Arterial Fibrous Proteins in Cynomolgus Monkeys after Atherogenic and Regression Diets

By Mark L. Armstrong and Marjorie B. Megan

ABSTRACT

Fibrous proteins were measured in five arterial beds in adult cynomolgus monkeys after administration of atherogenic and regression regimens. Atherosclerosis was induced by feeding the monkeys a hypercholesterolemic diet containing 1.2% cholesterol for 17 months. A low-fat, cholesterol-free regression diet was then given for 60 days, 200 days, and 20 months. In atherosclerosis, collagen concentration (mg/g dry weight) and collagen content (mg/cm length of artery) both increased. At 200 days of regression the collagen concentration, but not the collagen content, was higher than it was in atherosclerosis. In late regression (20 months), the collagen content was lower than it was in atherosclerosis, although in the five arterial beds considered together the collagen concentration was not significantly lower. Both the elastin concentration and the elastin content rose in atherosclerosis and decreased in regression. These mass data suggest that fibrous proteins are lost from the arterial wall during a regression regimen. Correlative evidence suggests that younger intimal fibers may be chiefly susceptible to fibrolytic activity, leaving dense intimal scars characteristic of regressed arteries.

KEY WORDS arterial collagen content and concentration arterial elastin content and concentration hypercholesterolemic diet experimental atherosclerosis

Collagen and elastin are of primary significance in arterial wall structure. In addition, these fibrous proteins play important roles in the response of arterial walls to injury, including the injury-response sequence characterized as atherosclerosis. Increases in arterial collagen and often elastin have been detected in human and experimental atherosclerosis at various stages between the formation of fatty streaks and fibrous plaques (1-6).

When experimental atherosclerosis is induced in primates by dietary means and subsequent regression occurs after the atherogenic diet is withdrawn, the residual lesions are altered by the significant depletion of lipid (7) and the persistence of connective tissue that contains both collagen and elastin fibers, but chiefly collagen (8). It is uncertain whether the regressing atherosclerotic artery tends to maintain or even accumulate excess fibrous proteins while it loses lipid or whether the fibrous protein mass decreases, as it does in involuting tissue (9) and apparently in dermal scars (10).

In the present investigation, fibrous protein changes in five arterial beds in a nonhuman primate highly susceptible to experimental atherosclerosis (11, 12) were studied after the induction of dietary atherosclerosis and at three intervals in a long regression trial.

Methods

Twenty adult cynomolgus (M. fascicularis [Thailand]) male monkeys (5.1 ± 0.17 kg) were fed a hypercholesterolemic diet (13) containing 1.2% cholesterol for 17 months. The monkeys were then divided into four groups of equal hypercholesterolemia. Group 1 was autopsied at the end of the atherogenic period. Groups 2, 3, and 4 were fed cholesterol-free commercial chow for regression periods of 60 days, 200 days, and 20 months, respectively, before autopsy. Five control monkeys given commercial chow were autopsied at the end of the study.

Selection and Preparation of Tissue

At autopsy the following segments of the arterial tree were measured for length in situ and then removed for analysis: the aorta from the left subclavian artery to the iliac bifurcation, the common carotid arteries, the femoral arteries, the subclavian arteries, and major portions of the extramural coronary arteries whose lengths were determined by measurement of the dissection grooves on the surface of the heart after removal of the segments.

Biochemical Studies

The specimens were freed of adventitia and clots, minced, homogenized, and extracted three times with 20 volumes of a chloroform-methanol solution (2:1) (14). The lipid extract was removed, and the residue was taken to constant dry weight at 50°C.
Samples of the residue were extracted with 0.1N NaOH for 50 minutes at 95°C (15, 16). After cooling, the solution was neutralized with 0.1N HCl. The insoluble elastin fraction was sedimented by centrifugation at 3,000 g for 15 minutes. Two washes of the elastin residue with distilled water were added to the removed supernatant solution containing alkali-soluble proteins. The supernatant solution was then subjected to acid hydrolysis with 6N HCl in sealed ampuls at 105°C for 48 hours. The hydroxyproline concentration of the supernatant solution was determined by the method of Woessner (17). The insoluble pellet was subjected to Kjeldahl digestion until 3 hours after the samples had cleared, and the nitrogen concentration was measured by the microdiffusion technique of Conway (18). Other samples of the lipid-extracted residue were taken directly to acid hydrolysis, and the total hydroxyproline concentration was determined. In still other samples, the hydroxyproline concentration of the alkali-insoluble, acid-soluble pellet was measured; in all instances it accounted for 1-2% of the mass.

Collagen was calculated from the hydroxyproline concentration measured in the alkali-soluble fraction, using a conversion factor of 1/0.189 (16), which is a midrange choice among factors used in the estimation of arterial collagen from hydroxyproline (16, 19, 20). Elastin was calculated from the protein nitrogen of the pellet, using a conversion factor of 1/0.129 (3), which is a midrange choice among factors used in the estimation of arterial collagen from hydroxyproline (16, 19, 20).

