Collagen Characteristics and Organization during the Progression of Cholesterol-Induced Atherosclerosis in Japanese Quail

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This study reports the concentration of collagen and its hydroxypyridinoline crosslinks, collagen fibril organization in the dorsal aortas, and systolic blood pressure during the progression of atherosclerosis in Japanese quail selected for cholesterol-induced atherosclerosis. The quail were placed on either a control or 0.5% cholesterol-added diet at approximately 16 weeks of age. The concentration of total collagen did not change in the control arteries during the course of the study, whereas at 5 and 10 weeks of cholesterol feeding, collagen levels decreased in the cholesterol-fed birds. Hydroxypyridinoline concentration increased during the duration of the study in the cholesterol-fed birds and by 15 and 20 weeks of cholesterol feeding, levels were significantly increased over those observed in the control arteries. Transmission electron microscopy showed changes in the organization of collagen fibrils. Increased systolic blood pressure was noted beginning at 10 weeks of cholesterol feeding, which is suggestive of other systemic changes induced by hypercholesterolemia. These results demonstrated remodeling of the collagen component of the dorsal aorta extracellular matrix during the progression of atherosclerosis and are suggestive of other systemic cardiovascular system changes. [Exp Biol Med Vol. 226(4):328–333, 2001]

Key words: atherosclerosis; cholesterol; collagen; extracellular matrix; hydroxypyridinoline

Atherosclerotic plaques develop through cell proliferation and extracellular matrix (ECM) deposition, resulting in restriction or blockage of blood flow. The ECM is not a static structure, but one that remodels itself to accommodate the functional requirements of organs. The major ECM molecules in atherosclerotic plaques include collagen and proteoglycans (1). The fiber forming collagen types I and III are the major collagen types found in arteries (2, 3). Type I and III collagen play an important role in arterial physiology by preventing arterial expansion beyond physiologic limits. Smooth muscle cells in the atherosclerotic arteries synthesize new ECM components, including collagen (4, 5). In atherosclerotic arteries collagen is crucial for plaque stability and its removal from the plaque's fibrous cap area may result in plaque rupture (6). In addition to providing the ECM with stability, the extracellular collagen network functions as a framework for the migration of smooth muscle cells into the intima where they proliferate and synthesize new ECM components. Since collagen plays key roles in plaque stability and cell migration properties, a comprehensive understanding of collagen expression and organization during the progression of atherosclerosis is essential.

To further investigate remodeling of the dorsal aorta collagen component during the development and progression of atherosclerosis, males from the cholesterol-induced atherosclerosis (CIA) line of Japanese quail were used. Previous research of Velleman et al. (7) and Jarrold et al. (8) have shown that the CIA line of Japanese quail is a valid animal model to study ECM remodeling induced by hypercholesterolemia. The cholesterol-fed CIA quail beginning after 6 weeks on a cholesterol-containing diet showed elevations in proteoglycan glycosaminoglycans in the atherosclerotic plaque, as well as the formation of foam cells (10). Jarrold et al. (11) demonstrated that the proteoglycan decorin was localized in the foam cell regions and collagen type I was found to surround the foam cells where decorin

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accumulated. In the present study, total collagen concentration, hydroxypyridinoline (HP) concentration, and collagen fibril organization surrounding the foam cells were studied in dorsal aortas, and systolic blood pressure was measured during the progression of atherosclerotic plaque formation.

Materials and Methods

Animal Model. The CIA quail used in this study were from stocks selected for the incidence of atherosclerosis after being fed diets containing added cholesterol as previously described in Velleman et al. (7). In the present study, 100 sexually mature male (approximately 16 weeks of age) CIA Japanese quail were randomly divided into two groups: a control group, which remained on a normal diet (no added cholesterol), and a cholesterol group, which was placed on a 0.5% cholesterol-added (Sigma, St. Louis, MO) diet. All procedures were approved by the Ohio Agricultural Research and Development Center Animal Care and Use Committee.

