Doxycycline ameliorates the susceptibility to aortic lesions in a mouse model for the vascular type of Ehlers-Danlos syndrome

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Running Title Page

VEDS-treatment by doxycycline

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Abbreviations: AAA, abdominal aortic aneurysm; ANOVA, analysis of variance; AR, adrenergic receptor; AU, arbitrary unit; BCA, bicinchoninic acid; COL3A1, alpha-1 chain of collagen type III; FDA, US Food and Drug Administration; HT, heterozygous knockout mouse; MMP, matrix metalloproteinase; OMIM, database Online Mendelian Inheritance in Man; PCR, polymerase chain reaction; SEM, standard error of the mean; vEDS, vascular form of Ehlers-Danlos syndrome; WT, wild-type mice

Section: Cardiovascular
Abstract

**Aims:** The vascular form of Ehlers-Danlos syndrome (vEDS), a rare disease with grave complications due to rupture of major arteries is caused by mutations of collagen type III (COL3A1). The only, recently proven, preventive strategy consists of reduction of arterial wall stress by beta-adrenergic blockers. Heterozygous Col3a1 knockout mouse (HT) has reduced expression of collagen III and recapitulates features of a mild presentation of the disease. The objective of this study was to determine whether change of the balance between synthesis and degradation of collagen by chronic treatment with doxycycline, a nonspecific matrix metalloproteinase (MMP) inhibitor, could prevent the development of vascular pathology in HT mice. **Methods and Results:** Following 3-mo treatment with doxycycline or placebo, 9-mo old HT or wild-type mice (WT) mice were subjected to a surgical stressing of the aorta. A three fold increase in stress-induced aortic lesions found in untreated HT one week after intervention (cumulative score 4.5±0.87 vs. 1.3±0.34 in wild-type, p<0.001) was fully prevented in the doxycycline treated group (1.1±0.56, p<0.001). The untreated HT showed increased MMP-9 activity in the carotid artery and decreased collagen content in the aorta, however, in doxycycline treated animals there was normalization to the levels observed in WT. **Conclusion:** Doxycycline treatment inhibits activity of tissue MMP and attenuates the decrease in the collagen content in aortas of mice haploinsufficient for collagen III, as well as prevents the development of stress-induced vessel pathology. The results suggest that doxycycline merits clinical testing as a treatment for vEDS.
Introduction

The vascular form of Ehlers-Danlos syndrome (vEDS, OMIM 130050) is a rare autosomal dominant disorder (incidence 1:100,000)(Germain, 2007) caused by mutations in the alpha-1 chain of type III collagen (COL3A1)(Pope et al., 1975; Smith et al., 1997; Pyeritz, 2000; Germain, 2007). Type III collagen is a homotrimeric fibrillar collagen found abundantly in the wall of arteries, the gastrointestinal tract, as well as in the uterus, and other tissues. The quantitative or qualitative deficit of structurally normal collagen III in vEDS and corresponding changes in the wall structure are responsible for major complications observed in afflicted individuals: arterial, bowel, and uterine ruptures. As a result of these dramatic complications, life expectancy in vEDS is shortened to a mean of <50 years (Pepin et al., 2000; Watanabe et al., 2007). Until very recently, there was no evidence based treatment or preventive strategy available (Watanabe and Shimada, 2008). However, in the just completed first multicenter randomized trial, almost 4 years (47 months) treatment with celiprolol – a long-acting \( \beta_1 \) antagonist with partial \( \beta_2 \) agonist properties used for treatment of hypertension – decreased the incidence of arterial ruptures in patients with clinical diagnosis of vEDS (Ong et al., 2010). Celiprolol was uptitrated every 6 months by steps of 100 mg to a maximum of 400 mg twice daily.

More than 150 mutations in the COL3A1 gene leading to synthesis of an abnormal type III procollagen protein have been presently identified (Stenson, 2009). Most of the mutations are single-nucleotide substitutions of the canonical glycine residues in the triple-helical domain of the pro\( \alpha_1 \) (III) chain, resulting in a regular quantity of abnormal collagen III; however haploinsufficiency of collagen III (reduced quantity of normal collagen III) is also reported in vEDS patients (Schwarze et al., 2001; Khalique et al., 2009).
We have shown recently that haploinsufficiency for Col3a1 in mice recapitulates mild presentation of vEDS in humans and thus can serve as an experimental model in the search for effective treatments (Cooper et al., 2010).

