Osteopontin Expression in Cardiomyocytes Induces Dilated Cardiomyopathy

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**Background**—Inflammatory processes play a critical role in myocarditis, dilated cardiomyopathy, and heart failure. The expression of the inflammatory chemokine osteopontin (OPN) is dramatically increased in cardiomyocytes and inflammatory cells during myocarditis and heart failure in human and animals. However, its role in the development of heart diseases is not known.

**Methods and Results**—To understand whether OPN is involved in cardiomyopathies, we generated a transgenic mouse (MHC-OPN) that specifically overexpresses OPN in cardiomyocytes with cardiac-specific promoter-directed OPN expression. Young MHC-OPN mice were phenotypically indistinguishable from their control littermates, but most of them died prematurely with a half-life of 12 weeks of age. Electrocardiography revealed conduction defects. Echocardiography showed left ventricular dilation and systolic dysfunction. Histological analysis revealed cardiomyocyte loss, severe fibrosis, and inflammatory cell infiltration. Most of these inflammatory cells were activated T cells with Th1 polarization and cytotoxic activity. Autoantibodies against OPN, cardiac myosin, or troponin I, were not found in the serum of MHC-OPN mice.

**Conclusions**—These data show that OPN expression in the heart induces in vivo T-cell recruitment and activation leading to chronic myocarditis, the consequence of which is myocyte destruction and hence, dilated cardiomyopathy. Thus, OPN might therefore constitute a potential therapeutic target to limit heart failure. (Circ Heart Fail. 2010;3:431-439.)

**Key Words:** heart disease ■ heart failure ■ myocarditis ■ inflammation ■ immune system

Dilated cardiomyopathy (DCM) is a major cause of nonischemic heart failure (HF). Common causes of DCM include acute ischemia and heart rhythm-related, pericardial, valvular, peripheral vascular, congenital, and familial disorders.1 Inflammatory nature of DCM also has long been recognized triggered by acute viral or autoimmune myocarditis.2,3 The current understanding of molecular pathogenesis after viral myocarditis involved a 3-stage process explaining the progression from acute injury to chronic DCM. These steps include activation of the innate immune system, instillation of a chronic autoimmune process inducing myocyte destruction, and finally, DCM.4

Clinical Perspective on p 439

Osteopontin (OPN), also called cytokine Eta-1, is a chemokine involved in tissue remodeling and immunity.5,6 OPN is highly expressed during chronic inflammatory and autoimmune diseases, and it is thought to play a role in inflammation through the recruitment and retention of macrophages and T cells to inflamed sites.7 Moreover, OPN regulates the production of inflammatory cytokines in these cells.8 OPN is also involved in the early Th1 response as demonstrated by the severely impaired type-1 immunity in OPN-deficient mice and by interferon-γ and CD40 ligand expression in OPN-stimulated human T cells.8,9 OPN also enhances the survival of activated T cells, suggesting that it might promote the progression of autoimmune diseases.10

Several studies have suggested a role for OPN in cardiovascular diseases, including atherosclerosis and valvular stenosis.7 In the heart, OPN expression is increased during both acute and chronic diseases.11 Indeed, after myocardial infarction, OPN expression was increased in macrophages and, during cardiac hypertrophy in both humans and rodents, cardiomyocytes seem to be the major source of OPN.12 This
expression probably plays a role in fibrillogenesis.\textsuperscript{13,14} OPN expression coincides also with the transition from myocardial hypertrophy to HF, and its level of expression is correlated with the severity of the HF.\textsuperscript{11} Moreover, OPN expression was increased during viral myocarditis in mouse models.\textsuperscript{15} Findings from several experiments have therefore converged to establish a link between cardiomyopathies, including DCM and OPN expression.\textsuperscript{14} However, the direct role of OPN in the development of DCM has not yet been determined. Here, we hypothesized that OPN might be involved in the underlying mechanisms of these diseases.

We designed a conditional transgenic mouse model that specifically expresses OPN in the heart to investigate the role of OPN in cardiomyopathies. We evidenced that expression of OPN in cardiomyocytes induces myocarditis-promoting T-cell–mediated inflammation and fibrosis in the heart, leading finally to DCM and HF.

Materials and Methods

Generation of TRE-OPN Transgenic Mice

This mouse model was constructed as previously described.\textsuperscript{6} The mouse OPN cDNA (bases 9 to 955, accession number BC057858) was introduced into the multiple cloning site of the tetracycline transactivator (TTA)-responsive promoter of pBI-G plasmid (Clontech, St. Germain en Laye, France). Transgenic mouse lines (TRE-OPN) were produced by microinjection of this construct. We obtained 3 independent transgenic lines. These lines were used after 7 breeding with C57Bl/6 background.

