Oncostatin M Induces FGF23 Expression in Cardiomyocytes

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Abstract

Background: It is well-known that elevated levels of Fibroblast Growth Factor-23 (FGF23), a bone derived hormone, in circulation are associated with renal failure. Recent studies emphasize the correlation between Heart Failure (HF) and FGF23, but the ability of cardiomyocytes themselves to express and secrete this phosphatonin is yet unknown. A further factor involved in HF is the cytokine oncostatin M (OSM). The aims of our study were: 1) to analyze the myocardium of HF patients in terms of FGF23 expression in cardiomyocytes and 2) to assess whether OSM is able to induce FGF23 production in cardiomyocytes.

Methods: Cultures of adult cardiomyocytes were treated with OSM and screened for the expression of FGF23 transcripts. FGF23 secretion was determined by Western blot and ELISA of cell culture supernatants. Heart explants of HF patients with Dilated Cardiomyopathy (DCM), Ischemic Cardiomyopathy (ICM) and myocarditis (Myo) were analyzed by immunofluorescence using FGF23 antibodies and compared with healthy controls. FGF23 levels were also determined in mice with a cardiac restricted overexpression of Monocyte Chemotactic Protein-1 (MCP1), which developed an "inflammatory" Heart Failure (iHF) due to macrophage infiltration.

Results: OSM massively induced the expression and secretion of FGF23 in cultured adult cardiomyocytes. Confocal microscopy revealed high amounts of FGF23 positive cardiomyocytes in the myocardium of patients with ischemic heart disease (IHD), myocarditis, dilated cardiomyopathy (DCM) and in mice with iHF.

Conclusions: The presence of FGF23 in the myocardium of patients with different types of HF and in mice with "inflammatory" HF suggests that macrophages are responsible for the FGF23 expression in cardiomyocytes via OSM. Whether FGF23 acts as a regeneration promoting factor and/or potentially serves as a HF/transplantation marker has to be clarified.

Introduction

About 2% of the population in western countries are affected by Heart Failure (HF), which is defined as the inability of the heart to adequately perfuse the organs with blood and is manifested as the final stage of various cardiac diseases including ischemic heart disease, hypertensive heart disease, valvular heart disease and dilated cardiomyopathy [1]. Treatment of HF is mostly symptomatic and heart transplantation is the only curative therapy available. The great lack of donor hearts restricts transplantation to few patients and the prevention of organ rejection necessitates the lifetime taking of drugs combined with the routine cardiologic follow-up examination [2].

Over the last decade's hemodynamic overload and humoral factors regarded as major causes of HF were under intense investigation. However, in recent years the development in gene and protein technology enabled a more comprehensive analysis of potential regulators/mediators such as cytokines and growth factors in the pathogenesis of HF. Thus, as an alternative to the hemodynamic overload model explaining heart failure as a vicious circle of constantly increasing hemodynamic load [3], the "cytokine model" has evolved. This alternative attributes the development of HF to a chronic and deleterious activation of cytokines after an initial insult [4]. Further evidence supporting the cytokine hypothesis is derived from various transgenic and knockout animal models. They convincingly demonstrate that the over expression or mutation of a single cytokine/chemokine/growth factor, a corresponding receptor or intracellular signalling molecule is sufficient to evoke HF [5-7]. However, these signalling cascades are not detrimental *per se* but constitute an evolutionary conserved defence mechanism [6,8,9]. Since the initiated "cell survival program" might turn into a "cell death program" by chronic activation, the blockade of cytokine/growth factor controlling signalling cascades becomes a promising therapeutic strategy. An additional important clinical aspect of cytokine based therapies is the function of cytokines as potential biomarkers. Ideally, a diagnostic marker should be a therapeutic target as well as an indicator of the disease status. Unfortunately presently known cardiac biomarkers such as natriuretic peptides do not sufficiently monitor the cardiac status prior to cardiac damage and might even be unspecific under certain circumstances [10]. As a result increasing numbers of proteome studies were performed worldwide in order to enlarge the arsenal of diagnostic tools [10]. Recent evidence suggests that fibroblast growth factor-23 (FGF23) is a new upcoming biomarker.

