. These results demonstrate that FasL released from infiltrating mononuclear cells plays a critical role in the detrimental effects of MCP-1 expression, and suggest that Fas/FasL signaling represents a novel therapeutic target for heart failure.

Keywords

Chemokines; Inflammation; Fas ligand; Apoptosis; Cardiomyopathy; Heart failure; Gene therapy; Soluble Fas

[★]This work was presented in part at the 2004 Scientific Sessions of the American Heart Association, New Orleans, Louisiana, USA, in November, 2004.

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1. Introduction

Over the past decade, inflammation has been recognized as a major contributor to the pathogenesis of ischemic heart disease [1,2]. The mechanisms underlying the role of inflammation in the development of cardiac dysfunction remain poorly understood. The recruitment and activation of monocytes/macrophages by monocyte chemoattractant protein-1 (MCP-1/CCL2) are thought to be an important event that contributes to the initiation and pathophysiology of ischemic heart disease [2–4]. MCP-1 is involved in the development of atherosclerosis [5], postischemic myocardial remodeling [6,7], and experimental and clinic heart failure [8]. In an attempt to mimic the inflammatory component implicated in the development of heart disease, we developed transgenic mice with cardiac-specific expression of MCP-1 [9]. These MCP-1 transgenic mice suffer a thrombotic occlusive arteriolar vasculopathy that results in ischemia, interstitial fibrosis, ventricular chamber dilation, impaired cardiac function, and increased mortality with congestive heart failure by about 6 months of age [9,10]. The clinical, histopathological, and molecular changes that occur in the hearts of these animals closely resemble the changes observed in human ischemic cardiomyopathy and heart failure [11,12]. The infiltrating mononuclear cells, vascular endothelial, and smooth muscle cells as well as some cardiac myocytes in the hearts of MCP mice display apoptotic cell death features.

How the chronic inflammation resulting from the production of MCP-1 leads to the development of heart failure is not known. We postulate that Fas ligand (FasL) derived from the dying mononuclear cells and activated macrophages causes Fas-mediated cell death and inflammatory response in the heart, and may contribute to cardiac damage in this model. The Fas/ FasL system has been identified as a major pathway involved in the induction of apoptotic cell death in many cell types, particularly cells related to the immune system [13,14]. Fas is widely expressed in many cell types, including cardiomyocytes, smooth muscle cells as well as endothelial cells, and there is increasing evidence suggesting that Fas-mediated apoptosis is associated with myocarditis [15], myocardial reperfusion injury [16], and postinfarction ventricular remodeling [17]. Recent studies indicate that binding of FasL to Fas can also induce production of proinflammatory cytokines [18,19] and thus amplify inflammatory response [14,20,21]. It has been shown that FasL derived from monocytes/macrophages functions as a pathogenic, death-inducing cytokine capable of promoting apoptosis of vascular smooth muscle cells in the atherosclerotic lesions [22]. These findings support our hypothesis concerning the central role played by FasL released by dying monocytes in the development of heart failure in this model. If this hypothesis is valid, cardiac expression of soluble Fas (sFas), a competitive inhibitor of FasL [23], should be able to prevent the apoptotic process as well as inflammatory response in the heart and thus prevent the development of cardiomyopathy and subsequent heart failure in this murine model. In this paper, we demonstrate that cardiomyocyte-targeted expression of sFas in MCP mice, in fact, prevents apoptotic cell death of cardiac cells and local inflammation in the heart, and rescues MCP mice from developing of heart failure.

2. Methods

2.1. Animals

Mice were bred and maintained at the WM Keck Genetic Research Facility of the Ohio State University and the University of Central Florida, and the experimental procedures in mice and protocols used in this study were approved by the Animal Care and Use Committee of both universities, conforming with the *Guide for the care and use of laboratory animals* published by US National Institutes of Health (NIH publication No. 85-23, revised 1996). For this study, we used homozygous male MCP-1 transgenic mice (line 6) [9], and age- and gender-matched FVB/N strain mice purchased from Harlan (Indianapolis, Indiana) were used as wild-type

controls. The wild-type and transgenic mice of 2, 4, and 6 months of age were examined during a 6-month study period. Five to six mice per genotype at each time point were used for experiments.