Transmission Electron Microscopy. A portion of each dorsal aorta sample was fixed overnight at 4°C in 2.5% glutaraldehyde in 0.2 mol/L sodium cacodylate, and 8 mmol/L calcium chloride, pH 7.2 to 7.4. After fixation, the tissue was treated in 1% osmium in 0.2 mol/L cacodylate for 1 hr, washed three times with distilled water, incubated in 0.15% tannic acid for 10 min, washed four times with distilled water, incubated in 2% uranyl acetate for 2 hr, rinsed two times in distilled water, and was finally embedded in Spurr's (Tedpella, Redding, CA). Thick 2-μm sections were viewed by light microscopy to determine the location of the intima in the controls and intima foam cell regions in the cholesterol-fed birds. The blocks were trimmed accordingly to centralize at the electron microscope level on the collagen surrounding the foam cell beds in the cholesterol-fed birds and the intima in the controls. The blocks were sectioned and viewed with a transmission electron microscope (TO1C, Phillips).

Collagen Concentration and Crosslink Analysis. Dorsal aortas from control and treated animals ($n = 10$) were collected at 0, 5, 10, 15, and 20 weeks after the initiation of cholesterol feeding and stored frozen at −70°C. The aortas containing the adventitia, media, and intima layers were baked overnight in a convection oven at 100°C, and the samples were then weighed and hydrolyzed in 6 N HCl at 110°C for 16 hr. An aliquot was removed for hydroxyproline determination (9). Collagen concentration (% dry weight) was calculated assuming collagen weighs 7.25 times the measured weight of hydroxyproline. The remaining hydrolyzate was subjected to HP crosslink analysis. Hydrolyzate HP was concentrated and separated from the bulk of the other amino acids by elution from a CF_1 cellulose column using the procedure described by Skinner (10). The dried fraction containing the HP was resuspended in 1% n-heptafluorobutyric acid containing pyridoxamine as an internal standard. Isolation and quantitation of the HP crosslink and pyridoxamine was accomplished by binary gradient reverse phase high pressure liquid chromatography using the procedure described by Eyre et al. (11). Identity of the HP peak was confirmed by comparison with a pure HP standard, which was prepared routinely from bovine articular cartilage by the method of Eyre et al. (11). Collagen HP crosslink concentration was expressed as mole of HP per mole of collagen, assuming that pyridoxamine has a molar fluorescence yield 3.1 times that of HP (11) and that the molecular weight of collagen is 300,000 daltons.

Measurement of Blood Pressure. Measurement of indirect systolic blood pressure of Japanese quail was done according to the method of Ely et al. (12). Systolic blood pressure was measured on ($n = 10$) cholesterol-fed and control animals the day prior to euthanasia for collagen analyses and transmission electron microscopy. In brief, a special stainless steel occlusion cuff was designed to fit around the thigh of the quail. The cuff was lined with a thin latex rubber sleeve that collapsed when exposed to 20 mmHg pressure or less. A transducer was placed on the thigh just below the occlusion cuff to detect heart pulsations. The quail was placed in a warming chamber (37°C) for 5 to 10 min to vasodilate the thigh arteries and then the bird was hand held while the occlusion cuff and transducer were slipped on and the pressure of the occlusion cuff was inflated to 200 mmHg and gradually released. Four to seven measurements were taken on each animal and averaged for a single systolic blood pressure measurement. The pulsations were recorded on a calibrated physiograph and were calculated for systolic pressure at the point of first pulsation after occlusion release.

Statistical Analysis. A *t*-test using the general linear model procedure of SAS (13) was used to estimate significant differences ($P < 0.05$) in collagen concentration, concentration of HP, and blood pressure between control and cholesterol-fed birds at each sampling interval.

Results

The organization of collagen fibrils in the control intima and surrounding the foam cell region in cholesterol-fed animals was examined by transmission electron microscopy. In the control group, collagen fibril organization remained relatively constant throughout the 20 weeks of the study. Morphologically, the control collagen fibrils in the arterial intima were characterized by tightly organized bundles (Figs. 1A, 2A, and 3A). At the initiation of the study the cholesterol group intima was similar to the control group in terms of collagen fibril organization (Fig. 1B).

