**Molecular Cardiology**

**Biglycan Deficiency Causes Spontaneous Aortic Dissection and Rupture in Mice**

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**Background**—For the majority of cases, the cause of spontaneous aortic dissection and rupture is unknown. An inherited risk is associated with Marfan syndrome, Ehlers-Danlos syndrome type IV, and loci mapped to diverse autosomal chromosomes. Analysis of pedigrees however has indicated that it may be also inherited as an X-linked trait. The biglycan gene, found on chromosome X in humans and mice, encodes a small leucine-rich proteoglycan involved in the integrity of the extracellular matrix. A vascular phenotype has never been described in mice deficient in the gene for small leucine-rich proteoglycans. In the breeding of BALB/cA mice homozygous for a null mutation of the biglycan gene, we observed that 50% of biglycan-deficient male mice died suddenly within the first 3 months of life.

**Methods and Results**—Necropsies revealed a major hemorrhage in the thoracic or abdominal cavity, and histology showed aortic rupture that involved an intimal and medial tear as well as dissection between the media and adventitia. By transmission electron microscopy and biomechanical testing, the aortas of biglycan-deficient mice showed structural abnormalities of collagen fibrils and reduced tensile strength. Similar collagen fibril changes were observed in male as well as in female biglycan-deficient mice, which implies a role of additional determinants such as gender-related response to stress in the development of this vascular catastrophe only in male mice.

**Conclusions**—The spontaneous death of biglycan-deficient male mice from aortic rupture implicates biglycan as essential for the structural and functional integrity of the aortic wall and suggests a potential role of biglycan gene defects in the pathogenesis of aortic dissection and rupture in humans. *(Circulation. 2007;115:2731-2738.)*

**Key Words:** aneurysm ■ biglycan ■ gene targeting

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The biglycan (*BGN*) gene, found on chromosome X in humans and mice,1 encodes a member of the expanding family of small leucine-rich proteoglycans (SLRPs). Their core protein is composed of tandem-linked repeats of \( \approx 25 \) amino acid long, leucine-rich motifs to which glycosaminoglycan chains are attached. BGN contains 10 leucine-rich repeats, and its 40-kDa core protein binds 2 chondroitin- or dermatan-sulfate glycosaminoglycan chains.2 In agreement with the high expression of BGN in bone,3 *bgn*-deficient mice have been reported to show a phenotype characterized by growth failure, reduced bone formation, and age-related severe osteopenia.4 This phenotype however has been described to be fully expressed in male but not in female mice.5 Recently, dental and muscular abnormalities, thinning of the skin, and osteoarthritis have been also described in these mice (for review see Young et al6). The demonstration of tissue-specific abnormalities of collagen fibrils in different collagenous matrices, such as bone, dermis, and tendon,7–9 indicates that BGN plays a critical role in the control of collagen fibrillogenesis and in the proper assembly of the extracellular matrix.

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Normal arteries are sites of expression and deposition of SLRPs, in particular decorin and BGN.3,9,10 Abnormalities in their expression or deposition have been described in diverse human vascular disease, such as aneurysm and dissection.11–14 For the majority of cases, the cause of aortic dissection in humans is unknown. Marfan syndrome and

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Ehlers-Danlos syndrome (EDS) type IV, and a few other, more rare, genetic disorders are associated with an inherited risk of aortic dissection. Nonsyndromic familial susceptibility to aortic dissection and rupture has also been established, and analysis of pedigrees has indicated that the risk for aortic dissection may be inherited as a X-linked trait. Interestingly, even though primary genetic defects in the BGN encoding gene have not been reported in humans, BGN mRNA and protein expression levels are altered in patients with Turner syndrome and vascular anomalies, such as aortic dissection and rupture, are frequently observed in these patients.

A vascular phenotype has never been described in SLRP-deficient mice, which include the original bgn-deficient (bgn-knockout (KO)) 129Sv/C57BL6 mice. In the breeding of BALB/cA female mice homozygous for a null mutation of the BGN encoding gene, we observed that male bgn-KO mice died suddenly before 2 to 3 months of age. To investigate the vascular phenotype of these mice we have conducted a histological, ultrastructural, and biomechanical analysis of the aortas of biglycan-deficient and wild-type (WT) mice.