2.2. Transgenic mouse breeding and selection

To express murine sFas gene in the myocardium of FVB/N mice, the extracellular domain with the secretory signal sequence of the murine Fas cDNA was cloned into pBS40 construct [9]. A Kozak sequence GCCACC was inserted prior to the ATG at base pair 50 of 484 bp-long mouse extracellular sFas domain. A stop sequence TGA was inserted after base pair 534. This construct was amplified by PCR and sequenced to confirm lack of mutations, and ligated to the 5.5-kb mouse α -myosin heavy chain (MHC) promoter of pBS40 construct at *SalI* and *KpnI* restriction sites. The expression cassette was released with *SalI/KpnI* and was purified for injection into the pronuclei of FVB/N zygotes to generate sFas transgenic mice, and homozygous sFas mice were produced by interbreeding. The homozygous sFas transgenic mice (termed sFas mice) were crossed with homozygous MCP-1 transgenic mice (termed MCP mice) to create double homozygous transgenic mice (termed MCP/sFas mice). The genotypes of MCP/sFas mice were identified by PCR with use of tail genomic DNA. Quantitative real-time PCR was performed to confirm expression of the transgenes. Primer pairs used were as follows: MCP-1, forward 5'-CAGGTCCTGT-CATGCTTCT-3', reverse 5'-TCTGGACCCATTCTTCTTG-3'; sFas, forward 5'-GTGCTTGCTGGCTCACAGTT-3', reverse 5'-TTACCAGGTTGGCATGTTG-3'. The plasma concentration of sFas protein was also measured by ELISA using a commercially available kit (R&D Systems, Minneapolis, USA) according to the manufacture's instructions.

2.3. Cardiac morphology and histomorphometric analysis

After blood samples were taken, the heart was removed, weighed, and fixed by immersion in 10% phosphate-buffered formaldehyde or snap-frozen in liquid nitrogen for RNA and protein analysis. A few photographs of whole hearts were taken with a Spot Insight digital camera (Diagnostic Instrument, Inc., Sterling Heights, MI) with a computerized Nikon SMZ1000 dissecting microscope. Equatorial regions of the heart were routinely processed and paraffin embedded. Sections (5- μ m) were stained with hematoxylin and eosin (H&E), and Masson's trichrome for histopathological analysis. Quantitative assessments for myocardial fibrotic area were performed on five sections in five randomly selected fields per section and expressed as interstitial collagen volume fraction. The collagen volume fraction was calculated as a percentage of the sum of all blue-stained areas to the total ventricular areas by using the Metamorph Series 6.2 image analysis program (Universal Imaging, West Chester, PA).

2.4. Immunohistochemical quantitative evaluation of cellular infiltrates

To identify infiltrating mononuclear cell populations, sections were incubated with anti-CD45 antibody (Pharmingen, San Diego, CA), anti-Mac-1 (CD11b, clone M1/70) antibody (Abcam, Inc., Cambridge, UK), anti-Mac-3 antibody (Pharmingen, San Diego, CA), or isotype control overnight at 4 °C, and then incubated with horseradish peroxidase-conjugated goat anti-rat antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Peroxidase activity was visualized with diaminobenzidine, and sections were counterstained with hematoxylin. Quantitative assessments for CD45-, Mac-1-, and Mac-3-positive cells were performed using the Metamorph Series 6.2 image analysis program, and the number of positive cells in five randomly selected fields of view (magnification, $\times 200$) (0.22 mm²) was calculated for each animal and five animals were studied per group per time point.