Results

PLASMA LIPIDS

Table 1 shows plasma cholesterol concentrations. In contrast to only minimal changes in plasma triglycerides, averaging less than 10 mg/dliter, in response to the atherogenic diet, cholesterol levels rose rapidly to an average of more than 500 mg/dliter. Lipoprotein electrophoresis showed a marked elevation in plasma low-density lipoproteins like that previously described by Kramsch and Hollander (11) and similar to the change found in cholesterol-fed rhesus monkeys (24, 25). During the regression period, average plasma cholesterol concentration declined to 228 ± 23 mg/dliter at 60 days but did not reach control levels until after 14 months, unlike the more rapid fall found in the rhesus monkey (8, 26).

ARTERIAL COLLAGEN

Collagen concentration (mg/g dry weight) was significantly higher in atherosclerotic monkeys (group 1) than it was in control monkeys (Table 2). The increase in elastic arteries (aorta, common carotid, and subclavian) varied from 50% to 75% above the control levels, and the control levels were exceeded by about 33% in muscular arteries (femoral and pooled coronary segments). In regression (groups 2-4), a further rise in collagen concentration was found in the five arterial beds (P < 0.001); however, as determined by Dunnett's test (27), only group 3 (studied at 200 days of regression) had significantly higher concentrations than did monkeys with base-line atherosclerosis (group 1). Group 4 did not differ significantly from group 1. In the regressing aorta, the collagen concentration was essentially the same at all regression intervals, averaging 12% more than atherosclerotic levels.

Collagen content (mg/cm length of artery) (Table 3) showed both concordant and discordant changes compared with collagen concentration data. In the atherosclerotic segments, the collagen content averaged 1.92 times that of the control segments, a highly significant increase. In regression, collagen content was significantly lower than it was in atherosclerosis (P < 0.025), but, as determined by Dunnett's test, only group 4 (studied at 20 months of regression) differed significantly from atherosclerotic monkeys (group 1) (P < 0.05). The order of decrease in mean collagen content among the five arterial beds (group 4 vs. group 1) was femoral > carotid > coronary = aorta > subclavian.

ARTERIAL ELASTIN

Highly significant rises in elastin concentration (mg/g dry weight) (Table 4) and elastin content (mg/cm length of artery) (Table 5) occurred in atherosclerosis. In regression, both concentration and content were significantly lower than they were in atherosclerosis (see Discussion). The order of decrease in mean elastin content among the five arterial beds (group 4 vs. group 1) was coronary > aorta > femoral > subclavian > carotid.

ARTERIAL TOTAL HYDROXYPROLINE

Measurements of total hydroxyproline content
confirmed the patterns of fibrous protein changes previously described. Changes in collagen content chiefly influence changes in total hydroxyproline content; the correlation found between these two variables was high ($r = 0.89$). When elastin (Table 5) was arbitrarily assumed to contain 1.5% hydroxyproline (an average value by this method), the correlation between the collagen content (Table 3) and the fraction of total hydroxyproline assignable to collagen showed 6% variability from a straight-line relationship ($r = 0.97$, $r^2 = 0.94$). Moreover, the collagen values calculated in this manner from total hydroxyproline were statistically identical to the values calculated from the hydroxyproline in the alkali-soluble fraction with an overall average difference of only 1%.

**Discussion**

Collagen increases are uniformly detected in experimental atherosclerosis by biochemical measurement except perhaps early in the induction period (28), but significant increases in elastin seem to be more variable. On rare occasions, in human atherosclerosis even a decrease in elastin concentration has been reported (29), but this atypical change has not been reproduced in any experimental studies of atherosclerosis to our knowledge, in spite of morphologic evidence of obvious damage and destruction of architectural elastin. The amount of new elastin created by the atherosclerotic lesion apparently equals or exceeds any losses of normal architectural elastin.

In the present study, the concentrations of arterial collagen and elastin in control monkeys and the subsequent increases in both fibrous proteins in atherosclerosis were quite similar to those found by Kramsch et al. (6) in their investigations of experimental atherosclerosis in this primate species. The concentration data indicate the fractional contribution of vascular wall components to the total (lipid-extracted) arterial weight. Arterial weight increased because of rises in nonfibrous protein resulting from factors such as intimal smooth muscle cell proliferation (30) and increased protein from higher concentrations of arterial lipoprotein (31). The rise in fibrous proteins was therefore absolute as well as relative to the unit weight of the artery: the absolute amount of collagen in the atherosclerotic aorta averaged 1.92 times that in the control aorta, and the collagen concentration was 1.49 times greater, the absolute amount of elastin in the atherosclerotic aorta averaged 1.55 times that in the control aorta, and the elastin concentration was 1.22 times the control level.

