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Cardiac Cell-specific Apoptotic and Cytokine Responses to Reovirus Infection: Determinants of Myocarditic Phenotype

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Abstract

Background—The pathophysiologic mechanisms underlying viral myocarditis are not well defined. As a result, effective treatments do not exist and viral myocarditis remains a potentially lethal infection of the heart.

Methods and Results—We used cultured rat cardiac myocytes and fibroblasts to investigate apoptosis and cytokine production in response to infection by myocarditic vs. non-myocarditic strains of reovirus. Myocarditic reovirus strain 8B and non-myocarditic strain DB188 replicate comparably in each cardiac cell type. However, strain 8B and related myocarditic reoviruses preferentially increase apoptosis of myocytes relative to fibroblasts, whereas DB188 and nonmyocarditic strains preferentially increase fibroblast apoptosis. Infection of cardiac fibroblasts with the nonmyocarditic strain DB188 elicits substantial increases in a panel of cytokines compared to fibroblasts infected with strain 8B or mock-infected controls. Analysis of culture supernatants using cytometric bead arrays revealed that DB188 enhanced release of interleukin (IL)-1 β , IL-4, IL-6, IL-10, IL-12(p70), GRO-KC, tumor necrosis factor- α , and MCP-1 relative to 8B or mock-infected controls (all $P < .05$).

Conclusion—We hypothesize that differential cytokine production and cell-specific apoptosis are important determinants of myocarditic potential of reoviral strains. Therapies that target the beneficial effects of cytokines in limiting cytopathic damage may offer an effective and novel treatment approach to viral myocarditis.

Keywords

Viral myocarditis; myocytes; fibroblasts

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Viral myocarditis is a potentially lethal infection of the heart without effective treatment. A significant proportion of surviving children and adults require chronic medical therapy or heart transplantation for resultant heart failure secondary to chronic dilated cardiomyopathy.^{1,2} Despite advances in the understanding of the pathophysiologic mechanisms underlying viral myocarditis, contemporary medical management remains supportive in nature with no significant progress made in altering the ultimate outcome of the disease.^{3–7} Rather, the infection is allowed to run its course and surviving patients are often left with debilitating heart failure and the need for life-long medical therapy. The poorly understood pathogenesis of viral myocarditis clearly contributes to the lack of evidence-based and efficacious therapeutic options.

Classically, myocarditis is defined as inflammation of the myocardium associated with myocyte damage or necrosis.⁸ Despite the simplicity of its definition, myocarditis is a complex disease.⁹ Various mechanisms have been implicated to a greater or lesser extent depending on the virus and animal model employed. The important role of direct cytopathic damage to cardiac myocytes has been appreciated in nearly all models of viral myocarditis.^{10,11} Secondary inflammatory responses or lymphocyte recognition of viral or self-antigens on myocardial cells and cytokine related injury have also been described.¹²

Reoviruses are ubiquitous, nonenveloped segmented double-stranded RNA viruses that have been extensively studied and are known to cause viral infection in mice and rats.^{13–19} Murine models have allowed detailed study of the pathogenesis of viral myocarditis, which is difficult to achieve from study of human tissues. Reoviral infection of neonatal mice is a well-characterized model of viral myocarditis.^{20–26} Reoviral strains differ in their potential to induce myocarditis in mice and the in vivo myocarditic potential of the various reoviral strains is well described.^{21,26–28} Comparative study of myocarditic and nonmyocarditic strains serves as a useful method for the elucidation of pertinent elements in the pathogenesis of disease.

Reovirus represents an ideal model with which to study events leading to direct myocardial injury and specifically the role of cytokines and other aspects of the innate immune system, because studies in severe combined immunodeficiency mice demonstrate that injury is a direct result of virus infection and does not depend on acquired (T-cell or B-cell) immune-mediated effects.²⁹ Hearts of mice infected with myocarditic strains of reovirus are noted to have disruption of the myocardial architecture and edema, but only a sparse cellular infiltrate that consists primarily of macrophages and some neutrophils.²⁰ This is in contrast to enteroviral and adenoviral myocarditis, which have been extensively studied in both animals and humans and are associated with a marked inflammatory cellular infiltrate, especially lymphocytes.³⁰

Prior studies demonstrate that reovirus acts directly on cardiac myocytes to cause apoptosis.^{20,21} Apoptosis is an important and distinct mechanism of cellular death, which is characterized by DNA fragmentation, condensation of nuclear chromatin, cell shrinkage, and membrane zeiosis.³¹ Apoptosis has been identified as an important mechanism of tissue injury in many noninfectious models of cardiac disease, such as ischemia-reperfusion,³² and may represent a final common pathway of cardiac injury. The role of apoptosis in viral myocarditis has been more recently appreciated.^{33,34} In vivo studies of reovirus myocarditis demonstrated that apoptosis occurs in the myocardium of reovirus-infected mice and colocalizes with areas of tissue injury and viral antigen.²¹ Apoptosis is an important mechanism of tissue damage in coxsackievirus myocarditis as well.^{35–37} Inhibition of specific apoptotic pathways has been demonstrated to be protective against the development of reovirus-induced myocarditis.^{20,21}

