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Abstract: BACKGROUND: Transgenic (tg) mice with chronic overexpression of the human erythropoietin gene are characterized by an increased hematocrit of about 0.80 in adulthood. This is accompanied by cardiac dysfunction and premature death. The aim of this study was to examine whether this cardiac dysfunction was accompanied by hypertrophy of the heart with remodeling of the extracellular matrix (ECM). METHODS: 3-months-old wild type (wt) and tg mice without cardiac hypertrophy were compared with the respective 7-months-old mice. The mRNA of brain natriuretic peptide (BNP), of the matrix metalloproteinases (MMP)-2, -8, -9, -13, of the tissue inhibitor of metalloproteinase (TIMP)-1, -2, -3, -4 and of collagen I and III was detected by ribonuclease protection assay. The activity of MMPs was measured by zymography. RESULTS: There was hypertrophy of both ventricles in 7-months-old tg mice, which was accompanied by elevated mRNA expression of BNP. MMP-2 activity was increased and MMP-9 activity was decreased in the left ventricle (LV) of 3-months-old tg mice. This was accompanied by elevated TIMP-4 expression, followed by a shift of collagen mRNA expression from type III to type I in this ventricle. CONCLUSION: The shift to collagen I in the heart of tg mice might be associated with a stiffer ventricle resulting in diastolic dysfunction. This may be responsible for a relative and intermittent LV- and right ventricle (RV)-insufficiency which was likely to have occurred as evidenced by the elevation of lung and liver weight with hemorrhage and interstitial fibrosis after 7 months.

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Cardiac Remodeling in Erythropoietin-Transgenic Mice

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Key Words

Brain natriuretic peptide • Matrix metalloproteinases • Extracellular matrix • Lung and liver fibrosis

Abstract

Background: Transgenic (tg) mice with chronic overexpression of the human erythropoietin gene are characterized by an increased hematocrit of about 0.80 in adulthood. This is accompanied by cardiac dysfunction and premature death. The aim of this study was to examine whether this cardiac dysfunction was accompanied by hypertrophy of the heart with remodeling of the extracellular matrix (ECM). Methods: 3-months-old wild type (wt) and tg mice without cardiac hypertrophy were compared with the respective 7-months-old mice. The mRNA of brain natriuretic peptide (BNP), of the matrix metalloproteinases (MMP)-2, -8, -9, -13, of the tissue inhibitor of metalloproteinase (TIMP)-1, -2, -3, -4 and of collagen I and III was detected by ribonuclease protection assay. The activity of MMPs was measured by zymography. Results: There was hypertrophy of both ventricles in 7-months-old tg mice, which was accompanied by elevated mRNA expression of BNP. MMP-2 activity was increased and MMP-9 activity was decreased in the left ventricle (LV) of 3-months-old tg mice. This was accompanied by elevated TIMP-4 expression, followed by a shift of collagen mRNA expression from type III to type I in this ventricle. Conclusion: The shift to collagen I in the heart of tg mice might be associated with a stiffer ventricle resulting in diastolic dysfunction. This may be responsible for a relative and intermittent LV- and right ventricle (RV)-insufficiency which was likely to have occurred as evidenced by the elevation of lung and liver weight with hemorrhage and interstitial fibrosis after 7 months.

Introduction

Erythrocytosis is characterized by an increased number of red cells in the peripheral blood. This form of polycythemia has been classified as primary erythrocytosis with truncation of the erythropoietin (EPO) receptor or polycythemia vera, as secondary and as idiopathic erythrocytosis [1]. There are congenital secondary forms of erythrocytosis such as high oxygen affinity hemoglobin or autonomous high EPO production

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and acquired secondary erythrocytosis by hypoxia or renal disease. Erythrocytosis has been regarded as one of the conditions in which hyperviscosity of the blood occurs. Under physiological conditions, the hyperviscosity state does not present a rheological problem, although platelet-vessel wall interactions are enhanced [2]. However, when circulatory adaptations in various types of erythrocytosis are limited or when general circulatory disturbances occur, blood hyperviscosity plays a critical role in reducing flow and causing ischemia and tissue damage.

Recently, a transgenic mouse line (EPO-tg6) has been generated that constitutively overexpresses the human EPO cDNA in an oxygen-independent manner [3]. This tg mice show excessive erythrocytosis with hematocrit values of 0.80. EPO is a 30.4 kDa glycoprotein that regulates red cell production [4]. In humans, peritubular cells in the adult kidney and hepatocytes in the fetus produce EPO. EPO binds to an erythroid progenitor cell surface receptor and acts primarily to rescue erythroid cells from apoptosis to increase their survival. EPO acts synergistically with several growth factors to cause maturation and proliferation of erythroid progenitor cells. This leads to hypoxia-mediated erythrocytosis with elevated hematocrit especially in high-altitude residents [5]. Adult EPO-tg6 animals do not develop hypertension or thromboembolic complications, a common reason for occlusive complications in erythrocytoses [6]. The expected rise in blood pressure and cardiovascular complications are prevented by a pronounced increase in NO bioavailability [3], and regulated elevation of blood viscosity is counteracted by increasing erythrocyte flexibility [7] in these animals. However, it has also been shown that the endothelin system is activated in these tg mice, suggesting an impairment of vasoactive mechanisms [8].