2.5. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) staining

TUNEL assay was performed in sections with use of the CardiaoTACS in situ Apoptosis Detection Kit (R&D Systems, Minneapolis, USA) according to the manufacture's recommendations. In this procedure, nuclei undergoing apoptosis were stained blue. Only cells with clear striations were scored as cardiac myocyte. The number of TUNEL-positive cardiac myocyte or infiltrating mononuclear cell was manually counted and expressed as a percentage of the total amount of cardiac myocytes or infiltrating cells in five randomly selected fields of view (magnification, $\times 200$) for each animal and five animals were studied per group per time point.

2.6. Real-time PCR assay for mRNA quantification

Total RNA was isolated from left ventricular (LV) tissues of transgenic mice and age-matched wild-type controls using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized from 1 μ g RNA using the SuperScript First-Strand Synthesis System (Invitrogen). Relative levels of mRNA transcripts for cytokines, tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-6 were quantified by real-time PCR. The quantity of an endogenous "housekeeping" messenger RNA (mouse β -actin) was measured and used to normalized the data. The expression levels of each targeted gene were normalized by subtracting the corresponding mouse β -actin threshold cycle (C_T) values by using the $\Delta\Delta C_T$ comparative method [24]. Primer pairs used were as follows: TNF- α , forward 5'-ACTCAACAACTGCCCTTCT-GAG-3', reverse 5'-TTACAGCTGGTTTCGATCCATTT-3'; IL-1 β , forward 5'-TGTGGCTGTGGAGAAGCTGT-3', reverse 5'-CAGCTCATATGGGTCCGAGA-3'; IL-6, forward 5'-CACGGCCTTCCCTACTTCAC-3', reverse TGCAAGTG-CATCATCGTTGT-3'; β -actin, forward 5'-GAAATCGTGCG-TGACATCAAAG-3', reverse 5'-TGTAGTTTCATGGATGC-CACAG-3'.

2.7. ELISA measurement of circulating C-reactive protein

The circulating levels of C-reactive protein (CRP) in plasma were measured with a mouse high-sensitive CRP ELISA kit (Kamiya Biomedical Company, Seattle, WA), which specifically detects mouse CRP.

2.8. Echocardiographic measurements

Echocardiographic studies were performed with an Agilent Technologies SONOS 4500 ultrasonography system with 15-MHz high frequency transducer (Philips Medical System, Germany). Mice were lightly anesthetized via a nose cone and maintained with 0.5 ~2.0% isoflurane (AErrane, Baxter, USA) mixed with oxygen. A two-dimensional short-axis view of the LV was obtained at the level of the papillary muscles, and two-dimensionally targeted M-mode tracings were recorded at a sweep speed of 100 mm/s. The percentage of LV fractional shortening (% FS) was calculated from M-mode-derived LV dimensions. Data from three to five consecutive selected cardiac cycles were analyzed and averaged.

2.9. Survival studies

Additional groups of mice, wild-type ($n = 24$), MCP ($n = 32$), MCP/sFas ($n = 26$), and sFas ($n = 28$) mice were included in survival analysis. During the study period of 28 weeks, the mice were allowed free access to food and water, and cages were inspected daily. Animals that developed obvious symptoms of end-stage heart failure consisting of deceased activity, tachypnea, and hunched posture were euthanized by CO₂ asphyxiation.

2.10. Statistical analysis

Data were presented as mean±standard error (SE). Results of functional and biochemical tests were compared between groups by one-way analysis of variance (ANOVA) followed by *t* tests. Survival analysis was performed by using a Kaplan–Meier method, and between-group difference in survival was tested by the log-rank test. Differences were considered significant at *P* value of <0.05.