Although significant research has focused on the cardiac myocyte response to reoviral infection, the distinct roles that specific non-myocyte cardiac cell populations play in the pathogenesis of viral myocarditis have not been fully elucidated. Sherry et al have described

the interferon-beta (IFN- β) response in cardiac myocytes and fibroblasts infected with reovirus.^{38,39} However, a more global assessment of cardiac cell-specific responses to viral infection is lacking. Fibroblasts are the predominant non-muscle cell type present in the heart, accounting for two thirds of the total cell population.⁴⁰ Activated fibroblasts contribute importantly in the heart as sentinels and amplifiers of immune and inflammatory signals, and in tissue repair and remodeling after myocardial injury. Fibroblasts maintain extracellular matrix homeostasis in the normal heart and play an important role in the response to myocardial injury by proliferating, migrating to the site of injury, matrix remodeling, and production of autocrine-paracrine cytokines.^{40,41}

We hypothesize that both cardiac fibroblast and myocyte responses to viral infection play critical roles in the pathogenesis of viral myocarditis, and improved understanding of these cell-specific effects is essential. We describe cardiac cell-specific responses to reoviral infection, including a comparison of the degree of apoptosis induction in isolated cardiac myocyte and fibroblast cell culture, as well as fibroblast cytokine induction patterns. We postulate that cardiac fibroblasts and their response to viral infection may represent an important potential target in the quest for an effective therapy for viral myocarditis.

Material and Methods

Virus

Growth of reovirus by propagation in L929 cells has been previously described.²¹ Viral titers (in plaque forming units, pfu/ml) were determined by plaque assays in monolayer cultures of L929 cells.²¹ After titration, viral samples were stored in aliquots at -80°C . Aliquots were then thawed and placed at 4°C for use.

Both prototypic and reassortant reoviral strains were used in the studies. The prototype reovirus serotype 3 strain Abney (T3A), serotype 3 strain Dearing (T3D), and serotype 1 strain Lang (T1L) were from laboratory stocks that were plaque purified and passaged twice in L cell monolayers to generate working stocks. T3A has been classified as myocarditic and T3D and T1L as nonmyocarditic. Reovirus strain 8B is a highly myocarditic strain that was originally derived as an in vivo reassortant after simultaneous inoculation of mice with the nonmyocarditic strains T1L and T3D.^{21,24,26,42} All DB reassortant viruses are derived from EW60 (myocarditic) and E3 (nonmyocarditic).⁴² DB188 and DB93A are nonmyocarditic reassortant viruses, whereas DB88 and DB181 are myocarditic. All EW reassortant viruses are derived from 8B and EB121. EW29 is nonmyocarditic. The 8B, DB88, DB188, DB181, DB93A, EW29, and EW60 were gifts of Dr. Barbara Sherry (North Carolina State University College of Veterinary Medicine) and were plaque-purified and passaged twice on L-cell monolayers to generate working stocks.

For the purposes of the flow cytometry experiments, an extended panel of reoviral strains that had been previously classified as myocarditic or nonmyocarditic were used for myocyte and fibroblast infection (Table 1).^{20–22,27,29,42} For the viral replication assay and cytokine detection experiments, 1 prototypical virus from each group (myocarditic and nonmyocarditic) was selected for analysis: 8B and DB188, respectively.

Cell Culture

Cardiac myocytes and fibroblasts were cultured from the hearts of neonatal Sprague-Dawley rats as previously described.^{43,44} All animal work was performed in compliance with the institutional standards of the University of Colorado Denver Health Sciences Center.

Cardiac Myocytes

Cardiac myocytes were isolated from neonatal rats after mincing and trypsin dissociation of the hearts, and cultured in a tissue culture incubator with 1% CO₂ overnight in Minimal Essential Eagle's Medium (MEM) containing calf serum (5%) to allow cell attachment. The following day, cultures were washed and changed to serum free MEM containing 10 µg/mL each of transferrin and insulin, 1 mg/mL of bovine serum albumin, and 0.1 mM bromodeoxyuridine to inhibit fibroblast proliferation. The addition of bromodeoxyuridine to the myocyte media resulted in nonmyocyte (fibroblast) contamination of ≤10% of total cell population. Reovirus infection of cardiac myocytes was initiated on culture day 1 after isolation.

Cardiac Fibroblasts

Cardiac fibroblasts were obtained by differential adherence from cardiac cell suspensions used for myocyte isolation according to established procedures.^{43,44} This procedure ensures isolation of highly enriched cultures of cardiac fibroblasts (>95%). Fibroblast primary cultures were plated in 100 mm petri dishes, and incubated in Dulbecco Modified Eagle's Medium (DMEM) containing 5% calf serum at 37°C (10% CO₂). Primary cultures were passaged at confluence with 0.2% trypsin-EDTA and seeded in DMEM with fetal bovine serum (10%) for experiments. At confluence, experimental cultures were changed into serum-free DMEM with bovine serum albumin (1 mg/mL) for 24–48 hours to induce quiescence before reoviral infection. Reoviral infection was typically initiated on day 8 after passage.