It has been demonstrated recently that the erythrocytosis-induced elevation of hematocrit leads to cardiac dysfunction with strongly reduced endurance upon exercise and to premature death [9]. However, there was no difference in heart function under baseline resting conditions [10]. The reduced exercise-endurance could be explained by acute heart failure since norepinephrine infusion in EPO-tg6 mice led to myocardial ischemia with diastolic dysfunction [10]. The hearts of tg mice were hypertrophied [9, 10]. Hypertrophy of the heart is often associated with cardiac fibrosis [11].

It was the aim of this study to investigate whether hypertrophy of the heart in EPO-tg6 mice is associated with cardiac fibrosis. Therefore extracellular matrix (ECM) remodeling was characterized by the analysis of the expression of collagen I and III, main components of the ECM, and the mRNA expression and activity of collagen degradation enzymes, the matrix metalloproteinases (MMP's) and their inhibitors (TIMP's). In addition, lung and liver were analyzed histologically and immunohistochemically for possible manifestations of the consequences of heart failure.

Materials and Methods

Animals

The transgenic mouse line termed tg6 carries the human erythropoietin (EPO) cDNA driven by the platelet-derived growth factor β (PDGF-β) promoter as described earlier [3]. The transgene is inherited as an autosomal dominant trait. Hemizygous transgenic (tg) males were mated to wild-type (wt) C57Bl/6 females, and the wt littermates served as controls in all experiments. 3 months old mice (wt: n = 5, tg: n = 5) were compared with 7 months old mice (wt: n = 19, tg: n = 18) The animals were maintained in accordance with the *Guide for Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). They were allowed to move freely in their cages with access to tap water and standard chow diet (Altromin C 100, Altromin GmbH, Lage, Germany), ad libitum.

The hearts were rapidly excised from the anesthetized animal (Trapanal 100 mg/kg i.p., Byk Gulden). The RV free wall was trimmed away. Both ventricles were weighed after freezing in liquid nitrogen. Lung and liver were excised, weighed, one part each was fixed in 4% buffered formaldehyde, and the other part was frozen in liquid nitrogen. Lung specimens of 9 animals were dried for 10 hours at 95°C. In these lung samples, the wet-to-dry weight ratio was determined as an indicator of potential edema.

Histological analysis

In another series, the hearts of 7 months old mice (wt: n = 5, tg: n = 5) were excised and fixed in 4% buffered formaldehyde, embedded in paraffin, and 5 μm sections were stained with hematoxylin-eosin (HE), PAS (periodic acid Schiff) and Sirius red (0.5% in saturated aqueous picric acid). The sections were quantified morphometrically with a computer-assisted image analysis system (VIDAS 25, Zeiss®, Germany) [12]. Collagen quantification was performed under a microscope fitted with cross-polarization filters by analyzing sections stained with Sirius red using the VIDAS system. Under polarization microscopy the collagen fibers which had been stained with Sirius red luminated in green or red-yellow colors. Cardiac fibrosis can be quantified due to these birefringent properties of fibrillar collagen.

The fixed and paraffin-embedded lung and liver samples were sectioned in 7 μm slices and stained with HE. The immunohistochemical analysis was performed as described recently [13]. For the immunohistochemical visualization of collagen Type I, III and IV, the following antibodies were used: collagen Type I: Batch 363205 (Quartett®, Berlin, GERMANY);

dil.: 1:50; collagen Type III: Clone HWD 1.1 (BioGenex®, Hamburg, GERMANY); dil.: 1:100; collagen Type IV: Clone CIV22 (DAKO®, Hamburg, GERMANY); dil.: 1:100. The reactivity of the antibodies was tested using sections of tissue with known positive reactivity in each batch. Negative controls were also performed by replacing the primary antibody with goat ascites fluid (Sigma-Aldrich Biochemicals®, St. Louis, MO). In all cases, liver and lung tissue from tg and wt animals was used. The staining of the sections was evaluated by an expert pathologist to rate the severity of histological changes without the knowledge which section was from wt or tg animal. The description of the histological changes represents the characteristic features of all preparations. For presentation, we chose typical examples, which were observed in all slices of the same subgroup.

