Lesion Regression and Protein Synthesis in Rabbits after Removal of Dietary Cholesterol

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This study was conducted to determine whether atherosclerotic lesions regressed in rabbits that were placed on a normal, low cholesterol diet. Rabbits (n = 16) were fed a 2% cholesterol diet for 90 days; nine rabbits were killed, and the remaining seven rabbits were fed a low cholesterol diet for 7 months. Appropriate noncholesterol-fed controls were used with each group. After 90 days of cholesterol feeding, the rabbits' thoracic aortas showed severe lesions, accompanied by significantly elevated rates of collagen and noncollagen protein synthesis and significantly increased amounts of cholesterol and soluble collagen. Cholesterol-fed rabbits that were fed a normal diet for 7 months had normal serum cholesterol levels, but exhibited extensive aortic lesions with elevated cholesterol content. Biochemically, the rate of aortic collagen synthesis remained significantly elevated above control values; however, the collagen content was not different from controls. This lack of collagen accumulation in the presence of a prolonged increase in synthetic rate was surprising. One possible explanation is that in the diseased aorta there is a rapid turnover of collagen, resulting in a redistribution or remodeling of the connective tissue, rather than an increased accumulation. (Arteriosclerosis 5:74-79, January/February 1985)

The reversibility of atherosclerotic lesions induced in the rabbit by a high-cholesterol diet has been well studied, but remains a controversial area. In early studies, Anitschkow reported a definite regression of lesions and a loss of lipid from aortic atherosclerotic plaques in rabbits 2 to 3 years after cessation of a cholesterol diet. Gupta et al. observed that aortic lesions induced by 2 months of cholesterol feeding (2 g/day) showed a biphasic change after cessation of dietary cholesterol. There was a rapid progression of lesion formation for 10 weeks after cholesterol withdrawal, as long as serum cholesterol remained elevated. In a group killed 20 weeks after cholesterol was withdrawn, serum cholesterol had returned to normal and there was a regression of atherosclerosis characterized by a reduction in cholesterol content and vascular lesion size. In a similar study, Friedman and Byers observed that after cholesterol withdrawal, the lesions continued to progress only as long as the animals were hypercholesterolemic, about 4 to 5 months. Once they were normocholesterolemic, the lesions did not increase.

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These data suggest that mild foam cell lesions can regress if the atherogenic stimulus is withdrawn. In contrast, other investigators have reported that when rabbits are fed a cholesterol diet for a sufficient period of time to induce the development of severe aortic lesions in the aorta, regression does not occur. As early as 1951, Duff and McMillan suggested that in the atherosclerotic rabbit small lesions may disappear, but the larger ones undergo partial replacement of the lipids by connective tissue and remain significant lesions. Constantinides et al. reported a worsening of rabbit lesions up to 2 years after conclusion of a 2-month period of cholesterol feeding, and an increase in the cholesterol content of the vessels and in the thickness of the lesions. Adams et al. also reported no regression of atheroma in rabbits fed cholesterol for 12 weeks followed by a normal, low cholesterol diet for 1 year, and said that the lesions became progressively more fibrous over the regression period. Eggan et al. in their study of diet-induced fatty streaks in Rhesus monkeys found that lipid decrease in the lesions was accompanied by an increase of fibrous tissue. Zilversmit has suggested that the fibrous cap that forms after the removal of cholesterol from the diet may prevent the reversal of the atherosclerotic lesion.

Our laboratory has been interested in studying the relationship of collagen metabolism to atherogenesis. Langner and Modrak have shown that feeding 2% cholesterol diet to rabbits for different times up
to 90 days resulted in the development of severe atherosclerotic lesions, which were characterized as having increased rates of collagen synthesis. Attempts to halt the development of aortic disease by removing the cholesterol from day 60 to day 90 caused a marked decrease in serum cholesterol levels, but no change in the severity of aortic lesion formation when compared to animals fed continuously for 90 days. These data suggest that short feeding periods of a normal low-cholesterol diet were ineffective in preventing or reversing pre-existing changes induced by relatively long periods of high cholesterol feeding.