Viral Infection

Cells were infected with fresh culture medium containing reovirus at a multiplicity of infection of 100 pfu/cell for all experiments, unless otherwise stated. Cell counts in a replicate culture were manually determined with a hemacytometer for the purpose of multiplicity of infection calculations. Mock-infection of cells for control was performed by adding a comparable amount of virus-free L929 cell lysate to the culture dish. Cells were incubated with virus (or in virus-free lysate) in appropriate conditions for the cell type as described previously. Cells were harvested at 48 hours post-infection unless specified otherwise.

Apoptosis Assays

Flow Cytometry—Cardiac cells were harvested with a 1:1 mixture of 0.2% trypsin-EDTA (BioSource): Accutase (Innovative Cell Technologies) from culture 48 hours after infection with reovirus. Cells were washed in phosphate buffered saline, then incubated in Annexin V incubation reagent for 15 minutes in the dark (TACS Annex V-FITC, Trevigen). Propidium iodide was added with wash buffer and populations of 10,000 cells were analyzed for annexin/propidium iodide binding by flow cytometry (University of Colorado Flow Cytometry Core). Staurosporine (2 µM) was added to primary cells as a positive control. Annexin binding represents plasma membrane phosphatidylserine inversion, whereas propidium iodide incorporation represents loss of cell viability.²¹

Viral Replication Assay—Cardiac cells were infected with 8B and DB188 reoviruses at multiplicity of infection = 5. The cells were incubated for 1 hour in conditions appropriate for the cell type, rocking the plate at 15-minute intervals. After 1 hour, the virus was removed and fresh media applied to the cells. At specified time points after infection (0, 24, and 48 hours), cell lysates were harvested and used for plaque assay determination of viral titer in L929 cells.⁴⁵

Cytokine mRNA Detection—Ribonuclease protection assays (RPAs) were used for the detection and quantification of specific cytokine mRNA expression for all in vitro experiments.

RNA was isolated from reovirus infected fibroblasts 48 hours after infection using TRIzol reagent (Life Technologies) according to manufacturer specifications. Single-stranded [^{32}P]-labeled antisense probes were synthesized as described previously using a commercially available multiprobe template (rck 2, BD-Pharmingen) and an RNA polymerase formulation (RPA II protocol, Ambion).^{46,47} These probes were hybridized with the RNA samples and digested with RNase A/T1 1:100, according to manufacturer specifications (RPA III protocol, Ambion). Protected fragments were separated on a 6% denaturing polyacrylamide gel. GAPDH housekeeping gene riboprobe was used as an internal control for hybridization efficiency and sample loading. Quantification of proband abundance was performed with PhosphorImager image acquisition and densitometry using ImageQuant 5.1 software. Specific cytokine mRNAs were corrected with the internal GAPDH signal and normalized to the corresponding values from mock-infected control cells.

Cytokine Protein Detection—Supernatant from reovirus-infected fibroblasts (48 and 72 hours post-infection) was collected following centrifugation to remove cell debris. Supernatant was concentrated (10:1) by ultrafiltration (Millipore Amicon Centriprep, 10,000 MW cutoff) and stored at -80°C . Samples were evaluated and quantitated for a panel of 14 cytokines by cytometric bead array analysis (Linco Research Company, St. Charles, Missouri). Quantification was determined relative to a standard curve with authentic recombinant cytokines provided by the manufacturer. Amount of protein was measured in pg/mL, with the lowest amount detectable being 4.9 pg/mL and the upper limit of detection as 20,000 pg/mL. For the purposes of analysis, all protein measured at <4.9 pg/mL is reported in the results as 4.9 pg/mL.

Statistics

Results of all experiments are expressed as mean $\pm$ standard error of the mean. Statistical comparisons are performed by analysis of variance with post-hoc test using commercial software (Graph-PAD Prism), with $P < .05$ to define significance.

Results

Cardiac Cell-Specific Responses to Reoviral Infection

Cardiac Myocyte Apoptosis—Reovirus strains have previously been classified into myocarditic and nonmyocarditic phenotypes, based on the degree of cardiac tissue injury as determined from in vivo infection experiments.^{21,27–29} To investigate the degree to which myocarditic phenotype in vivo correlates with induction of cardiac myocyte apoptosis in vitro, a panel of reovirus strains was used to infect cardiac myocytes and the amount of apoptosis was quantified by flow cytometry analysis of annexin binding and propidium iodide incorporation (Fig. 1A). Infection with all of the myocarditic viruses resulted in significantly more apoptosis in cardiac myocytes compared with mock-infection (mock: $21.8 \pm 3.4\%$, 8B: $72.1 \pm 6.3\%$, T3A: $51.2 \pm 1.0\%$, DB88: $56.3 \pm 6.9\%$, EW60: $53.9 \pm 3\%$, DB181: $50.8 \pm 5.6\%$; all with $P < .01$ vs. mock-infection). No increased myocyte apoptosis was seen after infection with the nonmyocarditic viruses T3D, EW29, and T1L compared with mock-infected cells (T3D: $24 \pm 0.8\%$, EW29: $30.5 \pm 1.9\%$, T1L: $20.0 \pm 2.0\%$; $P = \text{NS}$ vs. mock-infection). The nonmyocarditic viruses DB188 and DB93A induced intermediate levels of apoptosis (DB188: $42.3 \pm 4.6\%$, $P < .01$ vs. mock-infection and DB93A: $39.1 \pm 3.4\%$, $P < .05$ vs. mock-infection). Of note, 8B resulted in significantly more apoptosis compared with all of the other viral strains ($P < .01$ vs. all except DB88 $P < .05$).

