Transforming Growth Factor β1-regulated Xylosyltransferase I Activity in Human Cardiac Fibroblasts and Its Impact for Myocardial Remodeling*

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In cardiac fibrosis remodeling of the failing myocardium is associated with a complex reorganization of the extracellular matrix (ECM). Xylosyltransferase I and Xylosyltransferase II (XT-I and XT-II) are the key enzymes in proteoglycan biosynthesis, which are an important fraction of the ECM. XT-I was shown to be a measure for the proteoglycan biosynthesis rate and a biochemical fibrosis marker. Here, we investigated the XT-I and XT-II expression in cardiac fibroblasts and in patients with dilated cardiomyopathy and compared our findings with nonfailing donor hearts. We analyzed XT-I and XT-II expression and the glycosaminoglycan (GAG) content in human cardiac fibroblasts incubated with transforming growth factor (TGF)-β1 or exposed to cyclic mechanical stretch. In vitro and in vivo no significant changes in the XT-II expression were detected. For XT-I we found an increased expression in parallel with an elevated chondroitin sulfate-GAG content after incubation with TGF-β1 and after mechanical stretch. XT-I expression and subsequently increased levels of GAGs could be reduced with neutralizing anti-TGF-β1 antibodies or by specific inhibition of the activin receptor-like kinase 5 or the p38 mitogen-activated protein kinase pathway. Usage of XT-I small interfering RNA could specifically block the increased XT-I expression under mechanical stress and resulted in a significantly reduced chondroitin sulfate-GAG content. In the left and right ventricular samples of dilated cardiomyopathy patients, our data show increased amounts of XT-I mRNA compared with nonfailing controls. Patients had raised levels of XT-I enzyme activity and an elevated proteoglycan content. Myocardial remodeling is characterized by increased XT-I expression and enhanced proteoglycan deposition. TGF-β1 and mechanical stress induce XT-I expression in cardiac fibroblasts and have impact for ECM remodeling in the dilated heart. Specific blocking of XT-I expression confirmed that XT-I catalyzes a rate-limiting step during fibrotic GAG biosynthesis.

Cardiac fibrosis is a process that is characterized by a massive remodeling of the myocardial extracellular matrix (ECM) and the subsequent substitution of the functional tissue by inelastic fibrotic tissue. These alterations lead to an impaired organ function and finally to chronic heart failure. Multiple factors that serve as triggers for this process have already been identified, including increased mechanical force and consequently elevated ventricular loading.

The fibrotic remodeling of cardiac tissue during dilated cardiomyopathy (DCM) is characterized by a severe disruption of the ECM homeostasis. Changes in the ratio of type I and type III collagen and an up-regulation of proteoglycan expression is a main characteristic for the progression of this myocardial failure (1–4). During the fibrotic remodeling of the ventricular tissue, increased levels of the proteoglycans decorin and biglycan were found, confirming the importance of these matrix components in this process (5–7).

In addition to collagen fibrils, proteoglycans are the major components of the myocardial ECM. These polyanionic glycoproteins are expressed in every tissue, serve a wide range of functions, and are crucially involved in many physiological and pathological processes. Proteoglycans comprise a core protein that is post-translationally modified by lateral glycosaminoglycan (GAG) chains. These chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, or heparin chains are responsible for the high degree of structural variety of the proteoglycans. Both decorin and biglycan belong to the group of small leucine-rich proteoglycans and share similar core proteins substituted at the amino terminus with one or two chondroitin sulfate or dermatan sulfate chains, respectively. Decorin was shown to stabilize collagen fibrils in the ECM and orientate fibrillogenesis (8–11). It is also able to bind TGF-β1 and therefore may act as a negative effector molecule regulating increased TGF-β1 activity during cardiac fibrosis (12, 13).