Methods

Mice

bgn-KO mice with a 129Sv/C57BL6 background were backcrossed into a BALB/cA/BoTc (Taconic M&B A/S, Ry, Denmark) background for at least 7 generations. More than 300 offspring mice from backcross and intercross breeding were genotyped and observed for sudden death. The distribution of genotypes was as follows: 69 bgn-/-; 56 bgn+/-; 15 bgn+; 65 bgn-/-; 74 bgn-/-; 54 were not determined. The lower number of bgn-/- mice was the result of the breeding setup. During a period of 5 months, 22 mice were autopsied and further analyzed by histology. The mice were all housed together, and all litters were affected. Age-matched mice with the BALB/cA background derived from crossing heterozygous (bgn+/-) female mice with either WT (bgn+/-) or bgn-KO (bgn-/-) male mice were used for ex vivo studies. Four bgn-KO male, 4 WT male, 3 bgn-KO female, and 2 WT female mice (age 2.5 to 3 months) were used for ex vivo studies. Four bgn-KO male, 4 WT male, 3 bgn-KO female, and 2 WT female mice (age 2.5 to 3 months) were used for transmission electron microscopy analysis, and 13 four-month-old and 16 ten-month-old male mice were used for the strength measurements. All mice were euthanized. Animals were housed under standard conditions (free access to a standard mouse diet and water, 12-hour light/dark cycle) as approved by the Danish Ministry of Justice. Genotypes were determined by a polymerase chain reaction–based assay as previously described.

Tissue and Histology

Aortas with heart, lungs, and kidneys were isolated within 30 minutes of death from male bgn-KO mice that died spontaneously, and from 3 months old male and female bgn-KO and WT mice. The samples were fixed in 4% formaldehyde (freshly made from paraformaldehyde) in phosphate buffer (0.1 mol/L, pH 7.2), cross-sectioned, and routinely processed for paraffin embedding. Sections (5 μm thick) were stained with hematoxylin-eosin and Alcian blue (pH 2.5) for acid proteoglycans, Mallory’s trichrome for collagen fibers, and Weigert for elastic fibers.

Immunohistology

Immunolocalization of BGN was performed as described previously by means of an indirect immunoperoxidase protocol with a polyclonal antiserum to mouse BGN core protein (LF106). The LF106 antiserum was used at a dilution 1:100 in PBS–0.1% BSA for 2 hours at room temperature. Because the LF106 antiserum recognizes protein cores devoid of attached glycosaminoglycans chains, sections were treated with 1.25 U/mL chondroitin ABC lyase (pro tease-free, from Proteus vulgaris; ICN, Covina, Calif.) as described previously.

Transmission Electron Microscopy

Fixed samples were washed overnight in cacodylate buffer at 4°C. After post-fixation for 1 hour at 4°C with 1% osmium tetroxide in cacodylate buffer, samples were treated with tannic acid to enhance the contrast of elastic laminae as described previously. Samples were rinsed in water, dehydrated in a graded series of ethanol to propylene oxide and infiltrated and embedded in epoxy resin (Agar Scientific Ltd, Stansted, Essex, UK). Semithin sections were stained with Azur II-methylene blue and examined with a light microscope to select appropriate fields. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined with a CM Philips transmission electron microscope (KE Electronics, Tofts, UK). For quantitative studies, collagen fibril diameter and density were measured on photographic prints as described previously. Mean density values were calculated from 3 to 5 μm² per mouse from 4 bgn-KO males; 3 WT males; 3 bgn-KO females and 2 WT females before group means (± SD) was calculated. For diameter of collagen fibril profiles, measurements were obtained from a total of 3083 collagen fibril profiles (bgn-KO male, n = 769; WT male, n = 707; bgn-KO female, n = 799; WT female, n = 808) counted in 4 μm² of each group (2 animals per group) as described previously.