These experiments demonstrate that myocarditic reoviral strains cause marked myocyte apoptosis. However, some nonmyocarditic strains (DB188 and DB93A) also result in myocyte apoptosis. Based on these results, reovirus strains can be further subdivided into 3 groups: (1)

myocarditic, apoptotic in myocytes (8B, T3A, DB88, EW60, DB181); (2) nonmyocarditic, apoptotic in myocytes (DB188, DB93A); and (3) nonmyocarditic, nonapoptotic in myocytes (T3D, EW29, T1L) (Table 1). These findings support the hypothesis that myocyte apoptosis is necessary, but not sufficient in determining the myocarditic phenotype of a reovirus strain.

Cardiac Fibroblast Apoptosis—Experiments were designed to investigate whether the cardiac fibroblast response to reoviral infection plays a modulatory role in determining myocarditic potential of reoviral strains. The degree of virus-induced apoptosis in cardiac fibroblasts was quantified by flow cytometric analysis of annexin binding (Fig. 2).

Fibroblasts infected with nonmyocarditic viruses, DB188, DB93A, T3D, and the myocarditic viruses EW60 and DB181 showed significantly increased levels of apoptosis as compared with mock-infected cells (mock: $4.9 \pm 0.8\%$, DB188: $39.6 \pm 2.4\%$, DB93A: $46.0 \pm 2.7\%$, T3D: $15 \pm 4.5\%$, EW60: $19.1 \pm 1.2\%$, DB181: $36.2 \pm 3.2\%$; all with $P < .01$, except T3D with $P < .05$ vs. mock). In contrast, fibroblasts infected with myocarditic viruses 8B, T3A, DB88, and nonmyocarditic EW29 and T1L exhibited no significant difference in apoptosis compared with mock-infected cells (8B: $10.6 \pm 1.6\%$, T3A: $4.8 \pm 0.9\%$, DB88: $7.9 \pm 1.6\%$, EW29: $5.5 \pm 1.9\%$ and T1L: $4.8 \pm 0.9\%$; all $P = \text{NS}$ vs. mock). Of note, DB188 produced significantly more fibroblast apoptosis ($P < .01$) compared with all other viruses analyzed except DB181 and DB93A (Fig. 1B).

Apoptotic Index—To better understand these differences in virus-induced apoptotic response specific to cardiac cell populations, an apoptotic index (AI) was calculated as defined in the figure legend (Fig. 1C). Positive AI values for reoviral strains indicate myocyte-selective apoptosis, whereas negative values indicate fibroblast-selective apoptosis. The myocarditic viruses 8B, T3A, DB88, and EW60 resulted in more myocyte apoptosis relative to fibroblast apoptosis (AI for 8B: +44.6, T3A: +29.5, DB88: +31.5, EW60: +17.9), whereas the inverse was true for the nonmyocarditic viruses DB188, DB93A, and T3D (AI for DB188: -14.2, DB93A: -23.8, T3D: -7.9). The myocarditic virus DB181 and the nonmyocarditic viruses EW29 and T1L caused relatively equal degrees of apoptosis in the 2 cell types (AI for DB181: -2.3, EW29: +0.5 and T1L: -1.7). The mean AI for all myocarditic viruses was $+24.3 \pm 7.9$ (favoring myocyte apoptosis), whereas the mean AI for all nonmyocarditic viruses was -9.4 ± 4.4 (favoring fibroblast apoptosis, $P < .01$) (Fig. 1D).

Cardiac Cell-Specific Viral Replication Potential of Myocarditic (8B) and Non-Myocarditic (DB188) Viruses—To determine if the observed cell-type specific differences in response to reoviral infection could be explained by differences in viral growth within these cell types, growth of a representative myocarditic (8B) and non-myocarditic (DB188) virus was assessed in both cardiac myocytes and fibroblasts. There was no difference in growth of 8B or DB188 respectively at different time points post-infection in cardiac myocytes (0 hours: 8B 3.6 ± 0.5 vs. DB188 4.3 ± 0.1 log₁₀ PFU/mL, 24 hours: 5.7 ± 1.0 vs. 5.5 ± 0.3 , 48 hours: 6.5 ± 1.1 vs. 6.1 ± 0.4 ; $P = \text{NS}$) or in cardiac fibroblasts (0 hours: 3.9 ± 0.3 vs. 4.3 ± 0.1 , 24 hours: 5.4 ± 0.6 vs. 5.4 ± 0.4 , 48 hours: 5.9 ± 0.4 vs. 5.8 ± 0.2 ; $P = \text{NS}$). Therefore, nonmyocarditic strain DB188 propagated to similar extents as myocarditic strain 8B in each cardiac cell type (Fig. 3). These experiments indicate that the myocarditic phenotype of a reoviral strain is independent of the degree to which the virus replicates in a given cell type.