Originally identified as an endocrine hormone secreted by

osteocytes, FGF23 affects the mineral metabolism [11]. It decreases phosphate reabsorption in the kidney through downregulation of sodium-phosphate co-transporters, regulates the level of circulating vitamin D and inhibits the secretion of parathyroid hormone. Interestingly, FGF23 is also strongly linked to renal failure. Impairment in renal function leads to a decreased phosphate excretion which is believed to be compensated through increases in circulating FGF23 by inducing phosphate excretion and negative regulation of vitamin D synthesis. Recent studies demonstrated the association of FGF23 with left ventricular dysfunction [12], atrial fibrillation [12], left ventricular hypertrophy [13] and it might serve as a prognostic marker for all-cause mortality and surviving in stable systolic HF [14,15]. A correlation between Chronic Kidney Disease (CKD) and HF is well recognized. The worsening of renal function in heart failure patients is associated with poor prognosis and correlates with an increased mortality [16]. Furthermore, left ventricular hypertrophy commonly develops in patients with kidney disease and progresses with impairing kidney function [17].

Here, the question arises whether cardiomyocytes are able to express FGF23 under pathological conditions and, if so, which protein initiates this expression. To answer this question we analysed a number of growth factors and cytokines for their potential to induce FGF23 expression in cultured adult cardiomyocytes. Furthermore we screened myocardial tissues of HF patients with myocarditis, ischemic and dilated cardiomyopathy relative to the presence of FGF23.

Material and Method

Tissue samples

Human hearts were obtained from patients during the transplantation surgery. Patients suffering from final stage heart failure (ejection fraction ≤23%) due to DCM (18 patients), ICM (7 patients) and myocarditis (9 patients) were examined [18]. Samples from donor hearts with normal left ventricular function which could not be used for transplantation served as controls. The study was approved by the institutional ethical committee following standard procedure. The creation of monocyte chemotactic protein-1 (MCP-1) transgenic animals has been described elsewhere [5].

Western Blots, ELISA and Fluorescence Microscopy

Samples were processed for Western Blot analysis as previously described [6]. Immunoreactive proteins were analyzed with the QuantityOne software (BioRad) after visualization on the VersaDoc system (BioRad). Tissue samples were processed for immunocytochemistry and immunofluorescence analysis as reported previously [19]. Anti-FGF23 antibodies were obtained from R&D Systems, Inc (614 McKinley Place NE, Minneapolis, MN, 55413, United States).

RT-PCR of tissue samples was performed by standard methods utilizing forward (TCACCTTTTGGCCCCATCTC) and reverse (GCCTGACCATGTGGTTGTCT) primers for human FGF23 (NM_020638.2).

Cardiomyocyte culture

Ventricular adult cardiomyocytes from three-month-old Sprague Dawley rats were isolated and plated at a density of 1.5×10^4 cells/cm^2 on laminin-coated (10 µg/ml; Sigma) six well dishes (Nunc) as previously described [20]. Next day cultures were treated with albumin or 20ng/ml of oncostatin M (R&D systems). Culture media with 2% fetal calf serum were exchanged every other day for one week. From day 7 to day 12 cultures were kept in serum-free medium. Supernatants from days 7 to 12 were collected concentrated using centricons (Millipore) and processed for Western blot analysis by standard methods. In addition, FGF23 in cell supernatants was determined with the mouse FGF 23 (C-terminal) ELISA-kit (Immutopics) following the manufacture´s recommendations. Analysis of mRNA was performed utilizing Affymetrix gene chips of seven day old cultured cardiomyocytes.

Statistics

For statistical analysis the unpaired t-test or Mann-Whitney U test in case of missing normality and/or heterogeneous variance of the data was performed. P-values <0.05 were considered statistically significant.

