SHORT COMMUNICATION

Cardiac remodeling and MMPs on the model of chronic daunorubicin-induced cardiomyopathy in rabbits

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Summary

The matrix metalloproteinases (MMPs) play a key role during cardiac remodeling. The aim of the study was to investigate the changes in collagenous proteins and MMPs in the model of non-ischemic, anthracycline-induced chronic cardiomyopathy in rabbits using both biochemical and histological approaches. The study was carried out on three groups of Chinchilla male rabbits: 1) daunorubicin (3 mg/kg, once weekly for 10 weeks), 2) control (saline in the same schedule), 3) daunorubicin with the cardioprotectant dexrazoxane (60 mg/kg, before each DAU). Morphological changes in the myocardium of DAU-treated animals were characterized by focal myocardial interstitial fibrosis of different intensity. The subsequent proliferation of the fibrotic tissue was marked by an increased content of both collagen types I and III, which resulted in their typical coexpression in the majority of bundles of fibers forming either smaller or larger scars. Biochemical analysis showed a significantly increased concentration of hydroxyproline, mainly in the pepsin-insoluble fraction of collagenous proteins, in the DAU group (1.42 ± 0.12 mg/g) as compared with the control (1.03 ± 0.04 mg/g) and dexrazoxane (1.07 ± 0.07 mg/g) groups. Dexrazoxane co-administration remarkably reduced the cardiotoxic effects of DAU to the extent comparable with the controls in all evaluated parameters. Using zymography, it was possible to detect only a gelatinolytic band corresponding to MMP-2 (MMP-9 activity was not detectable). However, no significant changes in MMP-2 activity were determined between individual groups. Immunohistochemical analysis revealed increased MMP-2 expression in both cardiomyocytes and fibroblasts. Thus, this study has revealed specific alterations in the collagen network in chronic anthracycline cardiotoxicity in relationship to the expression and activity of major MMPs.

Key words

Anthracyclines - cardiomyopathy - remodeling - collagen - matrix metalloproteinases

Cardiac toxicity associated with repeated administration of anthracyclines represents a serious complication of their use in anticancer chemotherapy. So far, dexrazoxane is the only drug which has been clearly demonstrated to protect the myocardium from anthracycline-induced toxicity both in experimental and clinical settings (Herman *et al.* 1994, Marty 2006). Besides apparent clinical implications, anthracycline-induced cardiotoxicity is often used as a very useful experimental model of non-ischemic cardiomyopathy and congestive heart failure.

The development of heart failure is a chronic process associated with myocardial remodeling which involves necrosis and/or apoptosis of cardiomyocytes, proliferation of fibroblasts and alteration of both intracellular components of the myocardium, as well as of major structural proteins of the extracellular matrix (ECM) (Graham *et al.* 2008). Current understanding to the ECM remodeling attributes a pivotal role to the family of matrix metalloproteinases (MMP) (Spinale 2007). However, recent studies have shown that MMPs cleave not only the key protein components of the myocardial extracellular compartment but also numerous sarcomeric and cytoskeletal proteins within the cardiac myocytes (e.g., troponins, MLC-1 or desmin) (Schulz 2007). The structural damage of cardiomyocytes can be estimated by the release of cardiac biomarkers into the plasma. It has been previously shown that, e.g., cardiac troponins may serve as useful biomarkers of anthracycline-induced cardiac damage and they therefore become gradually utilized in clinical practice (Adamcová *et. al.* 2005, Adamcová *et. al.* 2007). However, a little attention has been devoted to the alterations of the extracellular matrix and the involvement of MMPs in anthracycline cardiotoxicity yet.

Hence, this study aimed to asses the changes in MMPs along with the determination of quantitative and qualitative alterations in the expression of collagenous proteins during cardiac remodeling induced by repeated administration of daunorubicin in rabbits.

