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OSM-Stimulated Cardiomyocytes Release a C-Terminal Fragment of FGF23

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Abstract

Background: Recent studies emphasize a correlation of increased FGF23 with the pathogenesis of heart diseases. Although it is widely assumed that the bone and not the heart is the major source of FGF23 we previously demonstrated that oncostatin M (OSM) activated cardiomyocytes strongly secrete FGF23. This phosphatonin can be released as intact molecule (iFGF23) as well as C-terminal (cFGF23) and N-terminal (nFGF23) fragments. Since cleavage does not only inactivate iFGF23 but might also exert antagonizing activity we wanted to determine which form is secreted by cardiomyocytes.

Methods: Adult cultured cardiomyocytes were stimulated with OSM or albumin as control. Supernatant and cell lysate were analyzed by Western blot (WB) and specific ELISAs against cFGF23 as well as iFGF23. Expression of FGF23 in cardiomyocytes of 6 patients with coronary heart diseases (CHD) was analyzed by confocal microscopy because OSM signaling cascades are activated after myocardial infarction.

Results: WB analysis identified cFGF23 as well as nFGF23 while iFGF23 was hardly detectable in the supernatant of OSM-stimulated cardiomyocytes. Analysis of the supernatant by ELISAs revealed that less than 3% of this secreted phosphatonin was intact. In patients with CHD the number of FGF23 positive cardiomyocytes increased from 0.2% in the remote zone to 4.4% in the border zone.

Conclusions: The expression and release of FGF23 by cardiomyocytes indicate local as well as systemic functions. The determination of the ratio of iFGF23/cFGF23 will be essential to understand the functional role of this growth factor in patients with cardiac diseases.

Keywords: Cardiomyocytes; ELISA; Fibroblast; Cardiac diseases

Introduction

The family of fibroblast growth factors (FGF) represents a group of 22 related polypeptides affecting a wide range of biological processes. The most recently discovered fibroblast growth factor-23 (FGF23) possesses unique biochemical properties mediated via its C-terminus [1]. It is grouped phylogenetically to the FGF19 subfamily and shares 22% and 24% amino acid homology with FGF19 and 21, respectively [2]. This subfamily lacks conventional heparin-binding domains and can act in a hormone-like manner by rapidly entering the circulation [3]. Its ability to decrease phosphate reabsorption and to reduce the level of vitamin D through inhibition of 1α-hydroxylase in the kidney distinguishes FGF23 functionally from other subfamily members [2].

Since osteoblasts/osteoclasts can highly express FGF23 the bone is regarded as major cause of increased serum levels and suggests the presence of a bone-kidney axis that coordinates renal handling of phosphate and vitamin D [1-4]. It is therefore not surprising that FGF23 is linked to chronic kidney disease (CKD). Recent studies emphasize a role of FGF23 in the development of heart failure since renal and cardiac diseases appear to be intimately connected. According to the United States Renal Data System [5] 15% of elderly patients with CKD suffered from a heart attack and almost half had a concomitant diagnosis of heart failure in 2005. Therefore, increased levels of FGF23 appear not only be associated with faster progression of chronic kidney disease and a higher mortality in hemodialysis patients but is also associated with left ventricular dysfunction, left ventricular hypertrophy, myocardial infarction and heart failure [5-11].

Despite the identification of transcripts in heart, brain, thymus and the small intestine [12] circulating FGF23 is still regarded under pathological conditions as bone-derived. Our observation of increased levels of FGF23 in patients with aortic stenosis, myocarditis and dilated cardiomyopathy [10,11] suggests that the heart itself functions as an additional source and furthermore indicates an inflammatory component of this growth factor.

We demonstrated in a mouse strain with a cardiac restricted overexpression of the monocyte-chemotactic protein-1 (MCP-1) that macrophage infiltration was associated with accumulation of FGF23 [10,11]. In search for FGF23 inducing cytokines and growth factors we identified the inflammatory cytokine oncostatin M (OSM) as a potent inducer of FGF23 expression and secretion [10,11,13,14]. Other

inflammatory cytokines such as tumour necrosis factors, interleukin-6, interleukin-1α and 1β did not stimulate the release of FGF23 by cardiomyocytes.

