

Experimental Hypercholesterolemia Induces Ultrastructural Changes in the Elastic Laminae of Rabbit Aortic Valve

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Atherosclerosis is the most severe problem in the high-pressure systemic circulation and similar changes also occur in the high-pressure loading valve. This study was designed to test the hypothesis that early atherosclerosis, induced by a high cholesterol diet in rabbits, is characterized by significant ultrastructural change in the elastic laminae of the aortic valve. However, it is not known whether this process is also taking place in the cardiac valve at the early stage of atherosclerosis. Animals were fed either a high cholesterol diet (n=5) or a control diet (n=5) for 10~12 weeks. Histologic analysis demonstrated that subendothelial thickening and foam-cell infiltration were evident in the arterialis of aortic valves. Confocal microscopy revealed an altered pattern characterized by fragmentation and disorganization of the arterialis elastic laminae of hypercholesterolemic valves. Computerized digital analysis of the images obtained by confocal scanning microscopy demonstrated that compared to normal valves, the arterialis elastic laminae of hypercholesterolemic valves decreased in percentage of their elastin content ($29.03 \pm 1.10\%$ vs. $42.94 \pm 1.35\%$, $p=0.023$). Immunohistochemical staining for matrix metalloproteinase-3 (MMP-3) revealed MMP-3 immunoreactivity was increased in hypercholesterolemic valves, predominantly in the arterialis. This study demonstrated that early atherosclerosis, induced by a high cholesterol diet in rabbits, is characterized by significant ultrastructural change in the elastic laminae of the aortic valve. The arterialis endothelium of the aortic valve may be a more atherosclerosis-prone area compared with the ventricularis. The presence of ultrastructural defect in the elastic laminae may play a role in chronic degenerative change and a resultant valvular dysfunction.

Key Words: Aortic valve, hypercholesterolemia, confocal microscopy, matrix metalloproteinase

Atherosclerosis is not confined to the intima of large and medium-size arteries; it also affects veins, cardiac valves, chordae tendineae, and endocardium.

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Moreover, the full thickness of the wall is affected, the severity diminishing from intima to adventitia. Fibromusculo-elastic intimal thickening with sclerosis, vascularization, medial thinning, hyalinization, loss of elastica, calcification, lipid accumulation, and luminal stenosis in small peripheral arteries, despite diminished severity, has distinct similarities. Several studies have demonstrated degradation and increased cross-links in elastin of the cardiovascular system, such as the aorta and carotid arteries during atherosclerosis (Yu, 1971; Sims, 1985; Nakatake and Yamamoto, 1987; Sims *et al.* 1989; Rekhter *et al.*

1991; Stehbens, 1995; Knox *et al.* 1997)

Atherosclerosis is the most severe problem in the high-pressure systemic circulation and similar changes occur also in the high-pressure loading valve (Stehbens, 1995). However, it is not known whether this process is also taking place in the cardiac valve at the early stage of atherosclerosis. The role and morphology of elastin remains poorly understood, despite the fact that it comprises up to 13% of the cusp dry weight (Scott and Vesely, 1996).

This study was designed to test the hypothesis that early atherosclerosis, induced by a high cholesterol diet in rabbits, is characterized by significant ultrastructural change in the elastic laminae of the aortic valve. The presence of an ultrastructural defect in the elastic laminae may allow a chronic, degenerative change and a result of valvular dysfunction. To test the hypothesis, we examined the ultrastructural changes of the subendothelial elastic laminae occurring in the rabbit model of the experimental hypercholesterolemic aortic valve without hemodynamic burden. The perfusion fixation and staining of the aortic valves with hematoxylin and eosin allowed us to visualize the ultrastructure of autofluorescent elastic laminae under confocal laser scanning microscopy imaging (Jester *et al.* 1991; de Carvalho and Toboga, 1996; Scott and Vesely, 1996; Wong and Langille, 1996). By means of this method, it was possible to evaluate the presence of morphological changes both qualitatively and quantitatively using a novel computerized digital imaging analysis technique.

