Osteopontin
A Fibrosis-Related Marker Molecule in Cardiac Remodeling of Enterovirus Myocarditis in the Susceptible Host

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Abstract—The characteristics of dilated cardiomyopathy (DCM) resulting from chronic viral myocarditis are remodeling processes of the extracellular matrix. Based on our findings of enhanced osteopontin (OPN) expression in inflamed human hearts, we further investigated in the murine model of acute and chronic coxsackievirus (CV)B3-myocarditis the role of OPN regarding its involvement in resolution of cardiac virus infection and fibrosis. In hearts of A.BY/SnJ mice susceptible to chronic CVB3-myocarditis, a pronounced increase of OPN expression levels was detected by microarray analysis and quantitative RT-PCR during acute stages of myocarditis. Combined immunohistochemistry and in situ hybridization identified infiltrating macrophages as main OPN producers. In contrast to resistant C57BL/6 and OPN gene–deficient mice, transcription levels of matrix metalloproteinase-3, TIMP1 (tissue inhibitor of metalloproteinases-1), uPA (urokinase-type plasminogen activator), and transforming growth factor β1 were elevated in susceptible mice, and as a consequence, procollagen-1 mRNA expression and fibrosis was considerably enhanced. Treatment of infected susceptible mice with the vitamin D analog ZK 191784 led to decreased myocardial expression levels of OPN, metalloproteinase-3, TIMP1, uPA, and procollagen-1α and subsequently to reduced fibrosis. Concurrently, the fibrosis-relevant signaling molecules pERK (phosphorylated extracellular signal-regulated kinase) and pAkt (phosphorylated Akt), increased in A.BY/SnJ mice, were diminished in ZK 191784–treated mice. Here, we show that high expression levels of OPN in acute myocarditis are associated with consecutive development of extensive fibrosis that can be reduced by treatment with a vitamin D analog. Thus, OPN may serve as a diagnostic tool as well as a potential therapeutic target to limit cardiac remodeling in chronic myocarditis. (Circ Res. 2009;104:851-859.)

Key Words: myocarditis infection inflammation remodeling osteopontin

Dilated cardiomyopathy (DCM), which is among the most common heart diseases, has various etiologies and clinical outcomes but is often a sequela of myocarditis and represents a major cause of morbidity and mortality worldwide. Up to 60% of patients with myocarditis and DCM are virus-positive.1 A critical step in development of DCM is the initiation of remodeling processes of the extracellular matrix. Pathological collagen synthesis leads to interstitial fibrosis and finally to cardiac dysfunction.2 Characteristics of DCM are left ventricular dilatation, a decreased ejection fraction, and a depressed wall motion attributable to fibrosis.2 This feature depicts the last stage of inflammatory heart disease, starting with acute myocarditis, passing to chronic myocarditis, and resulting in DCM.3–4 Viral infections of the heart have been associated with the development of DCM resulting from persistent infection and, consequently, chronic inflammatory processes following acute myocarditis.5,6 Among the cardiotropic viruses inducing myocarditis, enteroviruses, parvovirus B19, and human herpesvirus 6 represent the most commonly identified etiologic agents.5,6 Enteroviruses, especially coxsackieviruses of group (CV)B, belong to the best-studied pathogens evoking myocarditis. Recent studies demonstrate that the consequences of inflammatory responses in the heart caused by CVB3 infection play a decisive role in the development and progression of DCM.5,7,8 In the murine model of CVB3-induced myocarditis, it has been shown that CVB3 infection influences the regulation of extracellular matrix architecture via interference by inflammatory mediators.7,9 Proinflammatory cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor-α, and transforming growth factor (TGF)β are involved in the regulation of matrix metalloproteinases (MMPs) and their inhibitors, TIMPs (tissue inhibitors of MMPs); the urokinase-type plasminogen activator (uPA); and connective tissue growth factor (CTGF). Disturbance of the fine balance of these factors contribute to cardiac fibrosis and hence to cardiac dysfunction and dilatation.9–11

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.109.193805
More recently, another protein that has been described to be involved in inflammatory responses and in the maintenance or reconfiguration of tissue integrity, is osteopontin (OPN), a phosphorylated glycoprotein that exists as an immobilized matrix protein and as a cytokine. OPN is produced by a variety of cell types including fibroblasts, macrophages, and T cells and is expressed in a multitude of biological processes. The diverse functions of OPN comprise bone mineralization, chemotaxis, support of adhesion, modulation of T-cell responses, cell survival, and wound repair. In the normal myocardium, OPN expression is absent but is increased under pathological conditions such as coronary heart disease, infarction, hypertrophy, and ischemia. Whereas a major source of OPN in the heart is probably interstitial fibroblasts, in inflammatory cardiac diseases macrophages can be added to the list of OPN producers.