Our observation that OSM induces massively the secretion of FGF23 from cardiomyocytes was surprising since this phosphatonin and oncostatin M exert opposing effects on heart muscle cells. While FGF23 acts as a hypertrophic factor [6] chronic oncostatin M signaling induces strongly the loss of sarcomeres [14,15]. In order to solve this apparent contradiction we wanted exactly to determine the ratio of intact (iFGF23) and fragmented FGF23 (cFGF23) by utilizing specific ELISAs. Fragmentation of FGF23 might not only inactivate its phosphouretic activity upon release but might also block renal phosphate waste of circulating intact FGF23 originating from other sources.

While hypertrophic activities of iFGF23 are not only documented in cardiomyocyte cultures but also in animal models [6,16] there is to our best knowledge nothing known about cardiac effects of fragmented FGF23 and it is tempting to speculate about potential anti-hypertrophic activities. Therefore, the need for transgenic mice overexpressing fragmented and intact FGF23 to clarify their role during cardiac pathogenesis and regeneration is obvious. In this study, we wanted to determine the major secreted FGF23 of OSM stimulated adult cardiomyocytes by utilizing specific ELISAs. Since iFGF23 and cFGF23 exert opposing activities in the kidney [17] we will also discuss potential functions and their regulation in the diseased heart.

Methods

Isolation and culture of adult ventricular myocytes

Cardiomyocytes (ARC) were isolated from hearts of adult rats (200 g to 250 g) and protein concentration was determined as previously described [**18**]. ARC were plated at a density of 1.5×10^4 cells/cm^2 on laminin (10 µg/cm^2, Sigma) coated 6 well (Nunc) in basic medium [**18**] for Western blot analysis and at a density of 0.75×10^4 cells/cm^2 on 24 well dishes for ELISA assays. Neonatal rat cardiomyocytes used as positive controls for cleaved PARP and caspase 3 (Cell Signaling) and were cultured as previously described [**19**]. Knock-down of FGF23 by 0.25 µM siRNA (L-094167-02; NM_130754.1) was performed according to the manufacturer´s instructions (ON-TARGETplus, Dharmacon). Mouse OSM was from R&D Systems and rat OSM from Preprotech.

Determination of FGF23 by ELISA and Western Blot analysis

The antibody directed against mouse FGF23 was from R&D Systems (Cat Nr. AF2629; the immunizing protein was iFGF23) and anti-human FGF23 was purchased from Abcam (Cat Nr. ab56326; the immunizing peptide corresponds to the human internal amino acid sequence 176-188). The rat/mouse FGF23 ELISA kits used for the determination of cFGF23 (Cat Nr. 60-6300) and iFGF23 (Cat Nr. 60-6800) were obtained from Immutopics (Figure 1D). Anti-sarcomeric α-actinin (ACTN2) was obtained from Sigma, anti-Ral A was from Becton Dickinson and anti-myomesin 2 was a kind gift of Dr. H. M. Eppenberger.

For Western blot analysis, primary antibodies were diluted 1:1000 and secondary antibodies conjugated to horseradish peroxidase were diluted 1:5000. Bands were visualized with the SuperSignal West Femto Maximum sensitivity substrate ((ThermoFisher) in the Chemidoc system (Bio-Rad).

Collection of tissue from patients with coronary heart diseases and confocal microscopy

Anti-collagen-1 was from Rockland. Myocardial tissue was obtained from six patients during the transplantation surgery (Figure 1). 4 control samples were obtained from hearts with normal left ventricular function. Before participation in the study all patients gave their informed consent. Following standard procedure, the study was approved by the ethics committee of the Hesse medical association (FF8-2011). The study was conducted in accordance with the Declaration of Helsinki. Tissue sections were fixed in 4% paraformaldehyde for 5 minutes. F-actin was visualized with phalloidin (1:100, Biotrend). Primary antibodies were diluted 1:100 and secondary 1:300 (Millipore). Confocal images were taken with the Leica SP8 microscope utilizing a 40x objective.

Statistical analysis

The Graph Pad Prism software was used for calculation of the unpaired t-test with Welch`s correction and p values less than 0.05 are considered as statistically significant.