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2 The abbreviations used are: ECM, extracellular matrix; XT, xylosyltransferase; DCM, dilated cardiomyopathy; ALK, activin receptor-like kinase; GAG, glycosaminoglycan; siRNA, small interfering RNA; LV, left ventricle; HCF, human cardiac fibroblast; TGF, transforming growth factor; NF, nonfailing; CS, chondroitin sulfate; MAP, mitogen-activated protein; ColI1A1, type I collagen; β2m, β2-microglobulin.
Xylosyltransferase I in Cardiac Fibrosis

The interaction of the proteoglycans with a large variety of ligands is mediated by the polysulfated GAG chains, which are covalently attached to the core protein. These lateral chains determine the biophysical character of the proteoglycans and are mainly responsible for their biological function including cytokine binding, hydratative capacity, or cell-cell interaction. Thus, the biological activity of proteoglycans is closely related to the GAG biosynthesis (14).

The proteoglycan core protein is post-translationally modified by the addition of lateral GAG chains. The GAGs chondroitin sulfate, dermatan sulfate, heparan sulfate, and heparin are bound to the proteoglycan core protein by a xylose-galactose-galactose binding region. Xylosyltransferase I (XT-I) and xylosyltransferase II (XT-II) are the initiating and apparently rate-limiting enzymes involved in GAG biosynthesis catalyzing the transfer of UDP-xylose to specific serine residues of the core proteins (15–17). We have shown previously that the XT-I enzyme is secreted from the endoplasmic reticulum into the extracellular space together with chondroitin sulfate (CS) proteoglycans (18). Our group has demonstrated that the XT-I activity is a measure for the proteoglycan biosynthesis rate and is a reliable biochemical serum marker for the assessment of fibrotic processes in systemic sclerosis (18).

However, little is known regarding the role of both xylosyltransferases and their impact during the myocardial remodeling. Thus, in the present study we examined for the first time whether myocardial ECM remodeling has an effect on XT-I and XT-II expression and, consequently, on the GAG content in cardiac tissue.

DCM is characterized by an elevated ventricular loading, which also results in increased mechanical stress on the ventricular wall. As a simplified model for cardiac fibrosis in DCM, we used human cardiac fibroblasts (HCFs) subjected to cyclic mechanical stress to investigate the role of both XTs in cardiac ECM remodeling. TGF-β1 is known to be up-regulated under mechanical stress in cardiac fibroblasts where it was found to induce type I collagen (Col1A1) mRNA expression (19, 20).

We analyzed the role of TGF-β1 as a fibrotic cytokine in the transcriptional activation of XT-I and XT-II and determined the effect of an increased mechanical stress with elevated levels of TGF-β1 on XT expression and its effect on the GAG synthesis in HCFs. To determine the relevance of XTs during myocardial fibrosis in vivo, we analyzed the XT mRNA expression and the enzymatic XT-I activity in patients with DCM. This is the first report on XT-I and XT-II in myocardial fibrosis.

EXPERIMENTAL PROCEDURES

Preparation of Human Cardiac Fibroblasts—For the isolation of HCFs, myocardial samples of explanted failing human hearts and from atrial appendices taken at the time of cannulation during circulatory bypass were cut into small pieces and washed twice in phosphate-buffered saline. The tissue was digested at 37 °C for 90 min in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 5 mg/ml collagenase (Sigma) and 0.6 mg/ml protease type XIV (Sigma). Digested samples were pressed through a cell filter (Becton Dickinson) and centrifuged for 5 min at 450 × g. The cell pellet was washed twice with phosphate-buffered saline and plated on collagen-coated dishes (Dunn Laboratories, Asbach, Germany) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics (Sigma). The fibroblast culture typically reached a confluency of 70% after 1 week and was subsequently used for the further experiments.

Effect of TGF-β1, Anti-TGF-β1, SB203580, SB431542, and Actinomycin D on XT Expression—To determine whether TGF-β1 induces mRNA expression of XT-I, XT-II and type I collagen in HCFs cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. After the cells had reached a confluency of 70%, the culture medium was replaced with serum-free medium. Before the treatment with recombinant human TGF-β1 (Strathmann Biotec, Hannover, Germany) at a final concentration of 0.5–10 ng/ml, the cells were preincubated for 2 h with inhibitor or neutralizing antibodies. The inhibitors SB203580 (LC Laboratories, Woburn, MA) and SB431542 (Sigma) were used at a final concentration of 1–100 μM. Neutralizing anti-TGF-β1 antibodies were obtained from R & D Systems (Minneapolis, MN) and used at a final concentration of 25–150 ng/ml. Actinomycin D was obtained from Sigma and used at 5 μg/ml to block further transcription of XT-I mRNA.