MATERIALS AND METHODS

Animals

Ten male New Zealand White rabbits, aged 3-4 months, weighing 3~4 Kg were fed standard rabbit pellets and provided with water and libitum with (n=5; Group 1) or without (n=5; Group 2) the addition of 1% cholesterol for 10 to 12 weeks to induce experimental atherosclerosis. Rabbits were anesthetized by administration of 1 : 1 Ketalar (50 mg/mL; Parke-Davis) and Rompun Vet (20 mg/mL; Bayer AG), 0.35 mL/kg IV, and 0.17 mL/kg IV every 15 minutes thereafter. A thoracotomy, be-

tween the fourth and fifth ribs, was performed to place aortic cannulation for the measurement of mean aortic pressure and perfusion fixation.

The total plasma cholesterol level was determined by an enzymatic method previously described (Allain *et al.* 1974), using a commercial reagent (Roche). At the time of sacrifice, after plasma cholesterol level measurements, the animals were euthanized using an intravenous commercial euthanasia solution by the ear vein (5 ml Sleepaway, Fort Dodge Laboratories, Fort Dodge, IA, USA).

Tissue preparation and histologic analysis

Immediately after removal of the heart, each heart was placed on a perfusion fixation pump (4% formaldehyde) to maintain aortic morphologic integrity at a pressure of 100 mmHg. After fixation, the aortic valves were dissected from the aorta. Five aortic valves from the hypercholesterolemic group and 5 aortic valves from the control group were evaluated.

For histology, samples fixed in 10% formal saline were passed through a Shandon hypercenter enclosed tissue processor. After dehydration through graded alcohols, the samples were cleared in CNP 30 and embedded in paraffin wax. Sections were cut at 5 μ m and dewaxed in xylene prior to staining with Mayer's haematoxyline and eosin (H&E). Paraffin sections from the aortic to ventricular surface were stained with hematoxylin and eosin to assess the cellular component and to evaluate the elastin content, using confocal microscopy.

Immunohistochemistry for matrix metalloproteinase-3 (MMP-3)

Paraffin sections (5 μ m) were made and transferred to glass slides. Slides were deparaffinized and rehydrated through the following solutions: xylene twice for 5 minutes, 100% ethanol twice for 10 dips and 95% ethanol twice for 10 dips. Endogenous peroxidase activity was blocked for 10 minutes at room temperature in 50% volume H₂O₂/50% volume methanol and rinsed in running tap water. Non-specific protein binding sites were blocked by applying 5% normal goat serum diluted in PBS/0.05% Tween 20 (pH=7.2~7.4) to slides for 10 minutes at room temperature. The serum was blotted off and

the primary antibody (rabbit polyclonal antibodies for MMP-3, Merck Research) was diluted in 1% normal goat serum + PBS/0.05% Tween 20, applied and incubated overnight at 4°C in a humidity chamber. On day 2, the primary antibody was rinsed off in tap H₂O, blotted and the biotinylated secondary antisera cocktail (including goat anti mouse IgG and goat anti rabbit) diluted 1/400 was incubated on the slides for 30 minutes at room temperature. Slides were rinsed in running tap H₂O, blotted and streptavidin-horseradish peroxidase diluted 1/400 in PBS/0.05% Tween 20 + 1% normal goat serum was applied and incubated for 30 minutes at room temperature. The slides were rinsed in tap H₂O and color developed in 3-amino-9-ethylcarbazole substrate solution for 15 minutes at room temperature, counterstained in hematoxyline for 30 seconds and coverslip.

Stock Solutions

Tween 20	Pierce Chemical Co.
Normal Goat Serum	Dako (Carpinteria, CA, USA)
Biotinylated Mouse IgG	Dako
Biotinylated Rabbit IgG	Dako
Streptavidin-horseradish peroxidase	Dako
3-amino-9-ethylcarbasole	Sigma (St. Louis, MO, USA)

Confocal microscopy

Confocal microscopy was utilized for the identification of elastic fibers in hematoxylin-eosin staining rabbit aortic valves. The fluorescence properties of stained elastic fibers were due to eosin staining as revealed by fluorescence analysis of the dye in solution, with no or only a minor contribution by elastin auto-fluorescence (de Carvalho and Toboga, 1996; Wong and Langille, 1996). H&E-stained aortic valves were also used for comparison with light microscopic histologic architecture and morphological examination of elastin patterns under the confocal microscope, using the corresponding sections (Jester *et al.* 1991; de Carvalho and Toboga, 1996). Experiments were performed in duplicate and analyzed on a confocal laser scanning microscope

LMS310 (Carl Zeiss Inc., Germany) equipped with an argon/krypton laser. The elastic laminae of the aortic valve was visible by means of its autofluorescence with the laser tuned to an excitation wave length of 488/568 nm and the emissions collected through a 530/20 nm band-pass filter (pinhole =20). Images per specimen (hypercholesterolemic group, total number of fields=20; control group, n=20) were examined using a 10x neofluor lens with 0.3 n.a., digitized with a matrix of 512×512 pixels with a resolution of 1.25 mm per pixel and a field size of 0.4096 mm².