Results

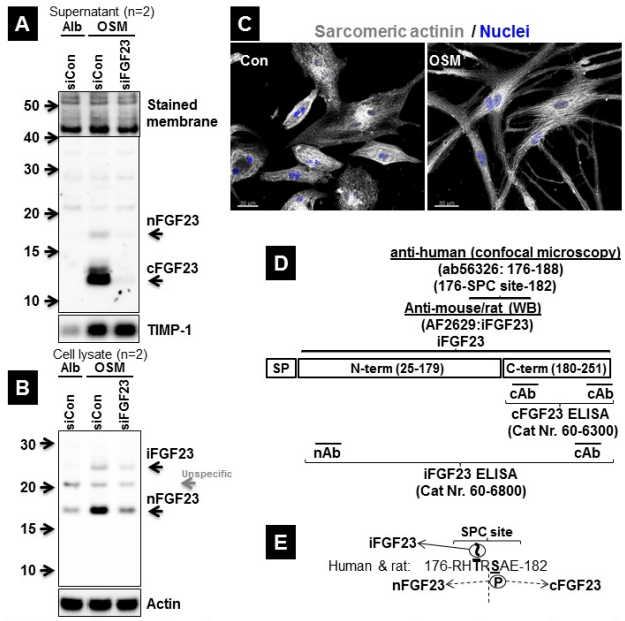
Western blot analysis reveals C- and N-terminal but not intact FGF23 in the supernatant of OSM treated cardiomyocytes

Adult rat cardiomyocytes were kept for the first 4 days in 5% fetal calf serum (FCS) and for further 3 days serum-free. Cultures were treated for 7 days with albumin or OSM. Concentrated serum-free medium (Centricon) of the last three days was used for Western blot analysis (Figure 1A). An antibody directed against the FGF23 identified the 18kDa N-terminal (nFGF23) and 12 kDa to 14 kDa C-terminal (cFGF23) fragments in the supernatant. The iFGF23 was hardly detectable indicating intracellular processing (Figure 1A).

In contrast, mainly nFGF23 and iFGF23 were detectable in cell lysates (Figure 1B). Knock-down of FGF23 by pretreatment of cultures for 3 days with siRNA demonstrated the specificity of the antibody. TIMP-1 served as a control of OSM activity since this metalloproteinase inhibitor is one of the major proteins regulated by OSM. Actin and the stained membrane serve as controls for cell lysates and supernatant, respectively. Cultures show no signs of deterioration but depict the rounding up and spreading of cells when serum is present (Figure 1C). OSM treated cardiomyocytes show characteristic cell elongation (Figure 1C). In order to determine the exact ratio of iFGF23 and cFGF23 we utilized specific ELISA kits.

Determination by ELISA Reveals that cFGF23 but not iFGF23 is the Major Secreted Form

We knew from previous time course studies of OSM stimulated cardiomyocyte that the peak of cFGF23 in the cell culture supernatant is reached within 2-4 days. In order to determine the maximum secretory activity after albumin and OSM treatment we collected conditioned medium from 2 days old cultures for ELISA measurements.



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	#	Fragment	Size	Molecular weight		Homology	Isoelectric point	Gene location
				Calculated	Observed			
Rat FGF23	iFGF	251aa	27kDa	28-32kDa Cardiomyocytes [11]	R/H= 72% R/M= 94%	9.89	Chr.4q42	
	cFGF	72aa	8kDa	12-14kDa, cardio-myocyte supernatant	R/H= 64% R/M= 96%	9.65		
	nFGF	155aa	18kDa	18kDa, cardiomyocyte supernatant	R/H= 76% R/M= 94%	9.79		
Human FGF23	iFGF	251aa	27kDa	32kDa [33-36]	H/M =72% H/R = 72%	9.17	Chr.12p13.	
	cFGF	72aa	7.5kDa	10-15kDa [33-36]	H/M= 64% H/R= 64%	5.18		
	nFGF	155aa	17.8kDa	16.5kDa [35,36]	H/M= 75% H/R= 76%	9.59		

Fig.1

Figure 1: Western blot analysis reveals fragmented but not intact FGF23 in the supernatant of remodeling cardiomyocytes after OSM stimulation. Adult cultured rat cardiomyocytes were treated as indicated with oncostatin M (OSM, 20 ng/ml) or with albumin (Alb, 20 ng/ml) for 7 days. During the last 3 days cells were cultured serum-free. Conditioned medium (supernatant) and culture lysates were used for Western blot analysis (WB). Intact, C-terminal and N-terminal FGF23 are indicated by iFGF23, cFGF23 and nFGF23, respectively. (A) WB analysis reveals secreted nFGF23 and cFGF23 but not iFGF23. (B) In contrast, mainly iFGF23 and nFGF23 were detectable in the cell lysate. (C) Confocal images demonstrate massive remodeling and characteristic elongation of OSM-stimulated cardiomyocytes. (D) Overview of the antibodies used in ELISAs, WBs and confocal microscopy for the determination of nFGF23 (nAb) and cFGF23 (cAb) as well as iFGF23 (ELISA). (E) Modification of the SPC site (subtilisin-like proprotein convertase site which is identical in rat and humans) determines either secretion (glycosylation of Thr) of intact molecule (iFGF23) or the intracellular cleavage (phosphorylation of Ser) of iFGF23 into cFGF23 and nFGF23. Further information is provided in the text. (F) Summary of the biochemical properties of rat and human FGF23. Note that data concerning rat cFGF23 and nFGF23 are calculated under the assumption that cleavage occurs in the SPC site as reported for human FGF23. For prediction of biochemical properties of intact, fragmented FGF23 and chromosomal localization we used UniProt, ExPASy, Phosphosite.org and NCBI.