Transfection of HCFs with siRNA against XT-I mRNA—HCFs were isolated and cultivated as described above. At a confluence of 40% the culture medium was replaced with serum-free medium, and the cells were treated with 500 μl of transfection mixture containing 5 μl of Lipofectamine 2000 (Invitrogen) and siRNA (final concentration, 20 nM) against XT-I or a scrambled RNA oligonucleotide (Ambion, Austin, TX). The medium was replaced 24 h after transfection, and the cells were used for further experiments.

Mechanical Stretch—Primary cultures of HCFs isolated from three different hearts were subjected to cyclic strain using a FlexCell vacuum system (Dunn Laboratories). The cells plated on BioFlex culture plates were placed in a humidified atmosphere with 5% CO2 at 37 °C, and then the cultures were stretched by 5% with a frequency of 1 Hz in a square wave pattern up to 24 h. HCFs from the same preparation and plated on the same culture dishes were used as controls.

XT-I Activity Assay—Using whole protein extracts from cardiac tissue or cell culture supernatant, the analysis of XT-I activity was carried out as described previously (21). The method is based on the incorporation of [14C]xylose with recombinant human bikunin as acceptor. Briefly, the reaction mixture for the assay contained in a total volume of 100 μl was: 50 μl of XT-I solution, 25 mM 4-morpholineethanesulfonic acid (pH 6.5), 25 mM KCl, 5 mM KF, 5 mM MgCl2, 5 mM MnCl2, 1.0 μM UDP-[14C]xylose (Du Pont, Homburg, Germany), and 1.5 μM recombinant bikunin. After incubation for 1.25 h at 37 °C, the reaction mixtures were placed on nitrocellulose discs. After drying, the discs were washed for 10 min with 10% trichloroacetic acid and three times with 5% trichloroacetic acid solution. Incorporated radioactivity was quantified after the addition of 5 ml of scintillation mixture (Beckman Coulter, Fullerton, CA) using an LS500TD liquid scintillation counter (Beckman Coulter). The radioactive signal of 35 ± 5 dpm (means ± S.D.) from a negative acceptor control was subtracted from all of the measured values.
Quantification of the GAG Content—Using cell lysate and culture supernatant, the total content of chondroitin and heparan sulfate GAGs was determined. The isolation of GAG chains from cell culture medium and cells was performed by adding four volumes of ethanol per sample volume. After precipitation at −20 °C for 12 h, all of the samples were dried and digested with Pronase (1 mg/ml) at 37 °C for 12 h. The peptides were removed by the addition of trichloroacetic acid to a final concentration of 10%. The precipitate was discarded, four volumes of ethanol containing 5% potassium acetate were added to the supernatant, and GAGs were allowed to precipitate at −20 °C overnight. The precipitate was dissolved in 0.5 ml of water and desalted by ultrafiltration (Vivascience, Hannover, Germany).

For the digestion of heparan sulfate and heparin chains, the samples were digested at 35 °C overnight with a mixture of heparin lyase 1, 2, and 3 (0.3 unit each) in 50 mM ammonium acetate (pH 7.1) and 4 mM calcium acetate. Disaccharides from chondroitin or dermatan sulfate chains were obtained by chondroitinase ABC (10 milliunits) in 50 mM ammonium acetate (pH 8) at 37 °C overnight. 2-Amino-acridone labeling of GAG disaccharides was performed according to Kitagawa et al. (22), and high pressure liquid chromatography analysis was carried out as described previously (23). Standard curves were generated for each 2-amino-acridone-labeled chondroitin sulfate and heparan sulfate disaccharide (Mobitec, Göttingen, Germany).

SDS-PAGE and Western Blot Analysis—Whole protein extracts from cardiac tissues (160 μg of protein/sample) were prepared, separated by SDS gel electrophoresis (8–16% SDS-PAGE), and transferred onto polyvinylidene difluoride membranes. Western blotting was performed using a semi-dry electro-blotting apparatus. After protein transfer, nonspecific binding sites were blocked with 5% milk powder in phosphate-buffered saline (pH 7.4), for 1 h at room temperature. The membrane was incubated with anti-decorin antibody (polyclonal rabbit IgG against human decorin core protein) for 1 h (24). Unbound antibody was washed from the membrane with Tris-buffered saline, pH 7.4. Detection was performed with peroxidase-conjugated secondary antibody. Monoclonal anti-α-tubulin antibody (Sigma) was used as a loading control.