RNase Protection Assay

Total RNA was isolated using TRIZOL™ reagent (Invitrogen™, Karlsruhe, Germany) according to the manufacturer's protocol. 2.5 or 7.5 µg of total RNA were used in the RNase protection assay (RPA) with the probe template mECM1 or mECM2, respectively, labeled with RiboQuant® *In Vitro* Transcription Kit (Pharmingen, Hamburg, Germany; final probe concentration: $4x10^5$ cpm/µl) and [α-^{32}P] UTP (3000 Ci/mmol, Amersham, Freiburg, Germany), as described by the manufacturer. After hybridization (56°C; 12-16 h), the unhybridized riboprobe was digested with a mixture of RNases A and T1 (RiboQuant® RPA Kit, Pharmingen, Hamburg, Germany), according to the manufacturer's instructions. Protected probes were displayed by electrophoresis on a denaturing gel containing 5% polyacrylamide/8 M urea, followed by visualization with the Molecular Imager (BioRad, München, Germany). The densitometric quantification of the individual bands of the RPA assays was performed by the Multi-Analyst program version 1.1 (BioRad, München, Germany). The probe template set mouse ECM number 1 and 2 (mECM1 and 2) were used. These contained the following mouse cDNA (probe length in bp/protected; GenBank Accession No., position). mECM1: CNP (432/380; NM_010933, 136-515), BNP (392/343; NM_008726, 1-343), colligin (350/306; NM_009825, 1016-1321), collagen I (250/200; U08020, 1914-2113), collagen III (200/149; X52046, 999-1147), L32 (165/113; M35397, 208-321) and GAPDH (150/96; NM_008084, 225-320);

mECM2: MMP-9 (480/430; S67830, 834-1263), MMP-2 (430/386; NM_008610, 432-817), TIMP-2 (390/341; NM_011594, 274-614), MMP-8 (325/274; NM_008611, 159-433), MMP-13 (290/240; NM_008607, 582-821), TIMP-3 (250/198; NM_011595, 355-552), TIMP-1 (215/160; NM_011593, 148-307), TIMP-4 (190/136; AF282730, 535-670), L32 and GAPDH as mentioned above. These cDNA probes were generated by RT-PCR essentially as previously described [14].

Preparation of cardiac tissue extract and zymography

Extracellular proteins of approximately 25 mg frozen tissue were extracted with 20-fold volume of extraction buffer (10 mM Tris-Cl pH 7.5, 150 mM NaCl, 20 mM $CaCl_2$, 1 µM $ZnSO_4$, 0.01% (v/v) Triton X-100, 1.5 mM NaN_3, 0.5% PMSF) over night at 4°C. These protein extracts contained approximately 1.5 mg/ml protein (BioRad Protein assay, BioRad, München, Germany). Myocardial matrix metalloproteinase activity in the gel was measured as previously described [15].

Statistical analysis

All data were analyzed and expressed as mean ± S.E.M. A multiple-sample comparison (ANOVA and multiple range test using the criterion of the least significant differences) was applied to test the differences between appropriate groups for significance. A value of $p < 0.05$ was considered to be significant. The program Statgraphics plus 4.1 (Statistical Graphics Corp.) was used for all statistical calculations.

Results

Heart

The ventricle weight/body weight (VW/BW) ratio, a macroscopic index of hypertrophy, of both ventricles was elevated in 7-months-old tg mice (Fig. 1A). Because increases in the expression of brain natriuretic peptide (BNP) gene is a marker of hypertrophy, we used RPA to quantify changes in BNP gene expression in wt and tg mouse hearts. BNP mRNA expression was elevated in tg animals at 7 months (Fig. 1B). The expression level of BNP was higher in the LV than in the RV in wild type and tg mice (Fig. 1B).

Hypertrophy of the heart requires remodeling of the ECM. Pathological remodeling leads to fibrosis with elevated expression of collagen and its deposition in the majority of experimental models. The collagen I expression tended to be increased in the LV and to be decreased in the RV of wt and tg mice both at 3 and 7 months (Fig. 2A). The collagen III mRNA expression was unchanged in the LV and decreased in the RV in 7-months-old wt and tg mice (Fig. 2B). The collagen type I/type III ratio was increased in the LV of 7-months-old tg animals (Fig. 2C). The prevalence of collagen I could have resulted in a stiffer heart, since collagen I is responsible for the formation of collagen fibers. There was a positive correlation between the expression of BNP and of collagen I in the LV of tg mice (Fig. 3).

To analyze the potential remodeling process of ECM, the MMP-2 and MMP-9 gelatinolytic activity was measured. There was an increase of MMP-2 activity and a slight decrease of MMP-9 activity in the LV of 3-months-old tg mice (Fig. 4A). The mRNA level of MMP-8 and MMP-13 was not detectable with 7.5 µg of total RNA of cardiac tissue. The MMP-9 signal was very weak. Therefore it was not possible to give a ratio to GAPDH. The mRNA expression of MMP-2 was not changed in both ventricles in wt and in tg mice (Tab. 1).