Chronic anthracycline cardiomyopathy was induced in Chinchilla male rabbits using a well described model (Geršl *et al.* 1994; Šimůnek *et al.* 2004) - daunorubicin (DAU, 3 mg/kg, i.v., Daunoblastina, Pharmacia Italia, Italy, n=11). Control animals received saline (1 ml/kg, i.v., n=8) in the same schedule. The third group of animals received a model cardioprotectant dexrazoxane (60 mg/kg, i.p., Cardioxane, Novartis, Switzerland) 30 min before each DAU administration. At the end of the experiment, invasive hemodynamic measurement (dP/dt_{max}) was performed using a Micro-Tip pressure catheter (2.3F, Millar Instruments, U.S.A) connected to a data acquisition system Powerlab and a Chart 5.4.2 software (ADInstruments Pty., Australia). The concentrations of cardiac troponin T (cTnT) were measured using an Elecsys Troponin T STAT Immunoassay (Roche Diagnostics, Switzerland). Cardiac troponin concentrations over the whole experimental period were expressed as medians of the area under the curve (AUC). For drug administration, blood sampling and other mini-invasive/non-invasive operations, a combination of short-acting anaesthetic agents was used (midazolam 1.25 mg/kg + ketamine 50 mg/kg, i.m.). Surgical anesthesia for the preparations and invasive hemodynamic measurements performed at the end of the experiment was induced with pentobarbital (30 mg/kg, i.v.). All the experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication, 1996).

Biochemical analysis of collagenous proteins was performed as described previously (Pelouch *et al.* 1995). Hydroxyproline concentrations were measured spectrophotometrically at 550 nm in both pepsin-soluble and pepsin-insoluble fractions. In addition, proteins were separated by 7.5 % SDS-PAGE and detected using silver staining. MMP enzyme activities were assayed by zymography using either gelatine (Type A, Sigma-Aldrich) or casein (β-casein, Sigma-Aldrich) or collagen (Collagen Type I, Sigma-Aldrich) as MMPs substrates.

The left ventricular samples were homogenized in the lysis buffer containing cacodylic acid, NaCl, $CaCl_2$, NaN_3, Triton X-100, and $ZnCl_2$.

Histological examination was made on paraffin transversal sections of the heart ventricles stained with H&E for basic evaluation, while Picro-sirius Red method was employed for collagen examination, particularly in polarized light. Immunohistochemical detection of MMP-2 was performed by standard peroxidase anti-peroxidase (PAP) technique using the primary rabbit anti-MMP-2 (Neomarkers, CA, USA; dilution 1:400) antibody and the swine anti-rabbit IgG (DAKO, Denmark; dilution 1:100) secondary antibody.

Statistical evaluation was performed with statistical softwares SigmaStat 3.5 (SPSS, U.S.A.) and NCSS 2004 (NCSS, U.S.A.). Significances were assessed with one-way ANOVA or Kruskal-Wallis ANOVA on ranks (with Bonferroni post-hoc analysis).

Invasive examination of the left ventricular systolic function (dP/dt_{max}) revealed progressive and significant impairment in the daunorubicin group in comparison with both control and dextrazoxane co-treated animals (control 1158.3 ± 76.0 kPa/s, DEX+DAU 1174.7 ± 100.3 kPa/s vs. DAU 773.6 ± 46.4 kPa/s, one way ANOVA, $p<0.05$). Concomitantly, *plasma concentractions of troponin T* (a biomarker of cardiomyocyte injury) were found to be significantly increased in the DAU-treated animals in comparison with the control and dexrazoxane groups (control 0.003 ng/ml; DEX+DAU 0.000 ng/ml vs. DAU 0.315 ng/ml, Kruskal-Wallis ANOVA, $p<0.01$).

The histological examination of the myocardium of controls revealed a normal morphology. Cardiomyocytes were arranged in typical columns surrounded by the loose interstitial connective tissue with capillaries in which fine reticular fibers, formed by collagen type III, prevailed. The dense connective tissue, composed mainly of thicker wavy bundles of collagen fibers (collagen type I), was arranged around larger vessels (Fig. 1A,D). In the DAU group, interstitial myofibrosis developed in places, where focal degeneration (death) of the

groups of cardiomyocytes occurred. The reparative process is characterized by the production of both types of collagen, but type III is produced predominately at the beginning of healing, whereas type I collagen is typical of the advanced stages of the process (Figs. 1B,E and 2B). Dexrazoxane co-administration apparently prevented the above-described DAU-induced changes (Fig. 1C,F), therefore the structure of the myocardium is fully comparable with intact controls (Fig. 1A,D).

Biochemical analysis of the myocardium from the DAU group showed a significantly ($p<0.01$) increased concentration of hydroxyproline mainly in the pepsin-insoluble fraction of collagenous proteins (1.42 ± 0.12 mg/g of wet tissue) as compared with the control (1.03 ± 0.04 mg/g of wet tissue) and dexrazoxane (1.07 ± 0.07 mg/g of wet tissue) groups (Fig. 2A). This points on the increased content of predominantly highly cross-linked collagenous aggregates in the DAU group. Using zymography, a predominant band of MMP-2 (72 kDa) was detected in all groups (Fig. 2D), whereas no activity of other MMPs was identified either on the casein or collagen containing zymograms. The average MMP-2 activity at the end of the experiment appeared to be somewhat higher in the DAU group as compared to both control and dexrazoxane groups; however, significant difference was not reached with respect to large interindividual variability of this parameter in the DAU group (Fig. 2C).

