Effect of Lanthanum Chloride on Established Atherosclerosis in the Cholesterol-fed Rabbit Mitral Valve as a Site for Assessment of Treatment Effects

Peter J. Gillies, Candy S. Robinson, Beverly Y. Cockrell, and G. Jay Graepel

The ability of lanthanum chloride (LaCl₃) to retard the progression of established atherosclerosis was investigated in cholesterol-fed rabbits. Rabbits were initially maintained on a high-fat plus cholesterol-supplemented diet for 10 weeks to induce lesions and were then changed to a low-fat diet or a low-fat diet supplemented with LaCl₃ for an additional 24 weeks to permit their serum cholesterol levels to normalize. LaCl₃ did not affect the rate at which serum cholesterol levels returned to normal. The dose of LaCl₃ was approximately 30 mg/kg body weight/day. In comparison with controls, LaCl₃-treated rabbits exhibited histologically less severe coronary artery and mitral valve atherosclerosis. Lesion severity in the carotid arteries was unaffected by LaCl₃ treatment. Although statistically significant, the salutary effects of LaCl₃ were relatively small. The data support the hypothesis that calcium antagonists can retard the progression of established atherosclerotic lesions. The data also illustrate the value of the mitral valve as a site to assess treatment effects on monocyte/macrophages in vivo. (Arteriosclerosis 9:253-260 March/April 1989)

Although current pharmacological approaches to atherosclerosis focus on the normalization of lipoprotein profiles, there is growing interest in the development of drugs specifically targeted at the arterial wall. In this regard, calcium antagonists are particularly noteworthy. There are many reports in the literature that calcium antagonists retard the atherogenic process in experimental animals (for reviews, see references 4, 5, and 6). While not unequivocal, the animal data are sufficiently convincing to foster the belief that calcium antagonists may be of value in the treatment of atherosclerosis in man. The International Nifedipine Trial on Antiatherosclerotic Therapy (INTACT) and a similar trial with nicardipine underscore this belief. These trials are clinical tests of the hypothesis that calcium antagonists can retard the progression or promote the regression of coronary atherosclerosis in man.

There are several aspects about the antiatherosclerotic effects of calcium antagonists for which there is little information. Some of these are: 1) effects on vascular beds other than the aorta, 2) effects on established lesions, and 3) histological quantitation of lesion amelioration. To provide information in these areas, the effects of lanthanum chloride (LaCl₃) on established lesions in carotid arteries, coronary arteries, and mitral valves were histologically quantitated in a cholesterol-fed rabbit model of atherosclerosis.

Methods

Animals and Diets

Animal studies were conducted in a facility fully accredited by the American Association for the Accreditation of Laboratory Animal Care. Male New Zealand rabbits (Hhr: [NZW]SPF, Hazelton Dutchland, Denver, PA) were housed in individual cages in a room controlled at 20±2°C, 50%-±10% humidity, and a 12-hour light/dark cycle. Rabbits were randomly assigned to groups as shown in Figure 1. During the lesion-induction phase, rabbits were maintained on a diet of pelleted rabbit food supplemented with 3% peanut oil (HF-diet) or 3% peanut oil plus 0.5% cholesterol (HFC-diet). Food and water were available ad libitum. After 10 weeks on these diets, rabbits were changed to a low-fat diet (LF-diet) or a low-fat diet supplemented with LaCl₃ (nominal concentration: 1.28 g LaCl₃/kg of diet) for an additional 24 weeks. During the drug-treatment phase, food was restricted to 125 g/day. Diets were formulated by ICN Nutritional Biochemicals, Cleveland, Ohio.

Serum Cholesterol

Total serum cholesterol was determined spectrophotometrically by using o-phthalaldehyde.

Necropsy

At the time of sacrifice, rabbits were anesthetized with sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL). The heart and carotid arteries were carefully excised and fixed in 10% formalin. The heart was sectioned at three levels (Figure 2). At level 1, the heart was cut longitudinally to include the mitral valve. At level 2, the heart was cut midventrally to include the papillary muscles. At level 3, the heart was cut at the apex below the papillary muscles. The carotid arteries were cut in cross section just
**Figure 1.** Study design.