It has been reported that in the third stage of aneurysm development, rapid expansion and increased risk of rupture are associated with accelerated degradation of collagen (Thompson and Baxter, 1999). Doxycycline, a tetracycline antibiotic and broad spectrum metalloproteinase (MMP) inhibitor, has been successfully tested in pilot clinical trials for the treatment of abdominal aortic aneurysms (Curci et al., 2000; Mosorin et al., 2001; Baxter et al., 2002; Lindeman et al., 2009). Doxycycline, given in a subantimicrobial dose, is also the only MMP inhibitor approved by FDA. It is currently used for treatment of periodontal disease (Wennstrom et al., 2001) and rosacea (Del Rosso et al., 2007). We hypothesized that MMP inhibition in the mouse experimental model of vEDS would shift the balance between collagen degradation and synthesis in the vascular wall and protect against vascular damage.

Methods

See Supplementary Methods for description of quantification of collagen content in skin and description of the colons biomechanical properties detection.

Subjects

Heterozygous Col3a1 deficient mice (strain C.129S4(B6)-Col3a1^tm1.Jae/J) (Liu et al., 1997) were rederived (Jackson Lab, Bar Harbor, ME) and bred in the vivarium of the National Institute on Aging. The Col3a1 genotype was determined by PCR (5’ CTTCTCACCCCTTCTTACATCC 3’, 5’ AGCCTGTTCATCAATCGGTACC 3' and neo 5’ GCTATCAGGACATAGCGTTG 3’) after weaning, and genotypes were reconfirmed at
the end of the experiments. Animals were housed and studied in conformance with the NIH Guide for the Care and Use of Laboratory Animals, Manual 3040-2 (1999), with institutional Animal Care and Use Committee approval (363-LCS). Mice were maintained on ad libitum food (NIH-07 Mouse/Rat diet) with permanent access to filtered water.

**Experimental protocol**

Two groups of six months old female HT mice were treated for 3 months with doxycycline (compound ID 5281011). Treatment was provided with food containing 200 mg/kg or 800 mg/kg of doxycycline (“Dox Diet” pellets, BioServ, Frenchtown, NJ). Since preliminary measured food intake of these mice was averaged at 3.5 g/day and average body weight of animals was 25 g, the average drug dose for low and high dose groups was 25 (Doxy25) or 100 (Doxy100) mg/kg per day, respectively. Untreated WT and HT mice were maintained on a regular diet (NIH-07 Mouse/Rat diet) and served as controls. After 3 months, under general inhalation anesthesia (2% of isoflurane in oxygen) and aseptic conditions, the abdominal aortas were surgically exposed and stressed by the following technique: the blood flow was stopped by occluding the abdominal aorta against the spinal column with a sterile cotton-tip applicator pressed at the level of the renal arteries. After 30 seconds a second applicator was pressed at the level of iliac bifurcation and the first applicator was abruptly released, followed by release of the second applicator. According to Weinbrenner et al (2002), who used a similar procedure for remote preconditioning in rats, the mean arterial pressure had been elevated by about 13% immediately after occlusion. The abdominal incision was sutured closed and mice returned to home cages. The treatment was continued after the intervention. One week after intervention mice were euthanized by an overdose of isoflurane. Blood was collected, and aortas and segments of colon and skin were harvested.
Tissue collection

Blood was collected from the left ventricle. The abdominal aorta was exposed and opened at the bifurcation. A 3% (w/v) agarose solution (SeaPlaque GTG Agarose, low-melt, Lonza, Allendale, NJ) diluted in physiological salt solution and colored with Evans blue (Sigma-Aldrich) at 37°C was injected through the left ventricle into the aorta to prevent collapse during tissue processing. The aorta was dissected free from the surrounding connective tissue and pinned onto a wax block before fixation in 10% formalin for 2 days. Cross sections of the aorta (2 mm in thickness) were ordered in 8% agar to create a block with an average of 20 sections of the aorta. The block was stored in 70% ethanol until it was processed and embedded in paraffin (AML Lab, Baltimore, MD). Samples of transverse colon were used for the determination of biomechanical properties. Left and right carotid arteries and a piece of the tail and the skin from the back were snap-frozen in liquid nitrogen.