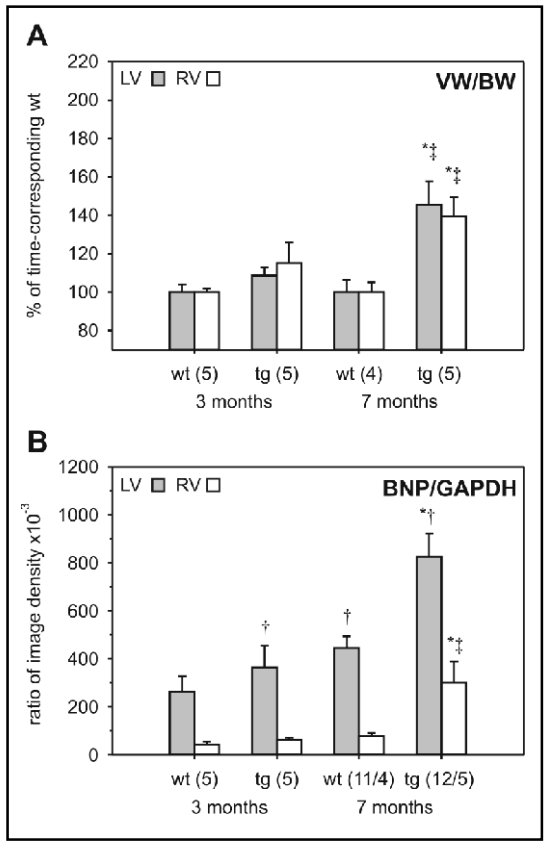

Fig. 1. Development of left ventricular (LV) and right ventricular (RV) hypertrophy in erythropoietin over-expressing transgenic (tg) mice. A) Ventricular weight (VW) relative to body weight (BW) of tg mice in relation to wild type (wt) controls after 3 and 7 months of age. Data are expressed as per cent of time-corresponding wt. B) The mRNA of the brain natriuretic peptide (BNP) related to the mRNA of GAPDH. Means ± S.E.M.; * $P < 0.05$ vs. the time-corresponding wt; † $P < 0.05$ vs. RV; ‡ $P < 0.05$ vs. corresponding 3 months results; number of measurements in parentheses (first for LV/second for RV in B).

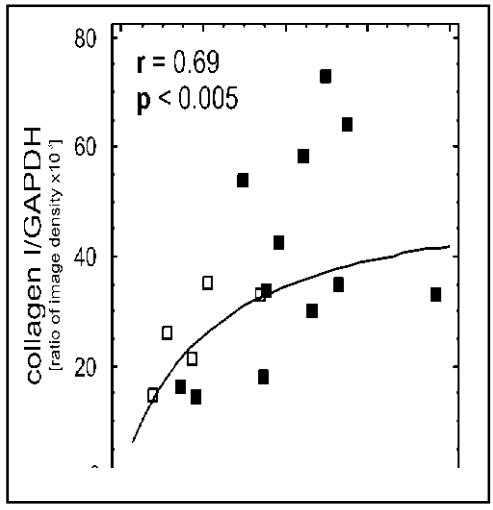

Fig. 3. The mRNA content of collagen I in the left ventricle of 3 months old (open squares) and 7 months old (filled squares) transgenic mice in correlation to the mRNA content of brain natriuretic peptide (BNP). The data were analysed with the double reciprocal model with a R-squared value of 47.6%: collagen I = 1/(0.019 + 8.124/BNP).