Furthermore, in order to digitally quantitate the average thickness and elastin content of the sub-endothelial elastic laminae of the aortic and ventricular sides respectively (Scott and Vesely, 1996), corresponding images were examined using a 40x neofluor lens with 0.75 n.a., digitized with a matrix of 512×512 pixels at a resolution of 0.625 mm per pixel and a field size of 0.1024mm². An image of the elastic laminae was stored using the LSM310 software on an IBM work station. Control experiments were performed in the same conditions. The results were analyzed independently by two analysts who were blinded to the diet regimen.

Digital image analysis and quantification of elastin content

The overlaid elastic laminae images obtained by confocal microscopy were captured and digitized on a computer station (Sun Microcomputer, Corte Madera, CA, USA). Quantitative morphometric measurements of the elastic laminae were obtained using the Analyze software (Version 7.5, Biomedical Imaging Resource, Mayo Foundation). Average elastin content of the subendothelial elastic lamina of the aortic side (arterialis elastic lamina) and ventricular side (ventricularis elastic lamina) (Scott and Vesely, 1996) were calculated respectively. The measurements were obtained as follows: A region of interest (ROI) was manually traced of the arterialis and ventricularis elastic lamina, excluding the sub-endothelial foam-cell area and amorphous elastin area of the ventricular side. Then an intensity histogram was selected, displaying the occurrence of gray levels (from white to black) within the ROI. Each elastic lamina area (displayed in white) was differ-

entiated against the black background, which represented the elastin fibers, by setting the lower threshold values for an intensity range of interest (IROI) that yielded the best identification of auto-fluorescent elastic laminae regions as judged by the analyst. The number of pixels that composed the area surrounded by the elastic fibers was then automatically counted by the computer. The values were divided by the total number of pixels within the image to obtain a percentage of the elastin content of the arterialis and ventricularis respectively.

The measurements of aortic valve thickness were obtained at the midportion of the leaflets under the low-magnifying digital images (final magnification X320).

Statistical analysis

Differences between groups in the plasma cholesterol values and in the morphologic measurements of the elastic laminae were assessed by a one-way ANOVA test followed by Mann-Whitney U-Wilcoxon Rank Sum W test. A value of $p < .05$ was considered significant in all analyses. All data in the text and figures are presented as mean \pm SEM.

RESULTS

The level of mean serum cholesterol from the control animals was 80.46 ± 8.10 mg/dl, while that from the animals fed with a high cholesterol diet was 257.67 ± 26.68 mg/dl. A significant increase in plasma cholesterol levels was present in the group of animals fed with a high cholesterol diet compared

with the control group (Table 1). Mean aortic pressure was not significantly different between the two groups (Table 1).

Normal aortic valve

The subendothelial elastic lamina of the aortic side (arterialis elastic lamina) was fine, regular and flat and the subendocardial elastic lamina of the ventricular side (ventricularis elastic lamina) was dense and interconnected with other elastic laminae (Fig. 1).

Digital quantitative analysis revealed that the percentage of the elastin content of the arterialis was $42.94 \pm 1.35\%$ while the ventricularis elastic lamina was $30.52 \pm 1.06\%$ (Table 1).

Experimental hypercholesterolemic aortic valve

The cholesterol-fed aortic valve demonstrated a marked increase in the leaflet thickness compared with the normal aortic valve (0.87 ± 0.05 vs 1.38 ± 0.08 mm, $p < 0.05$, Table 1). Foam-cell infiltration of thickened subendothelium was seen mainly in the arterialis and the amorphous elastin material was mixed with subendothelial foam cells. There was also inflammatory-cell infiltration in the arterialis which was associated with the fragmentation of elastic laminae (Fig. 2). Patch distribution of disorganized elastic laminae and a widening of the inter-elastic lamina space was mainly seen in the arterialis and protruded toward the aorta.