Fragmented FGF23 was determined by sandwich ELISA with two antibodies directed against different C-terminal regions of FGF23 while iFGF23 concentrations were determined with two antibodies targeting nFGF23 and cFGF23, respectively (Figure 1D). Stimulation with mouse OSM (mOSM; Figure 2A) strongly increased cFGF23 in comparison to the control group (16 pg/ml ± 7 pg/ml vs 405 pg/ml ± 21 pg/ml) while iFGF23 was hardly detectable in both groups (9.8 pg/ml ± 3.8 pg/ml vs 10 pg/ml ± 3.9 pg/ml). Calculation of the ratio of cFGF23 and iFGF23 showed that only 2.6% represented the uncleaved molecule. Surprisingly, stimulation with rat OSM (rOSM) was even more potent compared to mouse OSM (Figure 2A) and increased cFGF23 to more than 1 ng/ml (16 ± 7 control vs 1419 pg/ml ± 208 pg/ml).

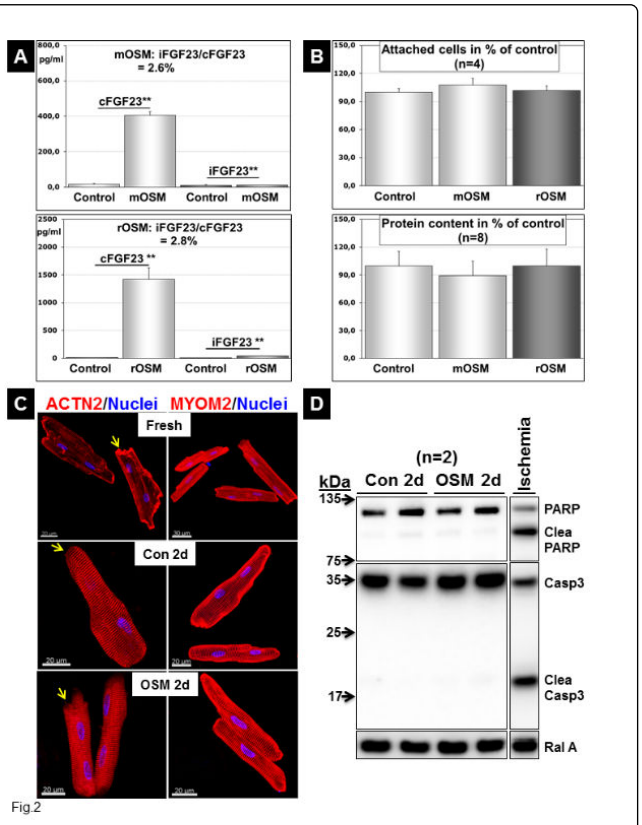

Fig.2

Figure 2: Determination of cFGF23 and iFGF23 by ELISA reveals cFGF23 as major secreted molecule in short-term cultures of OSM stimulated cardiomyocytes. (A) C-terminal (cFGF23) and intact (iFGF23) were determined two days after treatment with 20 ng/ml of mouse oncostatin M stimulation (mOSM) or 20 ng/ml of rat oncostatin M (rOSM). 20 ng/ml of albumin served as control. (B) Determination of the total protein content and cell number shows no differences between the groups. (C) Confocal images of myomesin 2 (MYOM2; a marker for mature sarcomeres) show an intact sarcomeric structure. Staining for sarcomeric α-actinin (ACTN2) visualizes some loss in the area of the intercalated discs which is more pronounced after OSM stimulation (see yellow arrows) (D) No signs of apoptosis such as cleaved PARP (cleaPARP) and cleaved caspase3 (cleaCasp3) are recognizable. As apoptotic controls serve cardiomyocytes cultured in PBS for 12 hours under 0.2% O2 (Ischemia). P-values (**) were smaller than 0.001.