By week 10 the cholesterol-fed animals showed initial stages of collagen fibril network remodeling around the foam cell bed region (Fig. 2B). By 20 weeks on the cholesterol-containing diet the collagen fibrils were dispersed throughout the foam cell region in the intima (Fig. 3B).

Total collagen concentration in the dorsal aortas from control birds did not differ during the study ($P < 0.05$; Fig.

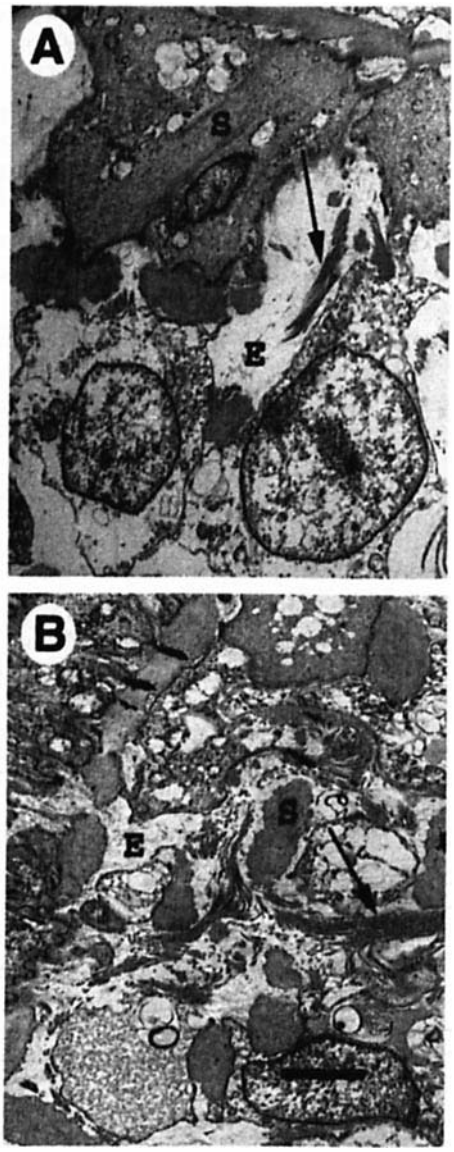

Figure 1. Transmission electron micrographs showing collagen fibril organization in dorsal aortas of male Japanese quail at week 0 of cholesterol feeding. (A) Control; (B) cholesterol-fed. The arrows highlight collagen fibrils in both the control and cholesterol-fed dorsal aortas. Smooth muscle cells are labeled with an S, and the extracellular matrix with an E. The scale bar represents 2 μm.

