Pressure Overload-induced Right Ventricular Failure is Associated with Re-expression of Myocardial Tenascin-C and Elevated Plasma Tenascin-C Levels

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Key Words

Right ventricular remodeling • Monocrotaline • Right ventricular failure • Tenascin-C • Integrins • Matrix metalloproteinases

Abstract

Background: We investigated whether (1) monocrotaline(MCT)-induced right ventricular (RV) dilatation is associated with re-expression of myocardial tenascin-C (TNC), (2) elevated plasma TNC levels can be used as a marker of ventricular dilatation, and (3) MCT-induced RV dilatation is associated with alterations of other remodeling-related proteins. **Methods:** Rats were treated with MCT in low dose (30 mg/kg, MCT30, n=10) to induce compensated RV hypertrophy, in high dose (80 mg/kg, MCT80, n=11) to induce RV failure, and with saline as control (CONT, n=9). After 4 weeks, RV function was assessed. TNC gene expression, protein levels, and plasma levels were assayed. Myocardial gene expression of integrin subunit $\alpha 6$ and $\beta 1$, and myocardial proMMP2 and proMMP9 levels were assessed. **Results:** TNC gene expression in the RV of MCT80 rats was significantly upregulated compared with RV of CONT (4-fold, $p < 0.001$) and MCT30 rats (3-fold, $p < 0.01$), whereas TNC gene expression was very low in LV and IVS of MCT80 rats, and absent in

those of CONT and MCT30 rats. Myocardial TNC protein levels were only observed in MCT80 rats, and were higher in RV (0.62 ± 0.64 ng/mg) than in LV (0.21 ± 0.44 ng/mg) or IVS (0.21 ± 0.09 ng/mg) ($p < 0.01$, RV vs LV and IVS). Plasma levels of TNC were significantly elevated in MCT80 rats compared with CONT ($p < 0.05$). RV volumes correlated positively with TNC plasma levels and RV ejection fraction correlated negatively with TNC plasma levels. MCT-induced RV failure was also associated with a significant downregulation of integrin $\alpha 6$ gene expression (by 48%, $p < 0.01$). **Conclusions:** MCT-induced RV failure is associated with an upregulation of TNC gene expression, resulting in re-expression of myocardial TNC protein levels, and elevated TNC plasma levels. As RV ejection fraction correlated significantly with TNC plasma levels, TNC plasma levels may serve as a marker of ventricular failure. De-adhesive properties of TNC in combination with a downregulation of integrin $\alpha 6$ may have caused cardiomyocyte slippage, leading to adverse ventricular remodeling.

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Introduction

Tenascin-C (TNC) is an extracellular matrix (ECM) glycoprotein that is highly expressed in embryonic tissue

during morphogenesis and sparsely in the adult. TNC is re-expressed during wound healing, inflammation, regeneration, and cancer invasion [1]. In the heart, TNC is primarily expressed during cardiogenesis but reappears in the adult myocardium under various pathological conditions, including acute myocardial infarction (AMI) [2], myocarditis [3], and dilated cardiomyopathy [4]. Elevated serum levels of TNC may reflect re-expression of TNC in myocardial tissue, and the specific pathological expression pattern of myocardial TNC suggests that plasma TNC levels may be useful as a marker of left ventricular remodeling in patients with AMI, dilated cardiomyopathy or heart failure [5-7].

The role of TNC in ventricular remodeling is complex. TNC is mainly synthesized by interstitial fibroblasts, and is upregulated by several factors, including growth factors, inflammatory cytokines, oxidative stress and mechanical stress [8-11]. Several studies suggested that TNC acts as an elastic ECM protein during mechanical stress [9, 12, 13]. Another important property of TNC is its ability to weaken the binding between cardiomyocytes and components of the ECM. Via this de-adhesive property, TNC facilitates cardiomyocytes to reorganize cell shape or arrangement in ventricular remodeling. TNC is able to bind to ECM components, as fibronectin [14], proteoglycans [15], and collagen [16], as well as to cellular integrins [17]. Interference of TNC with normal integrin-ECM interaction may also lead to cardiomyocyte slippage, ventricular dilatation and finally to impairment of cardiac function.

To investigate the role of TNC in right ventricular (RV) remodeling and dilatation we used the rat model of monocrotaline (MCT)-induced pulmonary hypertension. MCT is a toxin that selectively injures the vascular endothelium of the lung and leads to increased vascular resistance and increased pulmonary arterial pressure [18]. MCT-induced pulmonary hypertension is associated with the development of compensated RV hypertrophy that progresses to RV failure within weeks, depending on the dose of MCT [19]. Recently, we have demonstrated that MCT-induced RV pressure overload is associated with RV dilatation and an abnormal cardiac function that may be caused by cardiomyocyte slippage [20]. The purpose of the present study was to investigate whether (1) MCT-induced RV dilatation is associated with re-expression of myocardial TNC, (2) elevated TNC plasma levels can be used as marker of ventricular dilatation, and (3) MCT-induced RV dilatation is also associated with alterations of other remodeling-related proteins, such as integrin α 6, integrin β 1, MMP2, and MMP9.