Tissue Samples—Myocardial samples were obtained at the time of heart transplantation from 18 patients suffering from DCM. Nonfailing control samples were received from six organ donors without a detectable history of cardiac disease, whose hearts were not used for transplantation because of technical reasons. Using a standardized procedure transmural myocardial samples were taken from the free wall of explanted hearts of the left and right ventricles without apparent fibrotic scarring. All of the patients investigated were from the heart transplant program of the Herz- und Diabeteszentrum NRW. The investigation conforms to the principles outlined in the declaration of Helsinki. The sampling of the human specimens was done in all parts with approval of the local ethics committee.

RNA Extraction—The total RNA was isolated from 30–40 mg of frozen myocardial tissue using a commercial kit with additional on-column DNase I treatment for removing contaminating genomic DNA according to the manufacturer’s recommendations (Qiagen).

LightCycler Real Time Quantitative Reverse Transcription-PCR Analysis—The hybridization probes XT-I/II_anchor (5′-CTGGGGCTGAAGTGCACG-3′) and XT-I/II_sense (5′-LCRed-640-AAGGACATCGTGGAGACTGGT-Pho-3′) were designed with the LightCycler Probe Design software (Roche Applied Science) using the human XT-I and XT-II cDNA sequence (15). A specific detection of the target mRNA was realized using the intron spanning primers XT-I_F (5′-CTCCAGGACCTGATGG-3′), XT-I_R (5′-CCCAATGGTTTCTGAC-3′), XT-II_F (5′-TGCCCTGTAGAACCCCTCG), and XT-II_R (5′-AGAGGTGGGTCTTTGAGACT). LightCycler human β2-microglobulin (β2m) control reagent (Roche Applied Science) was used as an internal standard for fluorogenic detection of the β2m transcript. The mRNA expression of XT-I was analyzed by a fluorogenic one-step reverse transcription-PCR assay using the LightCycler System. Thermal cycling conditions included a cDNA synthesis at 50 °C for 10 min, activation of DNA polymerase at 95 °C for 2 min followed by 45 cycles of 94 °C for 2 s, 55 °C for 12 s, and 72 °C for 10 s. The PCR for the quantification of Col1A1 mRNA was performed using a SYBR green Taq DNA polymerase mixture (Platinum SYBR Green qPCR SuperMix-UDG; Invitrogen). Thermal cycling conditions included enzymatic degradation of uracil-containing DNA at 50 °C for 2 min, activation of the DNA polymerase at 95 °C for 2 min followed by 45 cycles 94 °C for 5 s, 58 °C for 15 s, and 72 °C for 15 s. The primers used were β2mF (5′-CTGTATGCGCACGAGA-3′), β2mR (5′-GACAAGTCTGATTGCTCC-3′), colIF (5′-CTCATCCAGACGAGA-3′), colIR (5′-GGTTAGCTTGATTGAGACT-3′), and col1R (5′-GTGGGAGATGGGAGGATT-TAC-3′). We used the RelQuant software (Roche Applied Science) for determination of the relative amounts of mRNA with β2m mRNA as a housekeeping gene. The constant transcription levels of β2m were verified for all of the tissue samples.

Densitometric Analysis of Alcian Blue-stained Sections—Alcian blue staining was used for the histological assessment of the total proteoglycan content. Tissue samples at a thickness of 5 μm were stained for 30 min with 1% Alcian blue in phosphate-buffered saline (pH 2.0), washed three times with Tris-buffered saline containing 2% Tween 100, and prepared with mounting medium for microscopy. For the semiquantitative and quantitative analyses of the relative Alcian blue content in the histological stainings, a densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD) as described below. The relative content of stained blue fractions was determined by color-selective conversion of the blue-stained areas and the subsequent analysis of stained pixels. Multiple representative sections (at least four) were analyzed by two independent researchers.