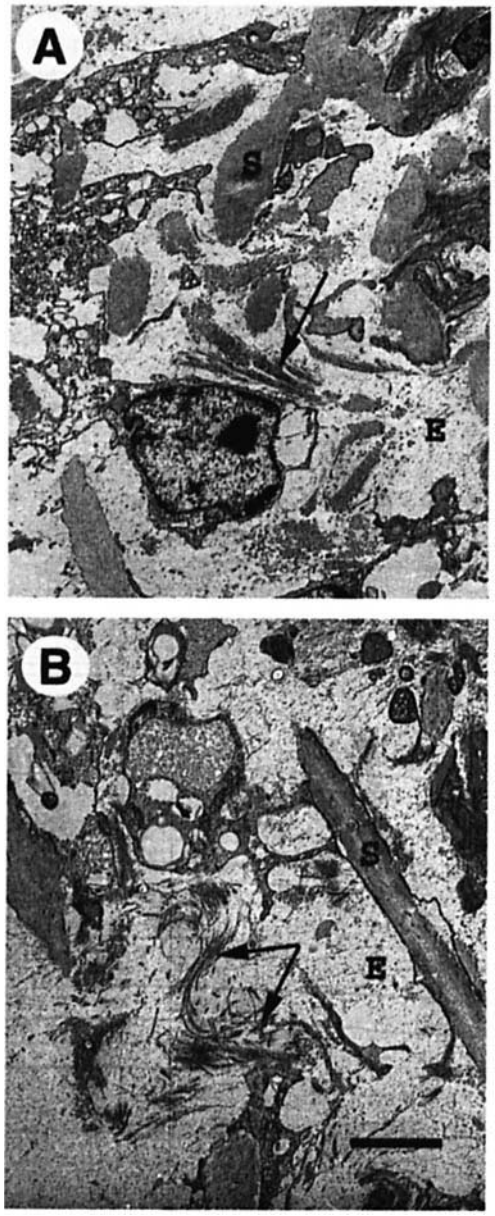

Figure 2. Transmission electron micrographs showing collagen fibril organization in dorsal aortas of male Japanese quail at week 10 of cholesterol feeding. (A) control; (B) cholesterol-fed. The arrows highlight collagen fibrils in both the control and cholesterol-fed dorsal aortas. Smooth muscle cells are labeled with an S, and the extracellular matrix with an E. The scale bar represents 2 μm.

4). The dorsal aortas collected from the cholesterol-fed birds at 5 and 10 weeks after the initiation of cholesterol feeding showed a significant decrease in the collagen concentration ($P < 0.05$; Fig. 4). However, there was a large increase in HP collagen crosslink concentration in the dorsal aortas from the cholesterol-fed birds at 15 and 20 weeks ($P < 0.05$; Fig. 5).

Blood pressure was measured in the control and cholesterol-fed CIA quail at each sampling time as an indication of cardiovascular function. The data in Figure 6 illustrate the difference in blood pressure between the control and cholesterol-fed birds. Beginning at 10 weeks and continuing through the duration of the study, the cholesterol-fed birds became hypertensive compared with the control birds. At 10 weeks the control birds had an average systolic blood pressure of 169 ± 13.7 mmHg and that of the cholesterol-fed birds was 199 ± 9.5 mmHg. By 20 weeks, the values were 164 ± 5.5 mmHg and 206 ± 17.4 mmHg, respectively.

Discussion

Results from the present study demonstrate a progressive change in intima collagen fibril organization, and total collagen and HP crosslink concentration. A decrease in collagen concentration in the cholesterol-fed animals compared with the normal animals was noted at 5 and 10 weeks, followed by an increase in HP concentration. These mea-

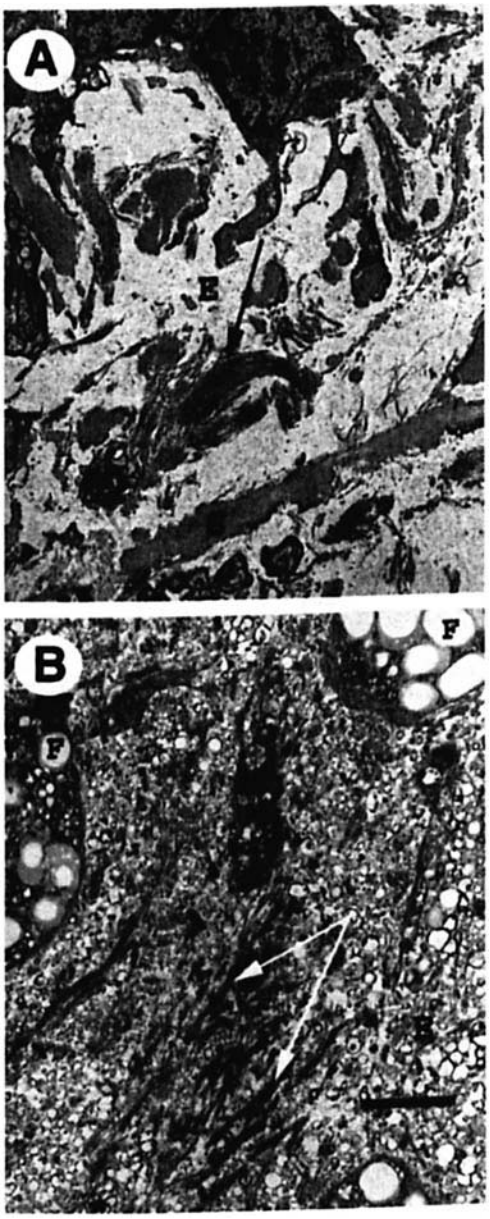

Figure 3. Transmission electron micrographs showing collagen fibril organization in dorsal aortas of male Japanese quail at week 20 of cholesterol feeding. (A) control; (B) cholesterol-fed. The arrows highlight collagen fibrils in both the control and cholesterol-fed dorsal aortas. Foam cells are labeled with an F, smooth muscle cells with an S, and the extracellular matrix with an E. The scale bar represents 2 μm.