**Figure 2.** Levels 1, 2, and 3 of the heart.

above the aortic arch. Paraffin tissue blocks were prepared by standard histological procedures, and tissue sections were stained with hematoxylin and eosin.

**Evaluation of Atherosclerotic Lesions**

Histological sections were randomly examined without knowledge of treatment and were graded according to the scale presented in Figure 3 and illustrated in Figure 4. The grading scale used in these studies represents an expanded version of a scale used by other investigators to evaluate the effects of calcium antagonists on coronary atherogenesis. The width of the plaque relative to the width of the unaffected vessel wall was the grading determinant, not the luminal occlusion. No distinction was made between epicardial and intramyocardial coronary arteries. When more than one coronary artery was observed in a plane of section, the most severely affected vessel was graded. The mitral valve was also graded on a 1 to 10 scale as illustrated in Figure 5.

**Severity Grade**

1.  
2.  
3.  
4.  
5.  
6.  
7.  
8.  
9.  
10.  

**Figure 3.** Atherosclerosis grading scale.

**Statistical Analysis**

Comparisons among groups with respect to serum cholesterol and body weight were made by analysis of variance; statistical significance was judged at the $p<0.05$ level. Comparisons between groups with respect to lesion severity were made with a two-sample median test or a two-sample Wilcoxon test depending on the nature of the tied values. The tests were one-tailed given the premise that LaCl$_3$ would decrease lesion severity. Statistical significance was judged at the $p<0.05$ level.

**Results**

**Diet and Drug Analysis**

The LF-diet was nominally 1% to 3% by weight lipid. Lipid analysis of the HF-supplemented diets indicated they were 6% by weight lipid; in addition, the HFC-diet contained the prescribed 0.5% cholesterol (Hazetton Laboratories America, Incorporated, Madison WI). Atomic absorption analysis of the LaCl$_3$-supplemented diet indicated it contained 534 ppm lanthanum (Galbraith Laboratories, Incorporated, Knoxville, TN). Over the course of the drug-treatment phase, the daily dose of LaCl$_3$ for rabbits in Groups IV and VI was approximately 30 mg/kg body weight.

**Clinical Observations**

A total of 62 rabbits were entered into the study. Three rabbits died of hemolytic spur cell anemia early in the drug-treatment phase. The remaining 59 rabbits gained and then maintained body weight on the restricted diet of 125 g/day. At the end of the lesion-induction and drug-treatment phases, rabbits in Groups I vs. II, III vs. IV, and V vs. VI were of comparable body weight (Table 1).

The HFC-diet induced a 56-fold increase in serum cholesterol by the end of the induction phase when compared with the HF-diet (1560±230 mg% vs. 28±5 mg%, Group II vs. Group I). The serum cholesterol levels of Group III vs. Group IV (29±2 mg% vs. 25±2 mg%) and Group V vs. Group VI (1853±209 mg% vs. 1966±176 mg%) were of comparable body weight.
mg%) were comparable. At the end of the drug-treatment phase, the serum cholesterol levels of Groups III and IV were significantly lower than at the end of the lesion induction phase but were not significantly different from each other (14±2 mg% vs. 13±1 mg%). The serum cholesterol levels of Groups V and VI had returned to normal by the end of the drug-treatment phase (29±8 mg% vs. 22±3 mg%). The area under the curves for Group III vs. Group IV (3417±245 mg% x days vs. 3031±277 mg% x days) and Group V vs. Group VI (61 448±8361 mg% x days vs. 61 030±8024 mg% x days) were not significantly different (Figure 6).

**Morphologic Observations**

At the end of the lesion-induction phase, Groups I and II were sacrificed to verify that the HFC-diet had induced the development of atherosclerosis. The carotid arteries, coronary arteries, and mitral valves of Group II rabbits exhibited extensive atheromatous lesion development; the Group I rabbits were lesion free. Because of the limited number of animals in Groups I and II, statistical analysis of the degree of lesion severity was not undertaken for these tissues.