Quantitative computerized digital analysis of images obtained with confocal microscopy demonstrated a statistically significant decrease of elastin content in hypercholesterolemic aortic valves, pre-

Table 1. Lipid profile, hemodynamic and morphometric parameters in the experimental groups

	Control (n=5)	Hypercholesterolemia (n=5)
Cholesterol (mg/dl)	80.46 ± 8.10	$257.67 \pm 26.68^*$
Mean aortic pressure (mmHg)	84.00 ± 4.04	83.27 ± 4.03
Thickness of aortic valve (mm)	0.87 ± 0.05	$1.38 \pm 0.08^*$
Percentage of elastin content		
Arterialis EL (AEL)	42.94 ± 1.35	30.52 ± 1.06
Ventricularis EL (VEL)	$29.03 \pm 1.10^*$	31.92 ± 1.00

EL: elastic lamina, *: $p < 0.05$

Ventricularis

Arterialis

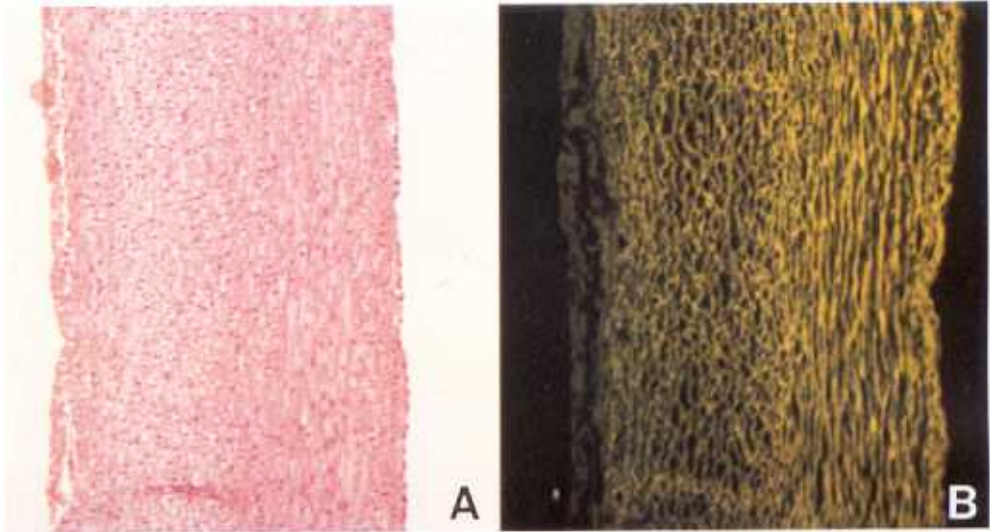


Fig. 1. *H & E staining (Panel A, final magnification X200) and Laser scanning confocal microscopy of the elastic lamina (Panel B, magnification X640) from a normal aortic valve. The arterialis elastic lamina was fine, regular and flat and the ventricularis elastic lamina was dense and interconnected with other elastic laminae.*