Immuo-Electron Microscopy

The technique was performed with an immunogold protocol as described previously. LF106 and polyclonal anti-type III collagen (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) were used at a 1:10 dilution in PBS–0.1% BSA. Ultrathin sections to be incubated with LF106 were pretreated with 1.25 U/mL chondroitin ABC lyase in 0.1 mol/L Tris, 0.05 mol/L calcium acetate, 0.01% BSA (pH 7.2) for 5 minutes at 37°C. After incubation with undiluted normal goat (Sigma, St. Louis, Mo.) or rabbit (Dako, Glostrup, Denmark) serum, ultrathin sections were incubated with LF106 or anti-type III collagen for 1 hour, and after repeated washes with PBS, with gold-labeled (particle size 10 nm) goat anti-rabbit (BioCell, Cardiff, UK) or rabbit anti-goat (Sigma) immunoglobulins, respectively, both diluted 1:20 in PBS–0.1% BSA for 30 minutes. Incubations were performed at room temperature. Control sections were labeled with a primary antibody of unrelated specificity or normal serum. Sections stained for type III collagen were used to estimate the density of gold particles on the collagen fibrils of the adventitia (number per μm²) as previously described.

Biomechanical Testing

Aortas were isolated from age-matched male bgn-KO and WT mice that were euthanized at 4 months and 10 months of age. Sampled aortas (6 to 9 per group of mice) were stored at −20°C until analysis in 50 mmol/L Tris/HCl (pH 7.4). The buffer was used throughout the testing procedure. Before testing, lower thoracic and upper abdominal aorta was cleaned of adhering fat and loose connective tissue. Aortic rings (1-mm high, 2 to 5 specimens per mouse, in average 4.4 specimens per mouse in the 4-month-old WT group, 4.1 specimens per mouse in the 4-month-old bgn-KO group, and 3 specimens per mouse in both 10-month-old groups) were cut between intercostal arteries and abdominal arterial branches. The ring height was measured in a microscope equipped with a calibrated ocular micrometer, and the mean radial thickness of the aortic wall was measured by an electronic length gauge (Heidenhain, Traunreut, Germany) on wet specimens flattened between 2 parallel surfaces under a fixed load. Specimens were mounted in a material testing machine (Alwetrion TCT5, Lorentzen & Wettre, Stockholm, Sweden) with 2 parallel steel wires (diameter 0.35 mm) through the lumen, and then radially stretched (10 mm/min) until failure while soaked in buffer at room temperature. After failure, the collagen content in the ring specimens was measured by hydroxyproline determination as previously described. Maximum load and stiffness were calculated.
values were divided by 2 to represent a single width of the aortic wall. The original circumference of the ring specimen was defined as the circumference at which the specimen attained a minimal load (1% of maximum). Mean values of the mechanical parameters as well as the aortic diameter, wall thickness, and collagen content were obtained for each mouse before group means (±SD) were calculated.

Statistical Analysis
Results for density of gold particles and diameter of collagen fibril profiles are presented as median and percentiles (25% and 75%) and those for density of collagen fibril profiles, mechanical parameters, aortic diameter, wall thickness, and collagen content as means (±SD). The difference in the density of gold particles between male \( bgn \)-KO and WT was analyzed by Student t test for unpaired data. Linear mixed-model analysis (PROC MIXED, SAS, Cary, N.C.) was used to test the effect of \( bgn \)-KO and gender on collagen fibril density and diameter with a random effect for animal. Two-way ANOVA was used to test the effect of \( bgn \)-KO and age on mechanical parameters, aortic diameter, wall thickness, and collagen content. Log transformation was performed as appropriate to obtain normality and variance homogeneity. A probability level of 5% was used to establish significance of differences.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agreed to the manuscript as written.

Results
Vascular Phenotype in Male \( bgn \)-KO Mice
About 50% of the male \( bgn \)-KO mice but neither the \( bgn \)-KO female nor the wild-type (WT) mice suddenly died before 2 to 3 months of age (average lifespan 9.3 ±0.3 weeks). One of the mice died while its ear was punched, and another died after placement with females for breeding. Necropsies performed on 22 \( bgn \)-KO male mice revealed that the mice died from a massive hemorrhage in the thoracic (82%) or abdominal cavity (18%) (Figure 1a). Histological studies (Figure 1b to 1h) showed aortic rupture with the rupture across the intima and the media and dissection of the aortic wall with blood collection between media and adventitia. Fragmentation of elastic layers, deposits of myxoid matrix, and dissection within the aortic media, which represent the hallmarks of Marfan-like dissection,33–34 were not observed in \( bgn \)-KO mice.

