Initiation of Atherosclerotic Lesions in Cholesterol-fed Rabbits

II. Selective Retention of LDL vs. Selective Increases in LDL Permeability in Susceptible Sites of Arteries

Dawn C. Schwenke and Thomas E. Carew

We asked if the arterial sites most prone to early lesions in cholesterol-fed rabbits have higher permeabilities to low density lipoprotein (LDL) in normolipidemic rabbits or if these sites become more permeable shortly after the onset of cholesterol feeding. We also considered whether the focal increases in the concentration of LDL within the arterial wall in lesion-susceptible sites before fatty streak formation can be explained by increased arterial permeability to LDL or by other mechanisms such as decreased rates of LDL efflux or degradation. 

The arterial sites with the largest increases in arterial LDL concentrations. Plasma LDL cholesterol concentration increased substantially and total LDL cholesterol delivery into the artery increased many fold. Since there was no differential change in permeability between susceptible and resistant sites, the increased entry of LDL did not explain the selective increases in arterial LDL concentration in susceptible sites. Kinetic analysis indicated that the fractional rate of degradation of the arterial LDL pool was lower in lesion-prone sites than in lesion-resistant sites in all animals. Fractional rates of efflux of arterial LDL decreased in lesion-susceptible branch sites of the abdominal aorta and were low in the lesion-susceptible aortic arch. These results suggest that the focal increases in LDL concentration observed in all lesion-susceptible sites of cholesterol-fed rabbits before fatty streak formation are due to localized differences in LDL retention and diminished fractional rates of LDL degradation, not to selectively increased permeability. (Arteriosclerosis 9:908–918, November/December 1989)

Morphologic studies of the initial cellular events in atherosclerosis suggest that the early accumulation of macrophage-derived foam cells occurs under an apparently intact endothelial layer and does not appear to require desquamation of endothelial cells and direct exposure of the subendothelium to blood elements.1-3 There is continuing speculation that subtler forms of endothelial injury not evident by scanning or transmission electron microscopy may play a role in the initiation of the atherosclerotic lesion.4 For example, studies have shown that under certain conditions, high levels of low density lipoprotein (LDL) can be cytotoxic to cells in vitro.8,9 Such cytotoxicity, if manifested in vivo, could alter the arterial permeability to lipoproteins. Indeed, there is thought to be a general association between the arterial sites most susceptible to early atherosclerosis in experimental animals and the sites of relatively high permeability to macromolecules as assessed by arterial uptake of Evans blue dye-labeled albumin.10-13 However, because the sites with relatively high permeability exist in the arteries of normal, nonatherosclerotic animals, it is unclear whether such differences should always be considered pathological.

In the accompanying article, we present evidence that LDL accumulates to a greater extent in lesion-susceptible than in lesion-resistant sites of the aorta during short-term cholesterol feeding. These site-specific differences might be due to increased rates of influx of LDL into the susceptible sites, decreased fractional rates of efflux (and
degradation), or both. We first considered the possibility that the enhanced concentration of intact LDL in lesion-susceptible aortic sites was attributable to an increase in intimal permeability to LDL during short-term cholesterol feeding. We sought to determine whether all sites having high concentrations of intact LDL also had high permeabilities to LDL and whether these high permeabilities were present before cholesterol feeding or only after cholesterol feeding. In this paper, we examine the initial rate of entry of $^{125}$I-tyramine cellobiose-labeled LDL ($^{125}$I-TC-LDL) into the artery to determine LDL permeability coefficients for lesion-susceptible and lesion-resistant sites in normal rabbits and in rabbits fed cholesterol for up to 16 days.

We present evidence that neither pre-existing differences in LDL permeability nor selective alterations in permeability due to hypercholesterolemia can adequately explain the selective accumulation of LDL in the susceptible arterial sites. In fact, with these short intervals of cholesterol feeding, we could not demonstrate any increase in the permeability coefficient for LDL at any arterial site over that existing for that site in normal animals. In addition, the arterial concentration of intact LDL was elevated in one lesion-susceptible site, (i.e., the aortic arch) where we could find no demonstrable increase in permeability coefficient relative to the adjacent lesion-resistant site either in normal animals or in cholesterol-fed animals. This evidence implies that the focal accumulation of LDL in susceptible sites during early cholesterol feeding before formation of fatty streak lesions must occur principally by selective retention of LDL, i.e., a decreased fractional rate of LDL efflux or a decreased fractional rate of degradation of arterial LDL.

**Methods**

**Plasma Lipoprotein Concentrations**

Cholesterol concentrations were determined in lipoprotein fractions isolated by ultracentrifugation. Plasma was obtained from blood collected into 0.01 volume of a solution 0.4 M in disodium EDTA and 4% in disodium azide. One to two ml aliquots of plasma from cholesterol-fed rabbits and 2 to 4 ml aliquots from normal animals were each adjusted to 1.006 g/ml, 1.022 g/ml, and 1.063 g/ml. After centrifugation at 50 000 rpm in a 50.3 rotor for 18, 18, or 24 hours, respectively, the tubes were sliced. The densities under the top fractions were 1.006, 1.020, and 1.061 g/ml. The plasma cholesterol concentrations and those of the density fractions after dialysis against a buffer containing 0.15 M NaCl, 2 mM EDTA, and 20 mM phosphate, pH 7.4 (Buffer A) were assayed by an enzymatic method.