Materials and Methods

Animals

All animals were treated in accordance with the national guidelines and with permission of the Animal Experiments Committee of the Leiden University Medical Center. A total of 30 male Wistar rats (200-250 g body wt; Harlan, Zeist, the Netherlands) were divided into three groups. The rats received a single subcutaneous injection of MCT (Sigma, Zwijndrecht, the Netherlands) diluted in phosphate-buffered saline (PBS) in a low dose (MCT30, 30 mg/kg body wt, n=10) or a high dose (MCT80, 80 mg/kg body wt, n=11). Control rats (CONT, n=9) were injected with an equal volume of PBS. After 4 weeks, RV function was assessed by a pressure-conductance catheter. Subsequently, rats were sacrificed and hearts and lungs were excised.

RV function

RV function was determined by recording ventricular pressure-volume relations as described previously [20]. Briefly, after anesthesia, a tracheotomy was performed, and rats were mechanically ventilated. Via a midline cervical incision the left jugular vein was cannulated for infusion of hypertonic saline (10%) to determine parallel conductance [21]. A midsternal thoracotomy was performed and a combined pressure-conductance catheter (SPR-878, Millar Instruments, Houston, TX, USA) was introduced via the apex into the RV, positioned along the long axis of the RV. The catheter was connected to a Sigma-SA signal processor (CD Leycom, Zoetermeer, The Netherlands) and RV pressures and volumes were recorded digitally. Calibration of the conductance catheter was performed by matching with cardiac output derived from an ultrasonic flow probe (Transonic Systems, Maastricht, The Netherlands) positioned around the ascending aorta. All data were acquired using Conduct-NT software (CD Leycom) at a sample rate of 2000 Hz and analyzed off-line by custom made software.

The hemodynamic data could be obtained in only 24 of the 30 animals. The remaining 6 animals (1 control and 5 MCT80 rats) died either shortly before the measurements (n=2, both MCT80 rats), during induction of anesthesia (n=2, both MCT80 rats), or during instrumentation (n=2, one CONT and one MCT80 rat).

Sample preparation

A) Blood samples. Four weeks after MCT-treatment, blood samples were collected in EDTA-containing tubes, and centrifuged (3000 x g). Plasma samples were stored at -80°C.

B) Tissue samples. After four weeks of MCT-treatment, heart and lungs were rapidly dissected and weighed. The right ventricle (RV), left ventricle (LV) and interventricular septum (IVS) were separated, weighed, and cut into four transmural blocks each. Two blocks were snap-frozen in liquid nitrogen and stored at -80°C for biochemical analysis. One block was stored in RNeasy lysis buffer (Qiagen, Crawley, UK) at 4°C for RNA isolation. The remaining block was fixed in 4% formalin for at least 24 h and embedded in paraffin for histochemical analysis. Myocardial samples for biochemical analysis were freeze-dried, and homogenized in homogenizing buffer containing 0.1 M

Tris-HCl and 0.1% (v/v) Tween-20, pH 7.5, using a Potter tube (Kimble/Kontes). Homogenates were stored in aliquots at -80°C. Protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Pierce, Etten-Leur, the Netherlands).

Total RNA isolation and semi-quantitative PCR

RNA was isolated from RV, LV and IVS of the three experimental groups, using TRIzol (Invitrogen, Breda, the Netherlands) according to the manufacturer's protocol. Total RNA was measured by A_{260} and the A_{260}/A_{280} ratio was used to check for contaminations. RNA samples were stored at -20°C before use. Following DNase treatment (Invitrogen), cDNA was synthesized using superscript II reverse transcriptase (Invitrogen) and random primers (Invitrogen). Semi-quantitative PCR was performed using primers (Invitrogen) specific for TNC, atrial natriuretic factor (ANF), integrin subunit $\alpha 6$ and integrin subunit $\beta 1$. A competitive PCR was performed on the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to correct for differences in cDNA concentrations. The following primers and annealing temperatures were used: GAPDH sense: GGT CGG TGT GAA CGG ATT TG; GAPDH antisense: GTG AGC CCC AGC CTT CTC CAT, 60°C; TNC sense: CGA CAG TTT TGT TAT CAG GAT CAG; TNC antisense: GGC ACA TAA GTA ATC CGG AAAT, 60°C; ANF sense: GGG GGT AGG ATT GAC AGG ATT; ANF antisense: TGG GTG GTG CTG AAG TTT ATT, 60°C; integrin $\alpha 6$ sense: GTG AGG GCG TCC ATA GAT GT, integrin $\alpha 6$ antisense: TGT GGT AGG TGG CAT CGT AA, 60°C; integrin $\beta 1$ sense: GGA GAA CCA CAG AAG TTT ACA, integrin $\beta 1$ antisense: GAA GTC TGA AGT AAT CCT CCT, 55°C. After agarose gel electrophoresis, PCR products were quantified by Scion image analysis software (Scion Corporation, Frederick, MD, USA).