Statistics—The data were analyzed with the algorithms included in the GraphPad Prism 4.00 for Windows (GraphPad Software, San Diego, CA). Multiple data points were obtained for each of the samples, and the Gaussian distribution of the data points was proven using the Kolmogorov-Smirnov test in each case. The variation between multiple data sets was tested with analysis of variance followed by an Tukey post-hoc test. Student’s t test and Mann-Whitney test were performed for single comparisons between two groups. p < 0.05 was consid-
RESULTS

TGF-β1-Mediated Regulation of XT Expression in Cardiac Fibroblasts—Previous work has shown that TGF-β1 is a potent inducer of the Col1A1 expression in HCFs. To examine whether TGF-β1 is able to induce XT-I or XT-II expression, HCFs were incubated with 0.5–10 ng/ml recombinant TGF-β1. After 24 h of incubation, we found increased levels of XT-I mRNA following a dose-response curve. For XT-I no changes in the mRNA abundance were observed. XT-I was found to be the dominant xylosyltransferase present in cardiac fibroblasts as judged from quantitative real time PCR data. Significantly elevated levels of XT-I mRNA were detected at concentrations of 1.5–10 ng/ml TGF-β1, with a maximum transcriptional increase using 10 ng/ml TGF-β1 (13.3-fold; S.D. 1.5; p < 0.001, 10 ng/ml TGF-β1 ) (Fig. 1A). In agreement with data from literature, we detected that TGF-β1 also induces Col1A1 mRNA expression up to 2-fold (5 ng/ml TGF-β1; data not shown). After 24 h of cultivation, increased levels of XT-I expression always correlated with an elevated abundance of the CS-GAG content in the cell lysate as well as in the culture supernatant. The concentration of HS-GAGs was at low levels (>5 pmol/well) without showing any significant changes.

Induced by 1.5 ng/ml TGF-β1, the stimulated expression of XT-I with increased levels of mRNA, enzymatic xylosyltransferase activity, and CS-GAG content could be reduced by application of different concentrations of neutralizing anti-TGF-β1 antibodies. The best results were obtained with the maximum dose of anti-TGF-β1 (150 ng/ml). Thereby, a significantly reduced XT-I mRNA (150 ng/ml anti-TGF-β1; –2.6-fold, S.D. 0.55, p < 0.001) abundance was in parallel with a –1.75-fold (S.D. 0.36, p < 0.01) lowered enzymatic activity in the supernatant and –2.05-fold (S.D. 0.24, p < 0.001) less CS-GAGs (S.D. 0.2, p < 0.001; 91 ± 5 pmol/well; 61% 0-S and 39% 2-S in the cell lysate and 14 ± 3.5 pmol/well; 31% 0-S, 51% 4-S, 11% 4,6-S and 7% 6-S, in the supernatant (Fig. 1). To determine which of the signaling pathways are involved in the TGF-β1-stimulated XT-I expression, inhibitors of different signaling pathways were used. Both SB431542, an inhibitor of the activin receptor-like kinase 5 (ALK5), and SB203580, which is a specific inhibitor of p38 MAP kinase, significantly reduced the TGF-β1-stimulated XT-I expression but had no effect on the XT-II expression. Following a dose-response curve, the attenuation of the TGF-β1-induced phosphorylation of ALK5 or p38 MAP kinase reduced the XT-I expression on the mRNA level (100 μM SB431542; –18-fold, S.D. 2.4, p < 0.001; 100 μM SB203580; –7.8-fold, S.D. 1.3, p < 0.001), on the enzymatic level (100 μM SB431542; –4.2-fold, S.D. 0.53 p < 0.001; 100 μM SB203580; –3.4-fold, S.D. 0.51, p < 0.001), and the total content of CS-GAGs (100 μM SB431542; –2.7-fold, S.D. 0.71, p < 0.01; 67 ± 14 pmol/well; 39% 0-S and 61% 2-S in the cell lysate and 11 ± 3.7 pmol/well; 75% 0-S and 25% 6-S in the supernatant) 100 μM SB203580; –1.5-fold, S.D. 0.16, p < 0.01; 121 ± 6.5 pmol/well; 42% 0-S and 58% 2-S in the cell lysate and 15 ± 3.1 pmol/well; 78% 0-S and 22% 6-S in the supernatant) (Fig. 1).
The effect of stretch-induced XT-I expression in HCFs could be reduced by using a neutralizing anti-TGF-β, antibody, SB431542 or SB203580. We detected significantly decreased levels of XT-I mRNA (150 ng/ml anti-TGF-β; −2.4-fold, S.D. 0.43, p < 0.001; 10 μM SB431542; −2.8-fold, S.D. 0.33, p < 0.001; 100 μM SB203580; −2.2-fold, S.D. 0.28; p < 0.001) in the cell lysate as well as decreased levels of XT-I enzymatic activity (150 ng/ml anti-TGF-β; −1.9-fold, S.D. 0.16, p < 0.001; 100 μM SB431542; −1.7-fold, S.D. 0.43, p < 0.01; 100 μM SB203580; −1.3-fold, S.D. 0.14, p < 0.01) and CS-GAGs (150 ng/ml anti-TGF-β; −1.7-fold, S.D. 0.56, p < 0.05; 132 ± 49 pmol/well; 38% 0-S and 62% 2-S in the cell lysate and 16 ± 2.4 pmol/well; 23% 0-S, 59% 4-S and 18% 6-S in the supernatant).

