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Atrial expression of the CCN1 and CCN2 proteins in chronic heart failure

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Abstract: Previous studies have reported the upregulation of CCN proteins early after acute heart injury. The aim of the present work was to evaluate the expression of the CCN1 and CCN2 proteins and their regulation by angiotensin II in the atrial myocardium of a chronically failing heart. Male adult mice were subjected to ligation of the left coronary artery to produce myocardial infarction (the MI group), and 16 of them were treated for 12 weeks with the AT1 receptor antagonist telmisartan (the MI-Tel group). Sham-operated mice served as controls. The expression of proteins was evaluated by immunohistochemistry 12 weeks after the operation. In sham-operated mice, stainings for CCN1 and CCN2 proteins were positive within atrial cardiomyocytes. CCN1-positive reaction revealed diffused cytoplasmic localization, while CCN2 was present mainly within the perinuclear cytoplasm. CCN1 was upregulated in the MI group, while CCN2 remained at basal level. Telmisartan prevented the upregulation of CCN1 and decreased CCN2 level. We compared the experimental data with the expression of CCN1 and CCN2 proteins in human right atrial appendages. We found an inverse, but not significant, relation between the level of either protein and the left ventricular ejection fraction. This suggests a similar atrial regulation of CCN1 and CCN2 expression also in humans. We conclude that in the murine atria, CCN1 and CCN2 proteins are expressed constitutively. In chronic heart failure, CCN proteins tend to be upregulated, which may be related to the action of angiotensin II. *(Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 1, 99–103)*

Key words: CCN1, CCN2, atria, heart failure, myocardial infarction

Introduction

CCN1 (CYR61, cysteine-rich angiogenic inducer 61) and CCN2 (CTGF, connective tissue growth factor) are secreted, cysteine-rich proteins associated with the extracellular matrix. Both of them modulate cellular responses through interaction with integrins and regulate adhesion, migration, proliferation, differentiation, survival of cells and apoptosis [1]. CCN proteins are necessary in the process of cardiac development and their expression in the ventricular myocardium is upregulated after myocardial infarction or in chronic heart failure [2, 3]. Basal CCN2 expression in the atria of adult mice has been described as being higher than its physiological ventricular level, while the expression of CCN1 in the atria has not been examined to date. Chronic impairment of the left ventricular function results in hemodynamic overload to the heart, including the atria. In addition, compromised

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cardiac function activates numerous neurohormonal mechanisms. Both mechanical and neurohormonal influences promote remodeling of the myocardium and worsen pumping performance of the heart [4, 5]. Atrial remodeling present in the failing heart is the substrate for supraventricular arrhythmias that may complicate the course of disease and worsen the prognosis [6].

CCN proteins are regulated by mechanical stress, as well as by angiotensin II or inflammatory cytokines and growth factors, that are overexpressed in chronic heart failure, and thus may be considered regulators of the process of unfavorable atrial remodeling [3].

The aim of the present work was to analyze the atrial expression of CCN1 and CCN2 proteins in the mouse model of left ventricular failure induced by experimental myocardial infarction and to evaluate the impact of angiotensin type 1 receptor blockade on the expression of these proteins.