This enhancement of FGF23 release might be due to the ability of rOSM to utilize the OSM as well as the LIF receptor complexes in contrast to mOSM which only utilizes the OSMR complex in rats [**20**]. In addition, the extracellular level of iFGF23 was increased in rOSM stimulated cardiomyocyte cultures (9.8 ± 3.8 control vs 40 pg/ml ± 8.4 pg/ml). However, the ratio of iFGF23 and cFGF23 was almost unchanged in comparison to mOSM (Figure 2A; mOSM 2.6% vs rOSM 2.8%) supporting our conclusion that cFGF23 but not iFGF23 is the major secreted molecule after oncostatin M stimulation.

The number of cardiomyocytes and the total protein content did not differ between groups (Figure 2B) and no signs of apoptosis were determined (Figure 2D). Sarcomeres appeared preserved as visualized by myomesin 2 staining (Figure 2C). In comparison to freshly isolated cells some rounding at cell ends were visible and in oncostatin M stimulated cultures. Sarcomeric α-actinin staining in the area of the intercalated discs was less pronounced and indicates the beginning of the formation of long extensions characteristic for OSM activity (Figure 2C vs. Figure 1C).

From these data, we conclude that the culturing process of cardiomyocytes has little influence on the level of secreted iFGF23. Since we previously demonstrated that the OSM receptor signaling cascade is strongly activated in an experimental model of myocardial infarction as well as in patients with myocardial infarction [14,21], we wanted to know whether FGF23 is increased in patients with coronary heart diseases (Table 1).

The number of FGF23 positive cardiomyocytes in patients with coronary heart disease is strongly elevated in the border zone

The remote (RZ) and the border zone (BZ) of six patients with coronary heart disease and myocardial infarction (MI) were analyzed (Table 1).

Magnetic resonance imaging of a patient clearly indicates the area of MI (yellow arrow) and reveals left ventricular thinning and dilation (Figure 3A). The RZ showed different degrees of fibrosis while massive fibrosis is visible in the BZ of the infarcted area (Figure 3D). Staining of the border zone with an anti-FGF23 antibody revealed a large number of positive cardiomyocytes (Figure 3B). The antibody is directed against the SPC site thus probably recognizing the intact molecule (Figure 1D). Counting of 7343 cardiomyocytes (average 1223 per patient) in the RZ identified less than 0.2% of FGF23 positive cells while from 4791 cardiomyocytes (average 799 per patient) 4.4% were stained for FGF23 in the BZ (Figure 3C; 0.17 ± 0.06 vs 4.42 ± 1.2 (n=6); p<0.003). The identification of the intracellular iFGF23 in cardiomyocytes of the BZ is in agreement with our previous observation of intact intracellular FGF23 in OSM stimulated myocytes [**11**]. For comparison data from hearts with preserved ejection fraction are integrated into the Figure 3.