Because the pattern of aortic dissection observed in male \( bgn \)-KO with blood collection between media and adventitia represented a phenocopy of that observed in \( col3a1 \)-KO mice,29 and depletion of a single noncollagenous protein in the extracellular matrix may profoundly alter the overall matrix stoichiometry, we asked whether a common molecular basis could underlie the aortic dissection and rupture in \( bgn \)- and \( col3a1 \)-KO mice. Specifically, we asked if a secondary reduction in type III collagen could be brought about by \( BGN \) deficiency. Quantitative transmission electron microscopy immunolocalization of type III collagen, however, revealed no difference in amount of labeling (Figure 1i) in the aortas of \( bgn \)-KO mice compared with WT mice.

Biglycan Immunolocalization in the Normal Mouse Aorta
To further investigate the link between the specific pattern of aortic rupture observed in \( bgn \)-KO mice and \( BGN \)-deficiency itself, the pattern of \( BGN \) localization in the aortas of WT mice was determined. Immunohistochemistry demonstrated that of the 3 layers of the aortic wall, the adventitia is in fact the major site of \( BGN \) deposition in the mouse. Prominent immunostaining of the adventitia and only minimal labeling in the media and intima were detected at the light microscopy level (Figure 2a and 2b). Transmission electron microscopy immunolocalization further demonstrated that most of \( BGN \)-immunoreactive sites in the aorta were associated with collagen fibrils (Figure 2c and 2d), which are much more numerous and densely spaced in the adventitia compared with the media. However, in keeping with previous studies that report the presence of \( BGN \) within elastin fibers,35 additional sites of \( BGN \) immunoreactivity were detected within the elastin-associated electron dense (microfibril) phase, which accounts for a minor proportion of the elastic fiber mass (Figure 2e and 2f). These findings predicted that the most prominent structural changes would be expected to occur in the adventitia as a result of \( BGN \) deficiency.

Structural Defects in the Aortas of \( bgn \)-KO Mice
Comparative light microscopy analysis of the aortas of male \( bgn \)-KO and WT mice failed to reveal abnormalities in the overall organization of the tunica media and adventitia (Figure 3a to 3d). In contrast, comparative transmission electron microscopy analysis revealed distinct abnormalities of collagen fibrils in the aortas of \( bgn \) KO mice (Figure 3e to 3h), including marked variations in size and shape, and ragged or notched cross-sectional profiles. These changes resemble those previously observed in dermis, bone and tendon of \( bgn \) mice7–8 and were highly reminiscent of those occurring in diverse human EDSs.36–37 Despite these collagen fibril changes, aortic diameter, wall thickness, and collagen content in the aortic ring specimens from 4- and 10-month-old male \( bgn \)-KO and WT mice (Table) were not different (\( P>0.14 \)).

Biomechanical Properties of the Aortas of \( bgn \)-KO Mice
Because collagen fibrils were found to be abnormal and collagen is the main determinant of vessel tensile strength, the resistance to radial elongation of aortic ring specimens from male \( bgn \)-KO and WT mice was compared. Mechanical testing of aortic rings subjected to radial elongation revealed that load-strain curves for \( bgn \)-KO and WT mice essentially coincided during initial (elastic) deformation (Figure 4a). This reflected the integrity of all arterial wall coats and the contribution of an intact media to overall energy absorption and deformation. However, the maximum load withstood by KO specimens (Figure 4b) and their maximum stiffness (Figure 4c) were significantly lower (50% to 70%) compared with WT. Energy absorption until failure was also reduced to a similar extent (47% to 58%) in the KO specimens compared with WT (2-way ANOVA: \( bgn \)-KO \( P<0.0001 \); age \( P>0.45 \)). Importantly, the vessel wall failed in a stepwise fashion, and failure at maximum load corresponded to the rupture of the medial coat alone, as observed by video recording during testing at a magnification of about \( ×40 \), and confirmed by amido black surface staining of the arterial coats (data not shown). After the failure of the media, the adventitia sustained further strain before failing in 1 or more steps. The
height of the load-strain curve for strain values after medial failure thus reflected solely the mechanical strength of the collagenous adventitia. Interestingly, as shown in Figure 4a, the difference in load sustained by the adventitia was closely similar to the difference in maximum load sustained by the entire aortic ring at failure of the media, which indicated that the reduced performance of the adventitia explains most of the observed reduced strength of KO aortic specimens.