Material and methods

Animal experimental procedure. Male C57BL/6J 14–16 week-old mice were used in the study. The animals were subjected to coronary artery ligation to produce myocardial infarction (n = 29) or to a sham operation (n = 10). The operation was performed as described previously with modifications [7]. Briefly, the animals were anesthetized with isoflurane (AErrane, Baxter, USA). The trachea was intubated via the oral cavity. Then the mice were put on a heated operating pad and ventilated with oxygen-enriched air using a rodent respirator (Minivent, Harvard Apparatus, USA). The chest was opened by a left-sided incision in the fifth intercostal space, followed by opening of the pericardial sac and exposure of the anterior wall of the heart. In the ten sham-operated animals, after ten further minutes the chest was closed in layers with reposition of the muscles. In the remaining animals, the left coronary artery was ligated just proximal to its main bifurcation, using a Prolene 7.0 suture with an atraumatic needle. The efficacy of myocardial ischemia was assessed visually as pale discoloration of the cardiac anterior wall. After closing of the chest wall, animals were further ventilated with oxygen-rich air without anesthetic until spontaneous breathing was evident (usually about two minutes). The mice were transferred to a 27°C cage and left for one hour for recovery. Sixteen animals subjected to coronary artery ligation were for the next 12 weeks treated with telmisartan (Cat No. T8949, Sigma) at a dose of 1 mg/kg/day in drinking water and this constituted the 'MI-Tel group', while the remaining mice after myocardial infarction (the 'MI group') and those sham operated were not subjected to further treatment. Animals were sacrificed by cervical dislocation after 12 weeks following the operation. Left and right atria were instantly harvested, rinsed in an ice-cold physiological saline solution and fixed in neutral buffered formalin.

Immunohistochemistry and microscopy. Formalin-fixed, paraffin-embedded tissue blocks were sectioned into 5 μm slices. After deparaffinization and rehydration, sections were pretreated with proteinase K solution and then blocked with 10% donkey serum in PBS for one hour at room temperature. The endogenous peroxidase activity was blocked with 3% H_2O_2. The primary antibodies against CCN1 (H-78; SantaCruz Biotechnology #sc-13100) and against CCN2 (L-20; SantaCruz Biotechnology #sc-14939) were applied in a concentration of 1:100 dissolved in phosphate buffered saline (PBS) for 90 minutes at room temperature. Then slides were incubated with proper secondary antibodies (Serotec Star54 over anti-CCN1 antibody and Serotec Star88 over anti-CCN2 antibody) conjugated with horseradish peroxidase at a concentration of 1:100 for one hour at room temperature. Next, sections were rinsed with PBS and incubated with DAB substrate (DAB Peroxidase Substrate Kit, Vector Laboratories) for ten minutes. Nuclei were counterstained with hematoxylin. Negative control of staining was performed on the corresponding sections by omitting the primary antibodies.

Protein level was assessed using the semi-quantitative scale, where '+' meant the lowest visible color reaction and '+ + + +' the highest color reaction noted.

Estimation of CCN1 and CCN2 abundance in human right atrial appendages. Specimens of the right atrial appendage were harvested from 11 patients undergoing cardiac surgery at the beginning of the operative procedure. Seven patients were operated because of stable ischemic heart disease, one due to mitral valve insufficiency, and the remaining three patients suffered from aortic valve disease. None of the patients had been treated with inhibitor of angiotensin converting enzyme or AT1 receptor blocker for at least one month before the operation. Tissue samples were snap frozen in liquid nitrogen and stored at –80°C. Then samples were homogenized in ice-cold RIPA buffer (Sigma) with the addition of protease inhibitors (Protease Inhibitor Cocktail for Mammalian Tissues, Sigma). Protein concentration in each sample was determined using the Bradford method (Bio-Rad) [8] and 25 μg of protein per lane was loaded for electrophoresis. The CCN1 and CCN2 protein levels were estimated using the Western blot method as described previously [9] and their relative abundances were adjusted for β-actin (Sigma, #A5316). Ejection fraction of the left ventricle, assessed by echocardiography according to Simpson's method, was used as a measure of left ventricular systolic function.

Statistical analysis of the correlations between protein abundance and ejection fraction was performed using a linear regression model with Statistica 8.0 PL software (StatSoft Polska).

All experimental procedures were carried out according to the European Council Directive of 24 November 1986 (6/609/EEC) and were approved by the Local Ethics Committee in Bialystok.