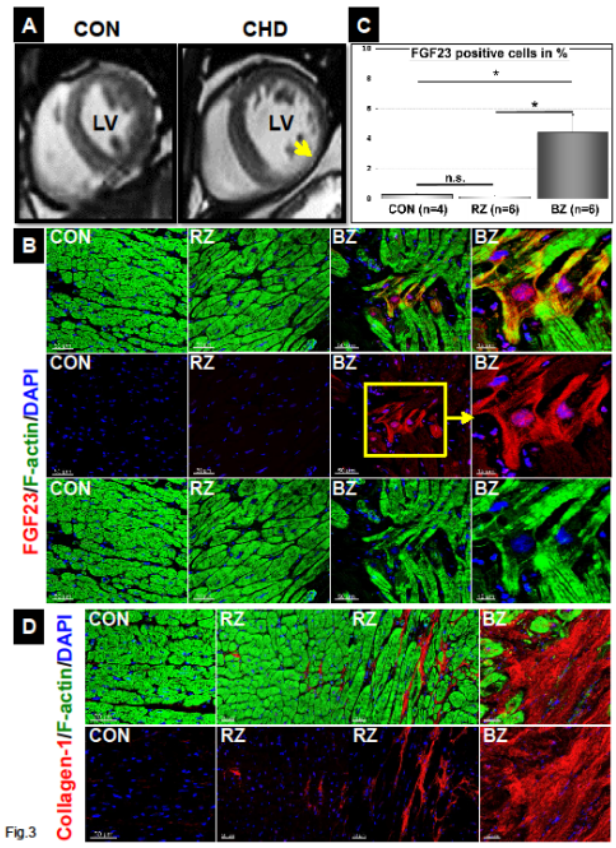

Figure 3: FGF23 is increased in the border zone of patients with coronary heart disease. (A) Magnetic resonance image of a patient with coronary heart disease (CHD) and myocardial infarction (yellow arrow). LV indicates left ventricle. CON indicates a healthy control heart. (B) Confocal images of the remote zone (RZ) and border zone (BZ) after myocardial infarction in patients with coronary heart disease. Cardiomyocytes of the border zone show a strongly increased number of FGF23 positive cardiomyocytes while very few cells of the remote zone are stained. (C) Number of positive cardiomyocytes in % of counted cells in the remote (7343 cells with an average of 1223 cells per patient) and border zone (4791 cardiomyocytes with an average of 799 cells per patient). For comparison images and data of myocardial samples from patients with normal ventricular function (CON) are shown (3321 cardiomyocytes were counted with an average of 831 cells). P-values (*) smaller than 0.05 were considered statistically significant. (D) Areas of the remote zone (RZ) show different degrees of fibrosis while in the border zone (BZ) massive fibrosis is visible.

Demographic and Risk Factors	Mean	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Number of myocardial infarctions	-	1	1	2	Multiple	Multiple	2
Etiology of CHD	-	AWI, LV-aneurysm	AWI, LV-aneurysm	CAD	CAD	CAD	CAD
Age (Years)	55.5 ± 2.3	59	57	52	61	58	46

Duration of Disease (Months)	-	>36	>36	>24	>36	>36	>24
Gender	-	Female	Male	Male	Male	Male	Male
NYHA Class	-	III	III	IV	III	IV	IV
Family history of coronary artery Disease	-	-	-	+	-	+	-
Prier myocardial infarction	-	1	1	+	+	+	+
Prior PCI or CABG	-	-	CABG MVR	PTCA and emergency CABG	CABG	PTCA	CABG; PTCA
Prior smoking	-	+	-	+	-	+	-
Prior hypertension	-	-	-	+	+	-	-
Prior diabetes mellitus	-	-	-	+	+	-	-
Prior hypercholesterolemia	-	-	+	+	-	+	+
Atrial fibrillation	-	-	-	+	-	-	-
Comedication							
Beta locker	-	-	-	+	+	+	+
ACE inhibitor and/or AT1 antagonist	-	+	-	-	-	+	+
Diuretic	-	+	+	+	+	+	+
Digitalis	-	+	+	-	+	+	-
Aldosterone antagonist	-	-	-	-	+	-	+
Statins	-	-	-	+	-	-	+
Laboratory Parameters							
Sodium (mmol/l)	137 ± 1.7	135	145	134	134	136	138
Potassium (mmol/l)	4.6 ± 0.12	4.6	4.7	5	4.7	4.2	4.3
Creatinine (mg/dl)	1.1 ± 0.16	1.83	1.1	0.77	0.97	0.86	1.02
Haemoglobin (g/dl)	13.8 ± 0.73	13.7	14.3	15.9	12.1	15.3	11.3
Haematokrit (%)	41 ± 2.2	39	40	49	37	46	34.7
CRP (mg/l)	1.6 ± 0.69	Na	Na	0.8	1.1	0.7	3.6
Leukocytes	7.5 ± 0.43	7.2	6.8	6.1	7.6	9.2	7.8
Total cholesterol (mg/dl)	221 ± 22.4	251	156	275	171	282	192
SGOT (IU/l)	12.7 ± 2.3	8	7	14	12	23	12
GPT (IU/l)	10.3 ± 2	7	5	19	9	13	9
Echocardiographic characteristics							
LVEF (%)	19 ± 3	15-20(17.5)	15-20(17.5)	20	20	10	30
LVESD (mm)	61 ± 2.3	66	67	58	55	55	65
LVEDD (mm)	64 ± 5	74	81	62	59	60	46

Hemodynamic Parameters							
Cardiac Index (l/min*m)	2 ± 0.12	1.9	1.8	1.7	2.5	2.25	2
Pulmonary vascular resistance (dynes*s/cm³)	150 ± 15.1	150	144	133	145	220	110
Pulmonary capillary wedge pressure (mmHg)	12.2 ± 1.5	12	13	7	18	10	
Physical Parameters							
Height (cm)	174 ± 2.8	165	180	172	172	171	184
Weight (Kg)	70.2 ± 5	62	85	71	58	60	85
BMI (kg/m²)	23 ± 1.1	22.8	26.2	24	19.6	20.5	25.1
Abbreviations used: AWI-anterior wall infarction; LV-left ventricle; NYHA-New York Heart Association; PCI-percutaneous coronary intervention; CABG-coronary artery bypass grafting; MVR-mitral valve repair; ACE-angiotensin-converting enzyme; AT1-angiotensin II receptor; CRP-C-reactive protein; SGOT-serum oxaloacetic transaminase; SGPT-serum glutamic pyruvic transaminase; LVEF-left ventricular ejection fraction; LVESD-left ventricular end-systolic diameter; LVEDD-left ventricular end-diastolic diameter; BMI-body mass index. Data are calculated in mean ± s.e.m.							