Gender Effect of BGN Deficiency

Because death caused by aortic rupture was apparently male-specific in bgn-KO mice, we asked whether the observed changes in collagen fibril structure were in turn male-specific. Both male and female bgn-KO mice demonstrated similar changes in fibril shape and range (Figure 5a) when compared with WT mice. The collagen fibrils were counted and mean density (per $\mu m^2$) were calculated for each
group (mean±SD; male bgn-KO: 187.4±6.9; male WT: 177.8±1.5; female bgn-KO: 191.3±8.4; female WT: 192.5±2.1). Linear mixed-model analysis revealed no significant differences between genotypes. However, significant differences in mean density were found between WT male and female mice (P<0.01). In contrast, when the collagen fibril diameter was analyzed with a linear mixed-model, significant differences were found between both genotypes and genders (P<0.0001). The collagen fibril diameter was also significantly lower in WT males compared with WT females, consistent with previous indications of a gender related difference in collagen fibril diameter in mice independent of the bgn-KO genotype.

Discussion
Either alone or when combined with deficiencies in other SLRPs, BGN deficiency in mice has been associated with skeletal, dental, muscular, and skin abnormalities, as well as with osteoarthritis and ectopic tendon ossification.5,6,7,8 Collagen fibril changes7,8 that are highly reminiscent of those observed in different tissues of diverse EDSs9,10 underlie the different tissue-specific phenotypes observed in bgn-KO mice. In the present article, we document that similar changes occur in the collagen fibrils of the aortic wall, which lead to reduced tensile strength and spontaneous dissection and rupture.

The pattern of aortic dissection observed in male bgn-KO mice is different from that commonly observed in Marfan-like human aortic dissection and similar to that observed in col3a1-deficient mice,29 the mouse model of human EDS-IV. In bgn-KO mice and in col3a1-deficient mice, dissection of the aortic wall occur between the media and the adventitia rather than within the media, which suggests that a secondary reduction in type III collagen could be brought about by BGN deficiency. However, no differences in the amount of labeling were detected in the aortas of bgn-KO mice compared with WT mice, which dispels a secondary change in type III collagen deposition and indicates a direct role of BGN in structural integrity and mechanical performance of the aortic wall.

To better clarify the link between the specific pattern of aortic dissection and rupture observed in bgn-KO mice and BGN deficiency itself, we have determined the pattern of BGN distribution in the aortas of WT mice. The demonstration that BGN deposition was mostly associated with collagen fibrils predicted that the most prominent structural and functional changes that resulted from BGN deficiency would be expected to occur in the aortic adventitia, the tunica in
which collagen fibrils are more abundant to sustain the bulk of hemodynamic stress. As previously observed in other collagenous extracellular matrices in the bgn-KO mice, ultrastructural analysis revealed marked variability in size and shape of aortic collagen fibrils. These changes were mirrored by the reduced tensile strength of the aorta, as demonstrated by the significantly lower maximum load and maximum stiffness withstood by KO aortic ring specimens compared with WT specimens. Interestingly, even though BGN deposition was also detected within the media and in particular within the elastin-associated electron-dense (microfibril) phase, structural changes in the elastic component of the aorta were not apparent in bgn-KO mice. This is in contrast to some mouse models of aortic aneurysm. For instance, targeted deletion of lysyl oxidase, an essential biosynthetic enzyme for collagen and elastin fibers, results in hazy and unruffled elastic lamellae by light microscopy. The ability of BGN to form complexes with microfibril-associated glycoprotein 1 and tropoelastin (the soluble precursor of mature elastin) suggests that BGN may be involved in the deposition of tropoelastin onto the surface of the microfibrils during elastinogenesis or, alternatively, in the stabilization of the mature elastic fibers. However, because tropoelastin can bind fibrillin-1 and -2, the main components of microfibrils, elastinogenesis and elastic fiber stabilization may also occur independently of BGN. In addition, the well established redundant functions of SLRP family, (in particular decorin, given its ability to bind tropoelastin, fibrillin-1, and microfibril-associated glycoprotein 1), may be able to substitute for BGN in both of these processes and further contribute to the explanation of the absence of structural changes in the aortic elastic lamellae of bgn-KO mice.