Results

In the atria of the sham-operated animals, the expression of both CCN1 and CCN2 proteins was present. CCN1 showed a diffused cytoplasmatic pattern of staining, but was non-uniform among different regions of the atrial wall. A higher reaction was noted in the subepicardial region of the muscle. CCN2 also showed non-uniform expression within the atrial wall, with regions of stronger and weaker reaction. In contrast to the CCN1 staining, which was present in the whole cytoplasm of the cell, CCN2 was localized mainly in the cytoplasm in the direct proximity of the nucleus, with a relatively low level in the subplasmalemmal portion. In animals after myocardial infarction that were not treated with telmisartan, staining for CCN1 revealed evident stronger color reaction, while CCN2 was only slightly increased with more diffused cytoplasmic staining. Telmisartan prevented upregulation of CCN1 protein after infarction, and caused lowering of CCN2 expression after infarction below the level observed in the sham-operated animals. Table 1 presents the results of the semiquantitative analysis of protein expression, and representative microphotographs are presented in Figure 1. Evaluation of the CCN1 and CCN2 proteins in human right atrial appendage specimens revealed an inverse relation between the abundance of CCN proteins and the left ventricular ejection fraction, although the negative correlation was not statistically significant (Figure 2).

Discussion

To the best of our knowledge, this is the first report regarding the expression of CCN1 in the murine atria in basal conditions and in chronic heart failure due