ELISA

A) *Tenascin-C*. TNC high molecular weight variant concentrations in myocardial homogenates and plasma samples were determined by a two-sided ELISA (ImmunoBiological Laboratories, Hamburg, Germany). TNC standards and samples were incubated in anti-human TNC antibody (clone 19C4MS) precoated 96-well plates at 37°C for 1 h and washed. After incubation with mouse HRP-conjugated anti-human TNC antibody (clone 4F10TT) for 30 min at 4°C, plates were washed, incubated with TMB/H₂O₂ and read at 630 nm. Detection limit of the assay was 0.01 ng/mL. Intra- and inter-assay variations were 6% and 5%, respectively.

B) *proMMP-2(TIMP-2)*. Concentrations of free proMMP2 and proMMP2 complexed to TIMP-2 in myocardial homogenates and plasma samples were determined by a precoated two-sided ELISA (GE Healthcare Europe, Diegem, Belgium). ProMMP-2 standards and samples were incubated for 2 h at 25°C in anti-MMP-2 antibody precoated 96-wells plates and washed. After incubation with HRP-conjugated anti-MMP-2 for 1 h, plates were washed, incubated with TMB/H₂O₂ and read at 630 nm. Detection limit for the assay was 0.37 ng/mL. The inter-assay variability was 9% and 12% at concentrations of 9.9 ng/mL and 2.4 ng/mL and the intra-assay variability was 6% at both high and low proMMP-2(TIMP)

concentrations.

Western Blotting

Myocardial homogenates and plasma samples were size-fractionated on NuPage Novex 3-8% Tris-Acetate NuPage gels (TNC) or on NuPage 12% Bis-Tris gels (MMP9) (Invitrogen), and transferred to Hybond PVDF membranes (GE Healthcare). After blocking non-specific binding sites, membranes were incubated for 1 h with the anti-TNC antibody conjugated to HRP (4F10TT, ImmunoBiological Laboratories, Hamburg, Germany) or were incubated for 1 h with an anti-MMP9 antibody (Sigma, Zwijndrecht, the Netherlands) and with a horseradish peroxidase(HRP)-labelled secondary antibody (rabbit anti-goat IgG, Santa Cruz Technology, Heidelberg, Germany) for another hour. A competitive Western blot for GAPDH was performed to correct for differences in protein concentrations. Chemiluminescence was induced by ECL Advance Detection reagent (GE Healthcare) and detected by exposure to Hyperfilm ECL (GE Healthcare). The intensity of the proMMP9 and GAPDH bands were quantified by Scion image analysis software (Scion Corporation, Frederick, MD, USA)

Histochemistry

After fixation, heart tissue samples were embedded in paraffin and tissue was cut into 4- μ m thick sections. Tissue sections were dried, deparaffinized in Ultraclear (Klinipath, Duiven, the Netherlands) and rehydrated in decreasing graded alcohols (100-25%).

To study the distribution of myocardial TNC, sections were exposed to immunohistochemistry. Endogenous peroxidase activity was inhibited in 0.3% hydrogen peroxide in PBS and sections were incubated in citrate buffer at 97°C for 10 min to retrieve antigens. Tissue sections were then incubated for 3 h at room temperature with the HRP-conjugated anti-TNC antibody (4F10TT, ImmunoBiological Laboratory, Hamburg, Germany) and TNC was visualized using diaminobenzidine (DAB). Nuclei were counterstained using hematoxylin.

Statistical analysis

The effect of treatment (CONT, MCT30 or MCT80) was evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. Correlations were determined using a parametric test (Pearson). Differences were considered significant at $p < 0.05$. SPSS11 for Windows (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. Data are presented as means $\pm$ SD.

Results

Monocrotaline-induced pulmonary hypertension

Table 1 summarizes the general and cardiac characteristics of the rats at the day they were sacrificed. After 4 weeks of MCT treatment, no signs of heart failure were observed in MCT30 rats and also body weights of these animals were not significantly different from those

Table 1. General and cardiac characteristics of the three groups of animals. Indicated are mean values $\pm$ SD. Abbreviations; RV, right ventricle; LV, left ventricle; IVS, interventricular septum. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. CONT, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. MCT30.