The expression of OPN is mediated by signaling via the vitamin D receptor. Transcriptional regulation of OPN is induced by the active vitamin D₃ metabolite 1,25(OH)₂D₃, also known as calcitriol, whose association to the vitamin D₃ receptor activates the binding of the vitamin D receptor 9-cis-retinoic acid receptor complex at the vitamin D response elements. Together with several coactivators, a multiprotein complex is built, which induces transcription of several genes among others is OPN and gene transcription is modulated in different signaling pathways. The expression of OPN is mediated by signaling via the vitamin D receptor. Transcriptional regulation of OPN is induced by the active vitamin D₃ metabolite 1,25(OH)₂D₃, also known as calcitriol, whose association to the vitamin D₃ receptor activates the binding of the vitamin D receptor 9-cis-retinoic acid receptor complex at the vitamin D response elements. Together with several coactivators, a multiprotein complex is built, which induces transcription of several genes among others is OPN and gene transcription can be enhanced by binding of calcitriol to G protein-coupled receptors. Besides other properties, calcitriol has profound effects on immunoregulation and its analog ZK 191784 has an immunosuppressive impact.

The aim of the present study was to investigate the contribution of OPN to remodeling processes in enterovirus-induced myocarditis and subsequent DCM. We used the murine model of CVB3-induced myocarditis, in which susceptible A.BY/SnJ mice develop chronic myocarditis and severe cardiac fibrosis following acute infection resulting from a restricted type of virus persistence, whereas resistant C57BL/6 mice eliminate the virus during acute infection without evolving significant fibrosis. Additionally, we investigated the impact of OPN on the outcome of CVB3-induced myocarditis and its relationship with the development of fibrosis using OPN gene-deficient (OPN−/−) mice (see the online data supplement, available at http://circres.ahajournals.org). Furthermore, we pursued downregulation of OPN expression in CVB3-infected susceptible A.BY/SnJ mice by application of the vitamin D analog ZK 191784 and studied the consequences of OPN downregulation.

Materials and Methods

In Situ Hybridization

Human OPN mRNA was detected in endomyocardial biopsies which were taken for diagnostic reasons with approval of the Ethics Committee of the SFB-TR19. 35S-Labeled antisense and sense hOPN RNA probes were synthesized by in vitro transcription from the dual-promoter plasmid pGEM-hOPN containing a 350-bp cDNA fragment. Pretreatment, hybridization, and washing conditions of dewaxed 5-μm paraffin tissue sections were performed as described previously. Slide preparations were subjected to autoradiography and counterstained with hematoxylin/eosin.

5S-Labeled antisense and sense RNA probes for detection of murine OPN mRNA were synthesized by in vitro transcription from the dual-promoter plasmid TOPo-mOPN containing an 879-bp OPN cDNA fragment. CVB3 positive-strand RNA was detected in tissues using single-stranded 35S-labeled RNA probes, which were synthesized from the dual-promoter plasmid pCVB3-R1. Control RNA probes were obtained from the vector pSPT18. Viral load was determined as previously described.

Virus and Viral Antigens

CVB3 used in this study was derived from the infectious cDNA copy of the cardiotropic Nancy strain. Virus stocks and viral antigen preparation were prepared as described previously.

Mice and Infection

C57Bl/6 mice, ABY/Snj mice, and OPN−/− mice (all H-2b) were purchased from The Jackson Laboratory. Animals were bred and kept under specific pathogen-free conditions at the animal facilities of the Department of Molecular Pathology, University Hospital Tübingen, and experiments were conducted according to the German animal protection law. Virus infection of mice and further processing of organs was performed as described previously.

DNA Microarray Hybridization and Analysis

Isolation of RNA from murine heart tissue and microarray analysis from Affymetrix (Santa Clara, Calif) MG-U74Av2 chips was performed by application of the Affymetrix gene expression software GeneChip, MicroDB, and Data Mining Tool as described previously.

