Transient Induction of Cytokine Production in Human Myocardial Fibroblasts by Coxsackievirus B3

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Abstract—Cytokine expression in enterovirus infections of the heart may trigger inflammation and have detrimental effects on myocytes. However, the induction of cytokines in human myocardial cells by cardiotropic enteroviruses, for example, Coxsackievirus B3 (CVB3), was not yet demonstrated. Fibroblasts are the predominant cell type of the myocardial interstitium before inflammatory infiltration develops. Hence, we investigated, by enzyme immunoassays, reverse transcription–quantitative polymerase chain reaction (RT-qPCR), and nucleic acid sequence–based amplification (NASBA), whether CVB3 induces cytokine expression in cultured human myocardial fibroblasts. As early as 3 hours after infection, RT-qPCR demonstrated a 2-fold increase of interleukin (IL)–6 and IL-8 mRNA compared with basal transcription, resulting in a significant increase of IL-6 and IL-8 to a median level of 1500 pg/mL (range, 1246 to 1858) and 529 pg/mL (range, 428 to 601) in culture supernatants, respectively. IL-6 and IL-8 expression returned to basal levels within 3 and 5 days, respectively, despite a persistent (carrier-state) CVB3 infection. For comparison, IL-6 and IL-8 were induced in dermal fibroblasts later than 3 days after CVB3 infection. Although the low-level IL-1α transcription of myocardial fibroblasts was not significantly increased, IL-1α was released from cells to culture supernatants 5 days after infection. Furthermore, a suppression of interferon-β transcription was demonstrated up to 24 hours after CVB3 infection of myocardial fibroblasts by highly sensitive NASBA. In conclusion, our results demonstrate a heart-specific pattern of a rapid and transient induction of proinflammatory cytokines after CVB3 infection, whereas the expression of protective interferon-β was suppressed by CVB3. (Circ Res. 2000;86:753-759.)

Key Words: fibroblasts ■ interferon ■ interleukin ■ myocarditis ■ gene expression

Many enterovirus infections are probably subclinical, but infections of the heart with cardiotropic serotypes may cause serious diseases, for example, myocarditis with life-threatening arrhythmias.1,2 Chronic enterovirus myocarditis may result in congestive heart failure, and the only remedy then may be heart transplantation.2,3 Recently, rapid diagnosis of myocardial enterovirus infections was achieved by endomyocardial biopsy with subsequent detection of enterovirus RNA.1,4-5 Despite the progress in diagnosing enterovirus heart disease, its pathogenesis is not yet understood completely. Besides direct lysis of myocytes and induction of autoimmunity to cardiac antigens,6-11 induction of cytokine expression by enterovirus replication may be crucial for the pathogenesis of myocarditis and heart failure.12,13 Cytokine expression in the myocardium may result in inflammatory infiltration, autoimmunity, myocyte hypertrophy, fibrosis, and negative inotropic effects.12-15 Elevated plasma concentrations of interleukin (IL)–1α, IL-1β, and tumor necrosis factor (TNF)-α were observed in patients suffering from myocarditis.16 Moreover, IL-1α, IL-1β, IL-4, IL-6, IL-8, and TNF-α gene expression was demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) in the myocardium of patients suffering from myocarditis or dilated cardiomyopathy,17,18 but the cytokine-expressing cells were not yet identified.

Immediately after an enterovirus infects the heart, cytokine and chemokine expression by infected myocardial cells may be a first trigger for inflammatory infiltration and immune response. However, it is unknown whether enteroviruses can induce cytokine expression in human myocardial cells. In vivo, an enterovirus infection of both myocytes and myocardial interstitial cells, obviously fibroblasts, was demonstrated by in situ hybridization.1,19 More than 90% of the interstitial cells are fibroblasts before an inflammatory infiltration develops.20 In vitro, cultures of myocardial fibroblasts can be infected with Coxsackievirus B3 (CVB3) but are not lysed as quickly as myocytes, because a persistent (carrier-state type) virus replication evolves.21 Therefore, we suspected that cytokine expression of human myocardial fibroblasts may be significant in enterovirus heart disease. In this study, we demonstrated the induction of proinflammatory cytokines after CVB3 infection of myocardial fibroblasts and the suppression of interferon (IFN)–β transcription.

