Viral Infection and the Pathogenesis of Dilated Cardiomyopathy

Tamara A. Martino, Peter Liu, Michael J. Sole

Abstract Long-term follow-up studies of patients with suspected viral myocarditis reveal progression to dilated cardiomyopathy (DCM) in a significant number of cases. Thus, an underlying viral etiology has been hypothesized in the pathogenesis of ongoing heart disease that leads to DCM. Recent application of molecular biology in clinical diagnosis has strengthened this hypothesis. By use of probe hybridization and polymerase chain reaction, enteroviral RNA has been detected in the myocardium of patients at all stages of the disease process: myocarditis, chronic heart disease, and DCM.

Acute myocarditis and its chronic complement, dilated cardiomyopathy (DCM), have gone through an evolutionary cycle in concept. Historically, it was recognized as a viral disease. However, the subsequent inability to consistently culture the virus or to detect viral antigens and the immunologic and pathological resemblance to transplant rejection opened the next era of the autoimmune etiology of myocarditis. Unfortunately, disappointment was set in when immune modulation failed to alter the course of the disease, and some recent immunodeficient models challenged the immune theory. With currently available molecular techniques, we are now again on the threshold of rediscovering the role of the virus and elucidating its interaction with the immune system and the cells of the myocardium. The present review will put in perspective clinical and experimental evidence on the viral etiology of myocarditis and DCM.

Clinical Studies: Serology and Viral Detection

Serological studies have long been used to examine viral involvement in heart disease. A fourfold rise in neutralizing antibodies to coxsackievirus B (CVB) in paired sera over a 2- to 4-week period has helped to establish infectious etiology in patients with myocarditis. Persistence of CVB-IgM antibody responses for up to 6 months in some patients with myocarditis and high CVB-IgM titers in 7% to 33% of DCM patients have contributed to the theory of a viral etiology underlying the pathogenesis of DCM.

Experimental murine models of enterovirus-induced heart disease provide a framework for examining the pathogenic mechanisms. Viral cytotoxicity, immunological responses, viral RNA persistence, and spasm of the coronary microvasculature are all implicated in the ongoing disease process. Abnormal cardiac function and heart failure are attributed to the pathological changes that occur. (Circ Res. 1994;74:182-188.)

Key Words • dilated cardiomyopathy • viral infection • coxsackievirus B • myocarditis

However, the high titers were significantly more common in patients with a recent clinical history of myocarditis. Furthermore, in many cases, an actual change in antibody titer was not observed using paired sera. Also, the heterotypic nature of CVB-neutralizing antibodies precluded the association of specific serotypes with ongoing heart disease. These findings raised the possibility that the antibodies were against a recent infection that was unrelated to the heart condition. Because of such difficulties in evaluating the serological data, these studies could only provide circumstantial evidence, but not definitive proof, of a viral etiology in DCM.

Isolation of Viruses From Heart Tissue

The presence of infectious virus or viral antigens within the heart tissue has also been examined. CVB has been cultured from the hearts of patients with neonatal myocarditis and adult pericarditis and myocarditis and pericarditis and from pericardial fluid. CVB proteins have been detected in the heart tissue of infants who died of myocarditis, using an indirect immunofluorescent technique. CVB-like particles were also identified in the myocardium of one cardiomyopathy patient on postmortem examination.

It should be noted, however, that isolation of viruses such as CVB from patients with heart disease is rare, particularly in the later stages of the disease process. Consequently, enteroviral etiology in heart disease cannot be firmly established using these techniques. In light of the relative infrequency of conventional viral detection, some investigators abandoned a role for the virus in the ongoing disease process and pursued instead alternative pathogenic mechanisms such as autoimmune responses. However, recent studies that have applied molecular technology to clinical diagnosis have once again revealed the importance of viral involvement in myocarditis and DCM. These developments are discussed in more detail below.
Clinical Studies: Molecular Detection of Entervoiral RNA

Detection of Viral RNA by Slot-Blot Probe Hybridization

The presence of virus in the heart tissue of patients with myocarditis and DCM has been demonstrated most recently by use of molecular techniques. The enteroviruses, CVB among them, contain a positive strand of RNA as their genetic material. Probes that are complementary to the viral genome can be used to detect the viral RNA within small patient samples such as endomyocardial biopsies.