In controls, MMP-2 expression was not detectable *immunohistochemically* (Fig. 1G). However, the DAU treatment resulted in a mild expression of MMP-2 in most of the animals in isolated (sporadic) both cardiomyocytes and fibroblasts within the foci with significant toxic damage (Fig. 1H). The expression tended to be more prominent in the animals with most pronounced functional and histopathological changes. MMP-2 expression was detected only in a half of the animals in the dexrazoxane group and the expression was generally mild and restricted to cardiomyocytes (Fig. 1I).

It has been recently revealed that the rabbit is a very suitable model for the study of human heart failure because the biophysical and biochemical characteristics of rabbit myofibers as well as the calcium handling closely resemble the properties of the human cardiomyocytes (Marian 2005). In our study, the histological examination of the left ventricular myocardium showed different stages of focal degeneration of cardiomyocyte in the DAU group. The cytoplasmic vacuolization and loss of myofibrils finally resulted in a total disintegration of the myocytes followed by the development of focal interstitial fibrosis. The newly synthesized collagen contained a greater proportion of the more compliant type III collagen, which is known to form less extensive cross-linking making the replacement collagen functionally inadequate (Graham *et al*. 2008). However, the collagen type I already prevailed in our samples because of maturation of connective tissue into fibrotic scar within the time-course of the chronic experiment. The above-described alterations in the collagen network create the basis for the development of typical fibrosis accompanying dilated cardiomyopathy.

MMPs are generally considered to be among the key regulators and executors of extracellular matrix remodeling under pathological conditions (Spinale 2007). It has been shown that MMP-2 and MMP-9 are significantly involved in ischemia/reperfusion injury and subsequent myocardial remodeling (Lalu *et al.* 2005). These MMPs are known to degrade gelatine, collagen type I and III which are the main components of the myocardial extracellular matrix. Similarly, an increased activity of these MMPs has been shown in acute anthracycline toxicity induced by a single supratherapeutical dose (Pacher *et al.* 2002, Bai *et al.* 2004). However, involvement of MMPs in the myocardial remodeling associated with a clinically-relevant chronic anthracycline cardiomyopathy development remained uncertain. In the present study, with the use of zymography, the present authors were able to detect only one gelatinolytic band which corresponded to MMP-2. However, the quantification of MMP-

2 activity did not show any significant changes between the groups in the study. No MMP-9 signal was detected, which may be due to the absence of a marked invasion of neutrophils (Schulz 2007). This finding is consistent with the recent data (Truter *et al.* 2009) arising from the investigation of the extracellular matrix remodeling in the chronic aortic regurgitation model. In this study, fibrosis was also not accompanied by a detectable quantity of MMP-9 at both protein and mRNA levels. The results of our study indicated that both main myocardial cell types (i.e. cardiomyocytes and fibroblasts) possess the capacity to express MMP-2 in rabbits. Although non-cardiomyocyte cell types have been particularly implicated as the sites for the synthesis of MMPs within the ECM, it should be noted that 90 % of the myocardial mass is composed of cardiomyocytes (Spinale 2007). Interestingly, no expression of gelatinolytic MMPs was detected in the extracellular matrix although gelatinases are known to be secreted by cardiac cells into the interstitium. These immunohistochemical findings were similar to those reported by Aupperle *et al.* (2007) in doxorubicin-treated rabbits, even though the myocardial fibrosis and the degenerative changes in cardiomyocytes were less pronounced there.

In the present study, the authors have shown that chronic anthracycline cardiotoxicity was associated with profound changes in the left ventricular morphology and function. These changes were accompanied by evident alterations in the collagen network which corresponds to the typical successive formation of the fibrotic scar tissue. Surprisingly, no significant changes in MMPs activity were found in the myocardium of rabbits with fully developed cardiomyopathy. In this way, the present authors' observations differ from previous reports on anthracycline cardiotoxicity available in the literature so far (Pacher *et al.* 2002, Bai *et al.* 2004). However, it should be noted that these reports dealt with the acute anthracycline toxicity induced by a single high dose and therefore their translatability to clinically relevant conditions remains uncertain. Although MMP-9 activity is involved in many other cardiac

pathologies, our data strongly suggest that MMP-9 is not involved in chronic anthracycline cardiotoxicity which might be associated with the absence of typical inflammatory infiltration. However, the investigation of the role of MMPs in the earlier phases of the development of chronic anthracycline cardiomyopathy merits a further study as the expression and activity of MMPs might undergo more dynamic changes during the time-course of cardiotoxicity development. Importantly, this study shows that the signs of DAU-induced myocardial remodeling could be efficiently prevented by dexrazoxane co-treatment.