surements of collagen and HP concentration were performed for the entire dorsal aorta. In studies by Rekhter et al. (14) using a rabbit atheroma model, similar changes were observed in collagen levels in the hypercholesterolemia animals. However, in contrast to the data reported in the current study, which stated that collagen crosslinking remained constant in the control animals, Rekhter et al. (14) observed HP concentration to increase in their controls. Differences in collagen crosslinking behavior observed in the control animals in the two studies may be due to species differences. The amount of collagen present in the aorta is the result of a balance between synthesis and degradation. Ma-

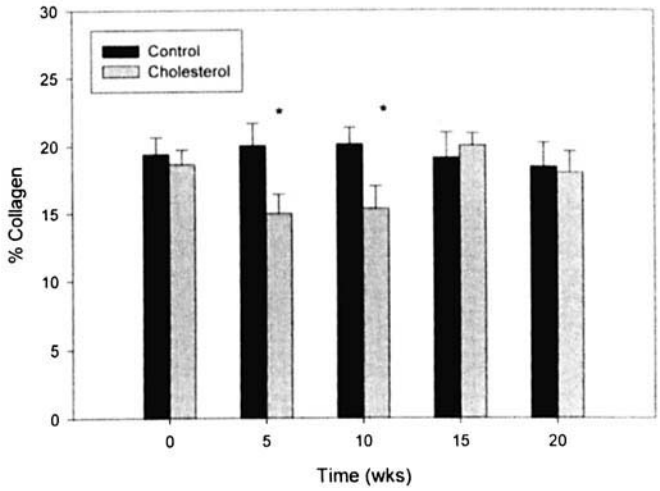

Figure 4. Collagen concentration (% dry weight) in male Japanese quail dorsal aortas throughout a 20-week period of cholesterol feeding. The asterisk indicates a significant difference ($P < 0.05$) between dorsal aortas from male control and cholesterol-fed animals at each sampling interval. Bars represent the standard error of the mean.

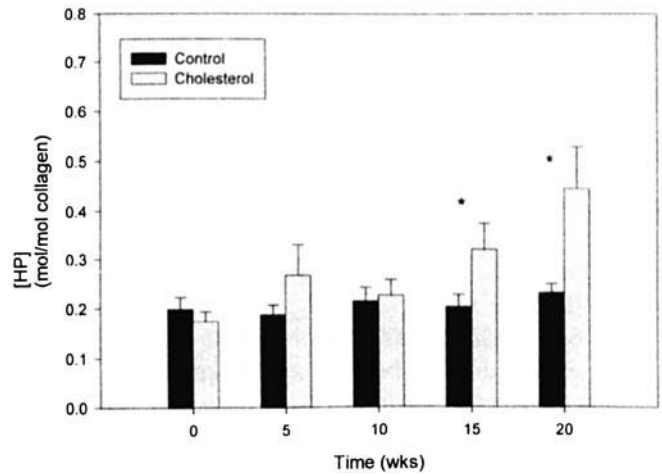

Figure 5. Hydroxypyridinoline (HP) crosslink concentration in male Japanese quail dorsal aortas throughout a 20-week period of cholesterol feeding. The asterisk indicates a significant difference ($P < 0.05$) between dorsal aortas from male control and cholesterol-fed animals at each sampling interval. Bars represent the standard error of the mean.

trix metalloproteinases degrade ECM components, as well as other structures including the basement membrane (15), which may be occurring in the cholesterol-fed dorsal aortas. The decrease in total collagen concentration is not reflective of data previously reported for dorsal aorta type I collagen levels in the CIA Japanese quail (8). In the study by Jarrold et al. (8), type I collagen levels increased in cholesterol-fed birds. The discrepancy in total collagen to type I collagen expression is likely due to changes in the ratio of type I to type III collagen with the progression of atherosclerosis that may be associated with a change in the synthesis and degradation of collagen types found in the dorsal aorta.