**Lipoprotein Preparation and Labeling**

LDL (d=1.020 to 1.057) was isolated as described.11 After dialysis against Buffer A, LDL protein was measured with the Folin phenol reagent12 with bovine serum albumin as a standard. Two preparations of LDL, 5 mg and 25 mg, were labeled with $^{125}$I-TC as described.11 Less than one TC residue was bound to each 100 000 Da of LDL protein. Specific activities were 1210 and 390 cpm/ng. The two LDL preparations were injected 2 to 3 days after labeling and 5 to 7 days after initial isolation. The trichloroacetic acid-soluble radioactivities in the preparations were 1% and 0.9%, and the radioactivities extractable into 2:1 chloroform/methanol13 were 6% and 1.2%. The first LDL preparation was characterized by sodium dodecyl sulfate (SDS) polyacrylamide gel and agarose gel electrophoresis. Less than 2% of the radioactivity was associated with proteins of a molecular size similar to apoprotein E, and 94% of the $^{125}$I-TC label was associated with a single beta-migrating peak.

**Animal Studies**

Seventeen female New Zealand White rabbits each weighing 2.52±0.04 kg (mean±SEM) were used in these studies. The initial rate of LDL flux into the aorta was measured in one group of eight rabbits consisting of two animals fed cholesterol for 4, 8, or 16 days and two control rabbits fed normal rabbit chow, and in a second group that contained an additional control animal. All members of a given group received the same amount of the same LDL preparation ($3.25±0.17×10^4$ $^{125}$I cpm/kg). After about 1 hour, during which time four serial blood samples were collected for determination of plasma radioactivity, each rabbit was heparinized and deeply anesthetized as described.11 Immediately thereafter and 1.15±0.01 hours after injection, the systemic circulation was perfused with 2 l of Buffer A.11 To better retain the $^{125}$I-TC-LDL that might be present in a superficial layer of the intima, we perfused the aorta in situ for 8 to 10 minutes with half-strength Karnovsky's fixative,14,15 while maintaining a perfusion pressure of 80 mm Hg. In some cases, sodium phosphate was used in lieu of sodium cacodylate as a buffer in the fixative, in which case no CaCl$_2$ was used. Parallel studies have indicated that this substitution of phosphate buffer for the cacodylate buffer made no difference in the retention of intact labeled LDL or TC-labeled degradation products of LDL in tissue. We have established in earlier experiments14,15 that fixation with half-strength Karnovsky's solution preserved nearly all of the arterial content of $^{125}$I-TC. The same was true whether cacodylate buffer or phosphate buffer was used. After fixation in situ, the aorta was removed and cleaned of loose adventitial tissue. The vessel was divided about 3 mm above the celiac orifice. Each segment was opened longitudinally, pinned flat on a rubber sheet, and fixed for a further 24 hours in half-strength Karnovsky's solution.14,15

**Tissue Sampling**

After fixation, the aortic arch and descending thoracic aorta were separated, and samples of lesion-susceptible sites were collected, weighed, and photographed as described.11 To express the arterial permeability data in the same terms as the arterial concentration and degradation data in the accompanying article (i.e., per gram of fresh tissue weight), we needed a small correction factor because fresh weights for these aortas could not be obtained directly. The fresh weight of each arterial sample was calculated by dividing its measured fixed weight by a correction constant based on the average of the corresponding fixed weight/wet weight ratio for the corresponding segment of arteries of 23 similar animals: 0.785 for the thoracic aortic segments including the arch and 0.867...
for the abdominal aortic segments. The surface area of each arterial specimen was determined by planimetry of tracings made directly from photographs or from magnified images of the photographs.

**Radioassay**

Arterial and plasma samples were radioassayed as described. The maximal counting error was 4.1% for in small samples; the typical error was less than 2%.

**Low Density Lipoprotein Transport Into Aorta**

The entry of LDL into the aorta was calculated from the arterial radioactivity accumulated during 1 hour of exposure to plasma 125I-TC-LDL. We had previously shown that 1 hour is a sufficiently short time to assess the initial rate of entry of LDL into the aorta of normal rabbits. The permeability coefficient of lesion-prone and lesion-resistant aortic sites to LDL was calculated by dividing the arterial 125I-TC-LDL radioactivity (cpm/g or cm²) by the area under the plasma 125I-TC-LDL curve (cpm×h/m). The area under the plasma 125I-TC-LDL curve was calculated by integrating a monoexponential equation fit to data for the declining concentration of 125I-TC-LDL in plasma from injection until death of that animal. Thus the permeability coefficient (μg/g/h or μg/cm²/h) expresses the rate of entry of LDL into the artery in terms of equivalent volume of plasma "cleared" of labeled LDL each hour. This calculation allowed us to compare the LDL transport in the aortic sites of different animals in a manner independent of plasma LDL levels. We also calculated the LDL influx in absolute terms by multiplying the permeability coefficient (μg/g/h or μg/cm²/h) by the plasma LDL cholesterol concentration (μg/μl) determined in each animal.