	CONT (n=9)	MCT30 (n=10)	MCT80 (n=11)
Body wt (g)	383 $\pm$ 33	360 $\pm$ 20	292 $\pm$ 31***,###
RV wt (g)	0.23 $\pm$ 0.04	0.26 $\pm$ 0.04	0.34 $\pm$ 0.05***,##
LV wt (g)	0.48 $\pm$ 0.07	0.50 $\pm$ 0.03	0.45 $\pm$ 0.06
IVS wt (g)	0.32 $\pm$ 0.05	0.28 $\pm$ 0.04	0.27 $\pm$ 0.03
RV wt / (LV+IVS) wt	0.29 $\pm$ 0.05	0.33 $\pm$ 0.04	0.48 $\pm$ 0.11***,##
RV wt / body wt (mg/g)	0.61 $\pm$ 0.09	0.72 $\pm$ 0.11	1.17 $\pm$ 0.17***,##

of CONT rats. However, MCT80 rats showed an obvious lack of physical activity, increased respiratory rate and reduced body weights (by 24% compared to CONT and by 19% compared to MCT30 rats, both $p < 0.001$). The degree of hypertrophy, determined as the ratio of RV weight over the sum of LV and IVS weights, tended to increase in MCT30 by 14%, and was significantly higher in MCT80 rats by 66% and 45% compared to CONT and MCT30 rats, respectively (both $p < 0.001$).

RV function

Table 2 summarizes the RV function characteristics in CONT, MCT30 and MCT80 rats. As expected, RV systolic pressures were significantly increased (approximately +80% compared to CONT) in the MCT80 rats, whereas in the MCT30 group the increases of RV systolic pressure were less pronounced and did not reach statistical significance. Cardiac output was unchanged in MCT30 rats and were slightly reduced in MCT80 rats (by 27% compared to CONT, n.s., and by 31% compared to MCT30, $p < 0.05$). RV volumes were significantly increased in the MCT30 and MCT80 groups. Ejection fraction indicated a significantly reduced systolic function in both MCT-treated groups.

Tenascin-C

A) Myocardial tenascin-C gene expression. Very low levels of TNC gene expression were detected in the RV of some CONT and MCT30 rats, but in the majority the expression was undetectable. In contrast, TNC gene expression was considerably upregulated in RV of all MCT80 rats as compared to CONT and MCT30 rats (Fig. 1a).

After quantification of signal intensities and normalization for GAPDH gene expression, a highly significant upregulation of TNC gene expression was demonstrated in RV of MCT80 rats as compared to RV of CONT (4-fold, $p < 0.001$) and MCT30 (3-fold, $p < 0.01$) rats (Fig. 1b). TNC gene expression could not be detected in the LV and IVS of CONT and MCT30 rats, whereas the LV and IVS of MCT80 rats showed low levels of TNC gene expression (Fig. 1b).

Fig. 1. Tenascin-C gene expression detected by PCR. Semi-quantitative PCR was performed to assess GAPDH and tenascin-C gene expression in the RV, LV and IVS of the three experimental groups. (a) Representative PCR of GAPDH and TNC gene expression in the RV of CONT, MCT30 and MCT80 rats. Lane 1: Marker; lane 2-6: CONT; lane 7-11: MCT30; lane 12-16: MCT80; lane 17: negative control. (b) Quantitative analysis of TNC gene expression in the RV of CONT (n=5), MCT30 (n=5) and MCT80 rats (n=5), in the LV of CONT (n=6), MCT30 (n=6) and MCT80 rats (n=5) and in the IVS of CONT (n=2), MCT30 (n=4) and MCT80 rats (n=4). Indicated are means $\pm$ SD. * $p < 0.001$ vs CONT and # $p < 0.01$ vs MCT30.

Fig. 2. Myocardial tenascin-C isoforms detected by Western Blotting in RV, LV and IVS of the three experimental groups.

B) Re-expression of myocardial TNC protein levels. To investigate whether upregulation of TNC gene expression resulted in re-expression of TNC protein levels, myocardial TNC levels were determined by Western Blot

Table 2. Cardiac and RV function of the three groups of animals. Indicated are mean values $\pm$ SD. Abbreviations; HR, heart rate; CO, cardiac output; V_{ES} , RV end-systolic volume; V_{ED} , RV end-diastolic volume; P_{PEAK} , RV peak pressure; P_{ES} , RV end-systolic pressure; P_{ED} , RV end-diastolic pressure; EF, RV ejection fraction. * $p < 0.05$, ** $p < 0.01$ vs. CONT # $p < 0.05$ vs. MCT30.