In the present study, increased systolic blood pressure with the development of atherosclerotic plaques was observed, which may play a role in modifying arterial collagen phenotype ratios. Changes in cardiac collagen type I to III

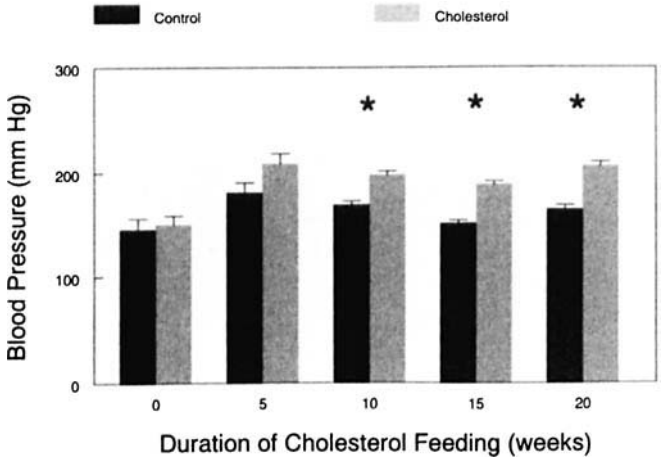

Figure 6. Difference in systolic blood pressure between control and cholesterol-fed Japanese quail throughout a 20-week period of cholesterol feeding. The asterisk indicates a significant difference ($P < 0.05$) between control and cholesterol-fed birds at each sampling interval.

ratios resulting from pressure-overload hypertrophy have been shown by others (16, 17). Coinciding with these biochemical observations, changes in collagen fibril organization surrounding the intima foam cell region were noted 10 weeks after the initiation of cholesterol feeding. In a previous study by Jarrold et al. (8), immunostaining for type I collagen showed a deposition of collagen surrounding the foam cell region.

Both collagen concentration and the organization of collagen fibrils are important in ECM remodeling because they affect the tensile strength and elasticity of the aorta. Molecular mechanisms responsible for the accumulation of collagen crosslinks in the aorta are not known. However, it has been noted that the formation of crosslinks is dependent upon collagen molecule spatial orientation and precise stereospecific alignment of crosslinking sites on adjacent collagen molecules and fibrils (18–20). The major fibrillar collagen phenotypes in the aorta are types I and III (3, 21); collagen fibrils may exist as either homo- or heteropolymers of the two types. The type I and III collagen molecules are joined by the HP crosslink (22). Hydroxypyridinoline is a trivalent crosslink and fibrillar collagen is a mixture of both types I and III, but flexibility exists in potential spatial arrangements of collagen molecules and in the composition of fibrils. As the ECM remodels during atherosclerotic plaque formation, it is necessary to have knowledge of both the changes in collagen phenotype and fibril organization. This is of importance because collagen types I and III are the predominant arterial collagens and have different concentrations of hydroxyproline, with type III having more than type I. If the collagen phenotype ratio changes with atherosclerotic plaque formation, this could be reflected in differences in hydroxyproline concentration and the level of collagen crosslinking. This will be the subject of ongoing research.

Collagen crosslinking provides the aortic wall with tensile strength and elasticity (23, 24). However, one of the consequences of increased arterial collagen crosslinking is a decrease in tissue elasticity. In the case of the CIA Japanese quail, the elevation of systolic blood pressure noted to begin at 10 weeks of cholesterol feeding and continuing for the duration of the study is likely due to increased stiffness of normally elastic trunk arteries, including the dorsal aorta. Interestingly, the elevation in blood pressure occurred at the same time that changes in ECM organization were observed (7, 8). Chronic pressure overload resulting from hypertension can lead to other cardiovascular system defects including myocardial hypertrophy and the remodeling of the muscular and ECM matrix compartments of the heart. These changes can result in decreased cardiac performance, leading to congestive heart failure. As the arterial wall is modified during the progression of atherosclerosis, it is likely that these changes result in other cardiovascular pathologies such as myocardial hypertrophy. Therefore, understanding how the ECM remodels during atherosclerosis is of critical importance in developing new therapies to regulate cardiovascular disease progression.

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