Protective Role for Interferon-β in Coxsackievirus B3 Infection

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Background—Coxsackievirus-induced myocarditis can be a serious cause of heart failure. In the absence of a specific antiviral therapy, modulating the host immune response may be protective. Interferons (IFNs)-α and -β perform a fundamental role in innate and adaptive antiviral responses, thereby presenting as candidate therapeutics for coxsackievirus infections.

Methods and Results—To examine the contribution of IFN-β in protection from coxsackievirus B3 (CVB3) infection, mice lacking the IFN-β gene were infected with 10^3 plaque-forming units of CVB3. In contrast to wild-type mice that exhibit an intact IFN-β response, we observed increased susceptibility to infection (70% mortality), a downregulation of IFN-stimulated gene targets (2'-5' oligoadenylate synthetase, serine/threonine protein kinase, the GTPase Mx), and cardiomyocyte breakdown and disruption in the IFN-β^-/- mice.

Conclusions—Viewed together, these results clearly demonstrate that IFN-β is important in mediating protection against CVB3-induced myocarditis. (Circulation. 2004;110:3540-3543.)

Key Words: interferon beta ■ coxsackievirus ■ interferon-stimulated genes

Coxsackievirus group B (CVB), enteroviruses of the Picornaviridae family, are one of the primary etiologic agents of viral myocarditis.¹ The pathogenesis of CVB3-induced myocarditis is triphasic: (1) virus infection that directly damages myocytes, (2) an immune phase with accompanying inflammatory cell infiltration, and (3) repair, fibrosis, and cardiac remodeling.² Evidence supporting CVB as an important causative agent in myocarditis includes in situ and polymerase chain reaction (PCR) detection of viral RNA in hearts of patients with myocarditis,³-⁵ isolation of infectious virus from acute myocarditic patients,⁶ and demonstration of virus-induced cell death in CVB3-infected myocytes.⁷

The type I IFNs (α, β, ω, and κ) are cytokines that interact with and signal through a common receptor complex composed of 2 transmembrane chains, IFNAR1 and IFNAR2. Signaling through the receptor invokes a phosphorylation cascade, culminating in the transcription of a group of genes collectively known as interferon-stimulated genes (ISGs).⁸ Studies with IFNAR1^-/- mice revealed that these mice were highly susceptible to CVB3 infection,⁹ suggestive of a protective role of type I IFNs in CVB3 pathogenesis and viral spread. In vivo¹⁰ and in vitro¹¹ studies demonstrate the efficacy of type I IFNs in inhibiting coxsackievirus replication. Notably, in vitro investigations identified IFN-β as exhibiting superior antiviral activity against CVB3 compared with IFN-α. To further distinguish the role of IFN-β from IFN-α, we examined the course of CVB3 infection in IFN-β^-/- mice.

Methods

Mouse Strains, Cells, and Virus

IFN-β^-/- and IFN-β^-/- mice (mixed 129/Sv × C57Bl/6 background, 129/Bl6F1)¹²,¹³ were maintained in a sterile, pathogen-free environment according to the Animal Care Committee guidelines of the Toronto General Research Institute. CVB3 strain, Charles Gauntt, was propagated in HeLa cells and cultured in RPMI supplemented with FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Progeny virus was purified from cell supernatants by centrifugation at 1000g for 10 minutes. Viral titer was determined by plaque assay.¹⁴

Infection of Mice and Measurement of Viral Load

Mice (8 to 10 weeks) were inoculated ip with 10^3 plaque-forming units (PFU) CVB3. At indicated times, mice were euthanized, and hearts, spleens, and livers were aseptically removed, weighed, and frozen in liquid nitrogen. After 3 freeze-thaw cycles, viral titers were determined by plaque assay in HeLa cells, expressed as PFU per gram of tissue.¹⁴ The lower limit of detection, 1 plaque, corresponds to 10 PFU/g.

Histopathology

Heart tissue harvested from CVB3-infected mice was fixed in 10% (vol/vol) formalin (Sigma), embedded in paraffin, and sectioned at 4 µm. Cross-sectioned tissues were stained with hematoxylin and eosin.