**Kinetics of Arterial Low Density Lipoprotein Metabolism**

To integrate the data on the initial rates of entry of LDL determined in these studies with the data on the accumulation of labeled intact LDL and labeled products of LDL degradation described in the companion article, we made use of the multicompartmental model shown in Figure 1. This model makes the following assumptions: 1) all intact LDL present in an aortic sample is located in one homogeneous compartment that exchanges with the plasma LDL pool; 2) intact LDL present in the artery is removed by two processes, irreversible degradation, resulting in accumulation of products of LDL degradation in a second compartment and efflux of intact LDL either via the luminal or adventitial surfaces; 3) products of arterial LDL degradation were assumed to "leak" from the second compartment at 0.4% per hour, a conservative estimate based on published data and our own unpublished observations in macroscopically normal portions of the aortas of rabbits fed cholesterol for 9 weeks.

The data shown below indicate that permeability to LDL at any given site did not change significantly during
16 days of cholesterol feeding. Therefore, the permeability coefficient, which is related to the fractional rate of entry, was assumed to be a constant equal to the mean value for all animals at that given site. The model was fitted to data for the arterial content of intact, labeled LDL and products of labeled LDL degradation for each of the five sites in the 20 animals that underwent studies with doubly labeled LDL (see the accompanying article). Each animal's own plasma decay curve was used in the modeling process.

The fitted values of intact labeled LDL and LDL degradation products from the kinetic analyses (20 animals × 5 sites = 100 separate cases) were extremely close to the observed values. This was not surprising in that the rate constants of this model are uniquely determined because of the limited number of observations per arterial compartment (i.e., one each). There are no extra degrees of freedom; either a solution to the model equation exists and it fits the observed points exactly or a physically realizable solution (one in which the rate constants are all non-negative) does not exist. It was also not surprising, therefore, that some sets of data were incompatible with the assumption of a particular mean value for the influx rate constant (k2) that was used. This happened in only four of the 100 cases. We found that re-analysis of these data, assuming a somewhat higher value for the initial rate constant (k2) that was used. This resulted in satisfactory fits of the observed data. As this adjustment was unnecessary in the majority of cases, we excluded these four cases (4% of the total) from further analysis.

ANOVA was applied to data for each aortic site individually across the times of cholesterol feeding. After a significant outcome of the ANOVA, we used t tests and the Bonferroni adjustment to compare the aortic sites in each cholesterol-fed group with the corresponding site in the normal animals. Linear regression was used to assess the overall trends in the measures of arterial LDL metabolism in each aortic site individually during cholesterol feeding. The effects of lesion susceptibility and time of cholesterol feeding on aortic LDL metabolism were investigated further by ANOVA of the data from multiple sites for all animals. This allowed detection of significant effects of lesion susceptibility and duration of cholesterol feeding in some cases where consideration of subsets of the data did not. The results of these latter analyses are shown only if they provide information beyond that in the previous analyses.

Plasma and lipoprotein cholesterol concentrations were also compared first by ANOVA and then where needed by t tests with the Bonferroni adjustment for multiple comparisons.

Results

Plasma and Lipoprotein Cholesterol Concentrations

Plasma lipoprotein cholesterol levels determined just before sacrifice are shown in Table 1. The total plasma cholesterol levels were increased by eightfold after 4 days of cholesterol feeding. Although the plasma cholesterol concentration continued to increase throughout the 16 days of cholesterol feeding, reaching a value 19 times higher than that in normal animals, the LDL cholesterol concentration reached a plateau after only 8 days of cholesterol feeding at a value nine times higher than that of normal rabbits. In normal rabbits, more of the plasma cholesterol was present in high density lipoprotein (HDL) than in any other fraction. In contrast, after as little as 4 days of cholesterol feeding, very low density lipoprotein (VLDL) carried the largest portion of the plasma cholesterol and the HDL fraction, the least. These relationships were maintained during the rest of the 16-day study period.

### Table 1. Lipoprotein Cholesterol Concentrations

<table>
<thead>
<tr>
<th>Duration of cholesterol feeding (days)</th>
<th>Plasma</th>
<th>VLDL</th>
<th>IDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (n=5)</td>
<td>68±12</td>
<td>9.7±3.5</td>
<td>13.8±3.3</td>
<td>21.4±5.8</td>
<td>23.0±2.8</td>
</tr>
<tr>
<td>(14.4±4.9)</td>
<td>(19.4±1.7)</td>
<td>(30.9±4.9)</td>
<td>(35.2±2.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 (n=4)</td>
<td>531±37*</td>
<td>223±19*</td>
<td>127±12*</td>
<td>148±16*</td>
<td>32.0±6.5</td>
</tr>
<tr>
<td>(42.2±2.6)</td>
<td>(23.9±1.6)</td>
<td>(28.0±3.0)</td>
<td>(5.9±1.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 (n=3)</td>
<td>820±91*</td>
<td>412±42*</td>
<td>186±19*</td>
<td>191±39*</td>
<td>30.6±7.0</td>
</tr>
<tr>
<td>(50.4±0.7)</td>
<td>(22.8±1.0)</td>
<td>(22.8±2.3)</td>
<td>(4.0±1.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 (n=4)</td>
<td>1264±94*</td>
<td>710±50*</td>
<td>301±31*</td>
<td>194±24*</td>
<td>59.6±8.41</td>
</tr>
<tr>
<td>(56.2±0.8)</td>
<td>(23.7±0.8)</td>
<td>(15.3±1.6)</td>
<td>(4.81±0.9)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means±SEM (mg/dl) corrected for recovery, which was 90.2%±1.5% (n=48). Values in parentheses are the percentages of total plasma cholesterol concentration.