Generation of MHC-OPN Mice

TRE-OPN mice were crossbred with cardiac α-myosin heavy chain (MHC)-tTA mice (FVB.Cg-Tg(Myh6-tTA)6Smbj/J, Jackson Laboratory) that express the transcriptional factor tTA under an MHC promoter. Crossbreeding produced the 4 following genotypes: wild type, single transgenic TRE-OPN, MHC-tTA mice, and double transgenic MHC-OPN mice. In all experiments using MHC-OPN mice, we have verified that the behavior of wild-type, MHC-tTA, and TRE-OPN littermates are not different in their level of OPN expression their survival, or show only minor differences compared with those associated with OPN expression (supplemental Figures I through III). The term “control” refers to a pool of wild type, MHC-tTA, and TRE-OPN littermates.

Mice were maintained in a conventional animal facility, on a 12-hour light/12-hour dark cycle. Food and water were available ad libitum. All procedures were carried out in compliance with the principles and guidelines established by the French National Institute of Medical Research (INSERM) and approved by the Institutional Animal Care and Use Committee. When required, doxycycline (Dox, Sigma-Aldrich, Belgium) was administered to induce OPN expression in vivo with experimental conditions and primers described in the Data Supplement.

Enterovirus Myocarditis Model

A.BY/SynJ mice were bred and kept in pathogen-free conditions at the Department of Molecular Pathology, University Hospital, Tübingen, Germany. Group B coxsackievirus infection of mice and further processing of heart and sera were performed as previously described.\textsuperscript{15}

Assessment of the Cardiac Function In Vivo

Electrocardiography

Six-lead surface ECGs were recorded with 25-gauge subcutaneous electrodes on a computer through an analog-digital converter (IOX 1.585, EMKA Technologies, Paris, France) for monitoring and later analysis (ECG Auto 1.5.7, EMKA Technologies). ECG channels were filtered between 0.5 and 250 Hz. Mice were anesthetized with an IP injection of etomidate (30 mg/kg; Janssen-Cilag, Berchem, Belgium). Body temperature was maintained at 37°C using a servo-controlled heating pad (Harvard Apparatus). The criteria used for measuring RR, PQ, QRS, and QT intervals as well as P-wave duration can be found elsewhere.\textsuperscript{16} The QT interval was corrected for heart rate using the formula, QTC = QT/(RR/100)\textsuperscript{12} established for mice; values of QT and RR are expressed in ms.\textsuperscript{17}

Transcoracic Echocardiography

Control (n=15) and MHC-OPN (n=5) mice were lightly anesthetized with 1.5% isoflurane. Echography was performed with a 15-MHz transducer (MI2L General Electric). The percentage of left ventricular (LV) fractional shortening (%FS) was defined as %FS = [(LVDd − LVDs)/LVDd]×100, where LVDd is the LV end-diastolic diameter, and LVDs is the LV end-systolic diameter.

Histology and Immunohistology

For histological analysis, tissue specimens were fixed with 3% paraformaldehyde in PBS. Sections were stained with Masson trichrome or Sirius red and observed with a white light microscope. Myocyte and fibrosis densities were quantified (Sigma Scan software) on digitized images of Sirius red–stained sections.

For immunodetection, paraffin sections (8 μm thick) were incubated for 2 hours with antibodies directed against OPN (Sigma Aldrich), collagen I (Novotec, Saint Martin La Garenne, France), CD31 and CD45 (Pharmingen, San Diego, Calif), CD3 (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif), CD4, CD8, tumor necrosis factor-α, and interleukin (IL)-17 (Biologend, San Diego, Calif). Bound biotin-conjugated secondary antibodies (Sigma Aldrich) were visualized using streptavidin peroxidase-diaminobenzidine (Vector, Peterborough, UK) or streptavidin Alexa Fluor 488/568 (Invitrogen, Paisley, UK). OPN area was measured on digitized images with Sigma Scan software.

Western Blot and ELISA

Autoantibodies against OPN, cardiac myosin, and troponin I in mouse serum were detected by Western blot using protein-blotted membrane, diluted mouse serum (1%), and biotinylated anti-mouse IgG. Cardiac myosin was prepared by high ionic solution extraction and water precipitation as previously described.\textsuperscript{18} Antitroponin I antibody and troponin I protein were provided by Dr Olivier Cazorla (Inserm U637, Montpellier, France) and Dr Gabriele Pfitzer (Institute of Vegetative Physiologuy, University of Cologne, Köln, Germany),\textsuperscript{19} respectively. Mice of OPN and OPN ELISA kit was purchased from R&D System (Abingdon, UK).