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RNA Extraction and cDNA Synthesis
Total RNA was extracted with Trizol (GIBCO, BRL). Complementary DNA was synthesized from 1 μg total RNA with AMV–reverse transcriptase in a 20-μL reaction. (Reverse Transcriptase System, Promega).

Real-Time Quantitative PCR
Real-time PCR analyses were carried out with the Prism 7700 Sequence Detection System (Applied Biosystems). PCR primer and probe sequences were designed with Primer Express software (Applied Biosystems). Primer and probe sequences were as follows: 2'-5' oligoadenylate synthetase (OAS): forward, TGAGGCG-C CCCCATCCT; reverse, CATGACCCAGGACTCAAAAGG; probe, AAGGGCTTGCTTGCT; probe, AGTCCTTTAGCTGTAACCTTA; reverse, GTTTACAA AGGGCTTGCTTGCT; probe, TCAGAATGTTGCCTTTAGACT- GTGG. Serine/threonine protein kinase (PKR): forward, GGCTC CTGTGTTGGGAAGTCA; reverse, TATGCAAAAGCCAGGACAGCTTCT; probe, AGAGCCCCCAAAGCACAATGGATG, GAPDH gene expression levels were determined with the Taqman rodent GAPDH VIC–labeled reagents kit (Applied Biosystems), specifically for standardizing gene expression levels to a housekeeping gene. Cycling reaction conditions consisted of the following: 50°C for 2 minutes, 95°C for 10 minutes, followed by 95°C for 15 seconds and 60°C for 1 minute cycled 40 times. Reactions were performed in 96-well MicroAmp Optical plates in a final volume of 25 μL. Each reaction mix included 200 ng cDNA, 1× Taqman master mix, 0.3 μmol/L forward and reverse primers, and 0.25 μmol/L FAM-labeled probe. Nontemplate controls and standards were included in each plate. All data were analyzed with the SDS 1.0 software (Applied Biosystems).

Results
Aggressive CVB3 Infection in IFN-β−/− Mice
To examine the role of IFN-β in susceptibility to CVB3 infection, we infected IFN-β−/− (n=6) and IFN-β+/− (n=6) mice with 10⁷ PFU of CVB3 ip. Three of 6 IFN-β−/− mice died within 4 days of infection, and by day 5, another mouse died (Figure 1A). In contrast, all of the IFN-β+/− mice survived to 15 days after infection, at which time all animals were euthanized. The difference in mortality rates between IFN-β+/− and IFN-β−/− mice is significant (P<0.001, Cox proportional hazards). In a subsequent time course experiment, we examined CVB3 tissue tropism during disease progression. By day 4 after CVB3 infection, virus was detected in the spleens, livers, and hearts of all animals (Figure 1B). Notably, viral titers were markedly elevated in the spleens, livers, and hearts of the IFN-β−/− mice compared with the IFN-β+/− mice. Although virus was cleared from the spleens and livers of the IFN-β+/− mice by day 7 after CVB3 infection, virus persisted in the spleens of IFN-β−/− mice to day 10 and in their livers to day 7. These findings are consistent with an earlier study in IFNAR1−/− mice, for which IFN-mediated viral inhibition is important for early clearance of hepatic virus.⁹ Viral titers measured in the hearts of the IFN-β−/− mice clearly increased until day 10, whereas titers decreased in the IFN-β+/− mice.
IFN-β⁻/⁻ Mice Exhibit a Compromised IFN-Induced Response to CVB3 Infection

Induction of ISGs is required for type I IFN-mediated viral clearance. Accordingly, we examined gene expression levels for antiviral-specific ISGs, namely the 2'-5'OAS, PKR, and the GTPase Mx in CVB3-infected heart tissues. In all cases, gene expression levels for 2'-5'OAS, PKR, and Mx were lower in IFN-β⁻/⁻ compared with IFN-β⁺/⁺ mice (Figure 1C). We observed that PKR gene expression is maximal at day 4 after CVB3 infection, whereas 2'-5'OAS and Mx gene expression levels peak at day 7 after CVB3 infection.