*p<0.0005, t p<0.025 indicate the significance of difference from untreated animals by t test with Bonferroni adjustment for multiple comparisons.

VLDL=very low density lipoprotein, IDL=intermediate density lipoprotein, LDL=low density lipoprotein, HDL=high density lipoprotein.
Figure 2. Initial rate of entry of low density lipoprotein (LDL) in lesion-prone and lesion-resistant aortic sites. LDL entry is expressed as the apparent volume of plasma cleared of LDL by the aorta per unit area per hour (nl/cm²/h). •—• = data for lesion-prone aortic sites; ○——○ = data for lesion-resistant areas. A. Lesion-prone aortic arch and lesion-resistant descending thoracic aorta. B. Lesion-prone branch sites and lesion-resistant uniform sites of the descending thoracic aorta. C. Lesion-prone branch sites and lesion-resistant uniform sites of abdominal aorta. The difference between the branch and uniform sites of abdominal aorta was significant (paired t test, p<0.05) in normal animals and at all times of cholesterol feeding. No other pairwise differences were significant, nor were significant trends in permeability detected by linear regression (Table 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>Aortic arch</th>
<th>Descending thoracic aorta</th>
<th>Abdominal aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Branch</td>
<td>Uniform</td>
<td>Branch</td>
</tr>
<tr>
<td>0 (untreated)</td>
<td>2.00±0.55</td>
<td>1.62±0.35</td>
<td>3.64±0.61*</td>
</tr>
<tr>
<td>4-day cholesterol-fed</td>
<td>1.38±0.16</td>
<td>1.35±0.20</td>
<td>1.91±0.34</td>
</tr>
<tr>
<td>8-day cholesterol-fed</td>
<td>1.49±0.25</td>
<td>2.32±0.53</td>
<td>4.07±0.82*</td>
</tr>
<tr>
<td>16-day cholesterol-fed</td>
<td>1.44±0.19</td>
<td>2.09±0.65</td>
<td>3.53±0.41†</td>
</tr>
<tr>
<td>Mean of all groups</td>
<td>1.60±0.18</td>
<td>1.83±0.22</td>
<td>3.31±0.33</td>
</tr>
<tr>
<td>Regression slope</td>
<td>-0.029</td>
<td>0.040</td>
<td>0.026</td>
</tr>
<tr>
<td>(permeability vs. time of feeding)</td>
<td>0.35</td>
<td>0.30</td>
<td>0.67</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means±SEM and are given as µl/h/g. Untreated rabbits, n=5; all other groups, n=4. The regression slope was nonsignificant in all groups.

*p<0.05, †p<0.01 indicate the paired comparisons of abdominal aortic branch sites with the uniform abdominal aorta.

**Arterial Permeability to Low Density Lipoprotein**

Figure 2 and Table 2 show the arterial permeability coefficients determined from the initial rate of entry of 125I-TC-LDL in lesion-susceptible and lesion-resistant aortic sites. The data are expressed as plasma clearance rates, which allow comparison of LDL influx into the artery independent of plasma LDL concentrations; in Figure 2, the data are expressed per square centimeter of surface area and in Table 2 in terms of clearance per gram of tissue weight. These data indicate that the permeability to LDL did not change during the 16 days of cholesterol feeding in any of the aortic sites considered. The slopes of least-squares regression lines for permeability coefficient vs. time of cholesterol feeding at any given site with data from all 17 animals were near zero and were not statistically significant at any site. In normal rabbits and at all times of cholesterol feeding, the arterial permeability in lesion-prone branch sites of the abdominal aorta was three- to fivefold higher than in the adjacent uniform abdominal aorta (all p<0.05). It was particularly interesting to note that the LDL permeability coefficient in the aortic arch, which is a site susceptible to early atherosclerotic lesions in cholesterol-fed rabbits, was no larger than that in the uniform area of the descending thoracic aorta, which is a relatively lesion-resistant site (not significant by ANOVA). Although the arterial permeability to LDL did not change during the first 16 days of cholesterol feeding, plasma LDL
concentrations increased considerably during this interval. Consequently, the LDL cholesterol delivery to each aortic site was four to nine times greater than the control at 4 days of cholesterol feeding (all \( p < 0.0005 \)), and delivery increased markedly in all aortic sites during the 16-day interval \( (p < 0.02, \text{time trend}) \) due to increasing hypercholesterolemia (Table 3).

**Kinetics of Entry, Efflux, and Accumulation of Low Density Lipoprotein in Lesion-prone and Lesion-resistant Sites in the Aorta**

We used a compartmental kinetic model to obtain further information about possible differences in arterial LDL transport and metabolism among lesion-susceptible and lesion-resistant sites during short-term cholesterol feeding. The tracer data for the initial rates of entry of LDL into artery and for the data described in the accompanying article for LDL degradation rates and aortic LDL concentrations were used to fit a multicompartimental kinetic model of arterial transport and metabolism as described in Methods (Figure 1). From this analysis, we obtained estimates of the fractional rates of degradation of the arterial LDL pool \( (k_d) \), the fractional rate of efflux of LDL from the artery \( (k_e) \), and the mass of the arterial LDL pool. Such estimates were obtained for five aortic sites in each animal for which data on arterial LDL degradation rate and arterial concentration of intact, labeled LDL were available. The average values of the arterial permeability coefficients for all the animals at a given site (Table 2) were used in these determinations.