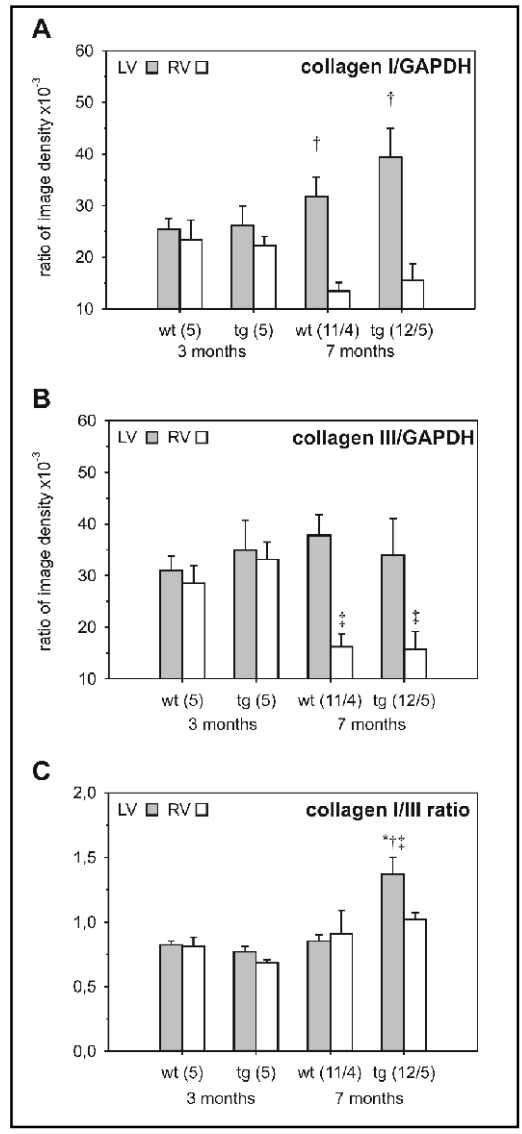

Fig. 2. Collagen type I (A) and type III (B) mRNA in the left (LV) and right (RV) ventricle in transgenic (tg) mice in comparison to wild type (wt) after 3 and 7 months, respectively. RNA was subjected to RNase protection assay. Data are given as mRNA abundance relative to GAPDH mRNA expression. C) The relative increase of collagen I mRNA in the LV of 7-months-old tg mice was shown by the increase in the collagen type I to type III ratio. Means ± S.E.M.; * $P < 0.05$ vs. the time-corresponding wt; † $P < 0.05$ vs. RV; ‡ $P < 0.05$ vs. corresponding 3 months results; number of measurements in parentheses (first for LV/second for RV).

TIMP-1 mRNA was not detectable. For all detectable TIMPs, there was a slightly higher expression rate in the RV than in the LV especially in wt controls (Tab. 1). This higher expression level was significant for TIMP-3 in 7-months-old wt mice. There was a significantly higher expression in the RV and a higher expression in comparison to mRNA in the RV of 3-months-old wt mice. The ratio between TIMP-2:TIMP-3:TIMP-4 was changed predominantly in the LV of 3-months-old tg mice in comparison to wt controls (Tab. 1). This change was characterized by a significantly increased TIMP-4 mRNA expression in the LV of 3

Table 1. Cardiac mRNA expression of matrix metalloproteinase (MMP) -2 and its tissue inhibitors (TIMP) -2, -3 and -4 in the left (LV) and right ventricle (RV) of transgenic (tg) mice overexpressing the human erythropoietin gene in comparison to wild type mice (wt).

		3 months		7 months	
		wt (n = 5)	tg (n = 5)	wt	tg
MMP-2 /GAPDH [ratio of image density x10^{-3}]					
	LV	7.5 ± 1.51	11.9 ± 1.94	6.8 ± 1.79 (n = 14)	5.5 ±0.48 (n = 13)
	RV	20.6 ± 6.55	14.2 ± 5.22	12.2 ±5.1 (n = 4)	4.4 ± 0.33 (n = 5)
TIMP-2 /GAPDH [ratio of image density x10^{-3}]					
	LV	24.4 ± 3.99	26.5 ± 1.83	19.2 ± 2.29 (n = 14)	18.7 ± 1.09 (n = 13)
	RV	32.2 ± 4.31	32.3 ± 6.63	28.2 ± 2.11 (n = 4)	27.3 ± 1.41 (n = 5)
TIMP-3 /GAPDH [ratio of image density x10^{-3}]					
	LV	23.9 ± 6.59	31.5 ± 1.67	43.1 ± 5.7 (n = 14)	47.7 ± 7.3 (n = 13)
	RV	31.7 ± 1.93	43.4 ± 13.49	73.5 ±9.81 (n = 4)†‡	66.1 ± 5.81 (n = 5)
TIMP-4 /GAPDH [ratio of image density x10^{-3}]					
	LV	3.7 ± 0.48	11.3 ± 2.2	4.9 ± 1.57 (n = 14)	5.3 ± 0.56 (n = 13)
	RV	8.5 ± 1.3	7.2 ± 1.65	10.5 ± 4.18 (n = 4)	5.5 ± 0.99 (n = 5)
ratio	TIMP-2:TIMP-3:TIMP-4				
	LV	7:6:1	2*:3*:1	4‡:9:1	4:9‡:1
	RV	4:4:1	4:6†:1	3:7:1	5:12*†‡:1

Values are mean ± S.E.M.;* P < 0.05 vs. time corresponding wt.; † P < 0.05 vs. LV; ‡ P < 0.05 vs. corresponding 3 months results

months old tg mice (Fig. 4B). The predominant TIMP in cardiac tissue was TIMP-3 in 7-months-old wt and tg mice in LV as well as in RV (Tab. 1).

The hypertrophy of the tg-heart was not accompanied by an increase of Sirius red-detectable collagen deposition. In the LV, the interstitial collagen content was 0.28 ± 0.03% in wt mice and 0.30 ± 0.03% in tg mice; in the RV, it was 0.35 ± 0.05% in wt and 0.38 ± 0.06% in tg animals ($p > 0.05$, $n = 5$ in wt and tg, respectively, results from Sirius red staining, data not shown).

Lung

The relative lung weight was increased in 7-months-old tg mice (Fig. 5A). However, this increase was not accompanied by an elevated wet-to-dry weight ratio of the lung (wt: 4.16 ± 0.03%, n = 4; tg: 4.49 ± 0.14%, n = 5; $p > 0.05$). There was slight edema but pronounced hemorrhage (Fig. 5D) without iron deposition (iron staining, data not shown). The interalveolar septa had a greater thickness in 7-month-old tg animals (Fig. 5D). This was accompanied by interstitial and perivascular fibrosis, indicated by elevated deposition of collagen I (Fig. 5E), collagen III and IV (data not shown).