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Materials and Methods

Cells and Virus
Human myocardial and dermal (FS4) fibroblasts were propagated as described previously. CVB3 was generated by transfection from the plasmid CVB3-M1. Infectious virus titers (plaques-forming units [PFU]/mL) in culture supernatants of human myocardial fibroblasts were determined by plaque assays.

Enzyme Immunoassays and Immunocytochemistry
Cytokine concentrations were determined using enzyme immunoassays for IL-1β, IL-6, IL-8, soluble IL-6 receptor (sIL-6R), IFN-β (Biosource), IL-1α, and TNF-α (R&D Systems). Immunostaining for IL-1α, IL-6, and IL-8 was performed as described previously with an alkaline phosphatase/anti–alkaline phosphatase technique.

Quantitative PCR (qPCR)
RNA was purified from myocardial fibroblasts, and cDNA was synthesized. In each cDNA sample, the concentration of a “housekeeping” gene transcript (GAPDH) was quantified by qPCR (Cytoxpress kit, Biosource). Before performing IL-1α, IL-6, and IL-8 qPCR, all samples were diluted to equal GAPDH cDNA concentrations. IL-6 qPCR was performed using a Cytoxpress kit (Biosource). IL-1α and IL-8 qPCRs were performed with serial 2-fold dilutions of competitive internal standard DNA (“MIMIC”, Clontech) and analyzed by ethidium bromide–stained agarose gel electrophoresis. Template concentrations were calculated under the assumption of equal template-band and MIMIC-band densities for equal template and MIMIC concentrations.

Nucleic Acid Sequence–Based Amplification (NASBA)
NASBA was performed for sensitive detection of IFN-β mRNA using the NASBA amplification kit (Organon Teknika), as described recently.

Superinduction of IFN-β Gene Expression
IFN-β expression was induced with 10 μg/mL poly-IC (double-stranded RNA [dsRNA]), 100 μg/mL cycloheximide, and 2 μg/mL actinomycin D.

General Experimental Outline and Statistical Analysis
Briefly, experiments were carried out with 2 different experimental designs. For the “short-term” design, 16 cultures were infected with CVB3 with a high (2.0 PFU/cell) multiplicity of infection (moi), and 16 cultures were mock-infected. At 3, 6, 9, and 24 hours after infection, supernatants of 4 CVB3-infected and 4 mock-infected cultures were collected, and cells were trypsinized for RNA extraction. To determine the influence of CVB3 infection, cytokine concentrations of CVB3-infected cultures were compared with time-matched controls. Mann-Whitney tests were performed because of the heterogeneity of group variances.

For the “long-term” experimental design, 4 cultures were infected with a low moi (0.1 PFU/cell), and 4 cultures were mock-infected. Media were changed daily. On day 7, cultures were trypsinized, and RNA was extracted. Cytokine concentrations were measured in culture supernatants, and the area under curve (AUC) was calculated. AUC values of infected cultures were compared with AUC values of controls either by performing t tests, if F tests did not indicate heterogeneity of group variances (GraphPad Prism and Statmate software, version 2.01), or by performing Mann-Whitney tests. Both experiments (short and long term) were repeated twice with different preparations of myocardial fibroblasts. For an overall analysis of the

Figure 1. Immunocytochemical staining of human myocardial fibroblasts with monoclonal antibodies specific for IL-6 (A), IL-8 (B), and a polyclonal antiserum to IL-1α (C). D, Negative control incubated with nonimmune mouse serum; phase contrast.
data of long-term experiments, the AUC values were transformed as follows: 

\[ \text{AUC}_{\text{transformed}} = \frac{\text{AUC}}{\text{mean AUC controls}} \]

Transformed AUCs of CVB3-infected cultures were compared with transformed AUCs of controls by performing a t test with Welch correction. Two-sided \( P \leq 0.05 \) was regarded as statistically significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

### Results

#### Cytokine Basal Expression

All cultured myocardial fibroblasts stained uniformly positive for IL-6, IL-8, and IL-1α (Figure 1). IL-1α staining was associated with the nucleus, whereas IL-6 and IL-8 staining was cytoplasmic (Figure 1). IL-6 and IL-8 were detected in culture supernatants of all 6 lines of myocardial fibroblasts tested (both \( >500 \) pg/mL after 24 hours), but concentrations varied with each cell line (compare controls in Figures 2A and 2B with Figure 3). Cytokine concentrations (Figures 2A and 2B, controls) increased with cell density, as fibroblasts proliferated from \( 2 \times 10^5 \) cells/well (≈ 30% confluency, day 1) to 100% confluence on day 7. In contrast to IL-6 and IL-8, IL-1α concentrations in culture supernatants were low (≈ 0.5 pg/mL), either slightly above or below the sensitivity threshold of the ELISA, depending on the line of myocardial fibroblasts and cell density. No basal expression of other cytokines (IL-1β, TNF-α, sIL-6R, and IFN-β) was detected in myocardial fibroblast cultures by enzyme immunoassays.