The first example was reported by Bowles et al. in 1986. The investigators used a slot-blot method, in which a radiolabeled probe was hybridized to RNA purified from endomyocardial biopsies. By use of this method, enteroviral RNA was detected in ≈50% of biopsies from patients with active or healing myocarditis or DCM. Over the years, however, it was recognized that the hybridization probe used in this study had some cross-reactivity to uninfected cardiac tissue. Consequently, the 50% positivity was likely an overestimation of enteroviral presence in myocardial tissue. Nevertheless, this study was very significant, for it confirmed viral presence in heart tissue and supported the hypothesis of an underlying viral etiology in the pathogenesis of myocarditis and DCM. The subsequent flurry of research interest sparked by this study led to additional confirmations by other groups using more refined molecular techniques.

Detection of Viral RNA by Polymerase Chain Reaction

One such molecular technique was the polymerase chain reaction (PCR). PCR allowed for the specific detection of enteroviral RNA from very small amounts of biopsy material. The specificity, which was accomplished by primer-mediated high-copy amplification of distinct genetic sequences, distinguished the viral RNA genome from the background genetic material in the tissue sample.

Our laboratory was the first to use PCR to detect enteroviral RNA in endomyocardial biopsies. Samples from 48 patients with clinically suspected myocarditis or DCM were examined. Enteroviral signals were detected in five cases: myocarditis, 1; postpartum cardiomyopathy, 1; and DCM, 3. Of particular importance was the observation that the three PCR-positive DCM patients had histories suggestive of myocarditis and that their biopsy specimens exhibited significant amounts of fibrosis and myocyte hypertrophy, which were classified as negative according to the strict Dallas criteria for pathological diagnosis of acute myocarditis yet were indicative of the description of resolved (healed) myocarditis. Taken together, these observations strongly supported the link between enteroviral infection and myocarditis and progression to DCM in certain patient subsets.

PCR is a more sensitive technique than conventional hybridization. For example, four of the PCR-positive cases mentioned above failed to demonstrate viral RNA signals when high-stringency slot-blot analysis was performed. However, the exquisite sensitivity of this molecular technique is dependent on many factors. The concentration and purity of reagents used, the specific technical protocols followed, the degree of tissue preservation, and inhibitors present in patient samples can all significantly affect the final yield of product.

Variations such as these likely contribute to the rather confusing range (0% to 45%) of PCR-enteroviral RNA positivity from endomyocardial biopsies now encountered in the literature. The inherent focal nature of myocardial heart disease and inadequate sampling through biopsies are also important contributing factors. To address these issues, we have designed a control enteroviral RNA standard fragment that can be amplified along with clinical specimens. The final yield of the control product provides quantitative information that can be used to objectively monitor the efficacy of the PCR test. The quantitative assay may also have diagnostic and prognostic value, which we are in the process of evaluating.

A lower range of enteroviral signals has also been reported using PCR on biopsy specimens from some patients with cardiac conditions not primarily associated with enteroviral infections. This may be the result of exogenous contamination in the laboratory (ie, work areas or work utilities contaminated through enteroviral work) or endogenous contaminants (ie, viremic virus from a recent unrelated enteroviral infection or even infected immune cells trapped in the biotome sample). These conflicting data may be resolved by PCR in combination with another molecular technique, namely, in situ hybridization.

Detection of Viral RNA by In Situ Probe Hybridization

In situ hybridization is technically more difficult than slot-blot or PCR; however, it offers the advantage of visualizing the specific cells infected with enteroviral RNA. As with slot-blot hybridization, probes complementary to the viral RNA are used, but they are hybridized directly onto histological sections cut from the biopsy samples.