Taken together, these results suggest that cardiac fibroblasts and myocytes exhibit markedly different responses to viral infection, which are not attributable to differences in the ability of the viruses to proliferate in these cell types. Myocarditic viral strains cause marked cardiac myocyte apoptosis in cell culture, with relatively less fibroblast death. Infection of cardiac cells with nonmyocarditic strains result in relatively more fibroblast death compared to myocyte

death. These cell-specific responses to viral infection likely play an important role in determining the myocarditic potential of a given reoviral strain.

Induction of Cytokine Expression by Reoviral Strains in Cardiac Fibroblasts

To investigate a possible mechanism by which fibroblast survival or death might contribute to myocarditic phenotype, further evaluation of fibroblast response to viral infection was performed. An important component of cardiac fibroblast activation following viral infection is the production of cytokines. Cytokine production was assessed at both the transcriptional and translational level. For the purposes of the cytokine expression experiments, studies focused on the prototypical myocarditic virus 8B and the nonmyocarditic virus DB188. These 2 viruses were specifically chosen for these experiments because they both induce high levels of cardiac myocyte death but contrast in their fibroblast death profile, providing a possible window into the mechanism for their observed difference in myocarditic potential.

Cytokine mRNA Expression—Cytokine mRNA expression was assessed by performing RPAs of cellular RNA harvested 48 hours after reoviral infection of cardiac fibroblasts in vitro. Increased mRNA expression of select inflammatory and non-inflammatory cytokines was noted in fibroblasts infected with nonmyocarditic virus DB188, as compared with infection with the myocarditic virus 8B and mock-infection (Fig. 4). DB188 infection of cardiac fibroblasts resulted in significant increases in mRNA production of IL-1 β (DB188: $4.2 \pm 0.2\%$ vs. mock: $1.2 \pm 0.1\%$, $P < .05$), IL-1 receptor antagonist (IL-1Ra) (DB188: $33 \pm 15\%$ vs. mock: $3.6 \pm 0.2\%$, $P < .05$), and IL-6 (DB188: $41.3 \pm 11.3\%$ vs. mock: $2.6 \pm 1.3\%$, $P < .01$). All values are expressed as a percentage of GAPDH. DB188 infection resulted in significantly more IL-1 β , IL-1Ra, and IL-6 mRNA production than 8B infection (8B-induced IL-1 β : $1.5 \pm 0.5\%$, $P < .05$, IL-1Ra: $2.2 \pm 0.6\%$, $P < .05$, and IL-6: $2.6 \pm 0.7\%$, $P < .01$). There was a trend toward increased mRNA expression of IL-1 α ($2.0 \pm 0.5\%$) and IL-18 ($5.7 \pm 1.7\%$) after DB188 infection, but these differences were not significant ($P = \text{NS}$). Fibroblast infection with the myocarditic virus 8B did not result in increased mRNA production of any of the cytokines studied. There was no consistently measurable expression of IL-10, IL-12(p35), IL-12(p40), or IFN- γ after fibroblast infection with either virus.

Cytokine Protein Quantification—To validate that the observed differences seen at the transcriptional level were biologically significant, quantification of cytokine protein production after viral infection was performed using cytometric bead assays. Fibroblast infection with nonmyocarditic virus DB188 resulted in significantly greater production of 10 of the 14 cytokines evaluated compared with mock infection (Fig. 5). DB188 infection of cardiac fibroblasts resulted in significant increases in production of IL-1 α (14.4 ± 4.2 pg/mL vs. mock: 4.9 ± 0 pg/mL), IL-1 β (33.9 ± 1.8 vs. mock: 4.9 ± 0), IL-4 (23.6 ± 1.6 vs. mock: 4.9 ± 0), IL-6 ($13,285 \pm 2241$ vs. mock: 43.0 ± 7.5), IL-10 (346.6 ± 18.3 vs. mock: 4.9 ± 0), IL-12 (p70) (10.8 ± 0.8 vs. mock: 4.9 ± 0), IL-18 (354.1 ± 38.2 vs. mock: 24.9 ± 11.6), GRO-KC ($20,000 \pm 0$ vs. mock: 1488 ± 338.4), tumor necrosis factor (TNF)- α (495.7 ± 136.4 vs. mock: 10.9 ± 4.1) and MCP-1 (7497 ± 544.4 vs. mock: 3164 ± 263.6), all with $P < .05$. There was no detectable production under any conditions of IL-2, IL-5, GM-CSF, or IFN- γ in cardiac fibroblasts. Infection of cardiac fibroblasts with myocarditic virus 8B resulted in significantly increased production of only 4 of the 14 cytokines measured vs. mock: IL-18 (366.9 ± 94.7), GRO-KC (4568 ± 505.3 pg/mL), and MCP-1 (5743 ± 111.4), IL-10 (57.1 ± 10.7)—all with $P < .05$.