#### Transient Induction of IL-6 and IL-8 by CVB3 Infection

IL-6 and IL-8 concentrations increased rapidly after CVB3 infection of human myocardial fibroblasts (Figures 2A and 2B). To determine the influence of CVB3 infection on cytokine concentrations early after infection, the AUC_{day 1–4} values of infected cultures were compared with AUC_{day 1–4} values of controls, indicating a significant increase of cytokine concentrations (IL-6, Figure 2A, \( P = 0.026 \), Mann-Whitney test; IL-8, Figure 2B, \( P < 0.001 \), t test). Moreover, a combined analysis of AUC_{day 1–4} data (depicted in Figures 2A and 2B) with AUC_{day 1–4} data from 2 other long-term experiments using different lines of myocardial fibroblasts (raw data not shown) confirmed an early increase of IL-6 concentrations (\( P = 0.0035 \), t test, \( n = 24 \)) and IL-8 concentrations (\( P < 0.001 \), t test, \( n = 24 \)) after CVB3 infection.

Despite a carrier-state infection of the cell culture (virus titer in culture supernatants on day 7, \( 1.6 \times 10^5 \) PFU/mL), IL-6 and IL-8 concentrations of CVB3-infected cultures returned to basal expression levels on days 4 and 6, respectively. Subsequently, IL-6 and IL-8 concentrations of CVB3-infected cultures remained unchanged or were slightly reduced in CVB3-infected cultures.

For comparison, human dermal fibroblasts (FS4) were infected with CVB3. In contrast to human myocardial fibroblasts, a late but persistent increase of IL-6 (\( P < 0.001 \), AUC, \( t \) test) and IL-8 (\( P < 0.001 \), AUC, \( t \) test) was demonstrated (Figures 2C and 2D).

To evaluate the kinetics of IL-6 and IL-8 induction, human myocardial fibroblasts were infected in the short-term experimental design with a high moi (2 PFU/cell) to assure that every cell had contact with the virus during the incubation period (1 hour). A significant increase of IL-6 and IL-8 concentrations was noted as early as 3 hours after infection compared with control cultures (Figure 3). This result was confirmed with 2 different lines of human myocardial fibroblasts (data not shown). Furthermore, IL-6 and IL-8 mRNA concentrations were significantly increased 3 hours (and 6 hours) after infection, as determined by qPCR, but returned to basal concentrations as early as 9 hours after infection (Figure 4, Table). As qPCRs were performed from identical cell numbers and after predilution of cDNA samples to equal concentrations of a housekeeping gene mRNA, they could be used to estimate the cytokine expression per cell. The
approximately equal IL-6 and IL-8 mRNA concentrations of infected cultures and controls on day 7 (Table) confirmed that the higher IL-6 and IL-8 concentrations of controls on day 7 (Figures 2A and 2B) were a result of a higher cell density, but not of an increased cytokine production per cell.

**IL-1α, IL-1β, sIL-6R, and TNF-α**

IL-1α concentrations in culture supernatants were starting to increase 4 days after CVB3 infection of myocardial fibroblasts compared with control cultures (Figure 5). However, IL-1α mRNA concentrations were unchanged in CVB3-infected cultures (data not shown), indicating an unchanged IL-1α gene transcription and a release of intracellular IL-1α from lysed cells. Furthermore, an induction of IL-1β, sIL-6R, and TNF-α expression was not observed after CVB3 infection of myocardial fibroblasts.