Apopotosis Assay

TUNEL assays were performed according to the manufacturer’s recommendations (Roche Diagnost, Meylan, France) using the in situ Cell Death Detection Kit.

Real-Time Quantitative Polymerase Chain Reaction (qPCR) Gene Expression Analysis

Quantitative PCR (qPCR) was performed by the Molecular Biology Platform of Angers University (available at: http://www.pfbiotech-angours.com/). OPN qPCR was performed using primers described in the Data Supplement.

Statistical Analysis

For survival study, a survival plot was used and log-rank test was performed for statistical analysis. Censored variable was survival status at the time of analysis. Statistical analysis of ECG parameters was performed using either 1-way ANOVA or 2-way ANOVA tests for repeated measures, both followed up by a Tukey test when appropriate. Other statistical analyses were performed using either ANOVA followed by a Student t test when only 2 groups have to be compared or Kruskal-Wallis 1-way ANOVA followed by Mann-Whitney U test. A P value <0.05 indicated significant difference between 2 groups. Results are expressed as mean values ± SEM. Data were analyzed with NCSS statistical software.
Results

Experimental Model

OPN is expressed at a very low level in the normal heart of wild-type mice. To specifically express the OPN gene in cardiomyocytes, we used double transgenic MHC-OPN mice obtained by crossing MHC-tTA mice with TRE-OPN mice that carry the mouse OPN gene under the control of the tTA-responsive promoter (Figure 1A). Unlike in the heart of control mice, OPN mRNA and protein were strongly expressed in the heart of MHC-OPN mice (Figure 1B and 1C; supplemental Figures I and II). However, the level of OPN mRNA and protein in the hearts of MHC-OPN mice remained in the same range than that observed in the hearts of mice during the acute phase of myocarditis induced by coxsackievirus infection15 (supplemental Figure II). Driven by MHC promoter, OPN was indeed mainly detected in cardiomyocytes where its distribution appeared patchy (Figure 1C and 1D). This expression did not change according to the age of the mouse from the 1st to the 16th week (supplemental Figure I). In blood of MHC-OPN mice, OPN concentration remained low, whereas it increased coxsackievirus infected mice undergoing myocarditis (Figure 1E).

OPN Expression in the Heart Induces Premature Death

During the first week of life, the phenotype (weight, heart/body weight ratio, and behavior) of MHC-OPN mice was indistinguishable from that of control mice. From then, most of the MHC-OPN mice died prematurely. Therefore, we monitored the survival rate of MHC-OPN mice and their control littermates, on a weekly basis for a period of 30 weeks (Figure 2). The lifespan curve showed that a huge increase in
the mortality of the MHC-OPN mice compared with control mice occurred between the 8th and 15th week after birth, with a half-life of 12 weeks, and no difference was observed between genders (supplemental Figure IIIB). To confirm the effect of OPN expression, we treated mother mice with Dox during both pregnancy and suckling and the offspring until the end of the experiment. In these conditions, OPN was not expressed in MHC-OPN embryo, pup, and adult mice, and the lifespan of these mice was identical to that of control mice up to 30 weeks of age (supplemental Table II).

OPN Expression in the Heart Alters ECG Parameters

A follow-up study of ECG parameters was performed in MHC-OPN and control mice from 9th to 26th week after birth. Figure 3A shows representative lead-1 ECGs. At 11 weeks, the MHC-OPN mouse exhibited a markedly prolonged PQ interval and a moderately prolonged QRS interval. Repolarization was also abnormal with disappearance of the early positive phase of the T wave commonly observed in control mice. However, the QT interval was not prolonged. At 15 weeks, this mouse that died during the week after this ECG recording, showed complete atrioventricular block, with complete dissociation between the sinus rhythm and the idioventricular escape rhythm. Also, note the markedly prolonged T-wave duration at this age. Figure 3A. The mean T-wave duration of ECG intervals measured during the last ECG recording (respectively at 9, 11, 11, 14, and 15 weeks) before death in MHC-OPN mice (black bars; n=5), in 15-week-old control mice (white bars; n=23), and in 15-week-old MHC-OPN mice, which survived until the end of the study (gray bars; n=3), is shown. Figure 3B. C, Evolution of PQ, QRS, QT, and QTc intervals as a function of age in control mice (white circles, n=23), in the MHC-OPN mice that survived until the end of the study (gray circles, n=3), and in the MHC-OPN mice that died during the study (black circles, n=5 at the beginning of the study; number of mice later on is given within parentheses). Statistical analysis of ECG parameters was performed using either 1-way ANOVA or 2-way ANOVA tests for repeated measures, both followed up by a Tukey test when appropriate.