	CONT (n=8)	MCT30 (n=10)	MCT80 (n=6)
HR (beats/min)	393 $\pm$ 26	371 $\pm$ 55	350 $\pm$ 34*
CO (mL/min)	94 $\pm$ 26	100 $\pm$ 25	69 $\pm$ 19 [#]
V_{ES} (μ L)	284 $\pm$ 82	553 $\pm$ 252*	576 $\pm$ 236*
V_{ED} (μ L)	493 $\pm$ 145	791 $\pm$ 298*	758 $\pm$ 229*
P_{PEAK} (mmHg)	32 $\pm$ 5	45 $\pm$ 17	57 $\pm$ 21*
P_{ES} (mmHg)	28 $\pm$ 5	38 $\pm$ 16	51 $\pm$ 17*
P_{ED} (mmHg)	4.7 $\pm$ 2.6	7.1 $\pm$ 2.2	4.3 $\pm$ 1.3
EF (%)	49 $\pm$ 6	36 $\pm$ 7*	28 $\pm$ 5** [#]

	CONT (n)	MCT30 (n)	MCT80 (n)
RV TNC (ng/mg tissue)	0.00 $\pm$ 0.00 (9)	0.00 $\pm$ 0.01 (10)	0.62 $\pm$ 0.64** ^{###} (10)
proMMP2 (ng/mg tissue)	0.51 $\pm$ 0.12 (9)	0.41 $\pm$ 0.08 (10)	0.28 $\pm$ 0.13*** [#] (10)
proMMP9 (%)	100 $\pm$ 6 (3)	127 $\pm$ 15* (4)	168 $\pm$ 50 (5)
LV TNC (ng/mg tissue)	0.01 $\pm$ 0.02 (9)	0.02 $\pm$ 0.08 (10)	0.21 $\pm$ 0.44 (10)
proMMP2 (ng/mg tissue)	0.46 $\pm$ 0.21 (9)	0.54 $\pm$ 0.35 (10)	0.32 $\pm$ 0.06 (10)
IVS TNC (ng/mg tissue)	0.00 $\pm$ 0.00 (8)	0.00 $\pm$ 0.00 (7)	0.21 $\pm$ 0.09*** ^{###} (9)
proMMP2 (ng/mg tissue)	0.27 $\pm$ 0.10 (7)	0.38 $\pm$ 0.25 (9)	0.32 $\pm$ 0.07 (9)

Table 3. Myocardial tenascin-C (TNC), promatrix metalloproteinase-2 (proMMP2) and proMMP9 levels in RV, LV and IVS of the three groups of animals after 4 weeks of treatment. Indicated are mean values $\pm$ SD (number). Abbreviations; RV, right ventricle; LV, left ventricle; IVS, interventricular septum. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. CONT. [#] $p < 0.01$ and ^{###} $p < 0.001$ vs. MCT30.

and ELISA (Fig. 2 and Table 3, respectively). Myocardial TNC in the heart tissue of CONT and MCT30 rats was undetected by Western Blot (Fig. 2). However, in the RV, LV, and IVS of MCT80 rats, three TNC bands were identified at 250, 200 and 80 kDa, representing two different TNC isoforms (250 and 200 kDa) and probably a TNC degradation product (80 kDa) in heart tissue [22]. In the RV of MCT80 rats, all TNC band intensities were approximately 2-3 times higher compared with corresponding levels in LV and IVS of MCT80 rats.

Similar results were found by ELISA. Myocardial TNC levels were hardly detected in heart tissue of CONT and MCT30, but were strongly elevated in that of MCT80 rats (Table 3). In these rats, TNC was re-expressed in the RV, LV and IVS, and was approximately 3 times higher in the RV than in the LV and IVS.

C) Distribution of TNC. In heart tissue of CONT and MCT30 rats, TNC was undetected by immunohistochemistry (data not shown). Images of transversal and longitudinal tissue sections of RV tissue of MCT80 rats clearly demonstrate that TNC was located in the extracellular space, exclusively (Fig. 3).

D) Plasma levels of TNC. Four weeks after MCT-treatment, plasma TNC levels were significantly higher (by 54%, $p < 0.05$) in MCT80 rats than those in CONT rats ($p < 0.05$), indicating that plasma levels of TNC may reflect re-expression of TNC in failing

	CONT (n)	MCT30 (n)	MCT80 (n)
TNC (ng/mL)	24 $\pm$ 9 (9)	31 $\pm$ 5 (10)	37 $\pm$ 16* (9)
proMMP2 (ng/mL)	14 $\pm$ 14 (4)	18 $\pm$ 8 (4)	13 $\pm$ 9 (4)

Table 4. Plasma tenascin-C (TNC) and promatrix metalloproteinase-2 (proMMP2) levels in the three groups of animals after 4 weeks of treatment. Indicated are mean values $\pm$ SD (number). * $p < 0.05$ vs CONT.

myocardium (Table 4).

E) Correlation of plasma TNC levels with RV function. RV end-systolic and end-diastolic volumes were correlated positively with plasma levels of TNC (Fig. 4a and 4b). RV ejection fraction was correlated negatively with plasma TNC levels (Fig. 4c). These results indicate that accumulation of TNC in plasma of MCT-treated rats is associated with RV failure. To test the diagnostic accuracy of plasma TNC we performed a receiver-operating characteristics (ROC) curve analysis. Results indicated that a cut-off value of 31.3 ng/mL detected RVEF below 40% with a sensitivity of 84.6% and a specificity of 72.7% (Fig. 5).