RT-PCR and Quantitative RT-PCR

Specific primers and probes were purchased from MWG Biotech. Primers were as follows:

- CVB3: forward, 5′-TATCCGGCAACTACTCTTGAA-3′; reverse, 5′-TGCCTGACATCATTGGGGA-3′
- mGAPDH: forward, 5′-AATGCCCTCGACACCACC-3′; reverse, 5′-GTCGGATGAGCAAGCCT-3′
- hMHPRT: forward, 5′-TTGGGCGGAAGCCG-3′; reverse, 5′-TAACTGTGCTCATCCTGTAATC-3′; probe, 5′-FAM-CGA-CGCCAGTCGCGTGCTA-TAMRA-3′
- mOPN: forward, 5′-GCGATTGCTCTCCTCCCTC-3′; reverse, 5′-GACGGCTGAAGATCTCTCC-3′; probe, 5′-FAM-CGTTGGAAGTGGAGCT-3′
- hMMP9: forward, 5′-TGGAGAACATGCGAGCTTTGGCC-3′; reverse, 5′-TGCATTAACTCTCTGTCAGG-3′; probe, 5′-FAM-GGAACAGCTGGCTGCTGATATG-3′
- tTIMP1, forward, 5′-TCCCTTTCTGTTGATGACTGAGT-3′; reverse, 5′-CGCTGTGATAAAGTGGTGTCGT-3′; probe, 5′-FAM-TCTCGCAACTCGGACCTGCAATG-3′
- hMGP: forward, 5′-TGACGTCACTGTGGGTTGACCC-3′; reverse, 5′-GTTCTGATCAGGAGTGGC-3′; probe, 5′-FAM-TTCAGCCGCTACGCTGCTGACAG-3′
- mGFPB1: forward, 5′-TGACGGTCTGAGGGTGGTTCG-3′; reverse, 5′-GGTCTGATACTGCTGCTGATATG-3′; probe, 5′-FAM-TTCAGCCGCTACGCTGCTGACAG-3′
- muPA: forward, 5′-AACAACTCTCTCGGCAAGT-3′; reverse, 5′-CTGTCCTCCTCTGACGACCTG-3′; probe, 5′-FAM-AGGAGGAGAGACTGTCGTTGTC-3′
- procollagen 1α1: forward, 5′-TCCGGCTCTGCTCCTTCTCTT-3′; reverse, 5′-GATATCGCTACGCTGCTGACAG-3′; probe, 5′-FAM-TTCAGCCGCTACGCTGCTGACAG-3′

In the first experiments for the evaluation of real-time RT-PCR, we compared mRNA expression from individual mice (n=5) in comparison with pooled mRNA from 5 mice to obtain information on the individual variation of expression levels in the different mouse strains. Because the expression variations of individual mRNA within 1 mouse strain were minimal and the mean values similar to pooled mRNA, we decided to use pooled mRNA for our experiments.

Determination of Cytokines by ELISA

Determination of interferon (IFN)γ and IL-10 levels was performed as previously described.
Histology and Immunohistochemical Staining
Histology and quantification of myocardial damage was performed as previously described.8 For immunohistochemistry, tissue sections were incubated for 1 hour at 25°C with goat anti-mouse antibodies recognizing murine OPN (R&D), rabbit antihuman OPN (Neomarker), with rat anti-mouse antibody recognizing Mac-3 (Becton Dickinson) on macrophages and with rabbit anti-mouse antibody recognizing CD3 (Neomarker) on T lymphocytes, respectively. Controls using normal goat, rabbit, or rat serum were run to exclude nonspecific staining. Slides were processed using streptABCComplexHRP (DAKO) and DAB (Vector Laboratories) as substrate. Quantification was assessed as number of positive cells per square millimeter myocardial lesion.21 For evaluation of fibrosis, picrosirius red staining was applied.

Concurrent Immunohistochemistry and In Situ Hybridization
For identification of OPN positive cells, tissue sections were processed by immunohistochemistry to detect Mac-3+ macrophages followed by in situ hybridization (ISH) to demonstrate OPN mRNA expression as described previously.21

Treatment With Vitamin D Analog ZK 191784
Mice were treated with 100 μg/kg the vitamin D analog ZK 191784 (a kind gift from Bayer Schering Pharma AG) subcutaneously 1 day before infection until day 12 postinfection (pi). CVB3 infection was performed as described above and mice were euthanized at day 8, day 12, and day 28 pi. As controls, ZK 191784–treated, uninfected mice as well as untreated, CVB3-infected mice were used. At indicated time points, hearts were removed and subjected to the investigations described above.

Statistical Analysis
When statistical analysis was performed, data were expressed as means±SEM. Significance was calculated by use of Mann–Whitney U test or unpaired t test using SPSS 11.0 software as indicated in the figure legends. An probability of P<0.05 was regarded as significant.