OPN Expression in the Heart Induces DCM

We evaluated the morphological and functional cardiac parameters of MHC-OPN mice by transthoracic echocardiography. Echocardiographic parameters of 6-week-old mice were identical to that of control mice (supplemental Figure V). However, at 11 weeks, the heart LV of MHC-OPN mice was severely dilated compared with that of control mice (Figure 4A through 4C). Moreover, during systole, the LV free wall of MHC-OPN mice was significantly thinner than in control hearts (Figure 4C), which suggests the development of a hypokinetic DCM. These structural defects led to systolic dysfunction, observed as reduced LV fractional shortening (MHC-OPN mice: 13.7±4.0%, n=5; control mice: 29.8±2.0%, n=13; P<0.05). In vivo imaging was confirmed by a direct observation of cardiac dilation in MHC-OPN mouse that died during anesthesia (Figure 5A and 5D).

OPN Expression in the Heart Induces Fibrosis and Myocyte Destruction

We then performed a histological analysis. At 6 weeks, the morphology of the hearts of MHC-OPN mice did not appear to be different from that of control mice (supplemental Figure IV).
After 11 weeks, the cellular organization in MHC-OPN mouse hearts was altered when compared with control hearts with evidence of myocyte loss (Figure 5B, 5E, and 5H), fibrosis (Figure 5C, 5F, and 5I) and interstitial cell infiltration (Figure 5B, inset) demonstrating that the disease developed very rapidly between the 6th and 11th week after birth. Moreover, although no difference in the number of apoptotic cells in the heart of control and 6-week-old MHC-OPN mice was observed, the number of apoptotic cells was increased at 11 weeks in the MHC-OPN mouse heart (Figure 5G).

OPN Expression Induced Heart Inflammation

The hearts of MHC-OPN mice were infiltrated by a large number of interstitial cells. Approximately 50% of infiltrating cells expressed the panleukocyte CD45 marker (Figure 6A). In MHC-OPN mice under 6-week-old, the number of CD45<sup>+ </sup>infiltrated cells in the heart was very low and was not different from that observed in control mice (Figure 6B). In early heart lesions, focal infiltration spots were observed first in the endomycocardium and the epicardium (not shown). Ten weeks after birth, CD45<sup>+</sup> cells were widespread in all parts of the myocardium with particular spots at the base of the mitral valve pillars in areas that were also associated with myocyte destruction (Figure 6C). As a consequence of the myocyte destruction, a strong fibroblast infiltration occurred characterized by vimentin expression (Figure 6A), explaining the extensive fibrosis.

Among infiltrated leukocytes (CD45<sup>+</sup>), only a very few, not different than in the control hearts, were neutrophil granulocytes, as characterized by myeloperoxidase activity (Figure 6D and 6J), or B lymphocytes (CD19<sup>+</sup>) (Figure 6J). More unexpectedly, macrophages (Mac-3<sup>+</sup>) were rarely observed only in rare small foci (Figure 6E and 6J). Most of the leukocytes were CD3<sup>+</sup> cells, thus demonstrating T-cell infiltration (Figure 6F and 6J). In this population, we identified helper T cells (CD4<sup>+</sup>) (Figure 6G) and cytotoxic T cells (CD8<sup>+</sup>) (Figure 6H) but no IL-17<sup>+</sup> T cells (data not shown). In the hearts from control mice, only a very few T cells (CD3<sup>+</sup>, CD4<sup>+</sup>, or CD8<sup>+</sup>) were detected regardless of age (data not shown). We also observed particularly strong CD44 expression in these inflammatory cells suggesting that they were in an activated stage (Figure 6I).
Expression of a set of Th1, Th2, and Th17 cytokines in the heart was assessed by qPCR (Figure 7). The strong induction of the IL-12 p40 subunit and interferon-γ and the absence of induction of IL-4, IL-10, IL-13, and IL-17 suggested that T cells were Th1-polarized. Moreover, the expression of perforin and granzyme confirmed the existence of a cytotoxic response as suggested by the presence of large numbers of cytotoxic CD8+ T cells (Figure 6H). Furthermore, expression of fractalkine, a molecule able to capture cytotoxic lymphocytes from blood flow and to promote their emigration, is also strongly increased (Figure 7).