In regression, intimal smooth muscle cell proliferation decreases toward control rates (32), and

### TABLE 2

**Collagen Concentration (mg/g dry weight)**

<table>
<thead>
<tr>
<th>Artery</th>
<th>Control</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>187 ± 12</td>
<td>280 ± 18</td>
<td>297 ± 13</td>
<td>338 ± 18</td>
<td>310 ± 7</td>
</tr>
<tr>
<td>Carotid</td>
<td>224 ± 14</td>
<td>391 ± 51</td>
<td>353 ± 22</td>
<td>435 ± 27</td>
<td>262 ± 36</td>
</tr>
<tr>
<td>Subclavian</td>
<td>197 ± 23</td>
<td>317 ± 32</td>
<td>379 ± 17</td>
<td>426 ± 35</td>
<td>308 ± 27</td>
</tr>
<tr>
<td>Femoral</td>
<td>308 ± 38</td>
<td>417 ± 15</td>
<td>438 ± 12</td>
<td>537 ± 53</td>
<td>329 ± 31</td>
</tr>
<tr>
<td>Coronary</td>
<td>354 ± 10</td>
<td>464 ± 62</td>
<td>519 ± 38</td>
<td>625 ± 36</td>
<td>428 ± 42</td>
</tr>
</tbody>
</table>

All values are means ± SE.
there is morphologic evidence (8) that cells participating in the lipogranulomatous response observed in induced primate atherosclerosis (33) are lost from the artery. Therefore, it is to be anticipated that both the arterial weight and the nonfibrous arterial protein will decrease in regression. In the present study, lipid-extracted arterial weight decreased per unit length 8%, 18%, and 23% for groups 2, 3, and 4, respectively, compared with group 1. The fibrous protein concentration will not remain constant under these conditions unless it decreases in parallel with the lipid-extracted arterial weight. A decreased concentration of fibrous proteins in regressing arteries obviously indicates a relatively lower content as well; a higher concentration does not clearly indicate whether the content is unchanged, increased, or decreased. In this study, a number of the changes in collagen concentration and content (Tables 2-3) were in opposite directions. Because of this lack of parallelism (a potential problem whenever arterial weight changes as a result of pathologic alteration [34]), content rather than concentration was used as the appropriate indicator of accrual or apparent loss of fibrous proteins in consonance with its prior use in studies of arterial collagen and elastin (35) and arterial lipid (7, 36-38).

Collagen content in regression was statistically similar to that in atherosclerosis during early regression (groups 2 and 3) but significantly lower during late regression (group 4). Elastin content was lower in both early and late regression than it was in atherosclerosis. Statistical discrimination of the fibrous protein changes among the five arterial beds is not possible unless other types of analyses with new assumptions and probability statements are used.

The decrease in elastin content in regression was notable. There was morphologic evidence of severe destruction of architectural elastin in both atherosclerosis and regression, but total elastin, biochemically determined, was decreased only in regression. Mass data cannot provide a definitive explanation for this disparity. However, the net increase in collagen over elastin in all arterial beds in atherosclerosis may be an important clue in our understanding of the decrease in elastin in regression: continuing preferential formation of collagen and a more rapid turnover of elastin (39) would, in combination, make collagen the increasingly dominant repair protein in regression. A decrease in elastin content to very low levels (e.g., in aorta), however, probably depends on the extent of the atherosclerotic destruction of architectural elastin,
which was much greater in the present study than it was in our previous studies on the rhesus monkey.

The significantly lower collagen content in late regression compared with that in atherosclerosis is also compatible with the notion of slower turnover for collagen (40), if it is stipulated that turnover can be unbalanced toward accrual or loss. Fibroplastic stimuli, which are present in atherosclerosis and commonly attributed to one or more extracellular lipid moieties in the atherosclerotic intima, are still present during the earlier phase of regression. With progressive depletion of lipid during regression, we propose that fibroplasia is attenuated. Collagenolytic activity is required, however, if an actual decrease in collagen content is to occur. It is reasonable to assume that collagenolytic activity takes place in the intima of a regressing artery as it does in normal arterial tissue, although no specific mechanisms (41, 42) by which lytic activity occurs have as yet been identified in arterial tissue. The regression data in the present study suggest that collagen formation is eventually exceeded by collagen loss from the artery.

If this interpretation of the mass data is correct, a distinction must be drawn about the type of collagen fibers affected by lysis. Thick, heavily cross-linked collagen fibers are quite resistant to lysis in vitro (43). It may well be that the range of collagenolytic activity is quite limited in regressing arteries and that collagen formation decreases slowly from a high rate in atherosclerosis to a rate below that of collagenolytic activity in the later phases of regression. In that situation, many young fibers would escape lysis and mature into a thick intimal scar. The concurrent morphologic studies show that the residual fibrous tissue in late regression is Van Gieson-positive to a marked degree, thus confirming the presence of large amounts of mature collagen. The collagen exhibits dystrophic features (12) that further underscore its age and probable resistance to lysis.

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