**IFN-β Expression**

As replicative CVB3 dsRNA should induce IFN-β in fibroblasts, we searched for IFN-β expression in myocardial fibroblasts. However, IFN-β was not detected in culture supernatants after CVB3 infection or mock infection (sensitivity 1 IU/mL). To exclude biologically active IFN-β concentrations, an IFN-β neutralizing antibody was added to CVB3-infected cultures. Neither virus titers nor cytokine concentrations were changed, indicating the absence of biologically active IFN-β in CVB3-infected cultures. Moreover, we searched for IFN-β gene transcription by highly sensitive NASBA. Thus, IFN-β gene transcription was demonstrated in mock-infected controls, but not in CVB3-infected cultures (moi 2 PFU/cell) up to 24 hours after infection (48 hours after trypsinization) (Figures 6A and 6B). Forty-eight hours after infection, IFN-β transcription was switched off in control cultures, but IFN-β mRNA was detected in CVB3-infected myocardial fibroblasts (Figures 6A and 6C). In contrast to CVB3 infection, superinduction of myocardial fibroblasts by dsRNA (poly-IC) demonstrated the capability of myocardial fibroblasts to express IFN-β (20 IU/mL culture supernatant).

**Discussion**

In this study, we demonstrated for the first time that a cardiotropic enterovirus, CVB3, transiently induced proinflammatory cytokines, IL-6 and IL-8, in human myocardial fibroblasts, a simplified but species-specific model to detect both cytokine expression and actions of cytokines. Moreover, the kinetics of CVB3-induced cytokine expression in myocardial fibroblasts was clearly divergent from dermal fibroblasts (Figure 2), indicating an organ-specific pattern of induction. Previously, the expression of IL-1β, IL-4, IL-6, IL-8, and TNF-α had been demonstrated by RT-PCR in the myocardium of patients suffering from enterovirus myocarditis, cryogenic myocarditis, and dilated cardiomyopathy, but neither the cytokine-expressing cells (myocardial or infiltrating) nor the stimulus (virus or secondary to infiltration) had been unequivocally identified. Recently, Seko et al compared the cytokine expression in murine CVB3 myocarditis with that of CVB3-infected murine heart cells in vitro. It was concluded that IL-1α, IL-5, IL-6, and IL-7 were predominantly expressed by heart cells, whereas IL-1β, IL-2, IL-3, IL-4, IL-10, TNF-α, TNF-β, and IFN-γ were predominantly expressed by inflammatory cells. However, the cytokine-expressing heart cells were not identified, as heart cell cultures consisted of myocytes, fibroblasts, and endothelial...
cells. By contrast, the propagation method used by us yielded human myocardial fibroblasts cultures that were free of other heart cells, and therefore fibroblasts were identified as cytokine-producing cells.

In vivo, both cytokine expression and action are supposed to be a complex network of interactions between myocardial cells and infiltrating cells. For example, sIL-6R may be released from infiltrating monocytes and interact with IL-6 produced by myocardial fibroblasts to induce myocyte hypertrophy. Although several cytokines, most importantly IL-1 and TNF, have several detrimental effects on the heart, IL-1α was released from infected fibroblasts (Figure 5). Although IL-1α is predominantly an intracellular cytokine, it has the same activities as IL-1β if released from cells. In vivo, IL-1 may trigger a postinfectious autoimmune myocarditis.

Nevertheless, chronic inflammatory infiltration and tissue destruction can follow the infection of the heart in vivo, and in vitro a persistent carrier-state infection of myocardial fibroblasts evolves. For example, IL-8 produced by fibroblasts may have detrimental effects, because IL-8 enhances cytopathic effects of enterovirus replication and suppresses the antiviral activity of IFN-α, which is active against CVB3 replication in myocardial fibroblasts. Moreover, IL-1α was released from infected fibroblasts (Figure 5). Although IL-1α is predominantly an intracellular cytokine, it has the same activities as IL-1β if released from cells. In vivo, IL-1 may trigger a postinfectious autoimmune myocarditis. In addition to CVB3-induced expression, we observed a basal expression of IL-6, IL-8, and IL-1α in myocardial fibroblasts. For comparison, IL-8 expression was described in explanted healthy donor hearts, but an IL-1α expression was not detected, and hearts were not tested for IL-6. However, a basal expression of both IL-1α and IL-6 was detected in heart tissue samples of healthy mice.

Interferons have a powerful antiviral activity compared with all other cytokines. Virus infection and dsRNA are well-established inducers of interferon production. For example, CVB3 infection induced interferon effectively (200 IU/mL culture supernatant) in human monocytes, and it was suspected that CVB3 infection should induce fibroblast IFN-β, in human myocardial fibroblasts. IFN-β is highly active against CVB3 replication in myocardial fibroblasts.