Effect of Mechanically Induced Stretch on the Expression of XT-I and XT-II in HCFs—Previous work has shown that TGF-β plays an important role in pressure overload-induced cardiac fibrosis. In cardiac fibroblasts increased mechanical stretch is associated with elevated levels of TGF-β (20, 25). Therefore, we analyzed the influence of mechanical strain on XT-I and XT-II expression in a cell culture model with HCFs. The in vitro cultivated cardiac fibroblasts responded to mechanical stress by 5% extension with an increased XT-I and Col1A1 expression. The Col1A1 response was similar to that observed in other studies (26). For the XT-II, no significant changes could be detected.

XT-I and Col1A1 showed a similar transcription increase: XT-I mRNA was 1.6-fold up-regulated (S.D. 0.14, p < 0.01) (Fig. 2A), and Col1A1 mRNA was 1.36-fold (S.D. 0.05, p < 0.05) after cyclic mechanical stress for 24 h. In parallel with an elevated XT-I mRNA content, we found increased levels of enzymatic XT-I activity (1.5-fold, S.D. 0.16, p < 0.01) and significantly elevated levels of CS-GAGs (1.5-fold, S.D. 0.14, p < 0.001; 251 ± 10 pmol/well; 44% 0-S and 56% 2-S in the cell lysate and 25 ± 2.5 pmol/well; 44% 0-S, 33% 4-S, 17% 6-S and 6% 2-S in the supernatant (Fig. 2, B and C). Similar results were obtained using cardiac fibroblasts isolated from DCM patients and from atrial appendices of patients under coronary artery bypass surgery (data not shown). For experiments with shorter periods of stretching, we did not detect significant changes in the expression of XT-I and Col1A1.

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Xylosyltransferase I in Cardiac Fibrosis

FIGURE 3. Decreased expression of XT-I and CS-GAGs using siRNA directed against XT-I. HCFs were transfected with siRNA against XT-I or with scrambled control. 24 h after transfection, HCFs were cultivated in BioFlex culture plates and stretched by 5% with a frequency of 1 Hz. A, relative mRNA levels of XT-I; B, enzymatic activity of XT-I in the cell culture supernatant. C, total content of CS-GAGs in the cell lysate and the cell culture supernatant. The values are the means of three independent experiments with corresponding standard deviation. *, p < 0.05 versus rigid control transfected with control siRNA; §§, p < 0.01 versus loaded control transfected with control siRNA. Bar – c, cells transfected with scrambled control siRNA, which were not subjected to mechanical stretch; bar – s, cells transfected with control siRNA, which were mechanically stretched; bar + c, cells transfected with XT-I siRNA, but not subjected to stretch; bar + s, cells transfected with XT-I siRNA and mechanically stretched.