Results
OPN Expression in Human Endomyocardial Biopsies With Chronic Myocarditis
We received first evidence that OPN might be relevant in the pathogenesis of chronic myocarditis when we performed in situ hybridization experiments and immunohistological stainings for visualization of OPN mRNA and protein in human endomyocardial biopsies. As exemplary illustrated in heart tissue sections from patients with histopathologically proven chronic myocarditis, we found a significant number of OPN mRNA (Figure 1A, arrows), as well as OPN protein–expressing cells (Figure 1B, arrows) in the interstitium of the myocardium. Clusters of OPN mRNA–positive mononuclear cells (Figure 1A), most probably representing macrophages, were found to be mainly located within areas of inflammation. In cardiac tissue of patients with DCM where an interstitial inflammatory response is usually absent, no significant OPN mRNA (Figure 1C) and protein (Figure 1D) expression was detected in cardiomyocytes or interstitial cells.

Expression Patterns of OPN in Murine Hearts in CVB3-Myocarditis
To gain further insight into OPN expression at different stages of myocarditis, we examined different CVB3-infected mouse strains. Highly upregulated OPN mRNA expression levels in infected mouse hearts were first noted in experiments identifying differential gene expression patterns by whole-genome microarray analysis using Affimetrix chips.23 During the acute phase of CVB3 infection, OPN mRNA expression was upregulated in the hearts of C57BL/6 mice undergoing mild acute myocarditis, as well as in A.BY/SnJ mice susceptible to severe acute and chronic myocarditis. Importantly, A.BY/SnJ mice revealed approximately 4 times higher expression levels than C57BL/6 mice during the acute phase of infection (Figure 2A). To further evaluate these findings, we performed quantitative RT-PCR of CVB3-infected A.BY/SnJ and C57BL/6 mouse hearts at day 4 pi (an early time point of infection), during the acute phase of myocarditis (day 8 and day 12 pi), and at day 28 pi when chronic myocarditis is established in A.BY/SnJ mice. As shown in Figure 2B and 2C, OPN mRNA expression was induced in the heart of both mouse strains after CVB3 infection with a peak during the acute phase of myocarditis, revealing more than 100-fold higher expression in susceptible mice. Thereafter, the OPN expression declined in both mouse strains until day 28 pi. In turn, the highly enhanced OPN mRNA expression in hearts of susceptible mice compared to resistant mice was detectable throughout the investigated time schedule (note the different scaling in Figure 2B and 2C). Determination of OPN protein in sera revealed a slightly higher secretion in susceptible mice; however, the clear differences seen in the heart were not reflected by sera concentrations (data not shown), stressing the importance of analyzing the local situation in inflamed hearts.

In the myocardium, OPN mRNA expression was spatially correlated with CVB3 RNA expression by ISH, as demonstrated in consecutive transverse heart tissue sections of the left and right ventricle (Figure 3A). OPN mRNA expression was present within areas of cardiac infiltrates in A.BY/SnJ mice and C57BL/6 mice and was found to correlate well with CVB3-positive areas within consecutive myocardial tissue sections (Figure 3B, I+II and IV+V). In situ hybridization further demonstrated that at the cellular level the macrophages of A.BY/SnJ mice express more OPN mRNA than those of C57BL/6 mice, suggesting that major histocompatibility complex–independent genetic factors influence OPN

Figure 1. Visualization of OPN mRNA (A) and protein (B) expression in mononuclear inflammatory cells within cardiac lesions by in situ hybridization in an endomyocardial biopsy of a patient with chronic viral myocarditis. In patients with DCM, no OPN mRNA (C) and protein (D) expression was observed.

Figure 2. OPN expression in C57BL/6 and A.BY/SnJ mice infected with CVB3 at different time points. (A) Bar graph showing OPN mRNA expression at different time points. (B) OPN mRNA expression at different time points. (C) OPN protein expression at different time points.
expression. In addition, as shown by immunohistochemistry in Figure 3B, III and VI, in susceptible mice, considerably more OPN protein expression per area fraction of cardiac lesions was found than in resistant mice, confirming the results of OPN mRNA expression. In the course of CVB3-myocarditis, first macrophages and then T cells infiltrate the myocardium. By using a double-labeling technique with ISH and immunohistochemistry to identify OPN-expressing cell types, macrophages (Mac-3–positive) were identified as the main OPN-expressing cells in the myocardium of CVB3-infected animals (C, I and II). ISH with control RNA probes did not reveal unspecific binding to labeled macrophages (Figure 3C, III) and also double labeling using isotype controls and uninfected myocardium gave negative results (data not shown). Hence, infiltrating cardiac macrophages were identified as major OPN-secreting cells in CVB3-myocarditis.