3. Results

3.1. Overexpression of sFas in the hearts of MCP mice

We generated sFas transgenic mice by using α -MHC promoter [9] to drive expression of murine sFas cDNA (Fig. 1A), and homozygous sFas mice were produced by interbreeding. The overexpression of murine sFas in the hearts of sFas transgenic mice was confirmed by quantitative real-time PCR (Fig. 1B) and ELISA (Fig. 1D). The endogenous sFas expression levels in the nontransgenic wild-type mice were undetectable (Fig. 1B and D). The homozygous sFas mice were crossed with homozygous MCP mice to generate mice expressing both MCP-1 and sFas in the heart. Since expression of both MCP-1 and sFas was driven by the same promoter, we tested whether expression of one negatively influenced the expression of the other. Real-time PCR analysis showed that MCP-1 and sFas transcript levels were the same in the hearts of animals that expressed only one and the animals that expressed both genes (Fig. 1B and C). Similar circulating levels of sFas were also observed in the sFas and MCP/sFas mice at 6 months of age (Fig. 1D).

3.2. Expression of sFas rescues MCP mice from developing cardiomyopathy

To evaluate whether expression of sFas could rescue MCP transgenic mice from developing cardiomyopathy, phenotypic analyses were performed on wild-type, MCP, and MCP/sFas mice. As shown in Fig. 2, the 6-month-old MCP mice presented significant cardiomegaly, as indicated by the increased heart/body weight ratio, which was already highly significant at 4 months of age (Fig. 2A and C). Cardiac-targeted sFas expression in the wild-type background showed no abnormalities; and the hearts of sFas mice were normal with no change in the heart/body weight ratio throughout each time-course (Fig. 2C). Expression of sFas in the MCP mice hearts was able to prevent cardiomegaly as indicated by the normalized heart/body weight ratio (Fig. 2A and C). Histological examinations showed the marked cardiac myocytes degeneration and collagen deposition in the hearts of 6-month-old MCP mice but not in age-matched wild-type controls and sFas mice, whereas the age-matched MCP/sFas mice displayed either no or significantly less cardiac myocytes degeneration and collagen deposition in the hearts than that seen in the MCP mice (Fig. 2B). The increased collagen volume fraction found in the hearts of MCP mice was completely restored to the wild-type levels by cardiac-expression of sFas (Fig. 2D). These results suggest that targeted cardiac-expression of sFas can rescue MCP mice from developing cardiomyopathy.

3.3. Expression of sFas suppresses myocardial inflammation in MCP mice

To determine whether expression of sFas prevents cardiomegaly of MCP mice by altering the inflammatory responses in the hearts, the cellular infiltrates were characterized by immunohistochemical analyses. Sections from LV of MCP, MCP/sFas and age-matched wild-type mice were immunostained with CD45, Mac-1, and Mac-3 antibodies (Fig. 3A). Whereas CD45 is a pan-leukocyte marker which labels neutrophils and weakly detects hematogenous macrophages, Mac-1 is expressed on both granulocytes and monocytes/macrophages, Mac-3 specifically is expressed by activated macrophages [25,26]. The numbers of CD45 and Mac-1 positive cells were clearly increased in the hearts of both MCP and MCP/sFas animals compared to the age-matched wild-type controls and sFas mice (Fig. 3B and C). This increase

persisted from 2 to 6 months of age. However, there were no significant differences in the number of CD45- and Mac-1-positive cells between MCP and MCP/sFas mice (Fig. 3B and C), indicating that the infiltration of leukocytes, granulocytes, and monocytes/macrophages was not affected by expression of sFas. In contrast, the number of Mac-3-positive cells was greatly increased in the hearts of MCP mice but was barely detectable in MCP/sFas mice (Fig. 3A and D). These results show that the activated macrophages were inhibited by the presence of sFas.

CRP, a marker of inflammation, has been consistently found to be associated with coronary heart disease and heart failure [27,28]. The circulating levels of CRP were measured by ELISA to estimate of the extent of inflammatory damage in MCP and MCP/sFas mice. As shown in Fig. 3E, the elevated levels of circulating CRP found in MCP mice with heart failure were restored to wild-type levels by cardiac-specific expression of sFas.