These data demonstrate for the first time in vivo that OPN expressed by cardiomyocytes triggers directly or indirectly T cells’ recruitment and activation and retains them in the heart. To verify whether OPN is just a trigger, we first blocked OPN expression in cardiomyocytes by administering Dox to 5-week-old MHC-OPN mice before the onset of T-cell infiltration. The survival curve of these mice during a 30-week period after birth was comparable with that of control mice, and their hearts were morphologically and histologically identical to control hearts (supplemental Table II).

In a second set of experiments, we blocked OPN expression in 11-week-old MHC-OPN mice when DCM was evidenced (LV diameter >0.41 mm) by echocardiography (Figure 8A). The survival rate of this population during the next 30 weeks was identical to that of control mice (supplemental Table II). Moreover, in Dox treated mice, OPN expression (Figure 8B, 8E, and 8H) and inflammation (Figure 8C, 8F, and 8I) were strongly reduced when compared with 11-week-old MHC-OPN mice. However, fibrosis (Figure 8D, 8G, and 8J) was still significantly increased demonstrating that these mice had experienced previous heart injury. The results of these experiments suggest that transient stimulation of OPN expression is not sufficient to induce or to sustain myocarditis and that chronic OPN expression in adult animals...
Figure 8. Chronic overexpression of OPN is required to induce DCM. A, MHC-OPN mice were bred in experimental conditions in which cardiac recombinant OPN was expressed (-Dox) up to 11 weeks after birth. The existence of DCM was investigated by echocardiography. Six mice with a diastolic LV diameter superior to 0.41 mm were selected. A subgroup of 3 mice was immediately euthanized (E through G). A second subgroup (n = 3) was given Dox in drinking water to inhibit recombinant OPN expression until the 41st week and then euthanized (H through J). Immunohistological analysis of heart sections with an anti-OPN antibody (B, E, and H) and an anti-CD45 antibody (C, F, and I) demonstrated that blocking OPN reverses the inflammatory process. However, fibrosis remained present (D, G, and J). Quantification was carried out after image digitization on 10 sections from each group (n = 3). C indicates control mice; 11, 11-week-old MHC-OPN mice; 41, 41-week-old MHC-OPN mice. Statistical analyses performed were nonparametric Kruskal Wallis followed up by Mann-Whitney U test. *P < 0.05.

is required to sustain the inflammatory process of myocarditis that leads to DCM.

**Chronic OPN Expression in Cardiomyocytes Did Not Induce a Humoral Autoimmune Response**

Experimental autoimmune myocarditis is characterized by the presence of autoantibodies directed against heart proteins including cardiac myosin and troponin I. Different studies have indicated that OPN promotes disease progression in autoimmune models including experimental autoimmune encephalomyelitis. Thus, we investigated whether OPN expression in cardiomyocytes induced autoimmune myocarditis. In the injured hearts from MHC-OPN mice, no presence of immune complexes was revealed using anti-mouse immunoglobulin (not shown). Moreover, we demonstrated that the serum of MHC-OPN mice did not contain antibodies directed against cardiac myosin, troponin I, or OPN proteins (supplemental Figure VI). Altogether, these experiments suggest that OPN-mediated chronic inflammation does not induce a humoral autoimmune response.

**Discussion**

OPN expression has been found to be increased in the heart during cardiac diseases that lead to HF, and its circulating concentration is now under consideration for use as a biomarker for the prognosis of HF. Studies with OPN−/− mice have underlined the potential role of OPN in heart disease. However, its role not only as a marker but as an actor triggering disease has not been fully established. To elucidate the role of heart-derived OPN, we generated MHC-OPN mice that express OPN conditionally in cardiomyocytes. In these mice, a strong increase in myocardial OPN mRNA and protein expression was observed. Nevertheless, this level remained relevant to physiopathology because it was close to that observed in the heart during coxsackievirus-induced myocarditis, one of the best-characterized myocarditis model. Interestingly, MHC-OPN mice died prematurely 10 to 15 weeks after birth. Clinical and histological diagnosis revealed that from the 6th week after birth, they developed spontaneous myocarditis followed by DCM with electrocardiographic abnormalities and heart block. Heart block that is found in some idiopathic myocarditis in humans is not a usual feature in mouse severe myocarditis models with DCM, stressing the interest of this new mouse model.