**OPN Is Not Involved in the Resolution of Myocarditis**

Because OPN can modulate T cell–dependent immune responses by acting as a chemokine and attracting T cells to the site of infection, we included OPN−/− C57BL/6 mice in our study. As a result, the composition and number of infiltrating macrophages and T cells per square millimeter inflammatory lesion were found to be comparable in hearts of OPN−/− and OPN+/+ C57BL/6 mouse strains. In addition, OPN−/− mice were found to eliminate cardiac virus infection during acute myocarditis.
myocarditis, indicating that OPN is not required to induce an effective immune response for the resolution of CVB3 infection. This observation is supported by the finding that the effective secretion of the protective cytokines IFNγ and IL-10 in OPN−/− mice reflects the typical patterns observed in resistant mice (see the expanded text and figures in the online data supplement).

**OPN in Cardiac Remodeling Processes in Chronic CVB3-Myocarditis**

Remodeling processes of cardiac extracellular matrix in the course of chronic CVB3-myocarditis are known to be associated with the expression of MMPs and TIMPs. The interplay between these factors contributes to the development of fibrosis in the inflamed heart. MMPs and especially MMP3 are well-known targets for OPN. To evaluate the relationship of MMP, TIMP, and OPN in CVB3-myocarditis, we compared cardiac MMP3 and TIMP1 expression in susceptible A.BY/SnJ mice and in resistant C57BL/6 mice. In susceptible mice, MMP3 mRNA expression was pronounced at day 8 and day 12 pi and also TIMP1 mRNA was highly expressed at day 8 pi, as determined by quantitative RT-PCR, whereas in resistant mice, a distinct lower expression of MMP3 and TIMP1 in the course of myocarditis was noted (Figure 4). Recently, it was shown that an additional factor important for degradation of interstitial matrix in myocarditis is the uPA. We found that transcript levels of uPA were elevated during acute myocarditis (8 and 12 days pi) in susceptible mice, whereas uPA mRNA expression was only minimally increased at day 8 pi in resistant mice (Figure 4). Next, we examined TGFβ1 mRNA levels, because TGFβ1 is known to be involved in the signal transduction cascade of OPN inducing fibrosis. In A.BY/SnJ mice, TGFβ1 mRNA expression was induced after CVB3 infection with a maximum at day 8 pi, whereas in C57BL/6 mice, only basal TGFβ1 expression could be detected throughout infection (Figure 4). Altogether, OPN associated fibrosis-related molecules were higher expressed in susceptible mice than in resistant mice. The results obtained in resistant mice were confirmed in OPN−/− mice (see Figures I and II in the online data supplement).

**Consequences of Treatment With the Vitamin D Analog ZK 191784**

To investigate whether downregulation of OPN expression can reduce CVB3-induced fibrosis, we applied the vitamin D analog ZK 191784 in susceptible A.BY/SnJ mice, because OPN transcription is mediated through signaling via the vitamin D receptor. By determination of area fractions of infection by ISH in hearts, we examined the influence of ZK 191784 on viral load and found no difference between the untreated and treated group (23.925±2419 μm²/mm² in CVB3 infected A.BY/SnJ mice versus 23.025±1658 μm²/mm² in treated animals 8 days pi), indicating that ZK 191784 does not influence virus replication. From hearts of CVB3-infected, ZK 191784–treated mice (day 8 pi), we performed quantitative RT-PCR to determine OPN, MMP3, TIMP1, and uPA mRNA expression, because at this time point, maximal RNA expression of these molecules was detectable in A.BY/SnJ mice. In CVB3-infected ZK 191784–treated A.BY/SnJ mice OPN mRNA expression was significantly diminished (P<0.05) compared to infected, untreated A.BY/SnJ mice and was accompanied by reduced MMP3, TIMP1, and uPA mRNA expression (Figure 5). Uninfected A.BY/SnJ mice treated with the analog showed the same expression levels of measured molecules such as uninfected controls and were used for calculation of the relative expression levels of treated mice.