Results

Adult cardiomyocytes express and secrete FGF23

In order to determine whether adult cardiomyocytes are able to synthesize FGF23 we stimulated cell cultures with a variety of growth factors including the cytokines of interleukin-6 (IL-6) family, tumor necrosis factors, monocyte chemotactic factors, growth differentiation factors, transforming growth factors, bone morphogenetic proteins and interleukins. Among all tested substances only oncostatin M was able to induce FGF23. Transcript analysis of seven-day-old cultures clearly demonstrates that adult cardiomyocytes potently express FGF23 after stimulation with 20 ng/ml oncostatin M (Figure 1A). In addition, determination by ELISA demonstrates a massive increase of FGF23 (25 pg/ml vs. 1140 pg/ml) in 7 days old culture supernatants after stimulation of cardiomyocytes with oncostatin M (Figure 1A).

Western blot analysis was performed with lysates of twelve-day-old cultures (Figure 1B). The cell supernatant of the last five days of culture was analyzed by Western blot (Figure 1C). The blot manifestly demonstrates the presence of FGF23 in oncostatin M stimulated cardiomyocytes while this protein is absent in LIF treated cultures and albumin controls (Figures 1B and 1C). Several bands of FGF23 can be observed probably due to proteolytic cleavage [21]. In cell lysates major FGF23 bands are visible at 28 kDa corresponding to the unmodified full length FGF23 and a further band at 17 kDa which corresponds to the N-terminal fragment (Figure 1B). In contrast, the full length FGF23 is absent in the supernatant while in addition to the 17 kDa band two further peptides appear at approximately 12 and 14 kDa which correspond to the C-terminal fragment (Figure 1C). For comparison the Ponceau stained membrane shows equal loading and the size of proteins can be recognized by the ladder in kDa.

Monocyte/macrophage infiltration is responsible for the accumulation of FGF23 in cardiomyocytes in inflammatory induced heart failure

Since in the damaged myocardium oncostatin M is restricted to infiltrating cells (mostly macrophages), we wondered whether macrophage infiltration alone in the absence of any initial damage like myocardial infarction is sufficient to induce FGF23 accumulation in cardiomyocytes. In order to examine this question we utilized a mouse strain with a cardiac restricted overexpression of monocyte chemotactic protein-1 (MCP-1). It is well-known that MCP-1 acts as a potent chemoattractant for monocytes/macrophages [5,6]. Therefore the overexpression of this chemokine leads to a massive infiltration of the myocardium by macrophages and causes an inflammatory heart failure (iHF) [5]. The heart of MCP-1 mice is characterized by the dilation of ventricles and poor ejection fraction resembling the

phenotype of dilated cardiomyopathy. Animals start to die at the age of 6 months because of final stage HF. In wild-type control mice FGF23 is hardly detectable by confocal microscopy (Figure 2A). Conversely, the infiltrated heart shows a strong and significant increase in FGF23 positive cardiomyocytes (Figure 2A). Since MCP-1 does not induce FGF23 in cultured cardiomyocytes (data not shown), we argue that cell infiltration is crucial for the increase of FGF23 in this transgene model and assume that oncostatin M contributes to the accumulation of FGF23 in the inflamed myocardium. Similar to this animal model human patients with myocarditis show macrophage infiltration and may develop dilated cardiomyopathy without resolution of inflammation [22]. Patients with myocarditis show the highest level of FGF23 accumulation (Figure 4A).