It was of interest that the estimate of the mass of intact LDL in the artery from this more complete kinetic analysis was in close agreement with that estimated by the isotope ratio of tissue to plasma at the time of sacrifice, \( C_t/C_p \) (see accompanying article). The largest difference was found in the aortic arch, where the estimate of the arterial LDL concentration derived from \( C_t/C_p \) was 17.3±2.3% \( (n=16) \) larger than that determined from the kinetic analysis. In the other aortic sites, the differences between these two measures of arterial intact LDL were 6.5±1.4% \( (\text{branch sites of descending thoracic aorta}) \), 5.2±1.2% \( (\text{uniform descending thoracic aorta}) \), 13.9±2.0% \( (\text{branch sites of abdominal aorta}) \), and 10.5±1.8% \( (\text{uniform abdominal aorta}) \). Such differences were not significantly affected by the duration of cholesterol feeding.

As shown in Table 4, the fractional rate of degradation of arterial intact LDL decreased in each aortic site during the interval of cholesterol feeding, consistent with downregulation or saturation of the LDL receptor-mediated LDL degradation. Due to the relatively larger changes in the branch sites than in the uniform areas of the abdominal aorta, the fractional degradation rates of arterial LDL in the branch sites decreased during the feeding interval from about one half that in the uniform aorta \( (p < 0.05) \) to a value one third that in the uniform aorta \( (p < 0.02) \). In both cholesterol-fed and normal animals, the fractional rate of efflux of arterial intact LDL from the lesion-prone aortic arch was only about one third that from the lesion-resistant uniform portion of the descending thoracic aorta \( (p < 0.02) \). In untreated rabbits, the fractional rate of efflux of intact LDL from the branch sites of abdominal aorta was actually about twice as large as in the uniform abdominal aorta \( (p < 0.05) \), somewhat paralleling the higher rate of LDL permeation at these sites \( (p < 0.02) \). However, the fractional rate of efflux of arterial LDL from the abdominal aorta branch sites decreased to one third the value in normals during the 16-day period of cholesterol feeding \( (p < 0.005 \text{ for the time trend}) \). Changes in the fractional rates of arterial LDL degradation and efflux in the branch and uniform sites of the descending thoracic aorta during early cholesterol feeding were similar in direction to those in the branch and uniform sites of the abdominal aorta, although of somewhat lower magnitude. This may be explained by the fact that the intercostal orifices were much smaller compared to the sample size than were the abdominal branch sites studied; thus more "normal" tis-

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**Table 3. Absolute Rates of Low Density Lipoprotein influx into Aorta**

<table>
<thead>
<tr>
<th>Group</th>
<th>Aortic arch</th>
<th>Descending thoracic aorta</th>
<th>Abdominal aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Branch</td>
<td>Uniform</td>
</tr>
<tr>
<td>LDL influx per unit weight (μg cholesterol/g/h)</td>
<td>0 (untreated)</td>
<td>0.40±0.12</td>
<td>0.32±0.09</td>
</tr>
<tr>
<td></td>
<td>4-day cholesterol-fed</td>
<td>2.05±0.36</td>
<td>2.05±0.42</td>
</tr>
<tr>
<td></td>
<td>8-day cholesterol-fed</td>
<td>2.98±0.58</td>
<td>4.36±0.84</td>
</tr>
<tr>
<td></td>
<td>16-day cholesterol-fed</td>
<td>2.87±0.68</td>
<td>4.45±1.78</td>
</tr>
<tr>
<td>LDL influx per unit area (ng cholesterol/cm²/h)</td>
<td>0 (untreated)</td>
<td>18.8±5.9</td>
<td>8.2±2.1</td>
</tr>
<tr>
<td></td>
<td>4-day cholesterol-fed</td>
<td>96±18</td>
<td>55±13</td>
</tr>
<tr>
<td></td>
<td>8-day cholesterol-fed</td>
<td>129±22</td>
<td>116±22</td>
</tr>
<tr>
<td></td>
<td>16-day cholesterol-fed</td>
<td>129±30</td>
<td>114±44</td>
</tr>
</tbody>
</table>

Values are means±SEM. Untreated rabbits, n=5; all other groups, n=4.

Regression analyses indicated significant linear increases in low density lipoprotein (LDL) influx with time of cholesterol feeding in each aortic site whether influx was expressed per unit weight or per unit area \( (p < 0.02) \). Because permeability coefficients for LDL at each aortic site did not change with duration of cholesterol feeding \( (p > 0.02) \), the increased LDL influx into the aorta was due entirely to increases in plasma LDL concentrations.