Because vitamin D receptor and G protein–coupled receptors signal via the MAPK-ERK pathway, we examined ERK expression in hearts of infected susceptible and resistant mice as well as in ZK 191784–treated A.BY/SnJ mice by immunohistochemistry. Phosphorylated ERK (pERK) expression was distinctly enhanced in hearts of susceptible mice compared to resistant mice (Figure 6A, I and III) and application of the vitamin D analog reduced pERK expression in infected hearts (Figure 6A, II), possibly contributing to the diminished OPN transcription. One consequence of diminished OPN expression was a decrease in fibrosis-related molecules, such as collagen and procollagen, in A.BY/SnJ mice treated with the vitamin D analog. These findings suggest that OPN plays a crucial role in the development of fibrosis in CVB3-myocarditis and that targeting OPN may be a potential strategy to prevent cardiac remodeling. Further studies are needed to clarify the exact mechanism by which OPN regulates fibrosis in CVB3-myocarditis and to evaluate the clinical relevance of these findings.
transcription may be less signaling via the OPN receptors integrin αvβ3 and CD44. OPN receptor signaling is conferred via the MAPK-ERK and also via the phosphorylated Akt (pAkt) pathway. To delineate these pathways in our myocarditis model, we performed immunohistochemical staining for pAkt in hearts of ZK 191784–treated and untreated A.BY/SnJ mice (Figure 6B). Cardiac pAkt expression was highest in susceptible A.BY/SnJ mice (Figure 6B, I), while resistant mice (Figure 6B, III) showed moderate expression and treated mice (Figure 6B, II) were intermediate. Control stainings with the respective irrelevant antibodies were negative (Figure 6B, IV).

**Fibrosis in CVB3-Infected Resistant, Susceptible, and ZK 191784–Treated Mice**

To study the impact of OPN expression on the development of fibrosis, we analyzed the expression of procollagen type I
The extent of fibrosis develops in susceptible mice but not in resistant mice or susceptible mice treated with a vitamin D analog. Compared to C57BL/6 or ZK 191784–treated A.BY/SnJ mice, procollagen 1α mRNA expression was enhanced in susceptible A.BY/SnJ mice at day 12 pi (A). RNA was pooled from 3 to 5 animals per mouse strain and experiments were repeated twice. A considerable replacement fibrosis was detected only in susceptible mice but not in C57BL/6 or ZK 191784–treated A.BY/SnJ mice at day 28 pi (B).

Discussion

Based on our findings of OPN expression in human hearts undergoing chronic myocarditis but not DCM, we aimed to investigate the impact of OPN for remodeling processes in murine acute and chronic CVB3-myocarditis. We found during acute disease high levels of OPN in CVB3-infected mice susceptible to chronic myocarditis, whereas in resistant C57BL/6 mice undergoing only mild acute myocarditis, OPN expression was only moderate. OPN transcription is mediated via the vitamin D receptor and G protein–coupled receptors, which signal via the MAPK-ERK pathway. In addition, it is most likely that CVB3 infection contributes to enhanced OPN transcription, as CVB3 is reported to phosphorylate ERK with higher activation levels in susceptible mice than in resistant mice.27 This notion was strengthened by our findings of reduced pERK expression in CVB3-infected susceptible mice by inhibition of vitamin D signaling.

The consequences of this high OPN expression may result in diverse implications, because OPN is involved in modulating immune responses as well as in fibrosis development.13,14,28 Concerning its role in the immune system, OPN might function as a protective chemokine, important for the attraction of macrophages and T cells to areas of infection. However, as previously reported for influenza virus or vaccinia virus infection,29 also in our model system, OPN was found not to be necessary for mounting an appropriate antiviral immune response. OPN–independent attraction of T cells and macrophages into the immunized heart was also observed in autoimmune myocarditis.30 Additionally, we found that OPN+/− mice were able to overcome cardiac infection during acute disease, thus preventing chronic myocarditis, which can be attributed very likely to an effective production of the protective cytokines IFNγ and IL-10, a finding which we already defined as resistance factors in immunocompetent C57Bl/6 mice.8 Importance of adequate cytokine secretion was also proven in herpes simplex virus type 1–infected OPN+/− mice, in which an impaired immune response was assigned to deregulated secretion of IL-12 and IL-10, as well as reduced IFNα production by plasmacytoid dendritic cells.31 In conclusion, our data suggest that OPN does not interfere with the antiviral immunity in CVB3-infected mice.