The purpose of the present study was to examine the biochemical and histological changes induced by prolonged feeding of a normal low-cholesterol diet to hypercholesterolemic rabbits. Friedman and Byers have estimated that it takes 4 to 5 months for the serum cholesterol levels to drop to control values following removal of cholesterol from the diet. Therefore, in our study, hypercholesterolemic rabbits were placed on a normal diet for 7 months so that we could determine biochemically: 1) the extent to which collagen content increases in the aorta, and 2) the effect of a long-term normal diet on the elevated synthetic rate of aortic collagen and noncollagen proteins, which is characteristic of hypercholesterolemic rabbits.

**Methods**

Male New Zealand rabbits weighing between 2.0 and 2.5 kg were housed two per cage and were given food and water ad libitum. The animals were randomly divided into four groups as follows: two of the groups served as control animals and were fed commercial Purina rabbit chow, while the remaining two groups were fed a 2% cholesterol diet for 90 days. The 2% cholesterol diet was prepared by dissolving cholesterol in chloroform, absorbing it onto commercial Purina rabbit chow and allowing it to air dry. At the end of the 90-day period, one control group and one cholesterol-treated group were sacrificed. The other cholesterol-treated group was transferred to a normal, low-cholesterol diet (regression group). At the end of the 9-month period, the animals in the two groups were killed. All procedures used in these studies were in accordance with institutional guidelines concerning use of experimental animals.

All animals were killed by cervical dislocation; the thoracic cavity was opened and the thoracic aorta from the beginning of the ascending aortic arch to the level of the celiac artery was quickly removed. The aorta was cleaned of loosely adhering tissue and opened longitudinally; the degree of the visible aortic lesions was estimated by using the 0–4 grading system described by Lorenzen. Grade 0 indicated the complete absence of lesions and Grade 4 indicated severe lesion development with lesions extending into the abdominal aorta. Histologic sections were taken from representative lesions located at the end of the aortic arch, fixed in buffered formalin, and stained with hematoxylin and eosin or Verhoeff’s stain. The remaining tissue was then weighed and placed in oxygen-rich Krebs bicarbonate buffer (pH 7.4) for a 20-minute preincubation period at 37°C under a 95% O2 and 5% CO2 atmosphere. Following preincubation, the tissues were transferred to incubation vessels containing fresh oxygenated Krebs bicarbonate buffer and 0.2 mM L-proline with 3 μCi of (U-14C) proline in a final volume of 10 ml. Following the incubation period (90 minutes), the aortas were immediately rinsed with ice-cold 5% trichloroacetic acid (TCA) to prevent further incorporation of 14C-proline.

The synthetic rates of collagen and noncollagen proteins were estimated by the hot TCA-extraction procedure described by Newman and Langner. Briefly, the procedure was as follows: following homogenization, an appropriate aliquot was washed with ice-cold 5% TCA to remove any unincorporated 14C-proline and was centrifuged. The resulting pellets were extracted twice with 5% TCA at 90°C for 60 minutes. The extracts were combined and hydrolyzed in 6N HCl at 125°C at 15 pounds of pressure for 18 hours. The resulting hydrolysates were then evaporated to dryness and redissolved in deionized-distilled water. In the present studies, we further purified the hot TCA extracts, which contained primarily collagen, by thin-layer chromatography using a modification of the technique described by Myhll and Jackson. In our procedure, an appropriate aliquot of the hydrolysate was washed four times with four volumes of ether to remove the TCA and was evaporated to dryness. The sample was redissoved in 70% EtOH and applied to glass plates precoated with Avicel (250 μ thick). The plates were then run twice in a butanol/acetic acid/water solvent (65:17.5:17.5) to ensure good separation of proline and hydroxyproline. After drying, the plates were developed with 1% Dansyl chloride and the hydroxyproline spots were dissolved in boiling water. An appropriate aliquot (2 ml) was then added to 5 ml Insta-Gel (Packard, Downers Grove, Illinois) for radiometric determination. In the Results section, the collagen synthetic rates are reported as the disintegrations per minute (DPM) of 14C-hydroxyproline.