Conflict of Interest

There is no conflict of interest.

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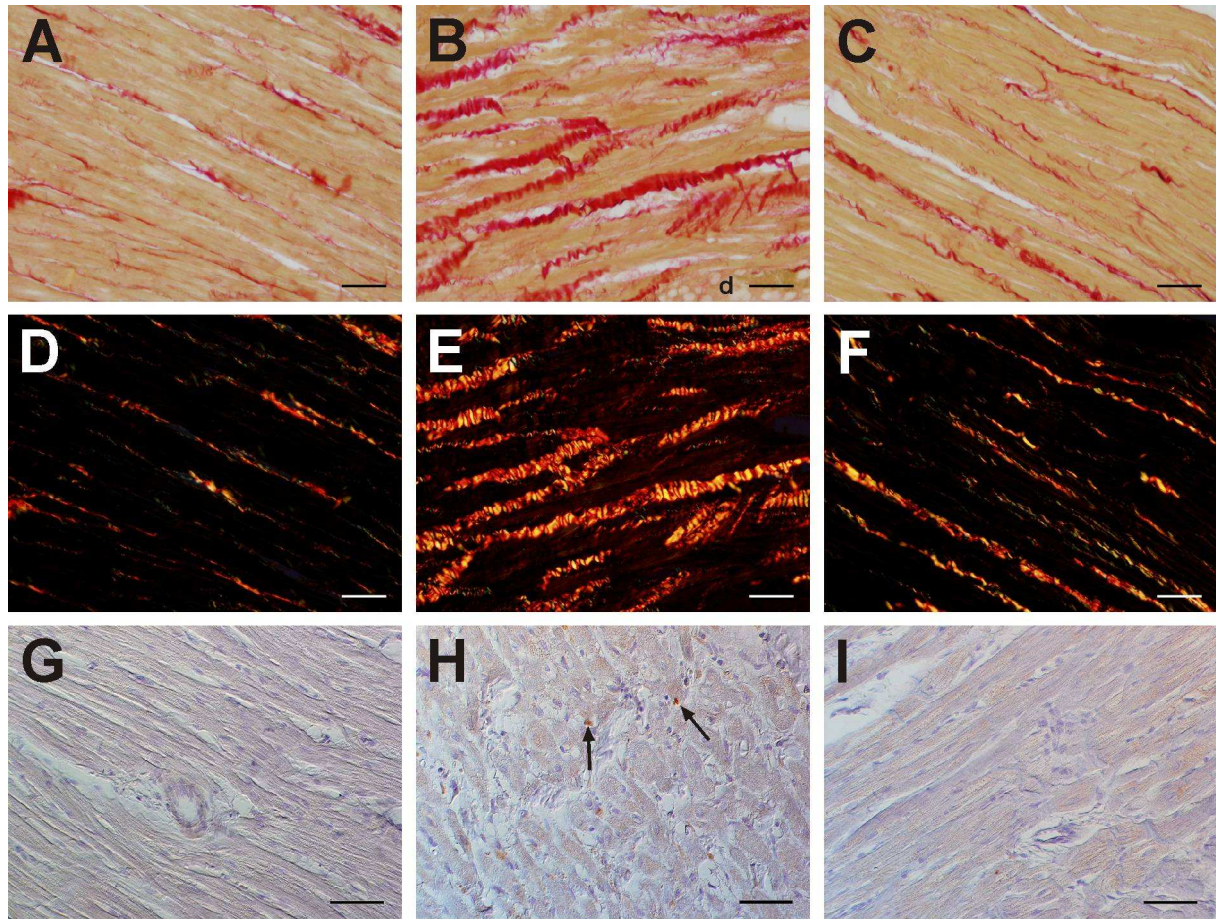
Fig. 1. Myocardium of the left ventricle wall

A,D,G: Control group – only fine loose connective tissue (**A** – red) with vessels, composed of fibres formed of both collagen type I (**D** – yellow/red) and III (**D** – green), surrounds the columns of cardiomyocytes (**A** – pale yellow); MMP-2 expression is not detectable (**G**).