Table 1: Selected clinical and demographic data of patients with coronary heart diseases (CHD). 6 patients with a disease duration of >24 months which previously underwent surgical revascularization were diagnosed with NYHA class III-IV. Magnetic resonance imaging, echocardiography and left as well as right heart catheterization revealed severe left ventricular dilation and impaired function. Patients were medically treated according to the guidelines of the European Society of Cardiology. Laboratory parameters appeared to be within physiological ranges before transplantation.

Discussion

Heart failure (HF) affects approximately 2% of the western population and is characterized by the inability of the heart to pump sufficient blood to meet the needs of the body. On the level of the cardiomyocyte as contractile unit HF comprises adverse restructuring of the contractile apparatus, rearrangement of the cytoskeleton and changes in energy metabolism [**22**]. We have previously demonstrated that OSM is a pivotal regulator of cardiac remodelling. Furthermore, we observed increased OSM receptor activation in patients with myocardial infarction and dilated cardiomyopathy as well as in the corresponding experimental models of [10,11,14,21-24]. Unfortunately, the restricted spatial and temporal appearance of OSM in the damaged myocardium makes this cytokine unsuitable to function as a circulating biomarker.

In order to identify biomarkers of OSM activity we found that increased circulating FGF23 correlate with OSMR activation during the transition to HF [10,11]. Increased cardiac FGF23 levels have been also observed in experimental models of myocardial infarction [25,26], which is in agreement with our observation of strongly increased FGF23 expression in cardiomyocytes of the border zone in patients with CHD. Furthermore, we have previously demonstrated significantly higher levels of FGF23 in the pericardial fluid than in serum indicating a cardiac origin of this growth factor during heart failure [**10**]. However, it has not been clarified whether cleaved or intact FGF23 is released by cardiomyocytes.

Modification of the human SPC site by glycosylation at T178 leads to secretion of iFGF23 while phosphorylation by Fam20C at S180 causes cleavage by furin resulting in nFGF23 and cFGF23 [**27**]. Human and rat FGF23 are 251 amino acids in length and the subtilisin-like proprotein convertase site (SPC) is identical in both species (Figures 1C and 1D).

Thus, intact and cleaved FGF23 in rats have probably the same length as in humans and might also undergo a similar processing mechanism (Figure 1D; Figure 4B). Interestingly, while the calculated molecular weights of nFGF23 and iFGF23 correspond to the detected sizes, the C-terminal fragment appears to be considerably larger than expected (7.5 vs. 12 kDa to 14 kDa) but are in agreement with the size reported for human cFGF23 [**28-31**] (Figure 1F). Since the glycosylation site T178 for secretion is lost during cleavage, we assume that additional posttranslational modifications [**27**] might be necessary for the release of cFGF23, which would explain the discrepancy between the calculated and observed molecular weight of cFGF23. On the other hand, we detected little nFGF23 in the supernatant of cardiomyocytes by Western blot analysis questioning whether this fragment plays an extracellular role.

Despite the increasing number of publications demonstrating the expression FGF23 in the myocardium, its function in the heart under pathological conditions is not clear [4,10,11,32,33] and we can only speculate on this. Since FGF23 is usually not expressed in normal adult cardiomyocytes but appears present in neonatal cardiomyocytes (unpublished) the reexpression of this phosphatonin might be part of the fetal gene program which then exerts resistance to ischemia by dedifferentiation and degradation of the oxygen consuming machinery [14,15,23]. These processes appear to be essential to restrict damage and reduce infarct expansion. Interestingly, FGF23 has been recently described as a factor which impairs neutrophil recruitment into the inflamed tissue [**34**]. Since oncostatin M is a major regulator of macrophage infiltration through the release of regenerating-islet derived proteins (Reg) by cardiomyocytes it is intriguing to speculate that the expression and release of cardiac FGF23 might be one means of OSM to orchestrate the shift from neutrophil invasion to macrophage infiltration after myocardial infarction.