Histological analysis

5 μm sections from each block of aortic sections were stained with hematoxylin and eosin (H&E) and Masson’s trichrome. Two veterinary pathologists (TKC, HJT) blinded to genotype independently evaluated the stained sections to count the number of lesions present in aortas and rate the severity of each lesion on a subjective 1-4 scale according to previously reported criteria (Cooper et al., 2010). Because of their mild nature, as well as a high frequency in both sexes and genotypes, grade 1 lesions were excluded from statistical analysis. The sum of the scores of lesions > grade 2 was added for each animal to produce a cumulative score.
Collagen detection by picro-sirius red staining

To examine collagen content in the vessel wall, 5 µm sections of abdominal aorta were stained with picro-sirius red. Digital images of stained sections were obtained from light microscopy using polarized filters and analyzed using a digital imaging analysis system (MCID, InterFocus Imaging Ltd, Cambridge, UK). The total collagen content in aortic wall as well as collagen content of tunicae adventitia and media separately were calculated as a percentage of the total area of the wall or its respective components (Seeland et al., 2007). Assessment was performed by a single individual (HJT) blinded to genotype of the animal.

Detection of MMP activity

Extracellular matrix proteins were isolated from right carotid artery and approximately 5 mg frozen skin from each animal. Proteins were extracted with 40 µl or 20-fold volume RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, 1 mM EDTA, 0.25% Na-deoxycholat, protease inhibitor mix M (SERVA Electrophoresis, Heidelberg, Germany), respectively), overnight at 4°C. Protein content was determined with the BCA method (BCA™ protein assay kit, Pierce, Rockford, IL).

Gelatinase activity was measured as described previously (Briest et al., 2001). Gelatin (0.1% (w/v), (SERVA Electrophoresis, Heidelberg, Germany) was added to standard Laemmli acrylamide polymerization mixture. Tissue extract was mixed 1:2 with sample buffer (250 mM Tris-Cl pH 6.8, 10% (w/v) SDS, 20% (v/v) glycerol, 0.005% (w/v) bromphenol blue). Serum was diluted 1:10 with electrophoresis buffer (2.5 mM Tris, 20 mM glycine, 0.005% SDS) and mixed 1:2 with sample buffer. Twenty microliters were loaded following 10 min incubation at room temperature without boiling. Following electrophoresis at 90 V, the gels were soaked in 2.5% (w/v) Triton X-100, incubated 2-3
days at 37°C in gelatin digestion buffer (50 mM Tris-Cl pH 8.0, 8 mM CaCl$_2$, 10 mM ZnSO$_4$, 0.02% (w/v) NaN$_3$), stained in 0.05% Coomassie blue R-250 (SERVA Electrophoresis, Heidelberg, Germany) in acetic acid:methanol:water (1:4.5:4.5 by volume), destained in 10% acetic acid, 5% methanol and scanned for lysis band intensity. The lysis band intensity is proportional to gelatinase activity and was quantified densitometrically by the program “one dimensional scan software” (Scanalytics, Rockville, MD, USA). The result, a number between 0.07 and 3.75, was normalized to the protein content by dividing the densitometry result with the relative optical density from the BCA protein assay kit result. The result was used for the analysis as the arbitrary unit (AU). For the total MMP activity results of lysis bands of proMMP-9, active MMP-9, proMMP-2 and active MMP-2 was added. A protein size marker (Page Ruler Plus, Fermentas, St. Leon-Rot, Germany) was used to determine the correct size.

**Statistical analysis**

All data are presented as mean ± SEM. The actual number of animals per group might vary in different measurements due to technical reasons and presented in the corresponding figures. A multiple-sample comparison (ANOVA and the multiple range test as post hoc test using the criterion of the least significant differences) was applied to test the differences between groups. Statistical significance was accepted at p<0.05.