The pellet remaining after the 90°C TCA extraction was digested overnight in 2 ml of Protosol (New England Nuclear, Boston, Massachusetts) at 50°C. A 1 ml aliquot of the pellet digest was added to 5 ml Insta-Gel for radiometric determination. In the Results section, the noncollagen synthesis was reported as the DPM of 14C-proline. All radioactive samples were corrected for counting efficiency.

The hydroxyproline content of the aortic homogenate was estimated by the method of Kivirikko et al. and was used as an index of collagen content. In the present studies, aortic collagen was separated into soluble and insoluble fractions as previously described. In this procedure an aliquot of aortic homogenate was combined with 10 volumes of ice-cold 0.45 M NaCl and shaken for 16 hours in a cold room.
The sample was centrifuged at 48,000 g for 1 hour. The supernatant, which contained soluble collagen, was evaporated to dryness and hydrolyzed in 2 ml of 6 N HCl for 18 hours at 15 lbs pressure and 125° C. The soluble fraction was assayed for hydroxyproline content as previously described.17 The protein assay described by Lowry et al.18 was used to estimate the noncollagen protein content of the aortic homogenates. Bovine serum albumin was used as the standard.

The cholesterol content of the aortic homogenate was determined following lipid extraction by a modification of the procedure described by Folch et al.19 After extraction of aortic tissues, free and esterified cholesterol were separated by thin-layer chromatography. Samples were applied to silica gel-coated plates (60 HR extra pure, 0.5 mm thick), developed in n-heptane/diethyl ether/acetic (75:25:2) and visualized in iodine vapor. The cholesterol content in each spot was determined using the ferric chloride-sulfuric acid method described by Franey and Amador.20 The absorbance of each sample was read in a Beckman DB-GT spectrophotometer at 560 nm.

Serum cholesterol was determined after 90 days and again after the 7-month regression period. Blood was collected from the marginal ear vein and serum cholesterol was determined on an ethanol extract of serum using the method described by Franey and Amador.20

The statistical significance of our results was determined by Student's t test for groups with equal variances, and by Satterthwaite's t test21 for groups with unequal variances.

Results

After 90 days of the 2% cholesterol diet, rabbits exhibited serum cholesterol values in excess of 2500 mg/100 ml (Table 1). In previous studies we demonstrated that when cholesterol was removed from the diet, serum cholesterol values fell rapidly but were still significantly elevated after 30 days.11 In this study, plasma cholesterol values in the regression group were at control levels by the time the animals were killed.

The control values shown in the tables represent a combining of the values from control animals killed at 90 days and those killed at the end of the 7-month regression period. The two control groups were analyzed separately, were found to be biochemically and morphologically similar, and were therefore combined into a single control group. Previous studies from our laboratory have suggested that following sexual maturity, the rate of aortic collagen synthesis remains relatively constant as the rabbit ages.22 The control data from the present experiments support that hypothesis.

After 90 days of cholesterol feeding, the rabbits exhibited severe lesion development involving 60% to 80% of the intimal surface of the thoracic aortas (Table 1). Histologically, the lesions had several layers of intimal foam cells with some disruption and fragmentation of the elastic lamellae at the base of the lesion. Fibrous cap formation was not evident in the lesioned areas.