Expression and release of other remodeling-related proteins

A) ANF gene expression. As expected, ANF gene expression in the RV of MCT80 rats was significantly upregulated compared to CONT (≈ 3 -fold, $p < 0.001$) and

Fig. 3. Distribution of myocardial TNC in the RV of MCT80 rats by immunohistochemistry (a) overview of RV tissue, 200x, (b) transverse section of RV tissue, 1000x, (c and d) longitudinal sections of RV tissue, 1000x.

MCT30 rats (≈ 2 -fold, $p < 0.001$) (Table 5), whereas ANF gene expression in the LV of MCT-treated rats remained unchanged.

B) Integrin gene expression. Integrin $\alpha 6$ gene expression in the RV of MCT80 rats was significantly downregulated by 48% compared to CONT ($p < 0.01$) and by 35% compared to MCT30 rats (80%) ($p < 0.05$) (Table 5), whereas integrin $\alpha 6$ gene expression in the LV of MCT-treated rats was unchanged. Also integrin $\beta 1$ gene expression levels tended to be downregulated (by 34%) in RV of MCT80 rats, but these results were not significant (Table 5).

C) Myocardial levels of proMMP2. In the RV of MCT80 rats, proMMP2 levels were significantly reduced by 45% compared to CONT ($p < 0.05$) and by 32% compared to MCT30 rats ($p < 0.05$) (Table 3), whereas in both the LV and IVS, no significant changes of proMMP2 levels were observed among the three groups.

D) Plasma levels of proMMP2. Plasma concentrations of proMMP2 were not significantly different between the three groups, suggesting that there are no differences in the release kinetics of proMMP2 between CONT and MCT-treated rats (Table 4).

E) Myocardial levels of proMMP9. In contrast to proMMP2, RV levels of proMMP9 were significantly higher in MCT30 rats than in CONT rats (by 27%, $p < 0.05$) and tended to be increased by 68% in MCT80 rats compared to CONT rats, but this increase just failed to reach statistical significance ($p = 0.06$) (Table 3).

Fig. 4. RV end-systolic volume (a) RV end-diastolic volume (b), and RV ejection fraction (c) are plotted against plasma TNC levels in CONT (black), MCT30 (grey) and MCT80 (white) rats.

Fig. 5. Receiver-operating characteristics (ROC) curve.

Table 5. Gene expression of atrial natriuretic factor (ANF), integrin α_6 and integrin β_1 in RV and LV of the three groups of animals. Indicated are mean values $\pm$ SD (number). Abbreviations; RV, right ventricle; LV, left ventricle. ** $p < 0.01$ vs. CONT. # $p < 0.05$ and ## $p < 0.01$ vs. MCT30.

		CONT (n)	MCT30 (n)	MCT80 (n)
RV	ANF (%)	100 $\pm$ 53 (4)	164 $\pm$ 36 (4)	281 $\pm$ 52**## (5)
	Integrin α_6 (%)	100 (2)	80 $\pm$ 17 (4)	52 $\pm$ 5*** (4)
	Integrin β_1 (%)	100 $\pm$ 32 (3)	72 $\pm$ 20 (4)	66 $\pm$ 20 (5)
LV	ANF (%)	100 $\pm$ 21 (3)	103 $\pm$ 25 (4)	140 $\pm$ 27 (4)
	Integrin α_6 (%)	100 $\pm$ 30 (3)	90 $\pm$ 16 (4)	93 $\pm$ 47 (4)
	Integrin β_1 (%)	100 $\pm$ 27 (3)	82 $\pm$ 21 (4)	107 $\pm$ 71 (4)

Discussion

The main findings of the current study can be summarized as follows. MCT-treatment induced a dose-dependent pulmonary hypertension, leading to progressive RV dysfunction and RV dilatation. MCT-induced RV dilatation in rats with heart failure (MCT80) was associated with a significant upregulation of TNC gene expression, resulting in re-expression of myocardial TNC protein levels and elevated TNC plasma levels. RV volumes and RV ejection fraction were correlated with plasma levels of TNC, indicating that plasma TNC can be used as a marker of ventricular failure. In addition to the re-expression of TNC, MCT-induced RV failure was also associated with a significant downregulation of integrin α_6 gene expression (to 52%) and a significant reduction of myocardial MMP2 ($p < 0.05$).

Re-expression and release of TNC in ventricular failure

Changes in expression levels of cardiac proteins during the development of heart failure may provide insight in the mechanisms of cardiac remodeling and may lead to new plasma markers of cardiac remodeling. The results of the present study support the hypothesis that TNC reappears during ventricular remodeling and contributes to cardiomyocyte slippage, ventricular dilatation, and impairment of heart function.