FGF23 is present in cardiomyocytes of patients with ischemic cardiomyopathy, myocarditis and dilated cardiomyopathy

Since MCP-1 mediates the recruitment of mononuclear cells and increases in ischemic cardiomyopathy (ICM) [23,24] we wanted to know whether patients with ICM also show increased levels of FGF23. In Figure 3A it can evidently be recognized that this growth factor is present in high quantity in cardiomyocytes of patients with ischemic cardiomyopathy, while in control samples FGF23 is not detectable (Figure 3A). Figure 3B shows the strong presence of FGF23 in cardiomyocytes at a larger magnification while it appears only in tiny amounts in the nucleus of surrounding non-cardiomyocytes (white arrows). This leads to the hypothesis that non-cardiomyocytes are targets of FGF23. Another heart failure phenotype showing macrophage infiltration and increased MCP-1 levels is dilated cardiomyopathy (DCM) [6,25-28]. DCM occurs in the absence of a coronary, valvular,
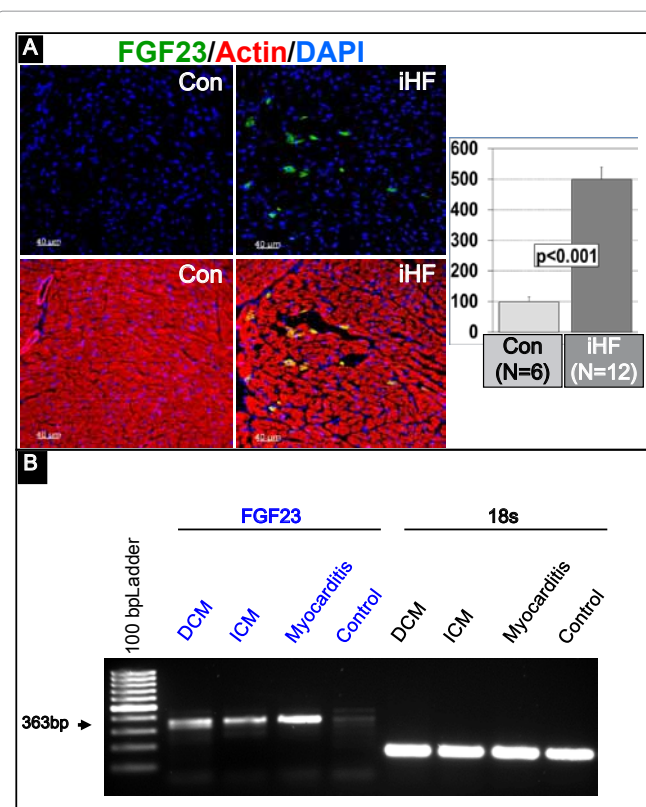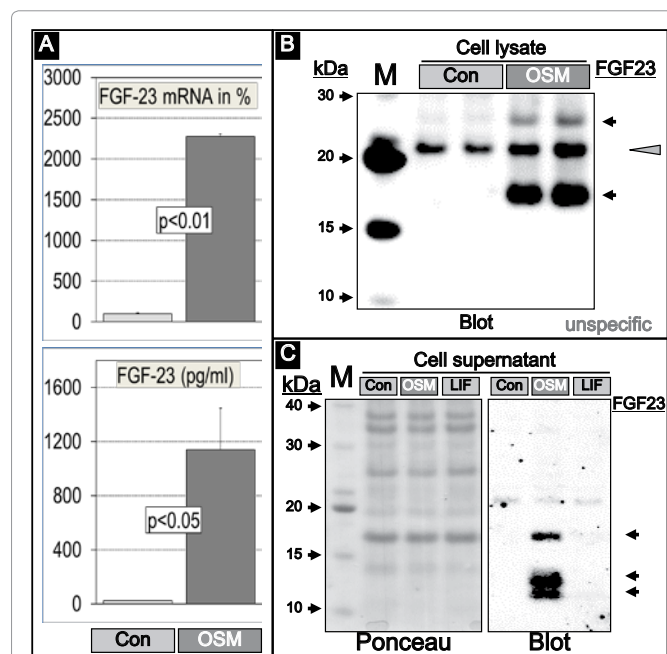