For each aortic site, LDL influx expressed either per unit weight or per unit area was significantly different from the untreated animals at each individual time of cholesterol feeding \( (p < 0.005, \text{Bonferroni adjustment for multiple comparisons}) \). *\( p < 0.025, \text{t}< 0.05 \) indicate paired comparisons of abdominal aortic branch sites with uniform abdominal aorta.
Table 4. Fractional Rates of Low Density Lipoprotein Degradation and Efflux Determined for a One Compartment Model of Arterial Intact Low Density Lipoprotein

<table>
<thead>
<tr>
<th>Group</th>
<th>Aortic arch</th>
<th>Descending thoracic aorta</th>
<th>Abdominal aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Branch</td>
<td>Uniform</td>
</tr>
<tr>
<td>Fractional degradation rate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (untreated)</td>
<td>0.083±0.009</td>
<td>0.130±0.021</td>
<td>0.128±0.021</td>
</tr>
<tr>
<td>4-day cholesterol-fed</td>
<td>0.071±0.013</td>
<td>0.146±0.022</td>
<td>0.152±0.027</td>
</tr>
<tr>
<td>8-day cholesterol-fed</td>
<td>0.063±0.010</td>
<td>0.091±0.018</td>
<td>0.091±0.029†</td>
</tr>
<tr>
<td>16-day cholesterol-fed</td>
<td>0.055±0.017</td>
<td>0.068±0.021§</td>
<td>0.088±0.021§</td>
</tr>
<tr>
<td>Fractional efflux rate, k_e</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (untreated)</td>
<td>0.16±0.04†</td>
<td>0.53±0.20</td>
<td>0.48±0.07</td>
</tr>
<tr>
<td>4-day cholesterol-fed</td>
<td>0.12±0.05†</td>
<td>0.62±0.14</td>
<td>0.67±0.11</td>
</tr>
<tr>
<td>8-day cholesterol-fed</td>
<td>0.11±0.01†</td>
<td>0.46±0.11</td>
<td>0.49±0.06</td>
</tr>
<tr>
<td>16-day cholesterol-fed</td>
<td>0.11±0.03†</td>
<td>0.31±0.06</td>
<td>0.50±0.03</td>
</tr>
</tbody>
</table>

All values are means±SEM given as fraction of arterial pool/hour. N=5 except for aortic arch of untreated and 4-day cholesterol-fed animals, n=3. Values of fractional degradation rate (k_d) and fractional efflux rate (k_e) were calculated from the model in Figure 2.

Simultaneous comparisons by analysis of variance of data for fractional degradation rates, k_d, from multiple sites of rabbits from each individual group (see Methods section) indicated that, overall, k_d was significantly lower in susceptible sites in each group (p<0.05). Analysis of variance could not detect a significant difference between the fractional efflux rates in lesion-prone and lesion-resistant aortic sites for the individual groups. However, when all experimental groups were considered together and grouped by time of cholesterol feeding, we could detect a significant effect of time of feeding (p<0.05) and of lesion susceptibility (p<0.0002). Linear regression indicated that the fractional degradation rate of LDL decreased significantly (p<0.025) in each aortic site during the interval of cholesterol feeding, while a statistically significant decrease in the fractional rate of LDL efflux could be detected only in branch sites of the abdominal aorta (p<0.005).

*p<0.05, †p<0.02 indicate paired comparisons with adjacent lesion-resistant aortic. ‡p<0.02, §p<0.005 indicate comparisons to same aortic site of untreated animals, t test with Bonferroni adjustment for multiple comparisons.

Table 5. Mean Residence Time of Intact Low Density Lipoprotein in Artery

<table>
<thead>
<tr>
<th>Group</th>
<th>Aortic arch</th>
<th>Descending thoracic aorta</th>
<th>Abdominal aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Branch</td>
<td>Uniform</td>
</tr>
<tr>
<td>0 (untreated)</td>
<td>4.5±0.8</td>
<td>1.9±0.4</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>4-day cholesterol-fed</td>
<td>6.3±1.9</td>
<td>1.5±0.2</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>8-day cholesterol-fed</td>
<td>5.9±0.4*</td>
<td>2.1±0.4</td>
<td>1.9±0.3</td>
</tr>
<tr>
<td>16-day cholesterol-fed</td>
<td>9.0±3.5</td>
<td>3.0±0.6</td>
<td>1.7±0.1</td>
</tr>
</tbody>
</table>

Values are means±SEM given in hours. N=5 except for aortic arch of untreated and 4-day cholesterol-fed animals where n=3. The mean residence time of low density lipoprotein (LDL) entering each aortic site was calculated from the data for the model shown in Figure 1 as described in the Methods section.

Analysis of variance for data in all aortic sites for each experimental group individually indicated a significant (p<0.05) prolongation of LDL residence time in lesion-prone sites for 4, 8, or 16 days of cholesterol feeding. Linear regression indicated a significant increase in LDL residence time in the branch sites of descending thoracic aorta (p<0.025) and abdominal aorta (p<0.002) during the interval of cholesterol feeding.

*p<0.001 for paired comparison with uniform descending thoracic aorta. †p<0.02 for comparison to abdominal branch sites of untreated animals, t test with Bonferroni adjustment for multiple comparisons.

Discussion

In this study, we measured the permeability of lesion-prone and lesion-resistant arterial sites, while in the accompanying study we measured the steady-state concentration of LDL in those sites. Both types of measurements were made from radiotracer data but were obtained in experiments of quite different duration; permeability was measured in experiments of 1-hour duration, while estimates of steady-state LDL concentrations were ob-
tained in experiments of 24- or 48-hour duration. It is necessary to make this distinction because the amount of intact labeled LDL in the artery many hours after injection is a reflection of the efflux and degradation pathways as well as of the rate of entry of the lipoprotein. To assess the rate of entry of LDL per se we measured those rates during a 1-hour interval after injection. We chose this time because previous work in our laboratory14 had shown that the arterial content of labeled LDL increased almost linearly with time during the first hour after injection, implying that relatively little of the labeled LDL taken up into the artery was lost during such a short interval so that we were measuring near initial rates of entry.