Liver

The relative liver weight was also increased in tg mice (Fig. 6A). The liver of 7-months-old tg mice was characterized by hemorrhage (Fig. 6D) with iron deposition (data not shown) and by thickening of the vessel wall (Fig. 6D), which was accompanied by perivascular and interstitial fibrosis. There was more collagen I (Fig. 6E), collagen III and collagen IV (data not shown) in the interstitial and perivascular space in 7-month-old tg mice.

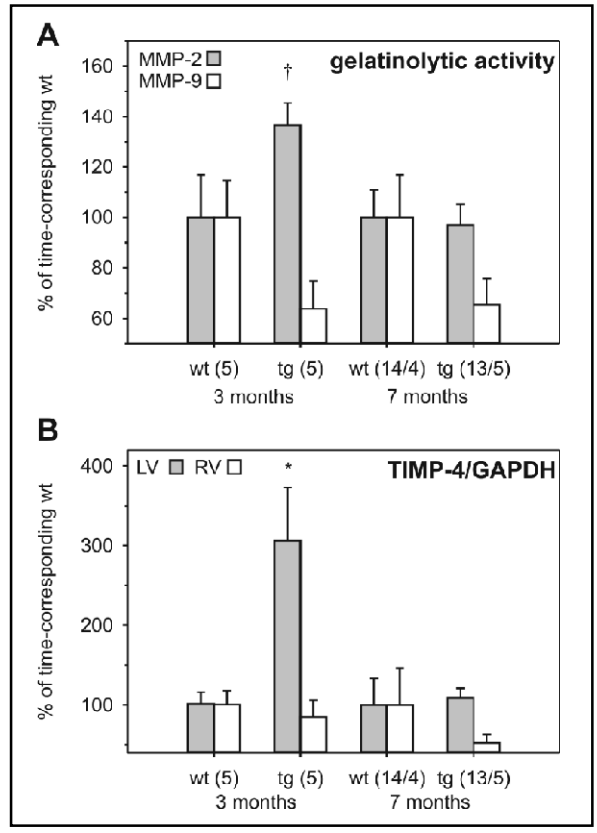

Fig. 4. A) Relative abundance of MMP-2 (filled bars) and MMP-9 (open bars) activity in the LV of wt and tg mice at 3 and 7 months of age obtained from zymography. Band intensities were compared with the protein concentration. B) TIMP-4 mRNA expression in the LV (filled bars) and RV (open bars) in tg mice in comparison to wt mice. RNA was subjected to RNase protection assay. All data are expressed as per cent of time-corresponding wt. Means ± S.E.M.; * P < 0.05 vs. the time-corresponding wt; † P < 0.05 vs. MMP-9; number of measurements in parentheses (first for MMP-2/second for MMP-9 in A, and first for LV/second for RV in B).

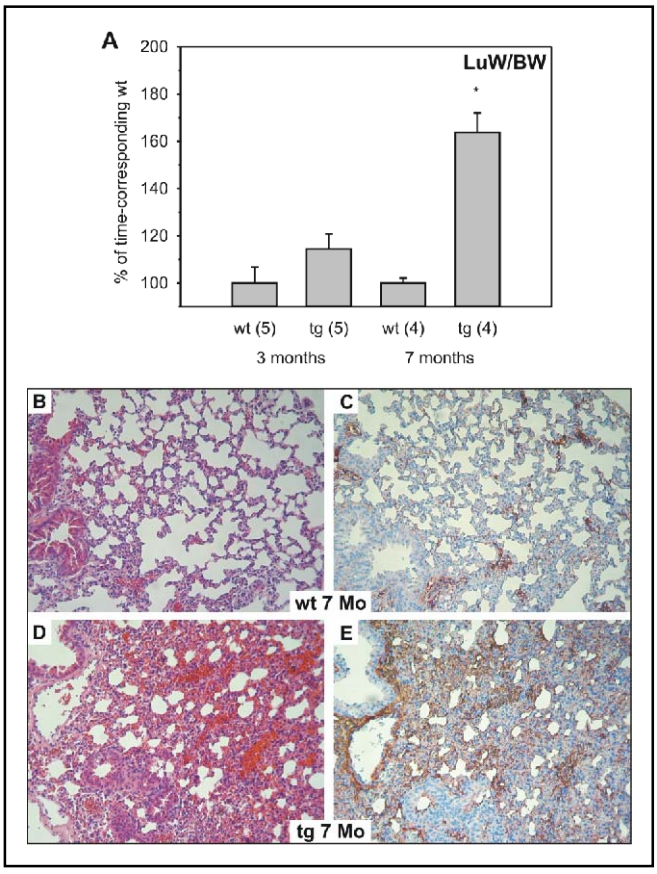

Fig. 5. Lung weight/body weight ratio (LuW/BW) in wild type (wt) and transgenic (tg) mice after 3 and 7 months. A) Results are expressed as per cent of time-corresponding wt. Means ± S.E.M.; * $P < 0.05$ vs. the time-corresponding wt; number of measurements in parentheses. Histological changes (B-E) in tg mice in comparison to wt in consecutive slices. Representative hematoxylin-eosin staining of 7 months old wt (B) and tg (D) mice. Representative immunohistochemical staining of collagen I of 7 months old wt (C) and tg (E) animals. After immunostaining, the slices were counterstained with hematoxylin (original magnification 28x).