The rabbits that were fed cholesterol for 90 days followed by 7 months of normal diet still exhibited very severe lesion development, even though their serum cholesterol values were at control levels. As seen in Table 1, three rabbits were judged as having Grade 3 lesions with approximately 60% to 70% of the entire thoracic aorta covered with lesions. The remaining four rabbits had Grade 4 lesions covering 80% to 90% of the thoracic aorta with additional lesions extending into the abdominal aorta. Histologically (as described by several authors5,4,9), these lesions exhibited a relative absence of foam cells and a more compact appearance, suggesting that the foam cells had been replaced by increased amounts of collagen. The aortic wet weight to body weight ratio was increased in the 90-day cholesterol-fed group, but had returned to control levels in the regression group after 7 months (Table 1).

The total cholesterol content was significantly elevated in the thoracic aortas of both the 90-day cholesterol group and the 7-month regression group.

Table 1. Aortic Lesions, Tissue Weights, and Plasma Cholesterol Levels in Cholesterol-Fed and Regressed Rabbits

<table>
<thead>
<tr>
<th>Rabbit group</th>
<th>No. of animals with lesion grade 0 1 2 3 4*</th>
<th>Thoracic aorta/body weight (g/g)</th>
<th>Plasma cholesterol (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 7)</td>
<td>5 0 0 0 0</td>
<td>0.32 ± 0.03</td>
<td>47.4 ± 6.67</td>
</tr>
<tr>
<td>90-cholesterol (n = 9)</td>
<td>0 0 6 3</td>
<td>0.47 ± 0.02†</td>
<td>2624.0 ± 309.8†</td>
</tr>
<tr>
<td>Regression (n = 7)</td>
<td>0 0 0 3 4</td>
<td>0.33 ± 0.04</td>
<td>30.0 ± 7.0</td>
</tr>
</tbody>
</table>

Control = combination of animals killed at 90 days and 10 months; 90-cholesterol = rabbits fed a 2% cholesterol diet for 90 days, and then killed; regression = rabbits fed a 2% cholesterol diet for 90 days followed by 7 months of a low cholesterol, normal diet, and then killed. All data are presented as means ± SE.

* Aortic lesions were graded on 0—4 scale, with 0 indicating the absence of lesions and 4 being the most severe.
† p < 0.05 compared to control and/or regression rabbits.
Table 2. Cholesterol Content in Thoracic Aortas from Cholesterol-Fed and Regressed Rabbits

<table>
<thead>
<tr>
<th>Rabbit group</th>
<th>Total cholesterol (mg/g tissue)</th>
<th>Free cholesterol (mg/g tissue)</th>
<th>Cholesterol ester (mg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 7)</td>
<td>1.04 ± 0.10</td>
<td>0.84 ± 0.07</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>90-cholesterol (n = 9)</td>
<td>12.77 ± 0.90*</td>
<td>3.62 ± 0.25*</td>
<td>9.15 ± 0.70*</td>
</tr>
<tr>
<td>Regression (n = 7)</td>
<td>10.68 ± 1.52*</td>
<td>6.07 ± 0.97†</td>
<td>4.61 ± 0.57†</td>
</tr>
</tbody>
</table>

See Table 1 for explanation of data.
* p < 0.05 compared to control rabbits.
† p < 0.05 compared to control or 90-cholesterol rabbits.

(Table 2). In the rabbits fed cholesterol for 90 days, approximately 70% of the cholesterol was esterified, while in the regression rabbits, more free cholesterol was found; only 40% was esterified cholesterol.

The rates of collagen and noncollagen protein synthesis are shown in Table 3. The separation of collagen 14C-proline and 14C-hydroxyproline from noncollagen 14C-proline was accomplished by extracting the aortic collagen into hot TCA.13 To insure that the collagen extracts contained no contamination of 14C-proline from noncollagen proteins, the 14C-hydroxyproline was separated from 14C-proline by thin-layer chromatography of the TCA extracts.14 Aortic collagen synthetic activity was significantly elevated in the animals fed cholesterol for 90 days and the regression animals. The aortic collagen synthetic rate appeared to be decreasing after the 7-month regression period, but was still elevated above control levels. Noncollagen protein synthesis was significantly elevated in the group fed cholesterol for 90 days, but returned to control levels in the regression group.