These data demonstrate that nonmyocarditic reovirus strain DB188 induces more profound inflammatory and noninflammatory cytokine production from cardiac fibroblasts than its myocarditic counterpart 8B. Because these viruses induce similar degrees of myocyte

apoptosis, these results suggest that virus-induced cardiac fibroblast cytokine production plays a potentially important role as a codeterminant of the myocarditic phenotype of a reoviral strain.

Discussion

Reovirus infection provides a valuable model for study of the mechanisms of viral myocarditis involving the innate immune system independent from adaptive (cellular) immune system activation. Although previous experiments demonstrated the importance of apoptotic cell death in association with reovirus myocarditis in vivo, the current study was designed to determine cardiac cell-specific responses to reovirus infection. Specifically, the purpose of this study was to define the response of the 2 primary cell types in the heart (myocytes and fibroblasts) to direct infection (in vitro) with viruses that have already been characterized (published data) with regard to their myocarditic potential during in vivo infection of the whole animal. Myocarditic reoviruses result in myocardial tissue injury and cardiac myocyte death after infection of mice in vivo (as typified by reovirus strain 8B). Conversely, nonmyocarditic viruses do not result in cardiac tissue injury and cardiac myocyte death, despite their ability to grow to equally high titer in the heart (as typified by reovirus strain DB188). In this study, we demonstrated that in vitro infection of cardiac myocytes with both reovirus strain 8B (myocarditic) and DB188 (nonmyocarditic) results in induction of cardiac myocyte apoptosis, despite the fact that infection of the whole animal with these viruses in vivo leads to very disparate outcomes on cardiac injury. Therefore, the ability of a virus to induce cardiac myocyte death cannot be the sole determinant of myocarditic phenotype in vivo. We hypothesized that fibroblast cytokine responses to virus infection could be an additional determinant of myocarditic phenotype. We demonstrated that infection of cardiac fibroblasts with nonmyocarditic virus (DB188) elicits production of cardioprotective cytokines, whereas infection with myocarditic virus (8B) does not. The resultant effect of cardiac myocyte and fibroblast responses to infection in vivo (whole animal) is net protection in the case of nonmyocarditic virus infection. Conversely, there is a net deleterious effect in the case of myocarditic virus infection, in which no protective cytokine response is elicited in fibroblasts to counter the pro-apoptotic effect of viral infection of cardiac myocytes. The major conclusion from these investigations is that, although a virus must induce significant levels of myocyte apoptosis to result in myocarditis, this alone is not sufficient to determine the myocarditic potential of a reoviral strain. The cardiac fibroblast response to reovirus infection, and more specifically, the relative levels of death induced in fibroblasts and myocytes are critical codeterminants of the myocarditic phenotype.

Given the findings of viral growth experiments, the differences in apoptosis in these cell types can not be attributed to a difference in replicative potential in vitro. Identical results were found in previous experiments at the in vivo level with whole heart plaque assays demonstrating equal growth of different reoviral strains suggesting that myocarditic potential is independent of viral growth.^{25,26}

The combination of these results suggests that although apoptosis plays an important role in the pathogenesis of reoviral myocarditis, other factors may be involved that contribute to the myocarditic potential of viral strains. The differential cell-specific responses to reoviral infection may explain why DB188 induces myocyte apoptosis in vitro, but does not cause myocarditis in vivo. In the whole animal, the response of the cardiac fibroblasts to infection with nonmyocarditic DB188 may have a protective effect on the myocytes, thereby preventing the development of myocarditis. Specifically, the finding that nonmyocarditic viruses such as DB188 preferentially induce fibroblast apoptosis in vitro suggests the possibility that cardiac fibroblast activation and apoptosis may reflect protective mechanisms in response to viral infection. Consistent with this hypothesis, our results demonstrate that nonmyocarditic reovirus DB188 elicits expression of an array of pro- and anti-inflammatory cytokines, suggesting the

importance of a robust host defense reaction. By contrast, myocarditic strains cause selective apoptosis of cardiac myocytes relative to cardiac fibroblasts, combined with blunted fibroblast cytokine induction, suggesting that these pathogens may be escaping fibroblast surveillance. Cytokines have been determined to play an important role in the pathogenesis of viral myocarditis, but expression patterns in response to myocarditic and nonmyocarditic reoviral infection had not previously been fully elucidated and compared.

In the setting of viral myocarditis, early cytokine production is from resident myocardial cells, whereas delayed cytokine production is from infiltrating cells (T lymphocytes and other mononuclear infiltrating cells).⁴⁸ One of the key features of the reovirus model of myocarditis is the *lack* of an infiltrative inflammatory process.²² Therefore, any cytokine response generated from reoviral infection of the heart is primarily a response of the resident myocardial cells. In the heart, although myocytes are known to produce some cytokines such as IL-6 and TNF- α , it is the nonmyocyte cell population (cardiac fibroblasts) that is responsible for the majority of cytokine production in response to injury or stress.^{49–53} Given this evidence and the fact that one of the main purposes of this study was to further define the role of the cardiac fibroblast in viral myocarditis, we chose to focus on cytokine production from cardiac fibroblasts specifically.