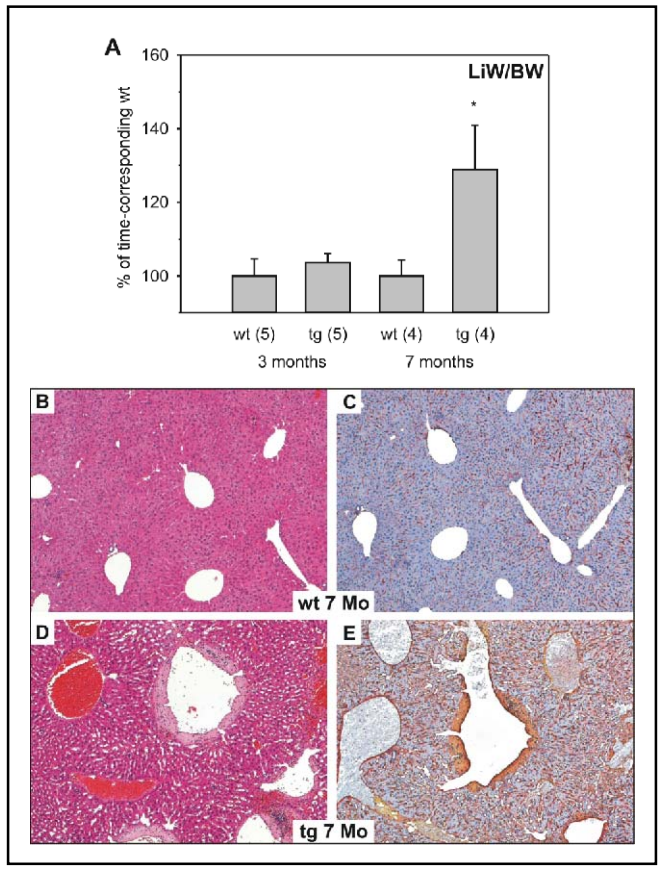

Fig. 6. Liver weight/body weight ratio (LiW/BW) in wild type (wt) and transgenic (tg) mice after 3 and 7 months. A) Results are expressed as per cent of time-corresponding wt. Means ± S.E.M.; * $P < 0.05$ vs. the time-corresponding wt; number of measurements in parentheses. Histological changes (B-E) in tg mice in comparison to wt in consecutive slices. Representative hematoxylin-eosin staining of 7 months old wt (B) and tg (D) mice. Representative immunohistochemical staining of collagen I of 7 months old wt (C) and tg (E) animals. After immunostaining, the slices were counterstained with hematoxylin (original magnification 20x).

Discussion

Erythrocytosis induced an increase of the heart weight of both ventricles in 7 months old tg mice (Fig. 1A). The hypertrophy of both ventricles (Fig. 1A) was accompanied by an elevation of LV BNP production (Fig. 1B). Brain natriuretic peptide (BNP), the more ventricle-specific natriuretic peptide is elevated in cardiac hypertrophy and was described as an "emergency" cardiac hormone against ventricular overload [16]. It has been shown that central venous pressure was elevated in EPO-tg6 mice [9]. Cardiac hypertrophy in 8 months old tg6 mice was also documented at the histological level by an increase of myocyte cross sectional area from 495 ± 32 to 644 ± 49 μm^2 ($p < 0.05$) [10]. In contrast, in rats in which the hematocrit was raised to 0.63 by EPO administration, no hypertrophy of the RV or LV was observed [17, 18]. However, moderate RV hypertrophy without LV hypertrophy had occurred after i.p. injection of packed red cells into mice [19, 20]. The hematocrit had been increased to 0.67-0.80. The hematocrit of 0.63 apparently is not high enough to induce cardiac hypertrophy. Increased hematocrit induces RV hypertrophy and hematocrit of 0.80 and higher induces hypertrophy of both ventricles.

The erytrocytosis-induced hypertrophy of the heart was accompanied by a change in the composition of the main components of the ECM, collagen I and III. There

was an age-dependent decrease in the mRNA expression of both collagens in the RV (Fig. 2). The expression of collagen III was changed to a lower expression and that of collagen I to a higher expression in tg mice, thus resulting in a relative elevation of the collagen I portion, especially in the LV of tg mice. There was more collagen III than collagen I in 3-months-old wt and tg mice as shown by the collagen type I to type III ratio of around 0.8 (Fig. 2C). The collagen type I to type III ratio was elevated to 1.4 in the LV of 7-months-old tg mice (Fig. 2C). This elevated collagen I mRNA content in the ECM could have been responsible for a higher stiffness of this ventricle, since collagen I is not as elastic as collagen III [21].