The amount of aortic noncollagen protein was estimated by using the protein assay described by Lowry et al.18 Since the color formed in the protein assay procedure is dependent upon the presence of amino acids that are essentially absent in collagen, this procedure was used as an estimate of the aortic noncollagen protein content. The total amount of protein appeared to be increasing in both the cholesterol and regression groups of rabbits (Table 4). Statistically, however, there was no significant difference between any of the groups. The collagen content of the aortas was estimated by measuring the aortic hydroxyproline content. As seen in Table 4, there was no statistical difference in total collagen content of the aortas among the three experimental groups. This is in contrast to our histological data which suggested an increase in aortic collagen content. The collagen content of the aortas was further examined by classifying it into soluble and nonsoluble forms. Newly synthesized collagen is in a form that is more readily extracted into cold salt solutions.
than are the older, mature collagen forms. As shown in Table 4, the amount of 0.45M NaCl-soluble collagen was significantly increased in the group fed cholesterol for 90 days, but had returned to control levels in the regression group.

**Discussion**

It is well documented that rabbits will develop severe atherosclerotic lesions when they are fed a high cholesterol diet. The reversibility of these diet-induced lesions after removal of the cholesterol remains unclear. Our results indicate that simple reduction of plasma cholesterol levels by prolonged feeding of a normal, low cholesterol diet does not result in a reversal of previously induced atherosclerosis. Following 7 months of a normal diet, the rabbits in our study still exhibited severe lesions in the aorta, even in the presence of normal serum cholesterol values. These results agree with those of Friedman and Byers, who estimated that return to normocholesterolemic serum occurred within 4 to 5 months, but observed that severe aortic atherosclerosis persisted even 9 months after cholesterol feeding had been discontinued. Constantinides et al. noted that lesions induced in the rabbit by 2 months of cholesterol feeding still remained as long as 2 years after a return to a normal diet. Therefore, these results demonstrate that cessation of cholesterol feeding, and the consequent reduction of serum cholesterol levels, does not induce regression of atherosclerotic lesions.

Controversy exists about whether the total amount of tissue cholesterol in the aorta actually decreases following removal of the cholesterol diet. Biochemically, our results show that removal of cholesterol from the diet for 7 months does not cause a rapid reduction in total cholesterol content in the aorta. Following the prolonged feeding of a normal, low cholesterol-containing diet, the regression group still exhibited as much cholesterol (10.68 ± 1.52 mg/g tissue) as those rabbits sacrificed after the 90 days of cholesterol feeding (12.77 ± 0.90 mg/g tissue).

There was, however, a significant shift in the ratio of free-to-esterified cholesterol during the regression period. Free cholesterol content increased from 3.62 mg/g tissue in the group fed cholesterol for 9 days to 6.07 mg/g tissue in the regression group, while cholesterol ester content decreased from 9.15 mg/g tissue to 4.61 mg/g tissue. Similar findings in the rabbit, Rhesus monkey, pigeon, and squirrel monkey have confirmed the shift from esterified cholesterol to free cholesterol in the atherosclerotic lesion following a period of low-cholesterol feeding. It is not clear whether the predominance of free cholesterol following return to a normal diet is due to a decrease in the rate of esterification of cholesterol, or to an increase in the rate of hydrolysis of cholesterol esters.