Figure 1: Adult rat cardiomyocytes express and secrete FGF23. Adult rat cardiomyocytes were treated with 20 ng/ml albumin (Con), 20 ng/ml of oncostatin M (OSM) or leukemia inhibitory factor (LIF). (**A**) Analysis of FGF23 mRNA expression in cultured adult cardiomyocytes after 7d in % of control and FGF23 ELISA (C-terminal) of the supernatant of the same cultures. (**B**) Cell lysates from day 14 were applied to Western Blot analysis. (**C**) Supernatants of cultured adult cardiomyocytes were collected from day 7 to 12, were concentrated (supernatant) and applied to Western Blot analysis. Note the unspecific band of the cell lysate in (**B**) which is marked by a grey triangle.

Figure 2: FGF23 is present in cardiomyocytes of mice with a cardiac restricted overexpression of the macrophage chemoattractant MCP-1. (**A**) Confocal images and statistical analysis of FGF23 in 6-month-old mouse myocardial tissues. Con indicates healthy wild-type controls and iHF represents the MCP-1 overexpressing strain which develops ventricular dilatation up to 6 months and dies because of heart failure. (**B**) RT-PCR of FGF23 from human tissue samples. DCM and ICM indicate dilated and ischemic cardiomyopathy, respectively.

congenital or a pericardial disease and ventricles show dilation and impaired systolic function [26]. Similar to patients with ICM and the MCP-1 transgenic mouse strain, patients with DCM show a marked enhancement of FGF23 expression in cardiomyocytes (Figure 4B). Furthermore, RT-PCR clearly demonstrates that the human heart tissue expresses FGF-23 under pathological conditions (Figure 2B). Increased transcript levels can be easily observed in patients with myocarditis, dilated and ischemic cardiomyopathy (Figure 2B).

Discussion

Chronic kidney disease (CKD) is often closely related to cardiac disease and heart failure. In 2005, almost half of the elderly patients with CKD had a concomitant diagnosis of HF and approximately 15% suffered from acute myocardial infarction [29]. Moreover, similar to HF patients inflammatory processes were present in the majority of patients with CKD [30]. It is therefore logical that circulating molecules, among them FGF23, are somehow connected with disease progression/protection of both organs.