Our discovery of the inflamed heart as a second major source of FGF23 raises further questions. From our viewpoint, there are three

major reasons contributing to the complex biology of FGF23 regulation and function. First, there are additional sources of FGF23 besides the bone and cardiomyocytes such as non-cardiomyocytes [**10**], fibroblasts [**26**] and macrophages [**35**] indicating that at least in theory FGF23 might be released in significant amounts from any other organ under certain pathological conditions. Second, FGF23 can be secreted within hours in high amounts from cardiomyocytes as well as non-cardiomyocytes [**10**] while most cardiovascular studies focus on chronic events thus neglecting potential important immediate early functions in the heart. Third, FGF23 might exert opposing effects, since this growth factor can be released as full length as well as protein fragments. Proteolytic cleavage of FGF23 does not only inactivate its biological activity but the resulting C-terminal fragment might also block signaling through inhibition of the FGF23/FGFR/Klotho complex formation [**17**] (Figure 4B).

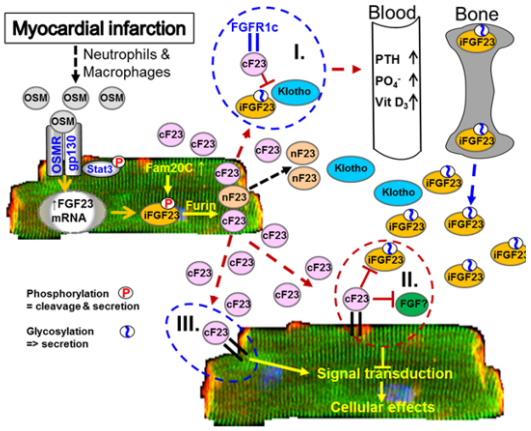

Figure 4: Simplified scheme of iFGF23 fragmentation and hypothetical functions of cardiac derived cFGF23 after acute myocardial infarction. After myocardial infarction infiltrating cells release oncostatin M (OSM) and activate Stat3 (P-Stat3) through the OSM receptor/gp130 complex which then induces FGF23 expression. Intact FGF23 (iFGF23) becomes phosphorylated by Fam20c and is cleaved through Furin thus creating N-terminal (nF23) and C-terminal (cF23) FGF23 fragments. Secreted cardiac cF23 might block complex formation of iFGF23/Klotho/FGFR1c (I.) which might lead to increases in concentrations of circulating vitamin D3 (Vit D3; Calcitriol), parathyroid hormone (PTH) and phosphate. In addition, one might speculate that cF23 also blocks iFGF23 induced cardiomyocyte hypertrophy (II.) or potentially exerts own cellular effects (III.).

In that context it is important to note that we determined full length FGF23 **10** in lysates of OSM treated cardiomyocytes while mainly C-terminal FGF23 were observed in the supernatant indicating intracellular proteolytic cleavage before release. ELISA-based determination of the exact concentrations of iFGF23 and cFGF23 clearly demonstrate that less than 3% of FGF23 in the supernatant of OSM-stimulated adult cardiomyocytes was intact. Therefore, it is tempting to speculate that the function of OSM-induced cardiac expression and cleavage of FGF23 is to prevent stimulation of cardiomyocyte hypertrophy by iFGF23 and to neutralize systemic iFGF23 (Figure 4B). Another issue which we have to keep in mind is that additional growth factors and cytokines might participate in OSM induced expression thus influencing the level of secreted FGF23 as well as the ratio of iFGF23/cFGF23. It is furthermore tempting to speculate, that cardiac cFGF23 might dock to an FGF receptor on myocytes thus creating intracellular effects or alternatively might block hypertrophic effects of iFGF23 and potentially other FGFs (Figure 4). Finally, the ratio of cardiac derived cFGF23 and systemic iFGF23 might also influence endothelial vaso relaxation, secretion of parathyroid hormone, vitamin D metabolism and the serum levels of phosphate [**36**].

The identification of cardiac derived FGF23 suggests that it does not act merely as regulator of bone-mineral metabolism. Instead, the massive release of FGF23 by cardiomyocytes after OSM-stimulation indicates local as well as systemic functions. The determination of the ratio of iFGF23/cFGF23 will be essential to understand the function not only of cardiac derived FGF23.

Conflicts of Interest

The authors declare no conflict of interest.

Disclosure Statement

The authors have no conflict of interest to disclose.

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