**Results**

**Doxycycline treatment normalized MMP-activity in carotid arteries**

Gelatinases MMP-2 and MMP-9 were detected in extracts from carotid arteries both as an inactive pro-form and as an active form (Fig. 1a). All 4 bands were summarized as total MMP, which was significantly elevated in untreated HT mice compared to WT, and were normalized by doxycycline treatment in HT (Fig. 1b). Pro-
forms of both MMPs (data not shown) and active MMP-2 (Fig. 1d) were unchanged in either untreated HT or in HT treated by doxycycline relative to WT. However, there was a significant increase in active MMP-9 in untreated HT, which was reduced in a dose-dependent manner by doxycycline (Fig. 1c). The MMP-9 activity in HT after doxycycline treatment was statistically not different from WT mice with normal diet.

**Doxycycline treatment attenuates the reduction of collagen in the aorta**

The collagen content was measured by picro-sirius staining in the abdominal aorta, since statistically significant differences in collagen content between wild-type and heterozygous mice had been detected previously specifically in that segment of aorta. \(\text{(Cooper et al., 2010)}\) In agreement with our previous findings there was significantly less total collagen detected in untreated HT mice than in WT (Fig. 2a). In the Doxy 25 group the collagen content remained significantly reduced compared to WT. However, in the Doxy100 group the collagen content increased and became similar to the WT. The change in the total collagen content of the aorta was a result of its reduction in the tunica media of untreated and Doxy 25 treated mice (Fig. 2b), while the collagen content in the tunica adventitia did not differ significantly between groups (Fig. 2c). The differences in collagen content were not accompanied by changes in luminal radius or thickness of the tunica media (Suppl. Fig. 1). The detection of collagen type I in the skin revealed no significant differences between wild-type and untreated heterozygous mice with normal diet. There was also no effect of doxycycline treatment on skin collagen (Suppl. Fig. 2).

**Doxycycline treatment prevented stress induced vessel pathology**

One week after surgical intervention, histological evaluation of aortas revealed 3 fold more vessel pathology in untreated HT than in WT mice \(p<0.001\), Fig. 3).
Doxycycline treatment prevented this increase in a dose-dependent fashion, significantly reducing cumulative score in doxycycline treated HT groups compared to untreated HT mice (p<0.001) and bringing the cumulative score to the level of WT (Fig. 3): There were no differences in the cumulative scores between WT and HT mice treated with doxycycline.

Colon biomechanics

The biomechanical properties of the colon were not changed by doxycycline treatment (data not shown). The stiffness and the maximal holding pressure of the colon from heterozygous animals with and without doxycycline treatment were both reduced in comparison to wild-type mice.

MMP-activity in blood serum and skin

The MMP activity was analyzed in the serum (Fig. 4a). There was a different level of detection of pro- and active MMPs compared to the carotid arteries extract: There was virtually no detectable pro-form of MMP-2 either in wild-type or heterozygous mice. Furthermore, there were no significant changes between WT and HT both untreated and treated with doxycycline (Fig. 4b).

In contrast to the serum, only pro-forms and no active MMP-9 were detectable in the skin (Fig. 5a). There was remarkably more pro-MMP-2 detectable, and some active MMP-2. Similar to the vessel tissue extract, total MMP (Fig. 5b) was significantly elevated in untreated HT. This was reduced to the level of WT in the HT group treated with the higher dose of doxycycline. The pattern of total MMP was reflected in the pattern of proMMP-2: It was elevated in untreated HT and normalized by high dose doxycycline (Fig. 5d). However proMMP-9 was not affected either by genotype or treatment (Fig. 5c).
Discussion

Consistent with expectations, we have shown that in the mouse model of collagen III haploinsufficiency chronic MMP-inhibition by doxycycline treatment prevented the increased MMP activity in the carotid artery and the skin present in untreated HT mice. The normalization of MMP activity was accompanied by partial normalization of collagen content in the tunica media of the abdominal aorta in HT mice. This mild increase in collagen accumulation seems to be sufficient to strengthen the vessel, because the increase in stress induced vessel pathology in untreated HT mice was prevented by doxycycline treatment. Therefore, on the basis of these results doxycycline therapy might be considered as a treatment for vEDS, at least of the haploinsufficient type.