In healthy humans intact FGF23 circulates in the blood at very low concentrations (30 pg/mL) but after a decrease of glomerular filtration rate the level of FGF23 in patients with renal failure progressively increases sometimes up to several hundredfold. It is assumed that the only source of circulating FGF23 in renal disease is the bone [21,31], in spite of the fact that FGF23 transcripts can be detected in liver, heart,
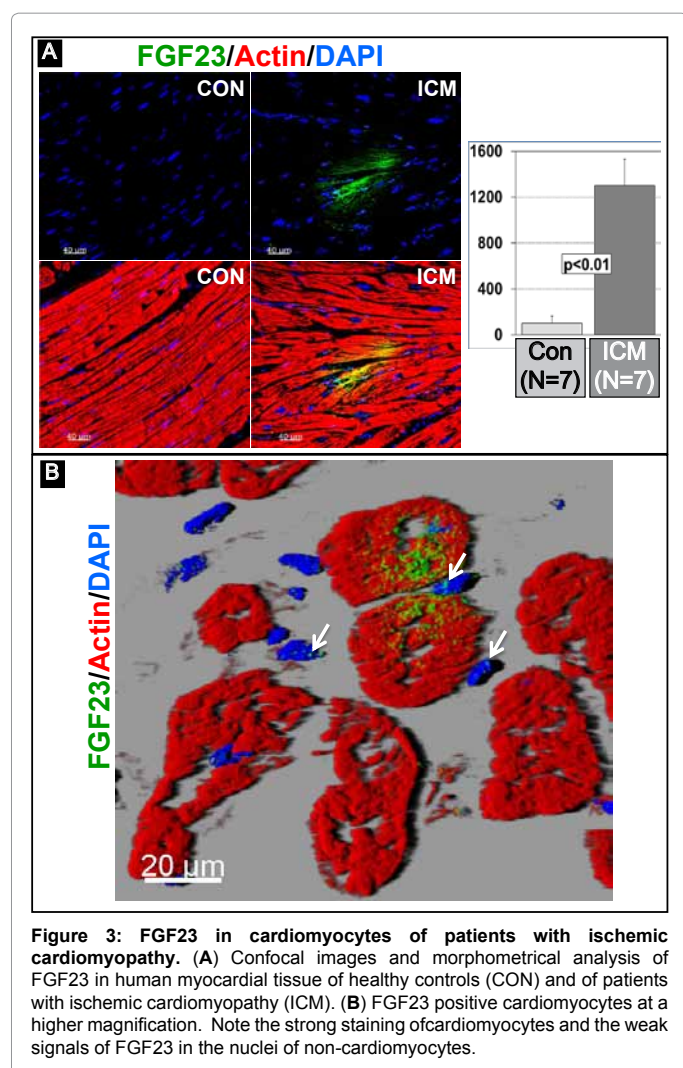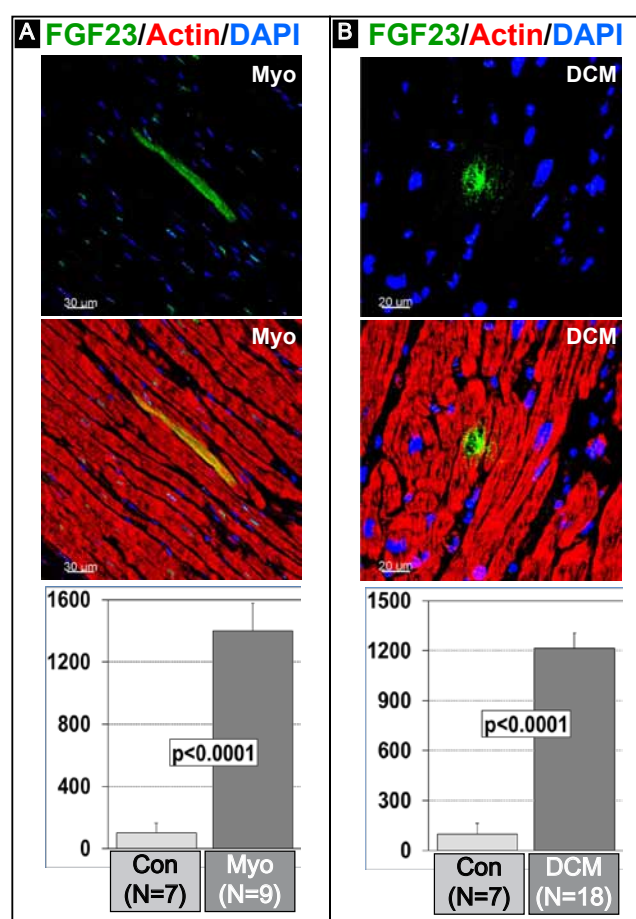
Figure 3: FGF23 in cardiomyocytes of patients with ischemic cardiomyopathy. (**A**) Confocal images and morphometrical analysis of FGF23 in human myocardial tissue of healthy controls (CON) and of patients with ischemic cardiomyopathy (ICM). (**B**) FGF23 positive cardiomyocytes at a higher magnification. Note the strong staining of cardiomyocytes and the weak signals of FGF23 in the nuclei of non-cardiomyocytes.

accumulation of oncostatin M secreting macrophages in the infiltrated myocardium [5,6]. In that context it is worth noting that observations in patients with renal disease and various animal models suggest a major role of MCP-1 in the progression of renal failure [32]. Our data clearly demonstrate that the accumulation of macrophages and oncostatin M signalling [6] in these transgenic mice correlates with increased amounts of FGF23 and substantiates the macrophage/OSM/FGF23 axis during the development of heart failure. Similarly, significant accumulation of FGF23 was observed in cardiomyocytes of patients with myocarditis, ischemic and dilative cardiomyopathy. We therefore suggest the following mechanism of FGF23 expression and secretion: Monocytes/macrophages invade the myocardium after an initial injury and release oncostatin M which induces a "cell survival program" via the oncostatin M receptor-β [6,9]. Under chronic extension of low level inflammation cardiomyocytes are constantly stimulated and produce FGF23 even if the myocardial performance deteriorates.

