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
Ehlers-Danlos syndrome (EDS) type IV, and a few other, more rare, genetic disorders are associated with an inherited risk of aortic dissection.15–16 Nonsyndromic familial susceptibility to aortic dissection and rupture has also been established,17–22 and analysis of pedigrees has indicated that the risk for aortic dissection may be inherited as a X-linked trait.23 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 syndrome24 and vascular anomalies, such as aortic dissection and rupture, are frequently observed in these patients.24

A vascular phenotype has never been described in SLRPs-deficient mice, which include the original bgn-deficient (bgn-knockout (KO)) 129Sv/C57BL6 mice.25 In the breeding of BALB/cA 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 background26 were backcrossed into a BALB/cA BomTac (Taconic M&B A/S, Ry, Denmark) background for at least 7 generations. More than 300 offspring from a 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 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 Experimental Animal Committee, The Danish Ministry of Justice. Genotypes were determined by a polymerase chain reaction–based assay as previously described.5

Tissue and Histology

Aortas with heart, lungs, and kidneys were isolated within 30 minutes of death from male bgn-KO mice that died spontaneously, or from 10-month-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.27

Immunohistology

Immunolocalization of BGN was performed as described previously28 by means of an indirect immunoperoxidase protocol with a polyclonal antiserum to mouse BGN core protein (LF106).29 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.30

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 as en bloc stain to enhance the contrast of elastic laminae as described previously.31 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.32 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.32

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 (Alwetrox 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.33 Maximum load and stiffness were calculated. The
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,23–34 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 col3al-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-KO and WT mice.27–34,37 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

Bioglycan 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 KO mice 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.

Figure 1. Vascular phenotype in male bgn-KO mice. a, Male bgn-KO mouse with a blood clot (arrow) on the posteromedial surface of the left kidney. b through f, Dissection of the abdominal and thoracic aorta are illustrated in b to d and e to f, respectively. The intima and media are ruptured (arrow in d and f) and blood-filled between media and adventitia (in b, c, and f). f, Detail of the boxed area in e. Stains for elastin (g) and collagen (h) fibers showed integrity of the elastic laminae of the tunica media (g) and the separation of the media from the adventitia (h). i, Quantitative electron microscopy immunolocalization of type III collagen (upper panels: staining for type III collagen; bottom panels: negative controls), calculated as density of gold particles (per $\mu m^2$) within the adventitia, failed to reveal significant difference between male bgn-KO and age- and gender-matched WT mice. Staining in b, d, e, and f: hematoxylin-eosin; staining in c and g: Weigert for elastic fibers; staining in h: Mallory for collagen fibers. Bar in i is 50 nm. Ao indicates aorta; adv, adventitia; and m, media.

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 difference in mean density was 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 significantly lower in bgn-KO males compared with WT males and bgn-KO females (Figure 5b). Interestingly, 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.38

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.4,6–8 Collagen fibril changes7,8 that are highly reminiscent of those observed in different tissues of diverse EDSs36,37 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,7,8 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,35 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.39 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.40 However, because tropoelastin can bind fibrillin-1 and -2,41 the main components of microfibrils, elastinogenesis and elastic fiber stabilization may also occur independently of BGN. In addition, the well established redundant functions of SLRPs,6,8,42 suggest that other members of the SLRP family, (in particular decorin, given its ability to bind tropoelastin, fibrillin-1, and microfibril-associated glycoprotein 140,43), 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.4,5 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,38 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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<td>WT</td>
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<tr>
<td>Diameter, mm*</td>
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<td>Wall thickness, μm*</td>
<td>25.20±1.50</td>
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<td>Collagen content, μg/mm*</td>
<td>6.00±1.80</td>
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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).
interpretation primarily to reflect either estrogen-mediated protection or androgen-mediated susceptibility against pathological conditions. Therefore, the evidence that 2 male mice died interpreted primarily to reflect either estrogen-mediated pro-

Figure 5. Gender effect of biglycan deficiency on collagen fibrils in male and female bgn-KO and WT mice. Morphological comparison of collagen fibril profiles in the aortic adventitia of bgn-KO and WT male and female mice. Shape and size variability of collagen fibril profiles are common to male and female gender. The panels on the right are the same images as shown in the panels on the left but were modified with Adobe Photoshop 9.0 to better visualize collagen fibril profiles. The bar is 50 nm. The total number of collagen fibril profiles measured in the different groups was similar and the range of their diameter was increased in both male and female bgn-KO compared with their gender-matched WT mice. b. Median and percentiles (25% and 75%) of the collagen fibril diameter measured in each group. Linear mixed-model analysis revealed that collagen fibril diameter was significantly lower in bgn-KO male compared with WT male (P < 0.0001) and bgn-KO female (P < 0.0001) mice and in WT male compared with WT female (P < 0.0001).

In humans, abnormalities in BGN expression or deposition have been described in diverse vascular diseases such as aortic aneurysm and dissection. For example, BGN mRNA and protein have been demonstrated to be reduced in aortas with aneurysms compared with normal aortas. 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. 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

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.

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.

Genetic defects in the biglycan gene have not been reported in humans, biglycan mRNA and protein expression is reduced in genetic disorders such as Marfan’s syndrome, muscular dystrophy, and corneal diseases. Inactivation of the lysyl oxidase gene Lox leads to aortic aneurysms, cardiovascular dysfunction, and perinatal death in mice. Familial thoracic aortic aneurysms and dissections: genetic heterogeneity with a major locus mapping to 5q13–14. This study confirms the genetic heterogeneity with a major locus mapping to 5q13–14 in Finnish families.

Gender differences in experimental aortic aneurysm formation. Interaction of tropoelastin with the amino-terminal domains of fibrillin-1 and fibrillin-2 suggests a role for the fibrillins in elastic fiber assembly. The effect of tannic acid on electron images of capillary endothelial cell membranes. Localization of bone sialoprotein (BSP) to Golgi and human and certain animal model bone matrix noncollagenous proteins.