The rational for the only available preventive strategy for vEDS by $\beta_1$-AR blocker was mainly mechanical – to reduce the arterial wall stress by controlling the rate of increase of pressure over time in the pulse wave ($dP/dt$) and, thus, to reduce “wearing and tearing” of arterial wall. The celiprolol, however, used in the successful clinical trial was not a typical $\beta_1$-AR blocker. It combines $\beta_1$-AR inhibition with some properties of $\beta_2$-AR stimulation and was proven beneficial for vEDS patients without affecting hemodynamic variables. (Ong et al., 2010) Heart rate or systolic and diastolic pressure were not decreased and pulse pressure was even elevated in patients on celiprolol. It was not tested if celiprolol would be able to reduce a stress response. A possible alternative mechanism of celiprolol’s protective effect in vEDS based on stimulation of transforming growth factor $\beta$ and subsequently stimulation of collagen synthesis was discussed (Brooke, 2010; Ong et al., 2010). However, this hypothesis was not proven.
The inhibition of MMPs – the rationale of this paper – targeted the balance of collagen homeostasis on the degradation side. Inhibition of MMPs by the broad-spectrum MMP inhibitor marimastat had been suggested as a potential therapy for vEDS (Sastry, 2002). There have been successful clinical trials testing doxycycline as a MMP inhibitor in patients with abdominal aortic aneurysm (Curci et al., 2000; Mosorin et al., 2001; Baxter et al., 2002; Lindeman et al., 2009). One week doxycycline treatment effectively suppressed the MMP-activity in the wall of the aortic aneurysm (Curci et al., 2000); however its actual clinical effect was not evaluated. In contrast to our results, a reduction of MMP-2 activity associated with doxycycline treatment was reported, while we observed a reduction of MMP-9 activity (Fig. 1c). The reason for this discrepancy might lay with the duration of doxycycline treatment, since another study showed a decrease in MMP-9 protein expression after 2 weeks of doxycycline treatment (Lindeman et al., 2009). Longer, 3 month duration therapy with doxycycline prevented the growth of the abdominal aortic aneurysm as measured in follow up testing 12 and 18 months after treatment (Mosorin et al., 2001). However, at 6 months follow up testing, no detectable effect was revealed yet. Side effects of long term treatment with 150 mg/day doxycycline in people were generally low: 8% incidence of cutaneous photosensitivity, 3% tooth discoloration, 3% yeast infection (Baxter et al., 2002). Even this low incidence of complications could probably be avoided when doxycycline is used in subantimicrobial doses, which also effectively reduce the MMP activity (Brown et al., 2004). Doxycycline is the only FDA approved MMP-inhibitor for the treatment of periodontal disease and rosacea. Periodontal disease is treated with 20 mg once daily doxycycline (Periostat®). Rosacea is treated with 30 mg immediate release and 10 mg delayed release beads of doxycycline (Oracea®). Some studies using either 20 mg twice daily (Bikowski, 2003; Thiboutot et al., 2009) or 40 mg once daily (Del Rosso et al., 2007) for the treatment of
rosacea reported no adverse events. The comparison of 100 mg versus 40 mg
doxycycline once daily for the treatment of rosacea revealed significant less adverse
events in the 40 mg group (Del Rosso et al., 2008). However, doxycycline long-term
therapy has been used safely in patients with rosacea and acne vulgaris. Minor side
effects are varied (summarized in (Valentin et al., 2009); serious side effects are rare
(Sloan and Scheinfeld, 2008). Furthermore, doxycycline therapy with 100 mg twice daily
up to 2 years decreased hemorrhagic risk in brain vascular malformations (Frenzel et al.,
2008). Doxycyclin therapy with the same dosage reduced aortic neck dilatation 6 months
after endovascular aneurysm repair (Hackmann et al., 2008). Clinical trials with other
applications of doxycycline are on the way.