The lack of overt fibrosis in erythrocytosis-induced hypertrophy can be explained by the fact that the concentration of collagen is not greater in volume overload hypertrophic hearts [22]. A higher cross-linking rate was postulated, since there was a rise in the activity of lysyl oxidase, which is the enzyme responsible for initiating the formation of cross-links in collagen. The decrease of the relaxation time index of the LV from 2.66 ± 0.11 in wt mice to 2.23 ± 0.14 ($p < 0.05$) [10] could be a result of the stiffer LV. Nevertheless, the baseline heart function of tg6 mice was not significantly different from wt mice apart from a decreased pressure amplitude in the aorta (wt: 26.6 ± 1.3, tg: 22.1 ± 0.9 mmHg, $p < 0.05$, [10]) and an increased central venous pressure (wt: 0, tg: 3 ± 2 mmHg, $p < 0.05$, [9]) in tg6 mice. However, tg6 mice had a reduced exercise performance [9]. If the mechanism of norepinephrine-stimulation is similar to that of reduced exercise performance in tg6 mice, then myocardial ischemia and ultimately acute heart failure is a typical feature [10].

There was a good correlation between BNP and the expression of collagen I in the LV (Abb. 3). This supports the significance of the relative elevation of collagen I expression in tg mice. This correlation can also be explained in the light of the known antifibrotic properties of BNP [23]. Focal fibrotic lesions were observed in ventricles from $BNP^{-/-}$ mice with a remarkable increase in ventricular mRNA expression of collagen I, which is implicated in the generation and progression of ventricular fibrosis. High mRNA levels of collagen I in EPO-tg6 mice might be responsible for the increased expression of BNP which in turn may prevent the deposition of collagen and thus fibrosis. This balance could have been responsible for the lack of interstitial fibrosis in older tg mice.

Tissue inhibitors of metalloproteinases (TIMPs) comprise a family of four distinct, but structurally and functionally similar proteins that (together with the MMP's) regulate the extracellular matrix in a variety of tissues and can lead to remodeling [24]. The most recently described member, TIMP-4 is the most cardiospecific [25, 24] and forms complexes with pro MMP-9 and MMP-2 [26]. Therefore, elevated TIMP-4 expression (Fig. 4B) could explain the change of MMP gelatinolytic activity from MMP-9 to MMP-2 in the LV of 3-months-old tg mice (Fig. 4A). TIMP-4 seems to have a higher potency to inhibit MMP-9 activity than to inhibit MMP-2 activity since the higher level of complexed TIMP-4 in sham-operated dogs was accompanied by lower MMP-9 activity in comparison to pacing-induced atrial cardiomyopathy [27]. The elevated expression of TIMP-4 only in the LV of 3 months old tg mice (Fig. 4B) which resulted in significant changes of the ratio between TIMP-2, -3 and -4 (Tab. 1) and a shift of MMP activity to MMP-2 (Fig. 4A) seems to be a sign for an early remodeling process of the ECM. The reason for the altered ratio between TIMP-2, -3 and -4 in the RV of 7 months old tg mice (Tab. 1) could probably be explained by a different nature of ECM remodeling in the RV, since TIMP-3 was more pronounced.

The increased lung weight (Fig. 5A) in combination with hemorrhage, thickening of interalveolar septa (Fig. 5D) and especially interstitial fibrosis (Fig. 5E) in 7-months-old tg mice may be an indication that there was an intermittent LV insufficiency. This appears to be a relative cardiac insufficiency which becomes manifest only when the animals are subjected to exercise [9] or NE infusion [10].

Also liver showed signs of hemorrhage (Fig. 6D) and was characterized by interstitial and perivascular fibrosis (Fig. 6E), with an increase of the relative liver weight in tg animals (Fig. 6A). The increase was not so pronounced as in lung. However, this might also be the result of relative RV insufficiency which was not as pronounced as in the LV.

In conclusion, the present study demonstrates that in chronic erythrocytosis there was remodeling of the heart with development of hypertrophy and alteration in the composition of the ECM in mice overexpressing EPO. Increased MMP-9 and decreased MMP-2 activity could be induced by elevated TIMP-4 expression in the LV of 3-months-old tg mice, followed by a shift of collagen mRNA expression from type III to type I in this ventricle without a change in the amount of interstitial collagen content in 7-months-old tg animals. The shift to collagen I might be associated with a stiffer ventricle and thus

lead to diastolic dysfunction. This could be responsible for a relative and intermittent LV and RV insufficiency, which in the long run leads to elevation of lung and liver weight with hemorrhage and interstitial fibrosis.

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