Inflammation has been recognized as a critical pathological component of a number of cardiac diseases. DCM, one of the most common heart diseases, is mainly the consequence of chronic myocarditis. In MHC-OPN mice, a chronic inflammation process characterized by CD4+ and CD8+ T-cell infiltration was observed. Both CD4+ and CD8+ T cells contribute to myocarditis: CD4+ help T cells are required to initiate the pathological process, whereas cytotoxic CD8+ T cells are directly involved in cardiomyocyte damage. The role of OPN in T-cell recruitment was clearly demonstrated in vitro. Indeed OPN induces T-cell chemotaxis, supports T-cell adhesion, and enhances their survival. In vivo, only a correlation between OPN expression with T-cell infiltration was shown. Our data clearly demonstrates that in vivo, local OPN expression induces T-cell recruitment at the site of OPN expression. This OPN effect could be direct through its chemotactic activity toward T cells or indirect through its ability to induce T-cell chemotactic factors such as fractalkine because OPN concentration was not increased in sera. This OPN-mediated T-cell recruitment in the heart and Th-1 polarization characterized by high expression of IL-12 and interferon-γ and low expression of IL-4, IL-10, IL-13, and IL-17 lead to myocarditis. Moreover, OPN induces the expression of the cytotoxic proteins perforin and granzyme, which are known to be involved in T-cell–mediated cardiomyocyte destruction and consequently DCM.

Myocarditis is described as a progressive disease with 3 distinct chronologically successive stages including an initial phase of myocardial injury and innate immune response, a second phase of autoimmune response, and finally, a third phase associated with DCM. OPN expression is increased in several autoimmune diseases including systemic lupus ery-
thromatosus, rheumatoid arthritis, and inflammatory bowel disease and appears as a critical factor in the development of experimental autoimmune encephalomyelitis.10,23 However, conflicting results have been published regarding the role of OPN in experimental autoimmune myocarditis. Indeed, OPN expression is dramatically increased,26 but impairment of this expression does not protect these mice from experimental autoimmune myocarditis.27 Our results show that chronic overexpression of OPN in the heart is not sufficient to trigger a humoral autoimmune response against this protein or against cardiac myosin or troponin I. These data strongly suggest that OPN does not trigger a humoral autoimmune disease but stimulates an immunoinflammatory cellular process. This conclusion is also supported by the different patterns of inflammatory cells found in experimental autoimmune myocarditis, in which macrophages represent the largest fraction of leukocytes,3 and in OPN-mediated myocarditis, in which only T cells are observed.

The level of circulating OPN is recognized as a marker for the shift from myocarditis to HF in experimental models and in humans.24 Because OPN is not increased in the sera of MHC-OPN mice, our work stressed the importance of local OPN in this disease that triggers inflammation by inducing T-cell recruitment, stimulating Th-1 differentiation and inducing production of cytotoxic compounds, the consequence of which is cardiomyocyte destruction. Moreover, the mouse rescue when recombinant OPN expression was inhibited by Dox at 6 or 11 weeks of age suggested that OPN is not only rescue when recombinant OPN expression was inhibited by Dox at 6 or 11 weeks of age suggested that OPN is not only

**Acknowledgments**

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**Disclosures**

None.

**References**


CLINICAL PERSPECTIVE

Osteopontin (OPN) is a chemokine expressed in chronic inflammatory and autoimmune diseases involved in immune cell recruitment and type 1 cytokine expression. It is also involved in tissue remodeling. Heart remodeling in response to chronic changes in hemodynamic load is a key process in the development of heart failure. It is mostly characterized by an increase in heart chambers’ volume and myocardial mass whose evaluation by various imaging techniques is of first importance for the management of patients and estimation of the progression of the disease with time and of prognosis. It is also a central therapeutic target. Changes in extracellular matrix structure and composition in the remodeling process are now well established. Its components include matricellular proteins like OPN with regulatory functions, whose expression is almost absent after birth but can be activated after tissue injury and participate to tissue remodeling. For these reasons, circulating concentration of OPN is now under consideration for use as a biomarker for the prognosis of heart failure. In this study, we suggest a potent causative/triggering role for OPN in the development of dilated cardiomyopathy. It shows that heart-derived OPN may trigger heart failure and induce a hypokinetic myocardopathy associating conduction defects and early signs of myocarditis preceding left ventricular dilation and death. Therefore, this study suggests that OPN may be used as a diagnostic tool and a potential therapeutic target to limit left ventricular remodeling in chronic myocarditis. Inhibition of OPN expression by vitamin D analogs could be tested in this context.
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Osteopontin expression in cardiomyocytes induces dilated cardiomyopathy