On the basis of the presented data it is difficult to distinguish whether increased
total MMP and active MMP-9 in carotid arteries of heterozygous mice was a result of
increased ECM turnover in heterozygous mice or a response to stressing of the vascular
system by physically manipulating the aorta. The former hypothesis is more probable,
because of elevated urine concentration of carboxy-terminal peptide of collagen I in
heterozygous males (Cooper et al., 2010), and increase of the total MMP activity in the
skin shown in our experiment, indicate that changes in collagen turnover are observed
not only in the vascular system, but outside it as well. However, to our knowledge there
have been no studies evaluating MMP activity in the serum of vEDS patients.
Nevertheless, an elevated MMP-9 activity was found in abdominal aortic aneurysms
among general population (Thompson et al., 1995; Yamashita et al., 2001). This activity
was localized to infiltrating adventitial macrophages (Thompson et al., 1995). The
absence of active MMP-9 in the skin in wild-type and heterozygous mice (Fig. 5) might
be explained by the absence of inflammation and macrophage infiltration. Matrix
metalloproteinase-2 is unique in its ability to degrade both elastin and fibrillar collagen
Mesenchymal cells (including vascular smooth muscle cells) constitutively express MMP-2, but other cell types like macrophages and fibroblasts produce small amounts of it. The main cellular sources of MMP-2 are located in the media and adventitia of the aortic wall facilitating the destruction of elastin and collagen fibres and the disorganization of the aortic wall structure in AAA (Kadoglou and Liapis, 2004). Failure to observe increased MMP-2 activity in our model (Fig. 1c) may be because the actual vascular lesions were not sufficiently severe. The activity was rarely elevated in vivo in abdominal aortic aneurysm in patients (Thompson et al., 1995). It was also increased in vitro in vascular smooth muscle cells isolated from abdominal aortic aneurysm (Crowther et al., 2000).

In our experiment, doxycycline prevented an increase in MMP activity in the vascular wall, as was seen for MMP-9 in carotid artery. These results are compatible with reported reduction of MMP-9 activity in serum of patients with abdominal aortic aneurysm treated with doxycycline (Baxter et al., 2002). Moreover, the activity of MMP-9 was reduced only in patients whose MMP-9 levels were elevated before the start of doxycycline treatment. Interestingly, the reported lack of doxycycline effect on normal MMP levels was in concordance with our observation on skin MMP activity: the normal levels of MMP activity in the skin of HT mice was not affected by doxycycline treatment and thus, accumulation of the collagen in the skin was not induced. Furthermore, doxycycline treatment did not alleviate the weakness of heterozygous mouse colon as determined by biomechanical measurements. The outcomes of this measurement support the hypothesis that effects of doxycycline might be tissue- or age- specific in preventing pathology. For instance, the biomechanical properties of the abdominal aorta were not yet different between HT and WT mice at 9 months, but did differ at 21 months of age (Cooper et al., 2010).
In our mouse model the reduction of MMP activity in the vessel was accompanied by reduced MMP concentration in the skin, while MMP concentration in serum was not affected. This suggests a possibility to monitor the progression of potential doxycycline treatment of vEDS patients by skin biopsy, if MMP activity would not be elevated in the serum of vEDS patients.

Doxycycline prevents the excess of stress induced vessel pathology in mice haploinsufficient for Col3a1 by inhibiting activity of tissue MMP-9 and thus, reducing the degradation of the elastic matrix. The results suggest that doxycycline merits clinical testing as a possible treatment for vEDS.

**Limitation**

The efficacy of proposed doxycycline therapy was tested in the haploinsufficient mouse model. Therefore, it definitely can be proposed as a potential treatment for only a small subset of vEDS patients with haploinsufficiency for COL3A1. Whether this treatment can be successful in the rest of vEDS patients shall be tested in the appropriate genetic mouse model yet to be developed. However, doxycycline treatment was successful in another mouse model with a mutation in a protein of the extracellular matrix causing aortic ruptures: a mouse model of Marfan syndrome based on a mutation in fibrillin-1 (Fbn1<sup>C1039+</sup>) (Chung et al., 2008; Xiong et al., 2008; Yang et al., 2010).