TNF- α and IL-6 are proinflammatory cytokines and the main inducers of secretion of CRP in the liver [28]. To determine whether the elevated levels of CRP reflect an increased production of proinflammatory cytokines, we determined the effect of sFas expression on the expression of proinflammatory cytokines TNF- α , IL-6, and IL-1 β by measuring their mRNA transcript levels by real-time PCR. The hearts of MCP/sFas mice showed greatly reduced levels of transcripts of these cytokines when compared with those of MCP mice, although the levels did not reach exactly the levels found in age-matched wild-type controls and sFas mice (Fig. 4A–C). Clearly the up-regulated expression of proinflammatory cytokines in the hearts of MCP mice was attenuated by expression of sFas. Taken together, these results show that cardiac-specific sFas expression suppressed inflammatory response in the hearts of MCP mice.

3.4. Expression of sFas inhibits apoptotic cell death in the hearts of MCP mice

To determine whether sFas expression inhibits apoptosis in different cell types in the hearts of MCP mice, apoptosis was assessed by TUNEL staining. As shown in Fig. 5, the majority of infiltrating mononuclear cells were TUNEL-positive in the MCP hearts manifesting heart failure (Fig. 5B). Cells infiltrating the hearts of MCP/sFas mice barely showed any TUNEL-positive cells with most fields entirely negative (Fig. 5C); the incidence of TUNEL-positive cells was similar to that seen in the age-matched wild-type controls and sFas mice (Fig. 5A and D). TUNEL-positive nuclei were detected in scattered cardiac myocytes in the hearts of MCP mice (Fig. 5B). In contrast, TUNEL-positive cardiac myocytes were barely detectable or absent in LV sections obtained from MCP/sFas and just as seen in the age-matched wild-type controls and sFas mice (Fig. 5A, C, and D). TUNEL-positive vascular cells (smooth muscle cells and endothelial cells) were also found in the hearts of MCP mice with heart failure but not in the hearts of age-matched wild-type controls, sFas, and MCP/sFas mice (Fig. 5E and F). As shown in Fig. 5G, quantitative analysis showed that majority of infiltrating mononuclear cells in the MCP hearts were apoptotic whereas there was no statistical difference in the percentage of TUNEL-positive cells between MCP/sFas and age-matched wild-type controls and sFas mice. There was an almost 12-fold increase in the number of TUNEL-positive myocytes in the MCP hearts compared with age-matched wild-type controls and sFas mice (Fig. 5H), whereas the frequency of occurrence of TUNEL-positive myocytes was comparable in MCP/sFas mice and wild-type controls. Taken together, these results suggest that cardiac-specific sFas expression protects cardiac cells from apoptotic cell death in the hearts of MCP mice.

3.5. Effects of expression of sFas on cardiac structure and function of MCP mice

To determine whether inhibition of apoptosis associated with attenuation of inflammatory response would benefit LV structure and function of MCP mice, serial echocardiographic measurements were performed in lightly anesthetized wild-type and transgenic mice. Examples

of M-mode echocardiogram obtained from the wild-type, MCP, MCP/sFas, and sFas mice at 6 months of age are presented in Fig. 6A. The MCP mice showed enlarged LV chambers, as shown by the progressive increase in LV end-diastolic dimensions (Fig. 6B) and severe cardiac dysfunction, as shown by the markedly reduced fractional shortening (Fig. 6C). Expression of sFas significantly attenuated progressive LV chamber dilatation and dysfunction caused by MCP-1 expression (Fig. 6A–C).

To further evaluate the beneficial effects of *sFas* expression on progression of heart failure in MCP mice, LV end-diastolic dimension and fractional shortening from echocardiograms were recorded at 6 and 8 months of age. Cardiac function in MCP mice was severely depressed at 6 months of age and none of MCP mice survived to the 8 months time point as they all died of heart failure. In contrast, cardiac function of the MCP/sFas mice at 8 months of age was comparable to that of wild-type (MCP/ sFas versus wild-type, % FS, 45.4 ± 1.1 versus 47.6 ± 0.7 , $P > 0.05$, $n = 5$).