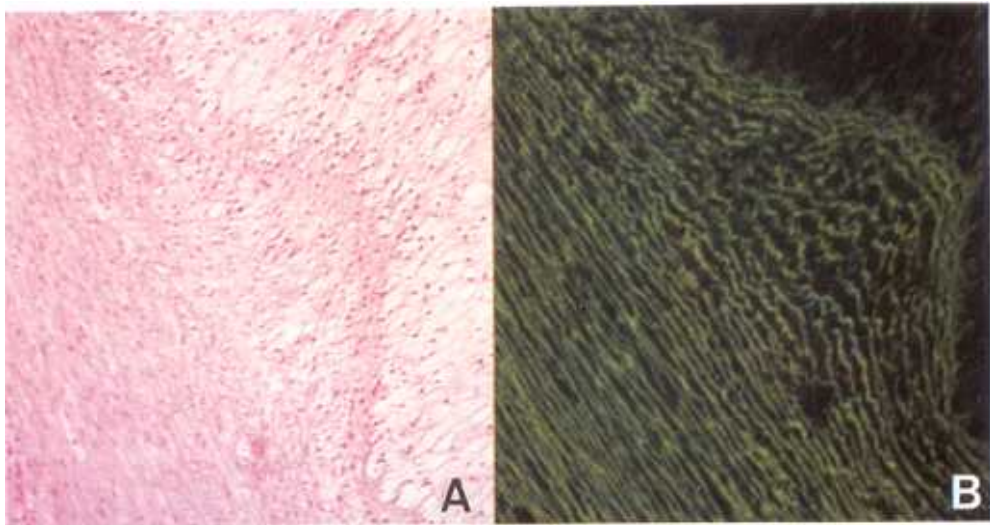


Fig. 2. *H & E staining (Panel A, final magnification X200) and Laser scanning confocal microscopy of the elastic lamina (Panel B) from a hypercholesterolemic aortic valve. Foam-cell infiltration of thickened subendothelium was seen mainly in the arterialis. There was also inflammatory-cell infiltration in the arterialis, which was associated with elastic lamina fragmentation. Images are projections that have been created by summing serial optical sections of the same area (final magnification X640). Patch distribution of disorganized elastic laminae and a widening of inter-elastic lamina space were mainly seen in the arterialis and protruded toward the aorta.*

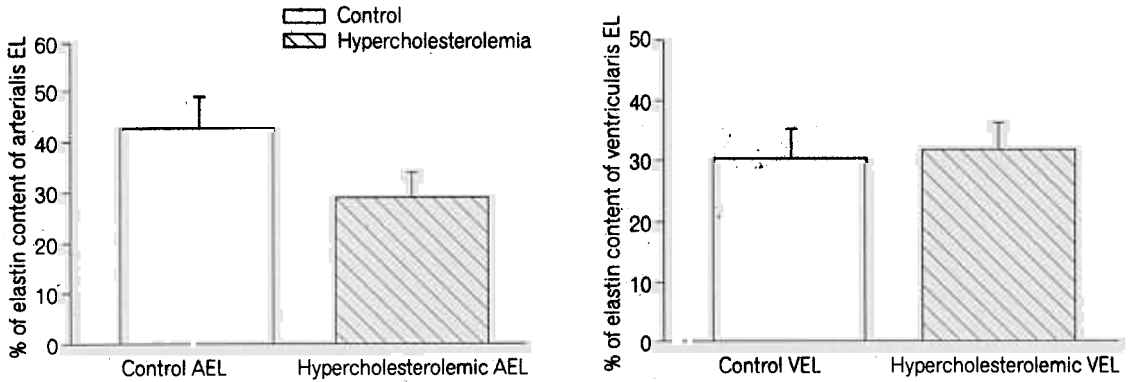


Fig. 3. Quantitative computerized digital analysis of images obtained with confocal microscopy demonstrated a statistically significant decrease of the elastin content in hypercholesterolemic aortic valves, predominantly in the arterialis elastic lamina, compared with normal valves ($29.03 \pm 1.10\%$ vs $42.94 \pm 1.35\%$). AEL: arterialis elastic lamina, VEL: ventricularis elastic lamina, *: $P < 0.05$