At the end of the drug-treatment phase, the carotid arteries, coronary arteries, and mitral valves of Groups III and IV were lesion free; in contrast, the same tissues from Groups V and VI exhibited significant atheromatous lesion development. The severity and frequency of carotid lesions in Groups V and VI are presented in Figure 7. The severity and frequency of coronary lesions in Groups V and VI at levels 1, 2, and 3 are presented in Figures 8, 9, and 10, respectively. With regard to the coronary arteries, the small intramyocardial arteries exhibited extensive atherosclerotic lesion development; the larger epicardial coronary arteries did not. Indeed, there was a notable sparing of the epicardial arteries. Thus, the data presented in
Analysis of the carotid artery data for Groups V and VI indicated that the two groups were of comparable lesion severity ($p<0.43$). In contrast, the same statistical analysis of the coronary artery data indicated that overall lesion development in Group VI was less severe than in Group V ($p<0.04$). When analyzed according to plane of section, lesion development in Group VI was significantly less severe than in Group V at levels 1 ($p<0.03$) and 2 ($p<0.01$) but not at level 3 ($p<0.08$). Analysis of the mitral valve data indicated that lesions in Group VI were less severe than in Group V ($p<0.005$).

In addition to vascular lesions, various myocardial lesions were noted in the study. Myocardial degeneration, chronic focal myocarditis, and intramyocardial lipid deposition were observed in Group IV; similar lesions were observed in Groups V and VI. Essentially no lesions were observed in Group III.

**Discussion**

After cessation of prolonged periods of cholesterol feeding, serum cholesterol levels slowly return to normal.$^{15,16}$ During the normalization period, atherosclerotic lesions continue to grow and undergo a fibrous transformation.$^{17,18}$ Thus, the present study constitutes an investigation of the effects of LaCl$_3$ on established, but still evolving, atherosclerotic lesions. At the end of the drug-treatment phase, lesion development was significantly less severe in the coronary arteries and mitral valves of LaCl$_3$-treated rabbits. Since the serum cholesterol elimination curves and the area under these curves were the same for control and LaCl$_3$-treated rabbits, it is tempting to attribute the
Table 1. Body Weights and Serum Cholesterol Levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Final Body weight (g)</th>
<th>Serum cholesterol 10 weeks (mg)</th>
<th>Serum cholesterol 34 weeks (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2141±70</td>
<td>3099±114</td>
<td>28±5</td>
<td>—</td>
</tr>
<tr>
<td>II</td>
<td>2287±63</td>
<td>2871±86</td>
<td>1560±230</td>
<td>—</td>
</tr>
<tr>
<td>III</td>
<td>2259±45</td>
<td>4309±126</td>
<td>29±2</td>
<td>14±2</td>
</tr>
<tr>
<td>IV</td>
<td>2277±42</td>
<td>4358±141</td>
<td>25±2</td>
<td>13±1</td>
</tr>
<tr>
<td>V</td>
<td>2258±35</td>
<td>3967±115</td>
<td>1853±209</td>
<td>29±8</td>
</tr>
<tr>
<td>VI</td>
<td>2342±91</td>
<td>3999±129</td>
<td>1966±176</td>
<td>22±3</td>
</tr>
</tbody>
</table>

Values are the means±SEM.

Figure 6. Serum cholesterol levels during the drug-treatment phase. Group III: O, y=−1.32e^-0.0x+1.42. Group IV: ●, y=−1.02e^-0.0x+1.34. Group V: □, y=−0.01x+3.17. Group VI: ■, y=−0.01x+3.21.

Figure 7. Severity and frequency of carotid artery lesions in control animals (Group V, hatched bars) and LaCl₃-treated animals (Group VI, open bars).