3.6. Expression of sFas improves survival of MCP mice

As shown in Fig. 7, a Kaplan–Meier analysis of survival showed that cumulative survival probability for MCP mice was greatly shortened. Most deaths were preceded by the development of ascites and edema. Over 28-week follow-up period, the probability of survival was approximately 7.24% for the MCP mice compared with 87.5% for wild-type controls and 91.6% for sFas mice. Over the same period, MCP/sFas mice showed a probability of survival of 86.3%, virtually identical to that of the wild-type mice. Many of the MCP/sFas mice were alive and well after 12 months follow-up but we did not continue their evaluation any further. These data clearly demonstrate that cardiac-specific expression of sFas drastically improved survival of MCP mice.

4. Discussion

The results presented here demonstrate that Fas-mediated apoptosis of infiltrating mononuclear cells plays a causative role in the development of heart failure in this model, as the blockage of Fas/FasL signaling through cardiac-specific sFas expression protected MCP mice from developing heart failure. Disruption of Fas/FasL interaction by cardiac-specific sFas expression inhibited apoptosis of infiltrating mononuclear cells in this model. This effect was associated with decreased macrophage activation and reduced production of proinflammatory cytokines in the hearts. sFas expression also protected vascular endothelial and smooth muscle cells as well as cardiomyocytes from apoptosis caused by chronic inflammation, leading to retention of normal myocardial structure and function with a drastic improvement in mean survival.

Early studies conducted on mouse myocardial infarction model showed that infiltrating inflammatory cells during the acute stage and granulation tissue cells during the subacute stage of myocardial infarction died via apoptosis [29], and suppression of granulation tissue cell apoptosis by use of pancaspase inhibitor or adenovirus encoding sFas gene delivery showed beneficial effects on left ventricle remodeling and heart failure [17,30]. A similar approach showed that transduction of adenovirus encoding rat soluble Fas gene into the aorta caused significant decreases in arteriopathic lesions in rat aortic allografts [31]. Our approach to block Fas/FasL system has several advantages. It is not associated with myocarditis that is associated with adenoviruses. In our approach, the degree of sFas expression is higher and distributed throughout the myocardium, which is independent of the virus transfection rate. Our data clearly show that the circulating levels of sFas protein remained steady for at least 6 months.

Monocytes/macrophages express Fas and FasL at low levels [13]. FasL is rapidly released in vitro by normal human monocytes/macrophages during phagocytosis and following activation

and thus can function as a cytokine to induce apoptosis in Fas expressing cells [32,33]. It has been shown that FasL derived from monocytes/macrophages is capable of inducing death in heterologous cells, specifically vascular smooth muscle cells (VSMCs) [22]. In the present study, apoptosis of infiltrating mononuclear cells in the hearts of MCP mice was prevented by cardiac-expression of sFas to decoy FasL released from dying and activated monocytes/macrophages. The death of cardiomyocytes and VSMCs that occurred in the hearts of MCP mice was completely prevented by expression of sFas and thus sFas was able to abrogate the development of thrombotic occlusive arteriolar vasculopathy and subsequent cardiomyopathy and heart failure.

