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ORIGINAL ARTICLE

Erythropoietin Modulates Imbalance of Matrix Metalloproteinase-2 and Tissue Inhibitor of Metalloproteinase-2 in Doxorubicin-induced Cardiotoxicity

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Background Doxorubicin (DOX) is a highly effective anti-cancer drug with limited clinical use due to its serious cardiotoxicity. Recent studies reported that erythropoietin (EPO) could exert a cardioprotective effect by non-erythropoietic effects. This study was to investigate fibrosis of DOX-induced cardiotoxicity and determine mechanisms of EPO against extracellular matrix (ECM) remodelling.

Methods Rats were grouped as the control group, the DOX group and the DOX+EPO group. DOX (2.5 mg/kg/dose, six doses for two weeks) was administered to induce cardiotoxicity by intraperitoneal injections in the DOX group and the DOX+EPO group, and EPO (2500U/kg/dose, six doses for two weeks) was administered simultaneously in the DOX+EPO group. Two weeks after the last administration, rats were killed with cardiac tissues used for histological analyses and immunological detections for matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-2 (TIMP-2).

Results Rats treated with DOX showed degenerative changes with cardiac fibrosis. Compared to the control group, the expression of MMP-2 was up-regulated whereas that of TIMP-2 was down-regulated in the DOX group. EPO administration improved cardiac fibrosis, decreased MMP-2 expression, increased TIMP-2 expression and ameliorated imbalance of MMP-2/TIMP-2 ratio.

Conclusions The present study suggests that EPO can exert a cardioprotective effect on DOX-induced cardiotoxicity which may be associated with improving MMP-2/TIMP-2 imbalance.

Keywords Doxorubicin • Cardiotoxicity • Erythropoietin • Fibrosis • Matrix metalloproteinase • Tissue inhibitor of metalloproteinase

Introduction

The cytokine erythropoietin (EPO) is classically recognised to be indispensable for the survival, proliferation and differentiation of erythroid progenitor cells. Recent studies have extended the traditional role of EPO to a cardioprotective role in acute coronary syndrome, ischaemia-reperfusion injury and kidney diseases [1–3]. It is suggested that EPO acts as a tissue-protective cytokine, prompting us to investigate its possible protective effect on heart disease of a non-ischaemic origin.

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Doxorubicin (DOX) is considered one of the most potent anti-neoplastic agents against solid and haematologic tumours. However, a dose-dependent cardiomyopathy limits its clinical potential. In the development of cardiomyopathy, a continuous remodelling process takes place in the myocardium and appears one of the major factors. Both cardiomyocytes apoptosis and interstitial alterations of myocardial architecture play important roles in determining the development of heart failure [4–7]. Several recent studies were focused on the close correlation between the deterioration of cardiac function and cardiomyocytes apoptosis, oxidative stress and progressive fibrosis in DOX-induced cardiotoxicity [8–10]. However, its possible molecular mechanism remains unclear in an involvement of the enzymatic systems of proteolysis and antiproteolysis in extracellular matrix (ECM) remodelling. We hypothesised that alterations of ECM metabolism in DOX-induced fibrosis were accompanied by the imbalance of matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-2 (TIMP-2), and may be influenced by preventive administration of EPO. Thus the aim of the present study was two-fold: 1) to investigate the expression of MMP-2 and TIMP-2 in the DOX-induced cardiotoxicity; 2) to determine the effect of EPO on ECM remodelling.

Materials and Methods

Experimental conditions

The experimental protocol of rats was approved by Animal Care and Use Committee of Tianjin Medical University and performed in conformity with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Male Wistar rats at 10 to 11 weeks of age were kept in a temperature and humidity controlled room with a 12/12-hour light/dark cycle. Twenty-four rats were divided into three groups: (1) the Control group (n=8) received normal saline only; (2) the DOX group (n=8) received DOX (2.5 mg/kg/dose) 0.5 hour after the same volume of normal saline by intraperitoneal injections; (3) the DOX +EPO group (n=8) received DOX (2.5 mg/kg/dose) 0.5 hour after administration of EPO (2500U/kg/dose) by intraperitoneal injections. DOX and EPO were administered in six equal doses over a period of two weeks. Two weeks after the last injection of DOX, rats were killed and cardiac tissues were sampled for molecular biological analyses.

Tissue preparation

The hearts of rats were quickly removed and cut transversely into slices, parallel to the atrioventricular groove. Some of the slices were placed in formaldehyde solution for histopathological examination, and others were frozen in liquid nitrogen and then stored at −70 °C for detection of proteins.

Histological analysis

For light microscopic evaluation, slices of each heart were fixed in 10% phosphate-buffered formalin and embedded in paraffin. 5 μm-thick sections were stained with Masson's trichrome and examined by a sample-blinded investigator. Quantitative assessments of fibrosis area were performed with Image Pro Plus 5.0 software (Media Cybernetics) on 20 randomly chosen high-power fields in each heart.

Immunohistochemical analysis

Deparaffinised sections were incubated with primary antibodies against MMP-2 and TIMP-2. After washing with phosphate-buffered saline, sections were subjected to an ABC standard kit according to the manufacturer's instructions. Diaminobezidine served as the substrate chromogen, followed by counterstaining for nuclei with haematoxylin. Staining intensity was semiquantitatively scored blindly as four grades: +/- when staining was very weak; + when staining was weak; ++ when staining was moderate; +++ when staining was strong.

Western blot

Fresh-frozen left ventricular myocardium was homogenised in lysis buffer. Equal amounts of proteins were fractionated by sodium dodecyl sulfate-polyacrylamide-gel electrophoresis, and transferred to polyvinylidene diflouride membranes. The membranes were incubated overnight with primary antibodies against MMP-2 (Santa Cruz) and TIMP-2 (Neomarkers). Blots were washed in Tris-buffered-saline with Tween (TBS-T) buffer containing 50mmol/L Tris-Cl, 150mmol/L NaCl and 0.05% Tween 20, and incubated with the second antibodies. Reactions were developed with an enhanced chemiluminescence kit (Pierce) and immunoblots were exposed on Kodak imaging films. MMP-2 and TIMP-2 were quantified densitometrically after normalisation to the β-actin (Santa Cruz) bands of each sample.

Statistical analysis

The results were expressed as mean±SEM. Group differences were analysed by one-way ANOVA with the Students-Newman-Keuls test as a post hoc test. A value of $P<0.05$ was considered significant.

Results

Pathological findings

No rats died of intervening measures during the whole experiment. Hearts of control rats showed normal morphology with abundantly normal myocardial cells and scarce interstitial fibres. Degenerative changes were apparent for rats in the DOX group including atrophic cardiomyoctes and some compensatorily hypertrophic cardiomyocytes, surrounded by significantly increased interstitial fibrosis. The area of interstitial fibrosis was significantly increased from 3.78±0.79% in the control group to 12.14±1.07% in the DOX group ($P<0.05$). However, EPO apparently improved DOX induced degenerative changes and significantly reduced the area of fibrosis in the DOX+EPO group (7.49±1.11%, $P<0.05$ vs the DOX group). See Fig. 1.

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