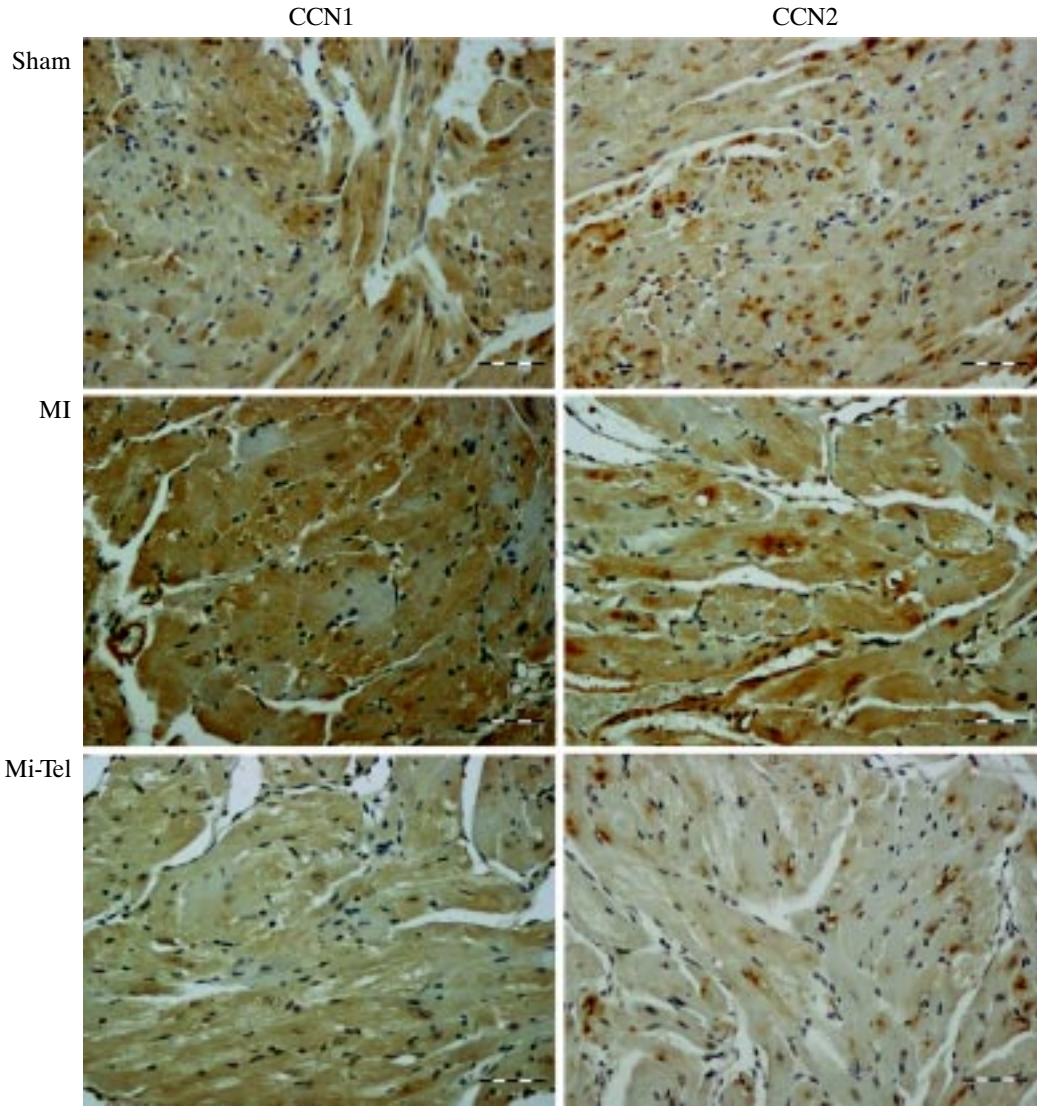

Figure 1. Representative immunohistochemical staining for detection of CCN1 and CCN2 proteins in the murine atria of each group. Nuclei are counterstained with hematoxylin. Magnification × 200 (bar = 50 μm)

Table 1. Expression of the CCN1 and CCN2 proteins in the atria of mice with chronic occlusion of the left coronary artery

Protein	Group		
	Sham	MI	MI-Tel
CCN1	+ + +	+ + + +	+
CCN2	+ + +	+ + +	+ +

The level of protein was assessed visually and is expressed in a semiquantitative scale from +, which was the minimal intensity of the color reaction, to + + + +, which corresponded to maximal staining, spanning most areas of the section

to myocardial infarction. In the human atria, elevation of atrial CCN1 has previously been described in patients subjected to cardiopulmonary bypass grafting [10]. Hilfiker-Kleiner et al. [3] described an elevated level of this protein in the ventricular myocardium early after myocardial infarction in mice. Upregulation of this protein was dependent on AT1 receptor stimulation, and protein kinase C or MEK/ /extracellular-regulated kinases inhibition were sufficient to completely abolish CCN1 overexpression. The same authors found CCN1 to be elevated in the ventricular cardiomyocytes of patients with end-stage ischemic cardiomyopathy, but not in non-failing human hearts. Our observations suggest upregulation of CCN1 also in the atria of hearts with depressed systolic left ventricular function.

CCN1 is believed to exert a beneficial effect on the myocardium. It promotes angiogenesis and protects cardiomyocytes from apoptosis after oxidative injury [11]. Thus, elevation of CCN1 seems to be a defensive mechanism against unfavorable influences. Our data is consistent with previous observations regarding this protein [3, 10]. In the experimental model, we found elevated expression of CCN1 that seems to be at least partially related to stimulation of the AT1 receptor.

CCN2 protein has been previously reported to be expressed in the mouse as well as in human atria [12, 13]. In human atrial fibrillation, CCN2 is upregulated and influences the expression of connexin 43 or N-cadherin in the atrial cells [13]. It has been previously postulated that CCN2 promotes fibroblast proliferation and hypertrophy of cardiac myocytes *in vitro* and *in vivo* [14, 15]. In the rat model of myocardial infarction, CCN2 was shown to be involved in fibrosis and its expression increased in the viable myocardium of the left ventricle late after the index event [16]. In the ventricular myocardium of the human failing heart, an increased CCN2 expression was observed, which correlated well with the abundance of the connective tissue within the ventricular cardiac