Beyond its apoptotic activity, Fas ligation may contribute to nonapoptotic signaling, including NF- κ B activation [34], early T cell development [35] and proliferation [36], monocyte/macrophages homeostasis [37], and strong proinflammatory activity [14,19–21]. The ligation of Fas to circulating monocytes and tissue macrophages may induce proinflammatory cytokine responses that can promote further inflammatory cells infiltration and amplify inflammatory responses [19–21]. Fas ligation promotes macrophages activation through IL-1R1 and TLR4 pathway that contribute to the pathogenesis and progression of chronic inflammation [38]. In the present study, no significant difference in the number of CD45- and Mac-1-positive cells was found between the hearts of MCP mice and MCP/sFas mice. The Mac-3-positive cells, which are major sources of TNF- α , IL-1 β , and IL-6 [38], present in the hearts of MCP mice were barely observed in the hearts of MCP/ sFas mice. The distinction between Mac-1- and Mac-3-positive cell populations probably reflects differences in their activation and/or differentiation status. A Fas-dependent, activated state of macrophages may be a major contributor to the progression of heart failure in this model. Thus, blocking Fas ligation by cardiac-expression of sFas prevents activation of macrophages in the hearts of MCP/sFas mice. Additionally, neutralization of FasL with expression of sFas also reduced the levels of TNF- α , IL-6, and IL-1 β mRNA expression in the hearts of MCP/sFas mice. Attenuation of TNF- α , IL-1 β , and IL-6 expression in the hearts of MCP mice by expression of sFas could be also through inhibition of apoptosis as apoptotic cells cause release of inflammatory cytokines [14].

Fas-signaling pathway could activate cytoplasmic mediators that regulate chemokine production and release. At least in vitro, FasL-induced secretion of proinflammatory cytokines can be uncoupled from cell death in a variety of different cell populations which implicates the Fas-signaling pathway in the induction of cytokine/chemokine production [19,20,39]. Macrophages would become activated with a release of cytokines when *Fas* is ligated [36, 38]. Thus, neutralization of FasL with sFas would prevent macrophage activation that would attenuate cytokine release from activated macrophages. The correlation between the death of infiltrating mononuclear cells and cytokine production as well as macrophages activation suggests that apoptotic infiltrating mononuclear cells were probably responsible for production of cytokines responsible for the activation of macrophages. Thus, the events leading to the development of heart failure in this murine model may be the following: monocytes/macrophages accumulating within myocardium would exceed the levels of antiapoptotic factors in their environment and consequently undergo apoptotic death and release FasL and probably other inflammatory cytokines. The FasL expressed on activated macrophages may bind to Fas antigen on VSMCs and endothelial cells as well as cardiomyocytes, leading to apoptosis of these targeted cells in the myocardium causing the onset of vasculopathy and thrombus formation resulting in ischemic injury. The accelerated apoptotic process could also enhance the inflammatory process and produce cytokines that contribute to the development of heart failure in this model. Our findings demonstrate that Fas signaling in cell death and in the physiological activation of macrophages contributes to the pathogenesis of heart failure, and provide new insights into how chronic inflammation contributes to the development of heart disease.

Acknowledgements

This work was supported by grant HL-69458 from the National Institutes of Health. Part of this work was done in Ohio State University.

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Fig. 1.

Characterization of mice with cardiac-targeted expression of both MCP-1 and sFas. (A) Molecular strategy used to express sFas gene with murine α -MHC promoter. (B, C) Real-time PCR demonstrates transgene mRNA expression in the hearts of wild-type and age-matched transgenic mice of 4 months of age ($n = 3$ per group). Equal expression of transgene was found in mice expressing only one transgene versus mice expressing both transgenes. ND, not detectable. (D) Circulating levels of sFas protein in mice at 6 months of age assayed by ELISA ($n = 3$ per group). ND, not detectable.

Fig. 2.

Cardiac-targeted expression of sFas in MCP mice prevents cardiac deterioration. (A) Representative photographs showing the whole hearts from the wild-type, MCP, MCP/sFas, and sFas mouse at 6 months of age. (B) Representative photomicrographs demonstrating morphology of heart sections from the 6-month-old wild-type and age-matched transgenic mice stained with H&E (upper panel) and Masson's trichrome (lower panel). Blue staining represents collagen deposition. (C) Heart weight to body weight (HW/BW) ratio in the wild-type and transgenic mice was depicted from 2 to 6 months of age. * $P < 0.001$, # $P < 0.05$ versus age-matched wild-type, sFas, and MCP/sFas mice, respectively. $n = 6$ per group per time point. (D) Quantitation analysis of myocardial collagen volume fraction in the 6-month-old wild-type

and age-matched transgenic mice. $*P < 0.001$ versus wild-type, sFas, and MCP/sFas mice. $n = 5$ per group.