Using in situ hybridization, Easton and Elgin (1988) detected enteroviral RNA in endomyocardial biopsy samples and noted two patterns of infectivity. First, the viral RNA was detected in the adventitia of blood vessels, presumably indicating initial viremia and perivascular delivery of the virus. Second, viral RNA was detected in cardiac muscle cells, possibly indicating the subsequent spread of virus through the cardiac muscle tissue. Kandolf et al. (1991) reported similar findings in patients with myocarditis and also noted that the viral RNA progressed from areas of inflammation. This was significant because it suggested that cell-to-cell spread of virus had occurred, which would be indicative of an actively replicating virus in the infected heart tissue. An explanation for viral RNA replication in the absence of detectable virions has been partly elucidated from animal model studies and is discussed in a later section.

Kandolf et al. (1991) further reported enteroviral RNA presence in serial biopsy specimens taken in the late stages of the disease process from patients with chronic myocarditis as well as from patients with DCM. In these cases, the viral signals were primarily associ-
ated with degeneration of myofibers, hypertrophy, and interstitial fibrosis, in the absence of inflammation. These observations were consistent with the pathological findings in DCM, further supporting the hypothesis that acute (or possibly subacute) viral myocarditis may evolve into chronic cardiac dysfunction.

**Clinical Significance of Enteroviral RNA Detection in Endomyocardial Biopsies**

The presence of enteroviral RNA in heart tissue has been associated with poorer patient prognosis. Enteroviral RNA has been detected in the myocardium of a higher proportion of patients who undergo cardiac transplantation because of DCM compared with control groups. Furthermore, in a follow-up study of patients with myocarditis, DCM, or other specific heart muscle disease, 26% of patients positive for enteroviral RNA in myocardial biopsy samples died compared with only 3% of patients that were enterovirus-negative. These two studies lend further support to the hypothesis of an etiologic role for enteroviruses detected within heart tissue.

**Murine Models of Viral Heart Disease**

Although enteroviruses such as CVB are strongly implicated in the pathogenesis of myocarditis and the evolution of DCM, their role in these conditions cannot be proven in a clinical setting to fulfill Koch's postulates. In this case, murine models of CVB infection provide a framework in which the general pathogenic mechanisms can be carefully studied.

**Pathological Changes**

Administration of CVB to mice results in heart disease that bears striking resemblance to the human condition of myocarditis. In the first 2 weeks after infection, severe pathological changes are noted. Focal myocardial lesions occur, which consist of a densely packed inflammatory cell infiltrate surrounding necrotic myofibers. Over a period of 6 months, histopathologic examination of the hearts reveals continuing inflammation and ongoing necrosis of myocardial muscle, which leads to fibrosis, calcification, and hypertrophy in the remaining myocytes. A model of human DCM becomes evident within 1 year. Heart failure occurs, which is attributed to pathological changes consisting of myocardial scarring in the absence of inflammation, endocardial and subendocardial thickening, and dilation of the cardiac chambers. Murine myocarditis is also associated with spasm or constriction of the coronary microvasculature similar to that seen in genetically myopathic hamsters. Treatment with the calcium channel blocker verapamil, the converting enzyme inhibitor captopril, or α-adrenergic blocking agents markedly ameliorates the microvascular spasm, preventing myocardial damage and evolution of DCM.

**Direct Virus-Mediated Destruction of Myofibers**

Virus- and immunocyte-mediated pathogenic mechanisms are both implicated in the destruction of myocardial tissue. In vivo studies that show a direct consequence of virus-induced cytotoxicity include the presence of necrotic myofibers in the absence of an inflammatory cell infiltrate 3 days after infection, extensive cardiac damage in severe combined immunodeficiency syndrome (SCID) and athymic mice, where the expected inflammatory cell infiltrate is genetically reduced or absent, and the lack of benefit of immunosuppressive therapy in several strains of inbred mice.