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Authorship Contribution

*Participated in research design:* Briest, McDonnell and Talan.

*Conducted experiments:* Briest, Cooper, Tae and Krawczyk.

*Performed data analysis:* Briest, Cooper, Tae and Krawczyk.

*Wrote or contributed to the writing of the manuscript:* Briest, Cooper, Tae and Talan.

The authors declared that they had no conflicts of interests with respect to their authorship or the publication of this article.
References


Stenson PD (2009) The Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff, in, Cardiff University.


Footnotes

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Legends for Figures

Figure 1 MMP activity in the carotid artery of wild-type (+/+) and heterozygous mice (+/-) was analyzed by zymography.

Representative zymogram of extracts from heterozygous mouse carotid artery fed with normal (doxy 0), doxy25 (25) and doxy100 (100) diet (A). All 4 bands were summarized as total MMP (B). Active MMP-9 (C) and active MMP-2 (D) were analyzed by densitometry. The result was normalized to the protein content. Data are means ±SEM. *P<0.05 vs. +/- normal diet, †P<0.05 vs. +/- normal diet, **P<0.001 vs. +/- normal diet, ††P<0.001 vs. +/- normal diet. Number of animals in each group is indicated in the respective column in B: total MMP

Figure 2 Heterozygous mice (+/-) had significantly less collagen per unit area than wild-type in the abdominal aorta (A) as determined by picro-sirius red staining. This reduction was partially ameliorated in the high dose of doxycycline group. The reduction was seen in the tunica media (B) and not in the tunica adventitia (C). Data are means ±SEM. *P<0.05 vs. +/- normal diet. Number of animals in each group is indicated in the respective column.

Figure 3 Cumulative lesion score (lesions ≥ grade 2) in each group demonstrates an increase in untreated heterozygotes and a dose dependent decrease of vessel pathology in doxycycline treated groups. 

A) Section of abdominal aorta from a wild-type control animal. Masson’s trichrome. B) A representative grade 4 lesion (indicated by arrows) with a large defect in the internal elastic lamina and significant subintimal spindle cell proliferation with deposition of collagen is shown. C) Data are means (WT=17, HT=19, HT-Dox25=10, HT-Dox100=11) ±SEM. **P<0.001 vs. +/- normal diet, ††P<0.001 vs. +/- normal diet.
Figure 4 There was a different pattern in lysis bands in the zymography of serum from heterozygous and wild-type mice. Representative zymogram of serum from heterozygous (+/−) and wild-type (+/+ ) mice (A). All 4 bands were summarized as total MMP (B). Number of animals in each group is indicated in the respective column.

Figure 5 MMP activities in the skin were elevated in heterozygous mice, and normalized to the level of wild-type mice after high dose doxycycline treatment. Representative zymogram of extracts from heterozygote skin (A). All 4 bands were summarized as total MMP (B). Pro-form of MMP-9 (C) and MMP-2 (D) were analyzed by densitometry. The result was normalized to amount of tissue used (in mg).

Data are means ±SEM. *P<0.05 vs. +/+ normal diet, †P<0.05 vs. +/- normal diet, **P<0.001 vs. +/+ normal diet, ††P<0.001 vs. +/- normal diet. Number of animals in each group is indicated in the respective column.
Figure 2

A. total collagen in Abdominal Aorta

B. total collagen in T. Media

C. total collagen in T. Advent.
Figure 3

A

B

C

vessel pathology

<table>
<thead>
<tr>
<th>Group</th>
<th>Cumulative Score</th>
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<tr>
<td>+/+ normal diet</td>
<td>6</td>
</tr>
<tr>
<td>+/- doxy_{20}</td>
<td>4 **</td>
</tr>
<tr>
<td>doxy_{100}</td>
<td>2 +</td>
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** * p < 0.01
Figure 4

A

130 kDa
95 kDa
72 kDa
55 kDa

proMMP-9
active MMP-9
proMMP-2
active MMP-2

+/−  +/+  +/−  +/−

B

[AU]

2.8
2.6
2.4
2.2
2.0
1.8
1.6

+/+
normal diet

+/−
doxy_{25}
doxy_{100}