Fig. 3. Effect of cardiac-expression of sFas on cellular infiltration in the hearts of wild-type and age-matched transgenic mice. (A) Representative photomicrographs demonstrating immunohistochemical staining for CD45, Mac-1, and Mac-3 in the heart sections from 6-month-old wild-type and age-matched transgenic mice. Positive-stained cells were visualized with diaminobenzidine (brown). (B–D) Histograms showing the number of CD45-, Mac-1-, and Mac-3-positive cells in the hearts of wild-type and transgenic mice. * $P < 0.001$ versus age-matched wild-type and sFas mice; # $P < 0.001$ versus age-matched wild-type, sFas, and MCP/sFas mice, $n = 5$ per group per time point. (E) Circulating levels of CRP assayed by ELISA.

$n = 6$ per group per time point. $*P < 0.05$ versus age-matched wild-type, sFas, and MCP/sFas mice.

Fig. 4.

Effect of cardiac-expression of sFas on proinflammatory cytokines mRNA expression in the hearts of wild-type and age-matched transgenic mice. Relative levels of TNF- α (A), IL-1 β (B), and IL-6 (C) mRNA expression in the hearts of wild-type and age-matched transgenic mice were determined by real-time PCR, and were normalized by mouse β -actin mRNA. $n = 5$ per group per time point. * $P < 0.001$ versus age-matched wild-type, sFas, and MCP/sFas mice; # $P < 0.05$ versus age-matched wild-type and sFas mice.

Fig. 5.

Effect of cardiac-expression of sFas on apoptotic cell death in the hearts of wild-type and age-matched transgenic mice. Representative photomicrographs demonstrating TUNEL staining in the heart sections from 6-month-old wild-type (A) and age-matched MCP (B), MCP/sFas (C), and sFas (D) transgenic mice. Blue staining indicates that TUNEL-positive cells and the TUNEL-positive cardiac myocytes are indicated by arrows in panel B. The insert in panel E shows TUNEL-positive vascular cells magnified from the rectangle area from the heart of MCP mouse. The insert in panel F shows no TUNEL-positive vascular cells in the heart of age-matched MCP/sFas mouse. (G, H) Histograms showing the number of TUNEL-positive infiltrating cells and TUNEL-positive cardiomyocytes in the hearts of 6-month-old wild-type and age-matched transgenic mice. $n = 5$ per group per time point. * $P < 0.001$ versus wild-type, sFas, and MCP/sFas mice.

Fig. 6.

Effect of cardiac-expression of sFas on left ventricle (LV) morphology and function. (A) Representative M-mode echocardiograms from the 6-month-old wild-type and age-matched transgenic mice showing changes in LV dimensions. EDD and ESD indicate LV end-diastolic dimension and end-systolic dimension, respectively. (B, C) LV end-diastolic dimension (LVEDD) and percent functional shortening (FS) were depicted from 2 to 6 months of age. MCP mice had progressive LV dilatation and cardiac dysfunction compared to age-matched wild-type and sFas mice, whereas the MCP/sFas mice had slightly increased LVEDD and reduced FS, but were not significantly different from LVEDD and FS in the age-matched wild-type and sFas mice. $n = 6$ per group per time point, $*P < 0.001$ versus age-matched wild-type, sFas, and MCP/sFas mice, $^{\#}P < 0.05$ versus age-matched wild-type, sFas, and MCP/sFas mice.

Fig. 7. Kaplan–Meier survival analysis of wild-type ($n = 24$), MCP ($n = 32$), MCP/sFas ($n = 26$), and sFas ($n = 28$) mice showing drastic improvement in survival profile of the MCP/sFas mice compared with MCP mice. * $P < 0.001$ versus age-matched wild-type, sFas, and MCP/sFas mice.