In CONT and MCT30 rats having a normal or compensated cardiac function, respectively, we could not demonstrate any TNC gene expression or re-expression of myocardial TNC protein levels in the LV and IVS. Only a very weak TNC gene expression was demonstrated in the RV of a few CONT and MCT30 rats, but this weak TNC gene expression was not translated to myocardial TNC protein levels.

In MCT80 rats, TNC gene expression was evidently upregulated in the RV, and slightly upregulated in the LV and IVS. This upregulation resulted in re-expression of myocardial TNC protein levels, which were 2-3 times higher in the RV compared to the LV and IVS. These results indicate that TNC reappears in heart tissue of

rats with pulmonary hypertension-induced RV failure, most abundantly in the RV.

Numerous factors may be responsible for the increased expression of TNC, including growth factors, inflammatory cytokines, oxidative stress and mechanical stress [8-11, 23]. The TNC gene promoter contains a mechano-responsive element [8] and several studies demonstrated an increased TNC expression mediated by mechanical load [8, 9, 12, 24, 25], suggesting that increased mechanical stretch in MCT-treated rats may be responsible for the induction of TNC re-expression. Nevertheless, mechanical stretch cannot be completely responsible for the re-expression of TNC in the myocardium of MCT80 rats because myocardial TNC concentrations are also elevated in the IVS and LV of these rats. Although the IVS is part of the RV, the LV in MCT-treated rats is not exposed to pressure-overload or enhanced mechanical stretch [26], indicating that TNC re-expression in the LV of MCT80 rats is dependent upon another factor than mechanical stress. Angiotensin-II, inflammatory cytokines, and growth factors are also known to stimulate TNC synthesis [27, 28]. In MCT-induced pulmonary hypertension enhanced neuroendocrine stimulation causes elevated plasma levels of catecholamines, cytokines and angiotensin II [26, 29], and may lead to elevated levels of myocardial TNC in MCT80 rats.

In addition, the RV of MCT30 and MCT80 rats are both exposed to relatively high levels of volume overload (Table 2) whereas TNC is selectively re-expressed in the RV of MCT80 rats. Jarvinen et al. [9] suggested that mechanical loading itself can not induce the *de novo* expression of TNC in skeletal muscle unless tissue damage, i.e. disruption of mechanical integrity of the muscle fibers, has taken place. In the present study we demonstrate a similar phenomenon in the myocardium as Jarvinen et al. [9] observed in skeletal muscle. Mechanical load itself may not be responsible for *de novo* synthesis of TNC because TNC is only re-expressed in the RV of MCT80 rats with RV failure and not in the RV of MCT30 rats with a compensated function. Re-expression of TNC in MCT80 rats may be caused by mechanical load in

combination with myocardial damage in RV failure. Many studies reported that mechanical load is an important regulator of TNC expression but these studies were done in tissue that already express TNC. Mechanical load may not be responsible for the induction of *de novo* TNC expression (or TNC re-expression) in adult muscle tissue that normally does not express TNC. *De novo* synthesis of TNC may be related to tissue injury such as occurs in myocardial infarction, wound healing, cardiac inflammation, and heart failure. The exact mechanism for TNC re-expression under pathologic conditions such as in MCT-induced RV failure is still unknown.

In the adult heart, TNC reappears under pathological conditions, including AMI [2, 30], myocarditis [3, 31], and dilated cardiomyopathy [4]. Recently, Terasaki et al. [7] have reported that higher serum levels of TNC reflect the severity of heart failure, LV dysfunction and LV remodeling in patients with dilated cardiomyopathy. In addition, we recently showed that *reverse* ventricular remodeling in response to cardiac resynchronization therapy in heart failure patients was associated with significantly decreased TNC serum levels [5]. This patient study indicates that TNC is also useful to evaluate the response to cardiac resynchronization therapy (CRT) in patients with heart failure. In the present study we showed that re-expression of myocardial TNC levels in MCT rats with RV failure was associated with elevated TNC plasma levels. We demonstrated that RV end-systolic volume, RV end-diastolic volume and RV ejection fraction were correlated with plasma TNC levels. No significant correlation was observed between plasma TNC levels and RV peak pressure nor RV end-systolic pressure (data not shown). RV end-systolic pressure is frequently used as a surrogate of pressure overload. The results of this study show that TNC is not a marker of pressure overload *per se* but indicate that plasma TNC is a marker of pressure overload-induced RV failure. Receiver-operating characteristics curve analysis revealed that plasma TNC levels > 31.3 ng/mL can detect a RVEF < 40% with a sensitivity of 84.6% and a specificity of 72.7%.