The mechanism by which CVB induces myocytolysis has been examined in vitro by use of cultured fetal human and murine heart cells. The virus is capable of infecting myocytes, as evidenced by the presence of viral particles in the cell cytoplasm on ultrastructural examination. Viral replication in the myocytes was noted by the generation of high titers of infectious progeny. Damage to the myocytes occurred within 2 days of infection, by which time the majority had lost their contractile ability. Electron microscopy of affected myocytes revealed a pattern of classic cytopathic effect, characterized by the destruction of myofibrils and filaments, and the formation of vesicles and vacuoles throughout the cytoplasm.

**Clearance of Culturable Virus From the Myocardium**

Under normal conditions, direct virus-mediated damage occurs primarily at the start of the disease process. By 4 days after inoculation, infiltrating macrophages, natural killer cells, and protective antiviral antibodies begin clearing the virus from the myocardium.

During the acute stage, infiltrating T lymphocytes are also seen in the myocardium. Cytotoxic/suppressor T lymphocytes (Lyt2+) predominate to day 6 and are then exceeded in number by helper T lymphocytes (L3T4+). These infiltrating immunocytes also play a critical protective role by limiting virus spread in the heart tissue and by eliminating infected myocardial cells.

**Cell-Mediated Immune Pathogenicity**

The immune cell infiltrate peaks by day 12 after inoculation, coinciding with the most severe acute pathological damage. The inflammatory response then continues at lower intensity, surrounding sites of ongoing cardiac necrosis, even though culturable virus has been eliminated. In light of these observations, it has been suggested that cell-mediated immune mechanisms are then implicated over viral mechanisms in the ongoing destruction of cardiac tissue.

Further support for this theory comes from studies in which CVB-infected mice were T-cell–compromised. A decrease in myocardial damage was noted in nude mice, TXB mice (thymectomized, irradiated, reconstituted with bone marrow cells), mice pretreated with antibodies against specific T-cell populations, and some inbred strains given immunosuppressant drugs.

Cytotoxic T lymphocytes (CTLs) have also been shown to be capable of lysing virus-infected cardiocytes in vitro. A second population of CTLs, which are capable of lysing uninfected cardiocytes, indicates the presence of an autoimmune component as well. Cross-reacting autoantibodies and molecular mimicry are two additional mechanisms implicated in the autoimmune response.
Persisting Viral RNA

Culturable virus and viral capsid proteins are absent after the initial phase of the disease; consequently, an ongoing contributing role for the virus has been largely ignored. However, there is recent evidence to suggest that this lack of focus on viral mechanisms may have been an oversight and that it contributes not only to the acute phase of myocarditis but also to the evolution of ongoing heart disease.

The implication of viral mechanisms in ongoing cardiac disease was best demonstrated by Klingel et al (1992), who used a murine model of CVB3-induced myocarditis. During acute and chronic stages of infection, murine hearts were examined for viral RNA presence by in situ hybridization. The sites where viral RNA was detected were then related to the extent of myocardial damage and inflammatory cell response. In the first 3 days after infection, myocytes containing CVB3 RNA were randomly distributed through the myocardium. By day 6, infected myocytes were adjacent to the foci of inflammation. From day 6 to day 9, the greatest area of CVB3 RNA–infected myocardial cells was noted. This correlated with a significant increase in myocardial injury. From days 15 to 30 after infection, after culturable virus had been eliminated, persistently infected myocardial cells were still detected. Infected cells were found primarily within foci of myocardial lesions characterized by fibrosis, myocardial necrosis, and mononuclear cell infiltrates. These observations strongly implicate viral RNA presence in the development of the myocardial lesions.