FIGURE 4. mRNA expression of XT-I in myocardium samples from DCM patients. A, increased XT-I mRNA levels in patients with DCM in comparison with the control group. *, p < 0.05 DCM LV versus NF LV. The median (black line), the 25th and 75th percentile (box), and the minimum and maximum values are shown (whiskers). B, left ventricles from both DCM patients (n = 18) and nonfailing controls (n = 6) exhibit higher transcriptional XT-I levels than right ventricles. DCM RV, right ventricles from DCM patients; DCM LV, left ventricles from DCM patients; NF RV, right ventricles from nonfailing controls; NF LV, left ventricles from nonfailing controls.

In DCM patients the increased ventricular diameter results in an elevated mechanical stress on the ventricular wall. The quantitative data for XT-I mRNA is shown in Fig. 4A. We found a pronounced 1.8-fold (S.D. 0.7, range 1.5–5.5) increase of the XT-I mRNA level in left ventricles and a 1.6-fold (S.D. 0.6, range 1.2–2.5) increase in right ventricles of patients with DCM. The mRNA levels of XT-I in left ventricular myocardium were significantly increased (p < 0.05) in patients with myocardial failure (0.32 ± 0.12 arbitrary units) compared with nonfailing controls from donor hearts (0.18 ± 0.05 arbitrary units). No significant changes were observed for XT-II mRNA expression (data not shown). For both groups no age-related changes in the XT-I expression could be detected. Interestingly, we found in failing hearts and in nonfailing controls an increased XT-I mRNA level in left ventricular samples compared with those from right ventricular myocardium (ratio left/right for DCM 1.28, control 1.14; Fig. 4B).

Increased XT-I Activity and Elevated Levels of Proteoglycans in Dilated Cardiac Tissue—To investigate whether the observed increased XT-I mRNA levels result in an elevated content of XT-I protein, XT-I enzyme activity was measured in whole protein lysates from cardiac tissues. Representative tissue samples from patients with DCM exhibited a 1.22-fold (S.D. 0.42, n = 4) increased XT-I activity compared with nonfailing samples (Fig. 5B). Protein levels of the small leucine-rich proteoglycan decorin were determined using Western blot analysis to ascertain whether increased xylosyltransferase activity and increased levels of o-glycosylated proteoglycans can be detected in DCM. The decorin content, which represents a major component in the composition of cardiac proteoglycans, was strongly increased in the failing human myocardium (Fig. 5).

To detect the total content of GAGs in tissue samples from nonfailing persons and DCM patients, we used Alcian blue staining (Fig. 5). The quantitative analysis of blue-stained sections of four tissue samples from DCM patients showed an 2.3-fold (S.D. 0.5) increased content of GAGs compared with samples from nonfailing controls.

DISCUSSION
In addition to an overproduction of collagen, the increased biosynthesis of the proteoglycans decorin and biglycan from interstitial fibroblasts is a main characteristic of cardiac fibrosis. Thereby, increased levels of TGF-β play an important role during the progression of this intensive matrix deposition (27). Proteoglycans with their highly diverse GAG chain glycosylation are a major part of the ECM and exhibit multiple functions, e.g. modulation of growth factor activities, regulation of collagen fibrillogenesis, or tensile strength (9, 13, 28). The knowledge about the regulatory mechanisms leading to an increased production of GAG chains and proteoglycans still...
remains elusive. Our study now provides the first evidence that XT-I is a rate-limited enzyme that is responsible for the increased biosynthesis of CS-GAGs in cardiac tissue in pathological conditions.

The xylosyltransferases XT-I and XT-II catalyze an initial and rate-limiting step in the synthesis of glycosaminoglycan chains in chondroitin sulfate, dermatan sulfate, and heparan sulfate proteoglycans and therefore represent key enzymes for ECM assembly (29). Both enzymes are capable of initiating the biosynthesis of chondroitin sulfate, dermatan sulfate, and heparan sulfate glycosaminoglycan chains (17, 30, 31). Until now we and others have not found any differences of both xylosyltransferases in regards to acceptor specificity or a preference for the biosynthesis of either chondroitin sulfate or heparan sulfate proteoglycans. XT-I has been found to be the dominant xylosyltransferase in cardiac fibroblasts and to be regulated by pro-fibrotic cytokines like TGF-β1. Consequently, it is concluded that XT-I plays the major role in the elevated proteoglycan biosynthesis during cardiac fibrosis and heart tissue remodeling.