Friedman and Byers reported similar findings in rabbits allowed to survive for 9 months after cessation of the cholesterol diet. The aortic lesions in these animals contained almost four times as much cholesterol as the plaques from rabbits sacrificed immediately after cholesterol feeding, even though they estimated that these rabbits had returned to normocholesterolemic serum levels 4 to 5 months earlier. Although biochemically the data indicated a significantly increased cholesterol content in the aorta, these authors concluded that the lesions had regressed based on a loss of sudanophilia in the tissue. Other investigators have also reported a decrease in staining the Sudan red as indicative of a regression of the disease process. In our studies there was no apparent loss of total cholesterol, but there was a shift of esterified cholesterol to free cholesterol. Since reportedly Sudan red does not stain free cholesterol, it is possible that earlier studies that reported a reduction in lesion cholesterol were underestimating the actual amount of cholesterol in the lesion. In any event, our data definitely demonstrate that simple removal of cholesterol from the diet does not promote a removal of cholesterol from aortic tissue.

It is generally accepted that placing rabbits on a low cholesterol diet for varying periods of time produces a more fibrous type of lesion. While these lesions have been characterized histologically as having increased collagen content, few studies have biochemically documented increased amounts of collagen. The data in Tables 3 and 4 demonstrate that the rate of collagen synthesis is significantly elevated after 90 days of cholesterol feeding and is accompanied by a significant increase in the 0.45 M NaCl-soluble collagen pool. This change in collagen synthesis is not specific, since there was also a significant increase in noncollagen protein synthesis. After 7 months on the regression diet, aortic protein synthetic rates returned to normal, but aortic collagen synthetic rates remained significantly elevated, even though serum cholesterol was at control level for approximately 3 months (Table 3).

Histologically, the lesions that we observed in the two treatment groups were very different, as previously documented. Lesions from rabbits fed cholesterol for 90 days were composed primarily of foam cells, and exhibited fragmentation of the elastic lamellae. No fibrous cap was evident. In the regression group, the lesions were characterized by a relative absence of foam cells and a more compact appearance of the tissue, which suggests that the lipid observed after 90 days of cholesterol feeding had been replaced by connective tissue during the 7 months of normal diet. A fibrous cap covered the lesion.

Friedman and Byers, Duff and McMillan, and Adams et al. reported that lesions underwent a "fibrous transformation" when an atherogenic rabbit was taken off a cholesterol diet. These studies, which relied on a histological assessment of the lesions, did not biochemically demonstrate any increase in the collagen content of the tissue. The results of the present
study indicate that there was no increase in total collagen content of the aorta after 7 months off the cholesterol diet (Table 4). This is despite the compact appearance of the lesions, which suggests that more fibrous tissue was present, and despite the continuous elevation of collagen synthetic activity.

There are many theories that could explain the absence of an increased aortic collagen accumulation. One possibility is that during this period there is also an increased rate of collagen degradation. This would lead to an increased collagen turnover without any net accumulation, and could result in an architectural rearrangement of collagen within the lesion, producing a closer association of connective tissue components with the increased lipids already deposited in the aorta. An alternate possibility is that changes in the collagen fibril or in the type of collagen being synthesized may sufficiently alter staining characteristics so as to give the histological impression of increased aortic collagen content. A third possibility is that localized collagen accumulation in aortic lesions may not contribute significantly to the entire collagen pool. Any of these suggestions could explain the morphologic appearance of increased collagen content in the aortic lesion, without any net increase in total collagen content.

A recent review article by Malinow summarized that in the rabbit, cessation of cholesterol feeding results in the regression of early atherosclerotic lesions initially induced by short periods of high cholesterol feeding. Lesions induced by several months of high cholesterol feeding do not regress when cholesterol is removed from the diet. In previous studies from our laboratory, we have demonstrated that increased aortic collagen synthesis occurs only after 2 to 3 months of a high cholesterol diet, and have suggested that this is an important factor in transforming the lesion from a reversible to a nonreversible state. The results of our present study are consistent with this hypothesis and further suggest that it is not the amount of collagen present in the lesion that is important, rather, it is the arrangement and perhaps the type of collagen present that makes the fibrous lesion so resistant to regression.

References

15. Lukens LN. Evidence for the nature of the precursor that is hydroxylated during the biosynthesis of collagen hydroxyproline. J Bio Chem 1965;240:1661-1669
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