Expression and release of other remodeling-related proteins

For efficient function the heart requires a mechanical linkage that provides the transmission of contraction forces from sarcomeres to the ECM and that prevents slippage of cardiomyocytes during contraction. Cardiomyocytes adhere to the ECM at transmembrane adhesion complexes, called costameres [32]. These costamere complexes form a mechanical linkage, extending from

the sarcomeres, through the plasma membrane, to the ECM. They contain vinculin, talin, $\alpha 6 \beta 1$ integrin, laminin and several other extracellular and intracellular components [32, 33]. TNC has been shown to modulate cellular contacts with the ECM by interactions with ECM components, such as fibronectin and collagen, as well as with several cellular integrin isoforms [17, 34]. Imanaka-Yoshida et al. [2] have demonstrated that TNC modulates the cell-ECM attachment by inhibition of the formation of costameric adhesion complexes. In this study we were not able to investigate a causal relationship between TNC and integrins but we studied whether MCT-induced RV failure is associated with changes in $\alpha 6$ and $\beta 1$ integrin expression. In the RV of MCT80 rats we found a significant downregulation of $\alpha 6$ integrin gene expression, and a tendency of downregulation of $\beta 1$ gene expression. Downregulation of $\alpha 6$ integrins may be responsible for a reduction in functional costameric adhesion complexes. These results indicate that besides TNC re-expression, downregulation of $\alpha 6$ integrins contribute to cardiomyocyte slippage, ventricular dilatation, and impairment of cardiac function.

In the present study we also showed that RV failure in MCT80 rats is associated with a reduction in myocardial proMMP2 levels in RV but not in the LV and IVS of MCT80 rats. This reduction in myocardial levels of proMMP2 in the RV of MCT80 rats suggests that (1) the expression of myocardial proMMP2 has been downregulated, (2) myocardial proMMP2 has been converted to active MMP2, and/or (3) myocardial proMMP2 has been released into the circulation. However, plasma levels of proMMP2 were not significantly different among the three groups. Further research is needed to investigate whether myocardial proMMP2 in the RV of MCT-treated rats is downregulated or converted to active MMP2.

In contrast, levels of myocardial proMMP9 in the RV of MCT30 rats were significantly higher (by 27%) compared to CONT rats and levels of myocardial proMMP9 in the RV of MCT80 rats tended to be increased (by 68%) compared to CONT rats, but this increase did not reach statistical significance. However, these results suggest a different expression of proMMP2 and proMMP9 levels in the RV of MCT80 rats with RV failure. Nishikawa et al. [35] reported that MMP9 rather than MMP2 plays an important role in LV dilatation. We also found similar results in our previous studies with MCT-treated rats [36] and patients with heart failure [5]. In the RV of MCT80 rats with RV failure, the expression of MMP9 (2.9 fold compared to controls) was

significantly higher than the expression of MMP2 (1.8 fold compared to controls). Also in patients with heart failure, reverse left ventricular remodeling in response to cardiac resynchronization therapy was associated with reduced plasma MMP9 levels, whereas circulating levels of MMP2 levels remained unchanged. These studies indicate that MMP9 rather than MMP2 is important in the development of heart failure and were correlated with RV function. The expression and activation of MMPs is complex and further research is needed to investigate the mechanism of elevated MMP9 expression in MCT-rats. Some studies proposed that myocardial MMP9 becomes more relevant during cardiac inflammation due to an inducible expression of MMP9 in neutrophils and macrophages [37, 38]. Cardiac inflammation has been demonstrated to play a significant role in the development of heart failure [39, 40] and is associated with an increased infiltration of several inflammatory cells, including neutrophils and macrophages. Further research is needed to investigate the role of MMP9 in MCT-induced RV failure and whether there may be an interaction with TNC.

Conclusions

RV failure as a result of MCT-induced pulmonary hypertension is associated with upregulation of TNC gene expression, resulting in re-expression of myocardial TNC protein levels and elevated plasma TNC levels. RV

ejection fraction is correlated with TNC plasma levels, indicating that plasma TNC can be used as a marker of ventricular failure. The de-adhesive properties of TNC together with the downregulation of integrin $\alpha 6$ may cause cardiomyocyte slippage leading to ventricular dilatation and impairment of cardiac function.

Limitations of the study

In the present study we demonstrated a correlation between plasma TNC levels, RV volumes and RV ejection fraction, indicating that plasma TNC levels can be used as a marker of RV failure. Although we demonstrated that increased plasma levels of TNC were associated with myocardial TNC re-expression and correlated with RV function, we have no evidence that plasma TNC is exclusively derived from the heart. It may be possible that TNC is also re-expressed in other organs. For instance, as a result of lung fibrosis MCT-induced pulmonary hypertension is associated with structural changes in the pulmonary arteries and arterioles. Further research is necessary to investigate the contribution of TNC expression in other tissues than the heart.

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