It is of interest that death for aortic rupture was male-specific in bgn-KO mice. A gender difference was also observed in the skeletal phenotype, which occurs in male but not in female bgn-KO mice. To explain the occurrence of aortic rupture only in male mice, we asked if the changes in collagen fibrils were in turn male-specific. Ultrastructural analysis of the aortas of male and female bgn-KO mice revealed the occurrence of comparable abnormalities of collagen fibrils in both genders. The fibril mean diameter, however, was significantly lower in male bgn-KO mice compared with either KO female or WT male and female mice. Interestingly, the mean diameter was also significantly lower in male than in female WT mice. Even though a gender-related effect on collagen fibril diameter is well established in mice, an additive effect of BGN deficiency with respect to a native gender-related difference in fibril structure may be invoked to explain the male-specific nature of aortic dissection and rupture in bgn-KO mice. However, this finding does not exclude a contributory role of additional and/or synergic determinants. Gender differences influence the incidence of many diseases and in general they have been

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*Two-way ANOVA showed no effect of either bgn-KO (0.15<P<0.42) or age (0.17<P<0.95) on any of the parameters but showed an unaccountable bgn-KO/age interaction in wall thickness (P<0.05).
interpreted primarily to reflect either estrogen-mediated protection or androgen-mediated susceptibility against pathological conditions.44–47 The evidence that 2 male mice died while either its ear was punched or after placement with either its ear was punched or after placement with

In humans, abnormalities in BGN expression or deposition have been described in diverse vascular diseases such as aortic aneurysm and dissection.10–14 For example, BGN mRNA and protein have been demonstrated to be reduced in aortas with aneurysms compared with normal aortas.14 However, a direct causative link between BGN deficiency and ruptured aneurysm of the aorta has never been shown, and the cause of spontaneous aortic dissection and rupture remains unknown for the majority of cases. A X-linked mode of inheritance has been identified in some families, and a very high incidence of aortic dissection and rupture is observed in patients with Turner syndrome.24 Taken together, all these findings support our data on bgn-KO mice, which points to BGN as a candidate gene for non-Marfan, non-EDS IV–related risk of aortic dissection and rupture in humans.

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Disclosures
None.

References

**CLINICAL PERSPECTIVE**

For the majority of cases of spontaneous aortic dissection and rupture in humans, the cause is unknown. An inherited risk is associated with genetic syndromes and with loci mapped to diverse autosomal chromosomes. Analysis of pedigrees has indicated that the risk for aortic dissection may also be inherited as an X-linked trait. The biglycan gene, found on chromosome X in humans and mice, encodes a small leucine-rich proteoglycan involved in the integrity of the extracellular matrix. Abnormalities in biglycan expression have been described in diverse vascular disease, such as aortic aneurysms and dissection. Although primary genetic defects in the biglycan gene have not been reported in humans, biglycan mRNA and protein expression is reduced in patients with Turner syndrome, in which aortic dissection and rupture are common. We observed aortic dissection and rupture in male biglycan knockout mice. The aortas showed structural abnormalities of collagen fibrils and reduced tensile strength. Similar collagen fibril changes were observed in both male and female biglycan-deficient mice, a finding that implies additional determinants such as gender-related response to stress in the development of this vascular catastrophe only in male mice. This study points to the biglycan gene as a candidate gene for the risk of aortic dissection and rupture in humans. If confirmed, this could be useful for identifying patients at risk in the future.
Biglycan Deficiency Causes Spontaneous Aortic Dissection and Rupture in Mice
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