A number of lines of evidence support the idea that alterations in arterial permeability may play a role in the development of atherosclerosis. First, mechanical,23,24 chemical,25 and viral26 injury to the endothelial layer all promote atherosclerosis. Second, high concentrations of LDL relative to HDL or other proteins in the medium can damage endothelial cells in cell culture,27,28 suggesting that extreme hypercholesterolemia could cause endothelial damage and permit increased penetration by lipoproteins. Third, experimentally induced hypertension increases the intimal permeability to plasma protein29 and enhances atherosclerosis in cholesterol-fed animals.30 Finally, other workers30,31 have shown that the permeability of atherosclerotic aorta to lipoproteins was greater than that of nonatherosclerotic aorta. These data suggest a causative relationship between aortic permeability to lipoproteins and fatty streak initiation, but by no means prove it. If altered arterial permeability to lipoproteins is a necessary initiating factor in atherogenesis, then one might expect that an increase in arterial permeability would presage lesion development.

We had previously shown16 that the initial rate of permeation of LDL into the artery (presumably by transcytosis) is independent of LDL receptors and thus should not be down-regulated by high plasma lipoprotein levels. Thus, one would not expect decreases in permeability to LDL during cholesterol feeding. In the studies described in the accompanying article, we found that the arterial concentration of intact LDL was markedly and specifically elevated in lesion-prone sites before evident foam cell formation. In view of the previous reports of increased permeability of lesioned areas, one might have expected the increased arterial LDL concentration to be explained by parallel increases in the aortic permeability to LDL during short-term cholesterol feeding. It was surprising, therefore, that we found no significant alteration in arterial permeability to LDL during 16 days of cholesterol feeding. Another surprising result was that the aortic arch, which in the rabbit is a site highly susceptible to early lesion formation, was no more permeable to LDL than the lesion-resistant uniform portions of the descending thoracic aorta. The concentration of intact LDL in the aortic arch, nonetheless, was three to five times as high as in the descending thoracic aorta.11 The situation was quite different in the other highly susceptible sites of the rabbit aorta. The aortic permeability coefficient for LDL was about 3.5 times larger in the branch sites of the abdominal aorta than in the adjacent uniform abdominal aorta (Figure 2). In normal animals, the aortic concentration of intact LDL in the branch sites of abdominal aorta was twice that in the uniform abdominal aorta. This difference might be explained on the basis of their differing permeabilities to LDL. However, after 16 days of cholesterol feeding, the concentration of intact LDL in the branch sites of the abdominal aorta had increased to about 5.4 times that of the adjacent uniform aorta, even though the relative permeabilities of these two sites apparently did not change. Together these results indicate that the enhanced accumulation of intact LDL in the lesion-prone aortic sites during short-term cholesterol feeding cannot be explained on the basis of altered aortic permeability to LDL.

Other workers32,33 have shown that the permeability of atherosclerotic aorta to lipoproteins was greater than that of nonatherosclerotic aorta. However, that increased permeability may be a consequence of endothelial alterations that occur when fatty streaks are well under way34,35 rather than being an initiator of the atheromatous lesion. Using the same methods that we used to measure aortic permeability in short-term cholesterol feeding, we also measured the intimal permeabilities to LDL in fatty streaks and the adjacent uninvolved aorta in rabbits fed cholesterol for 9 weeks. We confirmed the finding that fatty streaks were several times as permeable to LDL as the adjacent uninvolved aorta (unpublished observations). The permeability of fatty streaks was also greater than that of the lesion-prone, but macroscopically normal, aortic sites examined.

Having determined that an alteration in arterial permeability to LDL could not explain the marked accumulation of LDL in lesion-prone sites, we investigated the contribution of the two processes that remove LDL from the aorta: efflux and irreversible degradation. The results of compartmental analysis indicated that the fractional rate of degradation of LDL from the arterial pool decreased in all aortic sites during the 16 days of cholesterol feeding, consistent with the down-regulation of aortic LDL receptors or sequestration of some LDL, as discussed earlier.11 The fractional rates of degradation of LDL from the arterial pool were generally lower in lesion-prone sites and decreased more in lesion-prone abdominal branch sites than in other areas of aorta during the interval of cholesterol feeding.

The decreased fractional rates of degradation of arterial LDL in susceptible sites, while certainly contributing to the increase in arterial LDL concentration, were clearly not sufficient to account for all of the increase in intact LDL in these sites. Because the total rate of loss of LDL from the artery is simply the sum of the rate of efflux, k\textsubscript{eff}, and rate of degradation, k\textsubscript{deg}, it is clear from Table 4 that direct efflux (without degradation) is the largest contributor to loss of arterial intact LDL in all sites. This implies that the efflux rate was a relatively more important determinant of the mass of intact LDL in the artery than degradation rate.