The association between viral RNA presence and the sites of ongoing pathology was observed in three different strains of immunocompetent inbred mice, all of which were capable of progression to chronic heart disease. In contrast, DBA/1J mice, which were capable of terminating the inflammatory processes through elimination of the virus from the heart, showed no evidence of viral RNA persisting in the myocardium. These results strongly indicate that the development of chronic heart disease was dependent on viral RNA persistence.

In our laboratory, we used PCR to show that viral RNA persists even beyond the chronic stage. Using a murine model of encephalomyocarditis infection, viral RNA signals were detected through day 42, at which point the inflammatory response had mostly subsided. A second group of researchers have used PCR to show that the viral RNA signals are even detectable beyond 90 days, which is reportedly well within end-stage DCM in this model.

Pathogenic Mechanisms of Persisting Virus

The molecular detection of viral RNA in the myocardium during the acute, chronic, and end stages of virus-induced heart disease is an important finding. However, this finding raises two critical questions: (1) How does the persisting viral RNA contribute to the disease process? (2) How can these findings be reconciled with the immune-mediated responses? One possible explanation is that viral RNA persistence in myocardial cells contributes to the pathogenesis of ongoing cardiac damage by continuous triggering of the immune inflammatory reaction, achieved through the generation of new antigenic molecules. From the viral perspective, there is evidence to suggest that the persisting viral RNA is capable of replication, in a restricted or altered manner. One consequence might be the production of new antigenic noninfectious or defective interfering viral particles. From the cellular perspective, it has long been hypothesized that de novo antigens might be formed in chronically infected myocytes and recently, a novel glycoprotein of cellular origin has been described in CVB-infected cells. Neoantigens of viral and cellular origin have been implicated in the responses of two distinct populations of cytotoxic T lymphocytes. Final identification of such products would contribute greatly to our understanding of the ongoing cellular assault against infected myocardial cells.

Alternatively, carrier state infections have been suggested as a mechanism by which the virus induces ongoing heart disease, particularly under conditions in which host defense mechanisms are depressed. Persistent viral infection has been observed in the spleen and lymph nodes during the chronic phase of the disease. Liver and pancreas may represent additional extracardiac reservoirs of virus. Carrier-state infection has also been established in vitro using human fetal aortic organ cultures, human fetal myocardial fibroblasts, human lymphoid cell lines, and SV40-transformed murine skin fibroblasts. Another consideration is that repeated exposures to enteroviruses are involved in the recurrence of acute infections, the exacerbation of myocardial inflammatory disease, and the development of chronic heart disease.

Susceptibility Factors

Virus-induced heart disease shows a wide range of expression in inbred mice, thus making it a suitable model for examining the heterotypic responses seen in humans. The genotype of the inciting viral infection is implicated as a critical factor in the expression of the disease. Reduced nutritional status of the animal contributes to virus susceptibility, and exercise exacerbates the illness. Additional factors such as the sex of the host, age, the presence of specific viral receptors, and the type of immunologic response also appear to modulate the expression of enterovirus-induced heart disease. Many of these factors have also been examined in patients with myocarditis and DCM. Identification of matching viral and host factors may help to elucidate patient populations most likely to progress to end-stage DCM and are also critically important for determining effective therapeutic treatments.

Conclusion

An underlying viral etiology in acute myocarditis that progresses to DCM has been investigated in clinical studies and animal models. The infecting virus initiates the disease process and then persists as an insidious molecular pathogen, inciting ongoing cardiac damage. A well-orchestrated but adverse attack leads to myocardial degeneration, microvascular dysfunction, and reparative fibrosis of the cellular matrix. In a significant number of cases, this results in myocardial hypertrophy,
dilation of the cardiac chambers, abnormal cardiac function, and eventually heart failure. Establishing a new paradigm for the pathogenesis of viral myocarditis and DCM using molecular techniques will likely lead to further insights into the mechanisms of virus-myocardium-immune interactions. In addition, these techniques may well point the way toward new approaches in the investigation and treatment of this elusive disease.

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