B,E,H: DAU group – intensive proliferation of interstitial and above all of perivascular fibrotic tissue (**B** – red) at first represented mainly by reticular fibres (collagen III), in later stages by collagen fibres (collagen type I – **E**: yellow/red), replaces destroyed cardiomyocytes and results in focal formation of smaller or larger fibrotic scars; (**E**) "d" – degenerated myocyte with markedly vacuolized cytoplasm; (**H**) the mild expression of MMP-2 is in both myofibrils (cardiomyocytes) and single fibroblasts (arrows).

C,F,I: DEX+DAU group – No conspicuous differences are found in comparison with the control group (**C,F**); the only difference is the mild focal expression of MMP-2 bound to the myofibrils but, similarly to the controls, the fibroblasts are not labeled (**I**).

A-C: Picro-sirius Red staining - bright-field microscopy; D-F: Picro-sirius Red staining – polarization microscopy; G-I: anti-MAP-2 peroxidase immunohistochemistry. Bar – 50μm
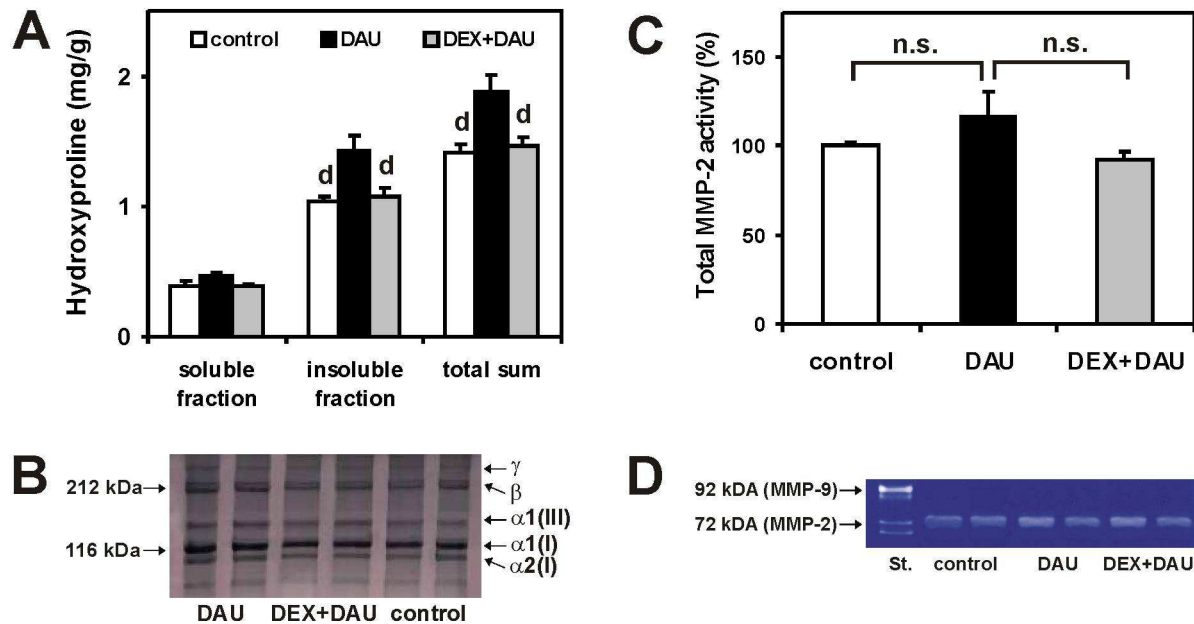

Fig. 2. Biochemical finding

A - Concentration of hydroxyproline in the fraction of pepsin-soluble and insoluble collagenous proteins in mg/g of wet tissue. Statistical significances (one-way ANOVA, $P<0.01$) in comparison with "d" daunorubicin group.

B - Typical gel electrophoresis profile of collagenous proteins. Typical gel electrophoresis profile γ fraction (chain polymers, collagen type I + III), β fraction (chain dimmers, collagen type I + III), α1 = mixture of the individual α1 chain (collagens type I + III), α2 = α2 chains (collagen type I).

C - Total activity of MMP-2 assessed by zymography.

D - Representative gelatinase zymography showing the MMP-activity at the end of the experiment in the comparison with the migration of standards MMP-2/MMP-9 (Chemicon, USA).