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Supplemental Methods

Mouse genotyping and qPCR
Transgenic mice carrying TRE-OPN and MHC-tTA DNA were identified by PCR using 5’-TCACTCCAATCGCTCCCTACA-3’ and 5’-GATGAGTTGAGCACAAACCAC-3’ primers and 5’-GCTGCTTAATTGAGGTCGG-3’ and 5’-CTCTGCACCTTGGTGATC-3’ primers respectively. PCR conditions were: 1 cycle of 3 min at 94°C, 35 cycles of 30 s at 60°C, 30 s at 72°C and 30 s at 94°C, and 1 cycle of 10 min at 72°C. For qPCR, total RNA was purified by phenol extraction (Tri-Reagent, MRC, Cincinnati, OH) according to the manufacturer’s protocol. OPN qPCR was performed using 5’-TCCTATAGCCACATGGCTGG-3’ and 5’-CAGAATCCTCGCTCTCTGCA-3’ OPN primers and 5’-GGAGGAGCTGGAAGCAGCC-3’ and 5’-GCTGTGCTACGTCGCCCTG-3’ β-actin primers. A CT value was obtained for OPN amplification for each sample. OPN data were adjusted to β-actin.

Electrocardiography
Small but significant differences were observed between single transgenic MHC-tTA mice (n=11) and WT (n=12) and TRE-OPN (n=12) concerning the PQ interval (average value of 40.3 ms in MHC-tTA versus 37.1 ms in WT and TRE-OPN mice over the whole study; p<0.001) and the QRS interval (average value of 11.6 ms in MHC-tTA versus 11.0 ms in WT and TRE-OPN mice over the whole study; p<0.05). Other ECG parameters were not modified. This confirms previous data suggesting that overexpression of tTA modifies the mouse cardiac phenotype¹. However, these effects were minor compared to those associated with OPN expression.

Supplemental reference
**Supplemental Tables**

**Table S1: Genotypes of offspring from a breeding scheme using the parental strains TRE-OPN and MHC-tTA.**

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Number (%) of mice of indicated sex</th>
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<td>Yes</td>
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<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Control</td>
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<td>No</td>
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<td>MHC-OPN</td>
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<tr>
<td>Control</td>
<td>15</td>
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<td>Yes at 5 weeks</td>
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<tr>
<td>MHC-OPN</td>
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<td>No</td>
<td>Yes at 5 weeks</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>No</td>
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OPN protein expression is detected with an anti-OPN antibody and labeled by immunoperoxydase complex. (A) OPN is not expressed in the heart of WT, MHC-tTA and TRE-OPN mice but only in MHC-OPN mice. Black bar represent 100 µm. (B) OPN expression is stable according to the age of the mice from the 1st to 16th week after birth. Cont 2W and Cont 12 W indicate control experiments omitting solely anti-OPN antibody. White bar represent 50 µm.
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B) OPN protein expression was assessed by western blot. Pieces of mice heart (MHC-OPN mice n=3; A.BY/SnJ infected mice n=3) were crushed using a Qiagen tissue lyser in RIPA buffer including antiprotease cocktail. OPN was detected using a goat anti-OPN antibody (Sigma Aldrich, Saint Quentin Fallavier, France) and an IRDye 800 coupled anti-goat IgG1 antibody (ScienceTec, Les Ulis, France). TroponinI (tnI) was detected with an anti tnI antibody (kind gift of O. Cazorla, Inserm U637, Montpellier, France) and and an IRDye 700 coupled anti-mouse IgG1 antibody (ScienceTec, Les Ulis, France).

C) Fluorescent labeling was detected and quantified using Odyssey infrared imaging system (Li-Cor, Lincoln, Nebraska). OPN expression in the different mice was adjusted to tnI expression. Since basal OPN expression in C57Bl/6 and A.BY/SnJ control mice are equivalent, data were expressed relative to the mean of control expression. The level of OPN protein in the heart of MHC-OPN mice was of the same range than in the heart undergoing acute coxsakievirus-induced myocarditis. A 30 fold OPN level increase was observed in the heart of both myocarditis model.

MHC-OPN mice (MHC-OPN Bl/6), control mice (Cont Bl/6), A.BY/SnJ infected mice (infected A.B), control non infected A.BY/SnJ mice (Cont A.B).
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Offspring from TRE-OPN x MHC-tTA mice were followed during 30 weeks. (A) There was no difference in the survival of control mice of the different genotypes (TRE-OPN, MHC-tTA and WT) or (B) in the lifespan according to the gender of MHC-OPN mice.

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Hearts from 11-week-old MHC-OPN and control mice were compared. Masson's trichrome staining shows myocyte loss and cell infiltration in the MHC-OPN heart. Sirius red staining shows increased fibrosis in the MHC-OPN heart. There was no difference in the cellular content of WT, MHC-tTA and TRE-OPN hearts in which no myocyte loss, cell infiltration or fibrosis were observed. Bars represent 50 µm.
Figure S5: Echocardiographic phenotyping of 6-week-old MHC-OPN mice.
Echocardiographic measurements demonstrated that the morphology of hearts of 6-week-old MHC-OPN mice and control mice are identical. Mean ± SEM, n = 5 per group.