### Table: IL-6 and IL-8 mRNA Concentrations of Myocardial Fibroblasts at Indicated Intervals After CVB3 Infection

<table>
<thead>
<tr>
<th>Time After Infection</th>
<th>IL-6</th>
<th>IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 hours</td>
<td>257</td>
<td>246–264</td>
</tr>
<tr>
<td>6 hours</td>
<td>200</td>
<td>158–254</td>
</tr>
<tr>
<td>9 hours</td>
<td>52</td>
<td>47–56</td>
</tr>
<tr>
<td>7 days</td>
<td>87</td>
<td>79–101</td>
</tr>
</tbody>
</table>

After reverse transcription, all cDNA samples were prediluted to equal concentrations of a housekeeping gene (GAPDH) mRNA. Mock-infected controls = 100%. Data are median percentage of 4 cultures. *Mann-Whitney test.

#### Figure 5. IL-1α in culture supernatants after CVB3 infection of myocardial fibroblasts. Shown are data from CVB3-infected cultures (4 cultures; *) and mock-infected cultures (4 cultures; ×). AUC<sub>infected cultures</sub> vs AUC<sub>control</sub>, t test. (IL-1α concentrations of days 2 to 7 were sensitivity threshold in mock-infected controls.)

#### Figure 6. Northern blot hybridization of IFN-β NASBA amplicons with an IFN-β-specific probe. A, Poly-IC-treated fibroblasts and in vitro-transcribed IFN-β RNA (100 molecules) served as positive controls. H<sub>2</sub>O as a negative control. MW indicates molecular weight standard; inf, infection. B, IFN-β transcription 3 hours after infection. C, IFN-β transcription in carrier-state infection.
probably because of a Janus kinase (Jak)-1 tyrosine kinase activation, which is not observed after exposure of myocardial fibroblasts to IFN-α. However, IFN-β was not detected in culture supernatants of CVB3-infected myocardial fibroblasts either early after infection or during carrier-state CVB3 persistence. As “superinduction” with dsRNA (poly-IC) demonstrated that myocardial fibroblasts are capable of expressing IFN-β, our results suggest that CVB3 has evolved strategies to avoid IFN-β induction in myocardial fibroblasts. To confirm this hypothesis, we searched for low-level IFN-β gene transcription. In case of intronless genes, as, for example, the IFN-β gene, intron-spanning primer pairs cannot be used to recognize false-positive RT-PCR results caused by amplification of genomic DNA. NASBA, which is as sensitive as RT-PCR, permits selective amplification of single-stranded mRNA in a background of genomic dsDNA. IFN-β mRNA was detected by NASBA in human myocardial fibroblasts as long as 24 hours after mock infection (48 hours after trypsinization), which may be nonspecific “stress” procedures inducing low levels of IFN-β transcription. On the other hand, IFN-β gene transcription was not observed (sensitivity, 10 copies of mRNA/100 ng cellular RNA) during the first 24 hours after CVB3 infection (Figures 6A and 6B), supporting the hypothesis that CVB3 has evolved strategies to avoid IFN-β induction in myocardial fibroblasts.

However, IFN-β transcription was detected in CVB3 carrier-state cultures as late as day 7 after infection, but not in mock-infected cultures (Figures 6A and 6C). Considering this result, it should be noted that IFN-β gene transcription in CVB3 carrier-state–infected cultures does not prove that IFN-β is transcribed by infected cells, as only 10% of the cells in a carrier-state culture are infected. For example, IFN-β transcription may be suppressed in CVB3-infected cells similar to the first 24 hours after infection, and IFN-β may be transcribed by noninfected fibroblasts of a CVB3-infected carrier-state culture as a reaction to cell components and factors released by infected cells. Hence, IFN-β transcription in late carrier-state infection may be a reaction of cells to nonviral stimuli, similar to the stress that induced IFN-β “basal” transcription shortly after the mock infection procedure. Analogously, it may be suspected that IL-6 and IL-8 are induced in noninfected cells after release of a speculative factor from the infected cells of a culture. However, the rapid increase of the IL-6 and IL-8 mRNA concentration as early as 3 hours after infection of a fibroblast culture (Table) strongly indicates either an increased transcription or a blocked IL-6 and IL-8 mRNA degradation by CVB3 in infected cells.

In conclusion, our results with species-specific heart cells demonstrated the induction of proinflammatory cytokines, but the suppression of protective IFN-β by a cardiotropic enterovirus, CVB3.

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References


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