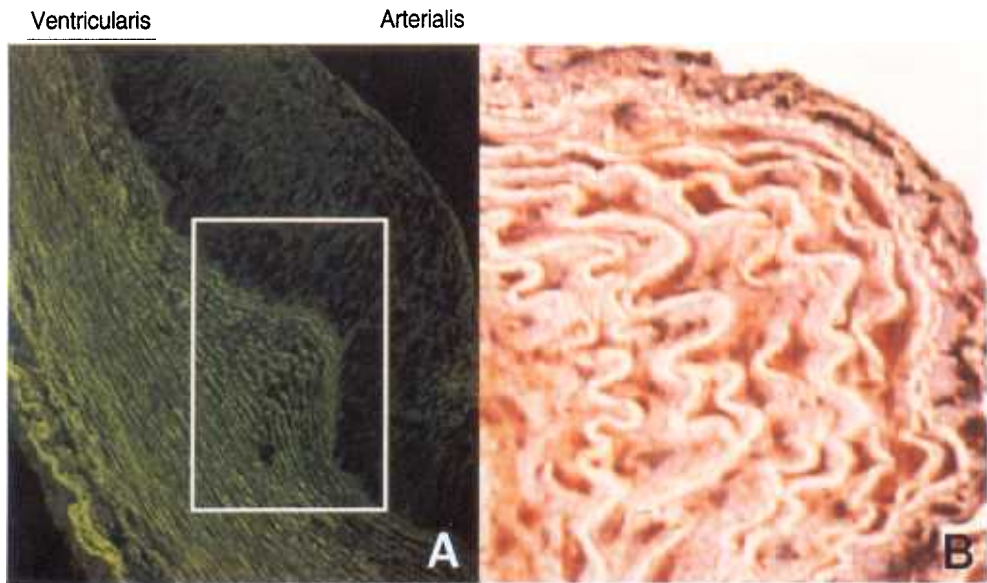


Fig. 4. Laser scanning confocal micrography (Panel A, final magnification X320) in hypercholesterolemic rabbit aortic valve revealed that the arterialis elastic lamina was fragmented and widened by elastolytic activity and deposition of the extracellular matrix. However, the ventricularis elastic lamina was preserved. Immunohistochemical demonstration of MMP-3 (Panel B, final magnification X200) in hypercholesterolemic rabbit aortic valve. MMP-3 immunoreactivity in the arterialis were distributed within multi-layered elastic laminae, which are fragmented and disorganized.

dominantly in the arterialis elastic laminae, compared with normal valves ($29.03 \pm 1.10\%$ vs $42.96 \pm 1.35\%$) (Fig. 3). The arterialis elastic laminae were fragmented and widened by elastolytic activity and deposition of the extracellular matrix. However, the

ventricularis elastic laminae were preserved (Fig. 4).

Compared with normal valves, hypercholesterolemic aortic valves demonstrated marked increases in immunoreactivity, predominantly in the subendothelial arterialis. Within hypercholesterolemic aortic

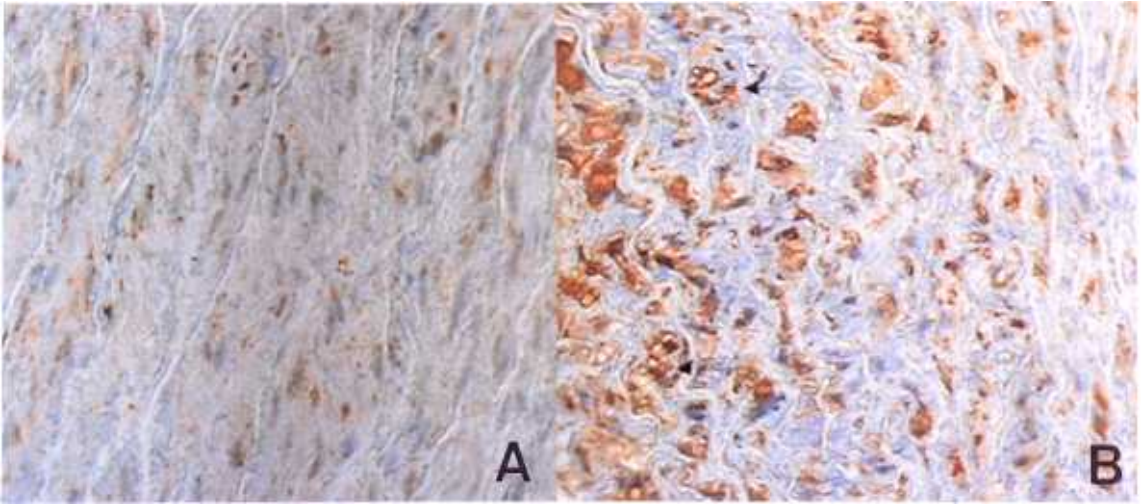


Fig. 5. Immunohistochemical demonstration of MMP-3 in normal (Panel A, final magnification X400) and cholesterol-fed (Panel B, final magnification X400) aortic valves. The cholesterol-fed aortic valve demonstrated a marked increase in MMP-3 immunoreactivity, predominantly in the arterial elastic lamina. Within the hypercholesterolemic aortic valve specimen, MMP-3 immunoreactivity was evident in the foam-cell and necrotic debris (arrow).

valves, MMP-3 immunoreactivity was evident in the arterialis. Moreover, MMP-3 immunoreactivity was evident in the foam cells and necrotic debris between the arterialis multi-layered elastic laminae. MMP-3 immunoreactivity in the arterialis was distributed within multi-layered elastic laminae, which were fragmented and disorganized arterialis elastic laminae (Fig. 5).