CVB3-Infected IFN-β⁻/⁻ Mice Exhibit More Severe Pathology

Histological examination of cardiac pathology confirmed a more pronounced severity of disease in IFN-β⁻/⁻ mice compared with IFN-β⁺/⁺ mice (Figure 2). By day 4 after CVB3 infection, myocyte architecture is clearly disrupted in tissue from IFN-β⁻/⁻ mice (Figure 2b and 2c) compared with tissue from IFN-β⁺/⁺ mice (Figure 2a). Examination of myocyte architecture in tissues from IFN-β⁻/⁻ mice that had died on day 4 (Figure 2c) or had survived (Figure 2b) identified similar loss of organization. Notably, by day 7 after infection, myocyte architecture is disrupted in all mice (Figure 2d and 2e). We observed the area of myocardial lesions to be more extensive in IFN-β⁻/⁻ than IFN-β⁺/⁺ tissue throughout the course of disease, with concomitant extensive cellular infiltration into the cardiac tissue of IFN-β⁺/⁺ mice. Cellular infiltration was maximal in IFN-β⁻/⁻ mice on day 7 and declined by day 10 after infection. At day 10, areas of pallor and persistent foci were consistently observed in IFN-β⁻/⁻ mice tissue, indicative of virally mediated myocyte necrosis, which was absent in tissue from IFN-β⁺/⁺ mice (Figure 2f and 2g).

Discussion

The rapid induction of IFN-β is a critical component of any innate immune response to viral infection. IFN-β–mediated antiviral and immune modulatory responses determine the extent of virus spread and disease progression. IFN-induced viral clearance from different organs requires both the direct antiviral effects of IFN, ie, inhibition of viral replication in infected cells, and the induction of specific immune responses. In this study, we show that cardiotropic CVB3 infection is more aggressive in IFN-β⁻/⁻ than in IFN-β⁺/⁺ mice, likely because of the blunted IFN response to infection. We provide evidence for an incomplete IFN response to CVB3 infection.
of heart tissue, specifically in the context of ISGs implicated in mediating IFN-inducible antiviral responses: 2'-5' OAS, Mx, and PKR. The dsRNA-activated 2'-5' OAS and PKR invoke the degradation of viral RNA and inhibit viral RNA translation, respectively,15,16 and dsRNA is an intermediate of viral replication. The GTPase Mx limits CVB infection by preventing the accumulation of viral RNA and capsid protein.17

In contrast to AJ mice, which are highly susceptible to CVB3 infection,18 129/Sv and C57Bl/6 mice are reportedly relatively resistant to infection.9,19 In agreement, our mixed-strain 129/Bl6F2 IFN-β−/− mice exhibited minimal symptoms of disease, as evidenced by no mortality and limited cardiac histopathology. Therefore, we infer that the aggressive disease observed in the IFN-β−/− mice specifically reflects the absence of IFN-β, unrelated to the mouse strain.

In accordance with findings in IFNAR1−/− mice infected with CVB3,9 we observed high viral loads in spleen, liver, and hearts early during the course of infection in IFN-β−/− mice. Apparently, these high viral loads correlate with severity of disease for both IFNAR1−/− and IFN-β−/− mice. Specifically, failure to clear virus rapidly from spleen and liver is associated with fatal disease. Notably, the mortality rate in IFNAR1−/− mice infected with CVB3 is higher than what we observe for IFN-β−/− mice. Because IFNAR1−/− mice cannot respond to either IFN-α or IFN-β yet IFN-β−/− mice will elicit a virus-inducible IFN-α response, albeit a severely restricted one,13 we attribute the lower mortality rate in the IFN-β−/− mice to a residual IFN-α antiviral response.

Given that cardiacmyocytes do not regenerate adequately, the extent of the antiviral and immune response to infection is critical in determining outcome with regard to cardiac damage and cardiac function. In our studies, we observe considerable cellular infiltration and myocarditis by day 7 after CVB3 infection for IFN-β−/− mice. In contrast, the extent of cellular infiltration and severity of myocardial lesions were less in the IFN-β−/− mice. Previous studies have identified T cells as the predominant cells infiltrating CVB3-infected myocardium, providing evidence for T cell–mediated cardiac damage.8 Specifically, T-cell effector functions may promote tissue damage via excessive cytokine activation and cytotoxic responses. Our findings in IFN-β−/− mice would support a role for destructive elements associated with extensive cellular infiltration of the myocardium. Viewed together, these data confirm a critical role for IFN-β in mediating protection from CVB3 infection and subsequent cardiomyopathy.

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References