Figure S6: Evaluation of humoral autoimmune responses in MHC-OPN mice.
The presence of autoantibodies against OPN, cardiac myosin (C-myosin) and troponin I (tnI) in the serum of control (n=3), cardiac myosin immunized (n=2) and MHC-OPN mice (n=4) was investigated by western blot.
A) Purified OPN, cardiac myosin (Myo) proteins were loaded on the gel, electrophoresized, transferred onto a blotting membrane. Membranes were incubated with the mouse serum samples (1/100 dilution) of OPN-MHC, control or C-myosin-immunized mice.
B) Purified tnI (kind gift of G Pfitzer, Institute of Vegetative Physiology, University of Cologne, Köln, Germany) was loaded on gel and membranes were incubated with the mouse serum (1/100 dilution) of OPN-MHC or control mice. TnI protein loading was assessed by incubating the previous membranes 1 and 2 with anti-tnI antibody.
Western blot images show that no autoantibodies against C-myosin, tnI and OPN are present in the serum of MHC-OPN mice.
Osteopontin expression in cardiomyocytes induces dilated cardiomyopathy

Marie-Ange Renault$^1$ PhD, Fanny Robbesyn$^1$ PhD, Patricia Réant$^{1,2}$ MD, Victorine Douin$^{3,4}$ PhD, Danièle Daret$^{1,2}$ PhD, Cécile Allières$^{1,2}$ BSc, Isabelle Belloc$^{1,2}$ BSc, Thierry Couffinhal$^{1,2}$ MD PhD, Jean-François Arnal$^{3,4}$ MD PhD, Karin Klingel$^5$ MD, Claude Desgranges$^{1,2}$ PhD, Pierre Dos Santos$^{1,2}$ MD PhD, Flavien Charpentier$^{6,7}$ PhD, Alain-Pierre Gadeau$^{1,2}$ PhD

1) INSERM, U828, Pessac, France; 2) University of Bordeaux Victor Ségalen, IFR4, Bordeaux, France; 3) INSERM, U858, Toulouse, France; 4) University of Toulouse Paul Sabatier, Toulouse, France; 5) Department of molecular pathology, Institute for pathology, University Hospital, Tübingen, Germany; 6) INSERM, U915, Institut du Thorax, Nantes, France; 7) University of Nantes and CNRS, ERL3147, Nantes, France.

Supplemental Methods

Mouse genotyping and qPCR
Transgenic mice carrying TRE-OPN and MHC-tTA DNA were identified by PCR using 5'-TCACTCCAATCGTCCCTACA-3' and 5'-GATGAGTTTGGACAAACCAC-3' primers and 5'-GCTGCTTAATGAGGTCGG-3' and 5'-CTCTGCACCTTGGTGATC-3' primers respectively. PCR conditions were: 1 cycle of 3 min at 94°C, 35 cycles of 30 s at 60°C, 30 s at 72°C and 30 s at 94°C, and 1 cycle of 10 min at 72°C.

For qPCR, total RNA was purified by phenol extraction (Tri-Reagent, MRC, Cincinnati, OH) according to the manufacturer’s protocol. OPN qPCR was performed using 5'-TCCTATAGCCACATGGCTGG-3' and 5'-CAGAATCCTCGCTCTGCA-3' OPN primers and 5'-GGAGGAGCTGGAAGCAGCC-3' and 5'-GCTGTGCTACGTCGCCCTG-3' β-actin primers. A CT value was obtained for OPN amplification for each sample. OPN data were adjusted to β-actin.

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Small but significant differences were observed between single transgenic MHC-tTA mice (n=11) and WT (n=12) and TRE-OPN (n=12) concerning the PQ interval (average value of 40.3 ms in MHC-tTA versus 37.1 ms in WT and TRE-OPN mice over the whole study; p<0.001) and the QRS interval (average value of 11.6 ms in MHC-tTA versus 11.0 ms in WT and TRE-OPN mice over the whole study; p<0.05). Other ECG parameters were not modified. This confirms previous data suggesting that overexpression of tTA modifies the mouse cardiac phenotype$^1$. However, these effects were minor compared to those associated with OPN expression.

Supplemental reference
## Supplemental Tables

### Table S1: Genotypes of offspring from a breeding scheme using the parental strains TRE-OPN and MHC-tTA.

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**B**

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