DISCUSSION

The present study demonstrated that cholesterol-fed aortic valves appeared to involve an active process with some similarities to atherosclerosis, including lipid deposition, macrophage infiltration and the formation of foam cells. The arterialis endothelium of the aortic valve may be a more atherosclerosis-prone area compared with the ventricularis. In this study, similar mean aortic pressure between the two groups suggested that experimental hypercholesterolemia without the effect of hemodynamic loadings to the aorta and aortic valve induced ultrastructural changes to the arterialis elastic laminae of the aortic valve by an elastolytic

activity. Hypercholesterolemia, similar to risk factors for atherosclerosis, induced ultrastructural defects in the elastic laminae and may play a role in chronic degenerative change and resultant valvular dysfunction (Stewart *et al.* 1997). Each cardiac valve has a central collagenous core, the fibrosa, which is continuous with the collagen-elastin of the cardiac skeleton. Both sides of the fibrosa are covered with the loose fibroelastic tissue, usually containing mucopolysaccharides, and the entire valve is covered with endothelium. The endothelium and connective tissue of the aortic valves are continuous with the aorta and ventricular endocardium. Gross and Kugel (Gross and Kugel, 1931) proposed that the loose connective tissue on the ventricular surface of the aortic valve be termed the *ventricularis* and that on the aortic side of the semilunar valve be termed the *arterialis*. Smooth and striated cardiac muscle may extend onto the proximal one-third of the arterialis in the aortic valve.

All valvular heart diseases place a hemodynamic burden on the left or right ventricle, or on both ventricles, which is initially tolerated as the cardiovascular system compensates for the overload (Carabello and Crawford, 1997). In the elderly, various aging changes may alter the semilunar

valves, particularly the aortic, and may result in low-grade systolic ejection murmurs. Beyond the age of 20 years, the annular circumference of the aortic valve increases linearly and is attended by mild cusp thickening and redundancy. The closing edges become thickened and, along the nodules of Arantius, may form whisker-like projections called Lambl's excrescence. Lunular fenestrations also tend to develop with aging. Degeneration of valvular collagen is often accompanied by lipidosis and focal calcification, even in the absence of significant aortic atherosclerosis, and most commonly involves the annulus, the nodules of Arantius and adjacent closing edges, and the basal portions of the valve pockets (Carabello and Crawford, 1997). While the structure and function of heart valve cusp collagen have been relatively well defined, the role and morphology of elastin remains poorly understood, despite the fact that it comprises up to 13% of the cusp dry weight. By imaging samples of digested fibrosa of the aortic valve, elastin in the fibrosa is much more complex, consisting of large tubes that emerge from the aortic attachment and extend circumferentially across the cusp. The tubes, constructed of an amorphous fenestrated sheet and loose-mesh elastin, likely surround the large circumferential collagen bundles observed in the fibrosa. Collagen and elastin are highly integrated. As a result, elastin plays an important functional role in the cusp and heart valve cusp mechanics must incorporate the contributions of both collagen and elastin (Scott and Vesely, 1996).

Several investigators have described an increase in elastolytic activity in aortic disease specimens (Cohen *et al.* 1992; Reilly *et al.* 1992; Knox *et al.* 1997). However, there has been no information regarding elastolytic changes in aortic valvular diseases.

Atherosclerosis is a chronic, degenerative disease of blood vessels. Furthermore, atherosclerosis is not confined to the intima of large and medium-size arteries, it also affects veins, cardiac valves, chordae tendineae, and the endocardium (Stehbens, 1995). Subendothelial fibroelastic thickenings commence in the neonatal cardiac valves, progressing throughout life and exhibiting sclerosis, hyalinization, elastic duplication and lipid accumulation. They are more pronounced on the left side of the heart and ac-

centuated by valve incompetence and cardiac hypertension. Lipid is predominantly extracellular (perifibrous) and associated with diminished cellularity. These phenomena have been attributed to a disturbed and turbulent blood flow. Superficial valve erosion, rupture and tears are associated with Lambl's excrescences, ulceration and small thrombi (Stehbens, 1990).