Concerning the evolvement of fibrosis associated with chronic inflammation, we investigated the contribution of OPN to cardiac remodeling processes as increased OPN expression is known to correlate with other fibrotic diseases, for example, with murine and human pulmonary fibrosis.32,33 Fibrosis following chronic myocarditis is a consequence of continuous influx of inflammatory cells triggered by persistent heart muscle infection.21 In our ISH experiments for the detection of OPN mRNA, macrophages were identified as major producers of OPN, whereas in human DCM, cardiomyocytes have been suggested as relevant OPN expressing cells.34,35 In our model system, there is a firm temporal and spatial correlation between invasion of macrophages early in enterovirus myocarditis and OPN expression. These observations are in agreement with findings in autoimmune myocarditis of rats, where OPN is expressed early after induction of inflammation, with CD11b+/− inflammatory monocytes as the main OPN-producing cell population.36,37

OPN is known to induce uPA, MMPs, and TIMPs, all molecules that have recently been shown to play a major role in cardiac injury, dysfunction, and fibrosis in viral myocarditis.10,38 Here, we demonstrate a high expression of these extracellular matrix molecules in CVB3-infected susceptible A.BY/SnJ mice during acute disease, whereas minor expression was detected in resistant C57BL/6 mice, OPN+/− mice and ZK 191784–treated A.BY/SnJ mice. Induction of these fibrosis relevant molecules was found to be dependent on the
contribution of OPN signaling via its receptor αvβ3 integrin. Integrins use multiple signaling pathways including the phosphatidylinositol 3-kinase/Akt cascade and the MEK-ERK cascade. In CVB3-infected hearts of OPN−/− mice, pAkt and also pERK expression was less pronounced in OPN−/− mice than in resistant C57BL/6 mice. For pERK activation, our results in susceptible mice point to 2 ways that might be operative for its activation: first, the enhancement via vitamin D signaling, leading to pronounced OPN expression; and second, the subsequent enhancement via OPN signaling. Because the OPN-ERK-Erk1 pathway, as well as the phosphatidylinositol 3-kinase, is well known to enhance type I collagen expression, it can be concluded that OPN contributes to the development of heart fibrosis in CVB3-infected animals.

In conclusion, OPN might represent a valuable tool to predict the evolvement of DCM following chronic cardiomyopathy, and inhibition of OPN expression by using a vitamin D analog might be a therapeutic option to reduce cardiac fibrosis and remodeling.

Acknowledgments
We thank Sandra Bundschuh, Carmen Ruoff, and Renate Junghaus for excellent technical assistance.

Sources of Funding
This work was supported by Deutsche Forschungsgemeinschaft grant SFB-TR19, Bundesministerium für Bildung und Forschung grant 01EZ0817, and the Dr Karl-Kuhn-Stiftung (to K.K.).

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Disclosures
None.

References
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Circ Res. 2009;104:851-859; originally published online February 26, 2009;
doi: 10.1161/CIRCRESAHA.109.193805
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/104/7/851

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2009/02/26/CIRCRESAHA.109.193805.DC1

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OPN deficiency in the outcome of CVB3-myocarditis

As OPN is well known to promote the release of IL-12 and to induce the development of protective cell-mediated immunity to viruses\(^1\) we aimed to investigate whether OPN influences the immune response and the consequences important for the resolution of cardiac CVB3 infection. For this purpose we infected OPN\(^{-/-}\) C57BL/6 mice with CVB3 and determined viral load (by ISH and RT-PCR), histological damage, the number of infiltrating T cells and macrophages/mm\(^2\) cardiac lesion as well as the production of IFN\(\gamma\) and IL-10. Hematoxylin/eosin staining of heart tissue sections of these mice revealed moderate influx of inflammatory cells at day 8 p.i. and day 12 p.i., (Online Figure IA, I), thereafter, no inflammatory cells were detected in the hearts (Online Figure IA, II). To quantify the extent of virus infection in the hearts, we determined the area fractions of infection by measuring the density of autoradiographic signals obtained by ISH.\(^2\) Area fractions of infection detected at day 4 [520±53\(\mu\)m\(^2\)/mm\(^2\)] and day 8 p.i. [1450±163\(\mu\)m\(^2\)/mm\(^2\)] in hearts of OPN\(^{-/-}\) mice were in the range of those recently published for C57BL/6 mice.\(^2\) Together with our finding that CVB3 RNA is only detected until the acute phase of infection by RT-PCR (Online Figure IB) and ISH (Online Figure IA, III,IV) we conclude that OPN\(^{-/-}\) mice are resistant to persistent infection and chronic myocarditis. This notion was strengthened by analyses of cytokine secretion patterns. Previously, we have shown for immunocompetent C57BL/6 mice that adequate secretion of IFN\(\gamma\) and IL-10 characterizes the resistant phenotype\(^2\) and in OPN\(^{-/-}\) mice we found IFN\(\gamma\) and IL-10 secretion patterns comparable to those of resistant C57BL/6 mice (Online Figure IC).