The kinetic analyses indicated that the fractional rate of efflux of intact LDL decreased significantly in the abdominal branch sites during 16 days of cholesterol feeding. The other lesion-susceptible sites, the aortic arch and branch sites of the descending thoracic aorta, also showed a similar trend. However, it should be noted that the
fractional rate of efflux of intact LDL was always much lower in the aortic arch than in the relatively lesion-resistant, uniform descending thoracic aorta, even in normal animals. All of the lesion-prone sites that we studied are areas where the arterial wall is somewhat thickened. On that basis, one might suggest that fractional rates of efflux of LDL might be lower and that the mean residence time of LDL might be increased in lesion-prone sites if LDL were to efflux from the artery primarily by passing through the arterial wall. The lower fractional efflux rate in the aortic arch and the trend toward a mean residence time of LDL in that site that is longer than in the adjacent uniform aorta would be consistent with that expectation. However, in normal rabbits, while the branch orifices of the abdominal aorta are approximately twice as thick as the adjacent uniform aorta, the fractional rate of efflux of LDL from branch sites was twice that of the adjacent uniform aorta. Furthermore, the thickened nature of the lesion-prone sites alone would not predict selective decreases in fractional efflux rates of LDL or selective increases in the retention of LDL in these sites during the early days of cholesterol feeding. Similarly, although a continual selective increase in the interstitial fluid space in these areas of artery during cholesterol feeding could explain the accumulation of intact LDL in these sites, the expansion of the interstitial space alone would not necessarily explain the continual decrease in the fractional rates of degradation and efflux of LDL in these sites.

A more plausible explanation for these differences in efflux and mean residence time might relate to differences in the interaction or binding of LDL to extracellular matrix components in susceptible and resistant sites. For example, immunochromatographic studies have shown that LDL is present within the arterial wall in close association with collagen and elastin. Collagen is present in greater amounts and in different forms in atherosclerotic lesions. Arterial glycosaminoglycans (GAG) also bind LDL and, indeed, GAG-lipoprotein complexes have been extracted from atherosclerotic aortas of rabbits and humans.

Studies of atherosclerosis-susceptible and atherosclerosis-resistant pigeon strains have identified a greater heterogeneity of GAG classes in pre-atherosclerotic lesion-prone sites of a susceptible strain and apparent structural differences between proteoglycans of the same general class isolated from embryos of lesion-susceptible and lesion-resistant strains. This may be relevant because subspecies of the same GAG class may have differing affinities for lipoproteins. Also, it is possible that hypercholesterolemia may actually induce changes in the matrix components secreted by arterial smooth muscle cells at lesion-susceptible aortic sites and that these changes, in turn, may facilitate lipoprotein binding to the extracellular matrix. Enhanced binding to matrix proteins or proteoglycans might explain why fractional rates of degradation of LDL in susceptible sites were lower than in resistant sites. Increased binding of LDL may not be the only effect of an altered matrix, however. Differences in molecular sieving of LDL particles within the tertiary structure of the matrix may also play a role in LDL retention.

Because both the fractional rates of degradation and efflux decreased more in susceptible sites than in resistant sites, the average time that an LDL particle spends within the wall must be greater in the susceptible sites. By 4 days of cholesterol feeding, the mean residence times of LDL in lesion-prone sites of the aorta were significantly greater than in their respective adjacent uniform areas (Table 5). By 16 days of feeding, the mean residence times in susceptible sites were even more prolonged, e.g., the mean residence time of LDL in abdominal branch sites was three times as long as that in the same site of normal animals.

In the kinetic calculations made in this study, we have made the assumption that intact LDL in a given aortic site is present in a single kinetically homogeneous compartment. However, if additional compartments (such as LDL bound to extracellular matrix) exist and if the exchange rate of LDL into and out of the compartments is long compared to the duration of our study, then the fractional residence times we calculated (Table 5) will underestimate the actual mean residence times in these areas, because the LDL present in the “bound” pool will not be labeled as readily. For the same reason, the mass of intact LDL calculated for lesion-prone aortic sites from the model used here or from C1/Cp11 will underestimate the actual masses of intact LDL in such areas.

The prolongation of the mean residence time of LDL in lesion-prone aortic sites may play an important role in atherogenesis. For example, the longer an LDL particle is present within the arterial wall, the greater the chance that it might be modified chemically or enzymatically to a form that alters its affinity for binding to extracellular matrix or its binding and uptake by cells. One such process, which has been extensively characterized in vitro, involves cell-mediated oxidative modification of LDL. Such modification could result in increased recruitment of monocytes, retention of macrophages, and increased macrophage uptake of the modified LDL. Recent work from our laboratory suggests that such oxidative processes may indeed occur within developing fatty streak lesions, since treatment with probucol, a potent inhibitor of this oxidative modification, slows the development of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit.

In conclusion, the present study demonstrated that aortic permeability to LDL was not altered during 16 days of cholesterol feeding at either lesion-susceptible or lesion-resistant sites in rabbits. In fact, one of the sites most susceptible to early atherosclerotic lesions, the aortic arch, was no more permeable to LDL than were the lesion-resistant, uniform portions of the descending thoracic aorta. Another susceptible site, the branch orifice areas of the abdominal aorta, was more permeable to LDL than adjacent sites, but this was also true in normcholesterolemic animals. Thus, we could find no evidence to support the idea that hypercholesterolemia per se leads to alterations in the permeability of the intimal surface before fatty streak formation. In contrast, retention of LDL was significantly enhanced in lesion-prone aortic sites during the interval of cholesterol feeding. It is possible that the selective retention of intact LDL in that sequestered
environment may be the key to permitting modification of the LDL to occur to the point that the LDL binds more avidly to the extracellular matrix or is more rapidly taken up by macrophages.

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