Although in clinical practice, cytokines are generally felt to be detrimental there is evidence in the setting of viral infection particularly that cytokines may play an important role in control of viral replication and clearance resulting in a protective phenotype. IL-1Ra (anti-inflammatory cytokine) gene transfer directly into the hearts of mice using a plasmid vector resulted in increased survival and decreased inflammation and fibrosis after coxsackie virus infection.⁵⁴ IL-1Ra mRNA expression was increased in response to nonmyocarditic DB188 infection of fibroblasts in our experiments and its role in the reovirus model warrants further study. IL-6 is conventionally considered an inflammatory cytokine based on its induction of acute phase proteins, but has been shown to be protective in viral myocarditis in animals. In an encephalomyocarditis virus (EMCV) mouse model, exogenous treatment with IL-6 demonstrated several beneficial effects.⁵⁵ IL-6-treated mice had a lower viral titer, decreased viral replication, increased EMCV neutralizing antibodies, decreased heart weight:body weight ratios, and decreased myocardial injury. In particular, decreased viral replication was partly attributed to decreased circulating TNF- α and natural killer cell activity, again underlying the potential importance of the innate immune system in the pathogenesis of viral myocarditis. The fact that DB188 induced significantly more IL-6 in infected fibroblasts may represent a protective response as well. IL-10 treatment in an EMCV mouse model resulted in increased survival, decreased myocardial lesions and decreased levels of TNF- α , but had no effect on myocardial virus concentration.⁵⁶ IL-18 has been demonstrated to have similar beneficial attributes: increased survival, decreased viral titer, decreased myocardial necrosis, increased IFN- γ expression in the heart, and increased natural killer cell activity in the spleen.⁵⁷ Specific to reovirus associated myocarditis, Sherry et al have identified IFN- β and the resulting cascade of effects as important factors in the control of viral replication.^{39,42,58,59} Given that reoviral myocarditis occurs independent of a cellular immune response, it was not surprising that no difference was found in the expression patterns of the 4 classically leukocyte-induced cytokines that we evaluated: GM-CSF, IL-2, IL-5, and IFN- γ .

The increased cytokine expression noted in response to nonmyocarditic reovirus DB188 is of particular interest. In this context, it is noteworthy that IL-1 β acts as a cardinal pro-inflammatory cytokine in rat cardiac fibroblasts. Stimulation with IL-1 β elicits coordinate production of additional pro- and anti-inflammatory cytokines, including IL-1 β itself (and smaller amounts of IL-1 α), IL-1Ra, IL-6, and IL-10.⁶⁰ The pattern of cytokine expression in response to reovirus infection strikingly resembles that in response to IL-1, suggesting that cytokine production mediated by cardiac fibroblasts may be an important component of innate

immune response, and may confer viral resistance to the cardiac myocytes. These findings point to an underappreciated and potentially important role of cardiac fibroblasts in the progression of viral myocarditis. From a therapeutic perspective, alteration in the cytokine milieu may offer the ability to change the myocarditic phenotype of a reoviral strain, therefore changing the natural history and outcome of the infection.

Much controversy surrounds the diagnosis and mechanism of heart failure in the setting of viral myocarditis. In human studies, histologic evidence of myocardial inflammation in patients with presumed viral myocarditis is quite rare.^{61,62} In a study of adults who died from myocarditis, only 25% of postmortem hearts had histologic evidence of an inflammatory infiltrate on a single biopsy consistent with the diagnosis of viral myocarditis.⁶³ In a study of children presenting with presumed myocarditis, although 76% had detectable viral genome present in the myocardial biopsy, 50% did *not* have histologic inflammation consistent with myocarditis.⁶⁴ The presence of viral genome in myocardial biopsies of patients with idiopathic dilated cardiomyopathy also implies that viral induced damage in the heart does not always occur in conjunction with a lymphocytic inflammatory response.^{65–67} The combination of these findings is consistent with the fact that systemic immunosuppressive therapies have failed to reliably alter the clinical course of viral myocarditis.^{68–70}