The mechanism of the ultrastructural changes in the elastic lamina in the current study may be multifactorial. Recently, several experimental studies have shown that in response to an increased presence of mildly oxidized lipoproteins which have been shown to be chemotactic for monocytes *in vitro*, an increased number of monocytes can enter the intima of the vessel wall forming macrophage foam cells under conditions of hypercholesterolemia. Despite our specimens being aortic valves, the present study was in agreement with these observations since infiltration of macrophage foam cells in aortic valves were frequently found during the histologic analysis. Different studies have shown by *in situ* zymography and *in situ* hybridization that there is an expression of the matrix metalloproteinases in human atherosclerotic plaques by both smooth muscle cells and foam cells (Henney *et al.* 1991; Galis *et al.* 1994). One member of the matrix metalloproteinase (MMP) family, MMP-3 (stromelysin), degrades proteoglycan core proteins, fibronectin, collagen IV and V, laminin, and gelatin, and can activate other MMPs. Proteoglycans are closely associated with matrix proteins and increase protein resistance to lysis. MMP-3 digestion of the proteoglycan core proteins can thereby enhance subsequent elastin degradation (Jones and Werb, 1980; Herron *et al.* 1986; Vine and Powell, 1991; Newman *et al.* 1994). Further evidence comes from work involving degenerative aortic disease that metalloproteinase expression is markedly increased in aorto-occlusive disease and aortic aneurysm samples, and an imbalance between metalloproteinases and their inhibitors results in similar proteolytic activity (Knox *et al.* 1997). Tissue inhibitor of metalloproteinases has been identified in association with the matrix in normal aorta by radioimmunoassay, raising the possibility that in pathological specimens, the delicate balance between MMPs and their co-regulated inhibitors has been lost (Brophy

et al. 1990). However, the contribution of proteolytic activity remains controversial whether it may be as an initiating process or as a secondary change in the degenerative process of aortic valvular diseases. Since the lesions in experimental hypercholesterolemia resemble those present in human atherosclerosis, it may be speculated that an increased production and release of metalloproteinase from macrophage foam cells may mediate the pathological ultrastructural changes of the elastic laminae in the aortic valve.

Although the prevalence of aortic disease increases with age, several lines of evidence suggest that degenerative aortic valve disease is not simply a consequence of aging. The early lesion of calcific aortic valve disease appears to involve an active process with some similarities to atherosclerosis, including lipid deposition, macrophage infiltration and production of osteopontin and other proteins, implicating atherosclerosis risk factors in the development of aortic valve disease (Olsson *et al.* 1994a, 1994b; Otto *et al.* 1994; Gotoh *et al.* 1995; O'Brien *et al.* 1995; O'Brien *et al.* 1996; Stewart *et al.* 1997). Gotoh *et al.* identified an association between Lp(a) levels and aortic valve sclerosis (Gotoh *et al.* 1995). Clinical factors associated with aortic sclerosis in 5,201 subjects enrolled in the Cardiovascular Health Study could be identified that age, gender, height, hypertension, smoking, Lp(a) and LDL-cholesterol are independent predictors of degenerative aortic valve disease and those are similar to risk factors for atherosclerosis (Stewart *et al.* 1997). Potential explanations for the association of these clinical factors with aortic valve disease include the possibility that hypertension results in abnormally high tensile stress on the aortic leaflets. Alternatively, turbulent flow patterns associated with high volume flow rates may lead to low shear stress, resulting in endothelial injury and disruption as is seen in atherosclerotic lesions (Thubrikar *et al.* 1986; Stebbins, 1990). Elevated levels of LDL-cholesterol and Lp(a) may facilitate lipid deposition after endothelial injury from any cause. However, the present findings were obtained from experimental hypercholesterolemia without the effect of hemodynamic loadings to the aortic skeleton. This study demonstrated that early atherosclerosis, induced by a high cholesterol diet in rabbits, is

characterized by significant ultrastructural change in the elastic laminae of the aortic valve. The arterialis endothelium of the aortic valve may be a more atherosclerosis-prone area compared with the ventricularis. The presence of an ultrastructural defect in the elastic laminae may play a role in chronic degenerative change and resultant valvular dysfunction.

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