The clear presence of FGF23 in all analysed patients raises the question concerning the role of this growth factor in cardiac protection and failure. Although increasing evidence suggests a causal relationship between FGF23 level and cardiovascular morbidity the

Note the strong presence of FGF23 in the nuclei of in patients with Myo.

Figure 4: FGF23 in cardiomyocytes of patients with myocarditis and dilative cardiomyopathy. (**A**) Confocal images and morphometrical analysis of FGF23 expression in human myocardial tissue of healthy controls (Con), patients with myocarditis (Myo) and (**B**) patients with dilated cardiomyopathy (DCM).

brain and thymus. However, with the increasing number of publications demonstrating the involvement of FGF23 in left ventricular dysfunction [12], atrial fibrillation [12] and left ventricular hypertrophy [13] it appears feasible that under pathological conditions secondary sources of FGF23 exist. In order to determine whether cardiomyocytes are able to express FGF23 we stimulated cultures with a variety of growth factors and cytokines. Under all tested substances only oncostatin M potently induced the expression and secretion of FGF23. Oncostatin M belongs to the interleukin-6 class of cytokines which also comprises leukemia inhibitory factor (LIF) and cardiotrophin. OSM exerts its activities in humans via the type I receptor, which is a heteromer of gp130, LIF receptor-α and type II receptor complex, consisting of gp130 and oncostatin M receptor-β. Since LIF only binds the type I receptor and does not induce FGF23 expression, we conclude that FGF23 production and secretion in cardiomyocytes are mediated via the type II receptor complex. Furthermore, taking into account that LIF is produced by the majority of cell types in mammals while the expression of OSM is mainly restricted to inflammatory cell infiltrates we hypothesized that under pathological conditions the expression of FGF23 in cardiomyocytes is associated with macrophage invasion. In order to test this hypothesis we utilized a mouse strain with a cardiac restricted over expression of MCP-1 [5]. This chemokine is a potent macrophage chemoattractant, which has been shown to cause the

present knowledge is insufficient in order to determine a function of FGF23 in the inflamed heart. The massive presence of FGF23 in stressed cardiomyocytes contrasted its almost complete absence in non-cardiomyocytes, where FGF23 was only observed in tiny amounts in the nucleus. The generally observed pattern suggests that at least a subset of non-cardiomyocytes in the myocardium is a target. A further issue concerns a signal communication between the heart and other organs, especially the kidney. Do cardiomyocytes sense kidney damage and release FGF23 in response or, conversely, does the release of FGF23 from damaged myocardium induce a heart saving reaction in the kidney? Hepatocyte growth factor (HGF) is suggested to be an important factor of the cardioprotective mechanism [33,34]. In ischemically damaged myocardium an upregulation of HGF receptor was reported. In kidney HGF transcripts had markedly increased after 3h and stayed elevated until 24h after damage coinciding with increased level of HGF protein in the serum. And vice versa the heart itself might probably induce a kidney saving reaction through the release of FGF23. In addition, inflammatory processes might apart from the heart create some secondary OSM responsive sources such as the liver and contribute to increased circulating levels of FGF23.

The identification of oncostatin M as a potent inducer of FGF23 expression in cardiomyocytes rather adds some knowledge to the complex pathology of failing heart than helps to solve urgent questions concerning cardiac function of FGF23. However, it is quite possible that FGF23 similar to oncostatin M initially acts as a mediator of a "cell survival program" and afterwards promotes heart failure under conditions of chronic inflammation.

Acknowledgement

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