The role of the cardiac fibroblast in viral myocarditis in humans is not well understood. Historically, the Dallas criteria were devised to standardize the pathologic diagnosis of viral myocarditis.⁸ This classic definition of viral myocarditis included the presence of an inflammatory infiltrate along with necrosis or degeneration of adjacent myocytes. The involvement of the cardiac fibroblasts is not considered in this classification scheme. More contemporary criteria for the diagnosis of viral myocarditis still do not include the fibroblast as part of the diagnostic criteria.⁷¹ Although the fate of the fibroblast *in vivo* is not well understood or described, the present data offer insight into the potential importance of the cardiac fibroblast based on an *in vitro* model. Endogenous protective mechanisms mediated by infected cardiac fibroblasts likely limit reovirus-induced tissue damage *in vivo*. Future studies using fibroblast/myocyte coculture and conditioned media experiments are planned to directly assess cardiac fibroblast and myocyte interaction in the setting of viral infection. In particular, we suggest antiviral effects of fibroblast-derived cytokines may limit cytopathic viral damage. Additionally, because cardiac fibroblasts can regenerate and proliferate, studies aimed at defining fibroblast fate *in vivo* would be useful to understand how the findings of this study translate into the whole animal. Further investigation of the cytokines identified here are warranted given that therapies that aim to take advantage of the beneficial effects of some of these important cytokines should be considered. Limiting tissue damage and protecting the host from further cytopathic injury could improve outcome and may offer an effective and novel treatment approach to viral myocarditis.

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Fig. 1.

Virus-induced cardiac cell apoptosis. (A) Percent of annexin positive cardiac myocytes measured by flow cytometry 48 hours after infection with a panel of reoviral strains. $*P < .01$ vs. mock; $†P < .05$ vs. mock. (B) Percent of annexin positive cardiac fibroblasts. $*P < .01$ vs. mock; $†P < .05$ vs. mock. (C) Apoptotic index (AI) representing relative degrees of cardiac myocyte vs. cardiac fibroblast apoptosis. $AI = (\text{virus induced} - \text{mock infected myocyte apoptosis}) - (\text{virus induced} - \text{mock infected fibroblast apoptosis})$. Apoptosis for the purposes of the index was defined as the mean percentage of annexin positive cells by flow cytometry 48 hours after viral infection. Positive AI values for reoviral strains indicate myocyte-selective apoptosis, whereas negative values indicate fibroblast-selective apoptosis. (D) Combined AI for myocarditic and nonmyocarditic reoviral strains, $*P < .01$. Virus classification key identifies the 3 categories of viruses based on their ability to cause myocarditis in vivo and their ability to cause cardiac myocyte apoptosis in vitro, which is denoted in the key as (+) myocyte apoptosis.

Fig. 2.

Apoptosis in cardiac fibroblasts detected by flow cytometry. Percent of annexin positive fibroblasts measured by flow cytometry 48 hours after infection with mock (A), 8B (B), and DB188 (C). Annexin binding, representing phosphatidylserine inversion is on the x-axis and propidium iodide (PI) incorporation on the y-axis represents cell death. Quadrant B3 contains live cells, B4 are live cells undergoing apoptosis, B2 are cells that died by apoptosis, and B1 are cells that died by necrosis. Cell numbers in quadrants B2 and B4 are added together to determine total percent of apoptotic cells.

Fig. 3.

Viral growth in cardiac myocytes and fibroblasts. As outlined in Methods, cardiac fibroblasts and myocytes were infected at a multiplicity of infection (MOI) = 5 and cell lysates were harvested at specified time points (0, 24, and 48 hours after infection). Plaque assay results are expressed in log₁₀ plaque forming units/mL (PFU/mL). (A) Viral proliferation in cardiac myocytes. (B) Viral proliferation in cardiac fibroblasts.

Fig. 4.

Fibroblast mRNA cytokine expression in response to viral infection. (A) Representative ribonuclease protection assay for comparison of cytokine mRNA expression in cardiac fibroblasts after infection with myocarditic (8B) and nonmyocarditic (DB188) reoviruses, and mock-infection. Lane 1 = mock infection; Lane 2 = 8B infection; Lane 3 = DB188 infection. (B) Quantitation of mRNA expression expressed as percentage normalized to GAPDH (loading control) signal. * $P < .05$ vs. mock; † $P < .05$ vs. 8B, ‡ $P < .01$ vs. mock; § $P < .01$ vs. 8B.

Fig. 5. Fibroblast cytokine production in response to viral infection. Protein amounts (pg/mL) of specified cytokines as determined by Luminex bead assay. Cytokine protein was measured from cardiac fibroblast supernatant harvested from mock infected cells, after infection with myocarditic virus 8B and nonmyocarditic virus DB188. * $P < .05$ vs. mock; † $P < .05$ vs. 8B.

Table 1

Reovirus strain characteristics

Reovirus Strain	Myocarditis in vivo *	Myocyte Apoptosis in vitro	Fibroblast Apoptosis in vitro	Apoptotic Index
8B	Yes	Yes	No	+24.3 ± 7.9 [†]
T3A	Yes	Yes	No	
DB88	Yes	Yes	No	
EW60	Yes	Yes	Yes	
DB181	Yes	Yes	Yes	-9.4 ± 4.4
DB188	No	Yes	Yes	
DB93A	No	Yes	Yes	
T3D	No	No	Yes	
EW29	No	No	No	
T1L	No	No	No	

An apoptotic index (AI) with a positive value denotes greater myocyte apoptosis, whereas an AI of negative value represents greater relative fibroblast apoptosis.

* Myocarditic potential as defined elsewhere.⁵²

[†] $P < .01$.