Moreover, the comparison of the composition of infiltrating cells in the hearts of infected OPN\(^{-/-}\) and OPN\(^{+/+}\) C57BL/6 mice revealed that OPN is not required for the adequate attraction of immune cells to the site of infection during acute disease. We observed similar numbers of Mac-3\(^+\) macrophages and CD3\(^+\) T cells per mm\(^2\) inflammatory lesion in the
knockout mice (CD3+ T cells: 2023 ± 72; Mac-3+ macrophages: 1778 ± 52) as in the immunocompetent mice (CD3+ T cells: 1944 ± 62, Mac-3+ macrophages: 1797 ± 53).

**OPN deficiency in the development of fibrosis in the course of CVB3-myocarditis**

To evaluate the impact of OPN deficiency on the development of fibrosis, we determined the mRNA expression levels of extracellular matrix proteins MMP-3, TIMP-1, and uPA by qRT-PCR. Similar to our data obtained in C57BL/6 mice, MMP-3, TIMP-1, and uPA mRNAs were only weakly expressed in the course of CVB3-myocarditis (Online Figure IIA). Also TGFß mRNA, which is involved in the signal transduction cascade of OPN inducing fibrosis, was nearly absent (Online Figure IIA). Consequently, the protein expression of the signal transduction molecules pERK and pAKT was also reduced in OPN−/− mice (Online Figure IIB) compared to A.BY/SnJ mice, susceptible for chronic myocarditis and cardiac remodeling.

We further examined the expression of procollagen type 1 alpha mRNA - an indicator for collagen synthesis - in the hearts of infected OPN−/− mice and determined the extent of fibrosis by picrosirius red staining. At day 8 p.i., mRNA of procollagen type 1 alpha was detected in the hearts of OPN−/− mice but thereafter no increase in expression was noted in the course of CVB3-myocarditis (Online Figure IIC). As a consequence of this moderate procollagen type 1 alpha expression, OPN−/− mice revealed minimal fibrotic lesions in the heart (Online Figure IIC).

**References**


Figure legends

Online Figure I: Outcome of myocarditis in CVB3-infected OPN<sup>−/−</sup> mice. Infiltrating cells in heart tissue sections are detected at day 8 (I) but not at day 28 (II) p.i. as determined by hematoxylin/eosin staining (A). CVB3 RNA is found until day 8 p.i. (A III and B), thereafter no CVB3 RNA is detected in the hearts as shown by ISH and RT-PCR (A IV and B). Spleens were collected at indicated time points and single cell suspensions were restimulated with heat-inactivated CVB3; after 4 days supernatants were collected and tested for IFN<sub>γ</sub> and IL-10 levels using ELISA-kits (C). Experiments were repeated twice with 5 mice per time point and data were presented as mean ± SEM. Values from infected hearts versus non-infected hearts differ significantly (p<0.01 for IFN<sub>γ</sub> and p<0.05 for IL-10) as determined by Kruskal-Wallis test.

Online Figure II: Diminished expression of fibrosis-inducing molecules and signal transduction molecules correlate well with minimal fibrosis in hearts of CVB3-infected OPN<sup>−/−</sup> mice. From hearts of infected OPN<sup>−/−</sup> mice RNA was isolated at indicated time points and tested for mRNA expression of MMP3, TIMP1, uPA, and TGFβ by quantitative RT-PCR (A). A comparably moderate expression of signal transducing molecules pERK and pAKT was detected by immunohistochemistry (B). Reduced mRNA expression of procollagen type 1 alpha and minimal replacement fibrosis was observed in OPN<sup>−/−</sup> mice following CVB3-infection (C). RNA was pooled from 5 animals and experiments were repeated twice with similar results.
**Online Figure I**

**Panel A**

Images showing tissue samples from OPN−/− mice. Top left: myocardial tissue. Top right: liver tissue. Bottom left: lungs. Bottom right: kidneys.

**Panel B**

Gel electrophoresis showing bands for CVB3 and PBDG RNA at days 4, 8, 12, and 28 post-infection.

**Panel C**

Bar graphs showing IFNγ and IL-10 production over time (days p.i.). IFNγ production peaks at day 12, while IL-10 production remains relatively constant.
**Online Figure II**

**OPN^-/- mice**

A

<table>
<thead>
<tr>
<th>Relative expression (2-ddCt)</th>
<th>(d4)</th>
<th>(d8)</th>
<th>(d12)</th>
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<tbody>
<tr>
<td>MMP-3 mRNA expression</td>
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<td>TIMP-1 mRNA expression</td>
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<tr>
<td>uPA mRNA expression</td>
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<td>TGFβ1 mRNA expression</td>
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B

- **pERK**
- **pAkt**

C

- **Procollagen 1alpha mRNA expression**
- **Fibrosis**