Like the other glycosyltransferases involved in the proteoglycan assembly, XT-I is a type II transmembrane glycosyltransferase and is located in the early Golgi compartments (32). However, a unique characteristic of XT-I is the shedding from the Golgi surface via an unknown mechanism and the release of a soluble form into the extracellular space. In previous studies we could show in in vitro studies that more than 90% of the enzyme activity is released from the Golgi apparatus and secreted into the cell culture supernatant (16). Furthermore, we could show that, for example, the increased proteoglycan biosynthesis in the generalized fibrotic process in systemic sclerosis leads to elevated XT-I activities in the peripheral blood (18). Although the molecular mechanisms of this shedding process are yet unknown, shedding of glycosyltransferases from the Golgi surface has been suggested to be a mechanism for regulating the intracellular glycosyltransferase activity (33). In our present study these characteristics of the xylosyltransferases are likely to provide a plausible explanation for the lower increase of XT-I activity in DCM samples compared with our in vitro experiments.

The present study reveals that XT-I expression is up-regulated by TGF-β1 in vitro and that increased levels of enzymatic XT-I activity are corresponding with an elevated synthesis of GAGs in cardiac tissues. The regulation of XT-I mRNA expression by TGF-β1 is not restricted to cardiac fibroblasts as other cells; for example, dermal fibroblasts also show a TGF-β1-induced XT-I expression.3 The attenuation of the TGF-β1-induced phosphorylation of the ALK5 and p38 MAP kinase significantly reduced the XT-I expression on mRNA and enzyme level as well as the GAG content. As described previously, TGF-β1 is able to induce biglycan expression through ALK5 and GADD45β (34). Therefore, there is evidence suggesting that a singular pathway is involved in XT-I expression.

A specific knock-down of the XT-I mRNA with siRNA resulted in a decreased enzymatic XT-I activity and in lowered levels of GAGs. This provides evidence for the central role of this enzyme during the fibrotic ECM remodeling.

TGF-β1 is significantly up-regulated in cardiac fibroblasts under mechanical stress (19, 20) and is a potent inducer for the expression of type I collagen (19) and several proteoglycans (12). We could show that the TGF-β1-mediated XT-I expression can be found during the pathological remodeling of the ECM in mechanically stressed HDFs in vitro and in vivo.

Previous studies have shown that decorin and biglycan are able to bind TGF-β1 (12, 13), which is known to induce the expression of these glycoproteins in cardiac fibroblasts (27) and therefore may act as a negative regulatory element during myocardial fibrosis. We could detect elevated levels of the decorin core protein in cardiac tissue from DCM patients but not in our in vitro experiments with stressed cardiac fibroblasts. The experiments were optimized for an maximum increase of XT-I expression on mRNA and enzymatic level. Therefore, the selected cultivation time of 24 h may be too short for an increased production of decorin. This has to be clarified in further studies.

Although type I collagen and XT-I respond to TGF-β1 with an elevated mRNA content, we could show that the time-dependent transcriptional regulation of both genes exhibits different maxima. In HDFs, we found a maximum for the XT-I mRNA content after 8 h of incubation with TGF-β1, whereas

3 S. Busch and C. Göttin, unpublished observations.
induction of XT-I transcription in human heart failure. However, in vivo mechanical loading in the dilated heart with increased levels of TGF-β1 is certainly not the only factor for the induction of XT-I transcription, because no significant correlation between the LV end diastolic dimension and XT-I mRNA levels was observed in DCM patients.

All in all, our data comprise new insights into pathological mechanisms of ECM remodeling during DCM. XT-I, the rate-limiting enzyme in proteoglycan assembly, is currently being investigated as a drug target for the inhibition of fibrosis. The regulatory function of TGF-β1 regarding the transcriptional regulation of XT-I has to be analyzed in further experiments. Additional studies, particularly in the context of the renin-angiotensin system, which together with TGF-β1 plays a pivotal role in cardiac remodeling, are necessary to shed light on the detailed function of XT-I in fibrotic degeneration during DCM.

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