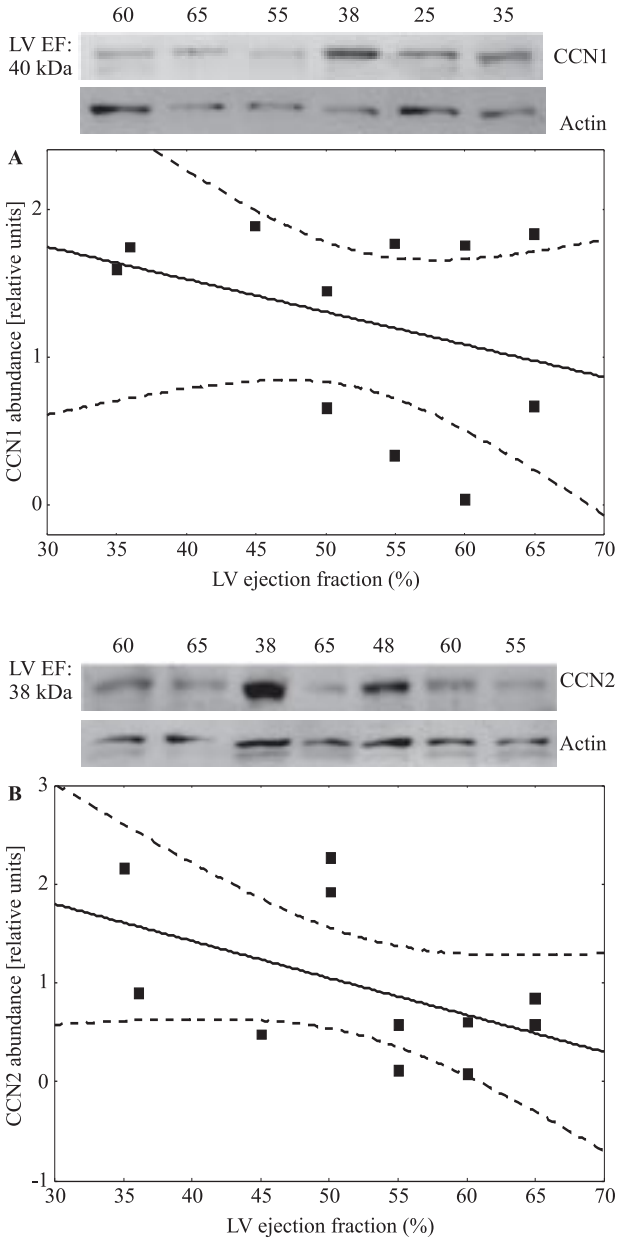

Figure 2. Relations between the abundance of CCN1 and CCN2 protein in the human right atrium and the left ventricular ejection fraction. Negative, but not statistically significant, relation between levels of both proteins and contractile performance of the left ventricle was observed. Dashed line delineates 95% confidence interval. Respective examples of representative Western blots are presented above each diagram

muscle [17], and our data suggests that similar CCN2 upregulation is present in the atria. In the ventricular myocardium, CCN2 was recently reported to exert a protective role in ischemic cardiac failure. The mechanism proposed includes CCN2 dependent upregulation of GRK5 activity, through which higher β-adrenoreceptor desensitization may be achieved

early after cardiac injury [18]. The role of angiotensin II for CCN2 upregulation has been shown *in vitro* as well as *in vivo*. The addition of angiotensin II to fibroblast culture elevated CCN2 mRNA and protein [19]. Pressure overload of the left ventricle of the heart caused increased expression of CCN2 protein, that was abolished by olmesartan — another AT1 receptor antagonist [20].

Although this protein may be responsible for increased deposition of connective tissue also in the atrial myocardium, there has been no direct investigation targeting this specific issue to date. Our observations reveal that CCN2 is upregulated in the atria of a failing heart, and that treatment with AT1 receptor antagonist prevents this effect.

Conclusion

CCN1 and CCN2 proteins are constitutively expressed in the cardiomyocytes of murine atria and are upregulated during chronic heart failure induced by permanent coronary artery ligation. Overexpression of CCN1 and CCN2 proteins can be inhibited by AT1 receptor blockade, suggesting a role for angiotensin II in the promotion of synthesis of both proteins in the atria of a failing heart. The influence on atrial CCN proteins may contribute to a beneficial effect of angiotensin converting enzyme/angiotensin type 1 receptor antagonists in the prevention of atrial fibrillation in heart failure patients.

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