Salutary effects of LaCl₃ to direct effects on the vessel wall. However, in the absence of detailed lipoprotein studies, the possibility cannot be excluded that subtle changes in the distribution or composition of the various lipoprotein classes also contributed to the amelioration in lesion severity. The antiatherosclerotic effects of LaCl₃ are particularly noteworthy because of its poor oral bioavailability and short half-life in the blood. The rapid clearance of La³⁺ from the blood may reflect its propensity for cell surface adsorption. Studies with ¹⁴¹Pm, a chemically similar lanthanide, illustrate the tenacity with which rare earth ions such as La³⁺ bind to cell surfaces. In future studies, it may be worthwhile trying to correlate arterial levels of La³⁺ with lesion severity.

LaCl₃ and other calcium antagonists, such as nifedipine and nicardipine, dramatically suppress atherogenesis when administered concomitantly with cholesterol-enriched diets. In contrast to these lesion-induction studies, the present study showed that LaCl₃ is far less effective in reducing lesion severity once the lesions have been established. A similar conclusion was reached in a study with verapamil, although more beneficial effects have been reported in earlier studies with LaCl₃ and ethanehydroxy-diphosphonate. In these studies, aortic rather than coronary atherosclerosis was investigated. Further studies are needed to determine the relative efficacy of calcium antagonists as antiatherogenic versus antiatherosclerotic agents.

In addition to the coronary arteries, LaCl₃ treatment also reduced atheromatous lesion development in the mitral valves. This observation merits a number of comments. First, compared to arterial lesions, lesions located on the ventricular surface of the mitral valve are considerably more homogeneous in their range of lesion severity and less fibrous in composition due to a paucity of constituent smooth muscle cells. Second, in the prelesion stages of development, lipid accumulation in the mitral valve is...
Lesion Severity

Figure 8. Severity and frequency of Level 1 coronary artery lesions in control animals (Group V, hatched bars) and LaCl₃-treated animals (Group VI, open bars).

Lesion Severity

Figure 9. Severity and frequency of Level 2 coronary artery lesions in control animals (Group V, hatched bars) and LaCl₃-treated animals (Group VI, open bars).

Lesion Severity

Figure 10. Severity and frequency of Level 3 coronary artery lesions in control animals (Group V, hatched bars) and LaCl₃-treated animals (Group VI, open bars).

Lesion Severity

Figure 11. Severity and frequency of mitral valve lesions in control animals (Group V, hatched bars) and LaCl₃-treated animals (Group VI, open bars).

Predominantly extracellular; however, in the more advanced stages of lesion development, lipid accumulation occurs primarily within monocyte-derived macrophages. Thus, studies of the mitral valve offer an excellent opportunity to investigate the effects of drugs on atheromatous lesions that: 1) exhibit well-defined topography, 2) are comprised primarily of macrophage-derived foam cells, and 3) exhibit a range of severity that tends to be normally distributed.
Within this paradigm, the observation that LaCl3 significantly decreases lesion severity in the mitral valve permits the speculation that, in addition to the many well-documented effects on smooth muscle cells (e.g., inhibition of cell division, migration, and collagen production), calcium antagonists may also exert their antithromogenic and antithrombotic effects by modulation of macrophage function. Two recently published cell culture studies provide mechanistic support for this hypothesis. In J774 macrophages, it was reported that verapamil decreased acyl CoA cholesterol transesterase-mediated esterification of cholesterol, an effect that should attenuate foam cell formation. In mouse peritoneal macrophages, it was reported that nifedipine promoted a high density lipoprotein-independent efflux of cholesterol, an event that represents another form of reverse cholesterol transport. The mitral valve may be an excellent site at which to evaluate the in vivo validity of these cell culture hypotheses.

While the coronary arteries and mitral valves were affected by LaCl3, the carotid arteries were not. Longitudinal sections of several carotid arteries revealed a marked variation in lesion severity along the length of the vessel wall. This variation may have resulted in a less sensitive statistical test. In future studies, it would be preferable to section the carotid artery not only at its ostium from the aortic branches, but also at the carotid bifurcation and carotid bulb where the severity of lesion development may be more consistent. Another possibility that should be considered is that the carotid arteries are less sensitive to the effects of LaCl3. In this regard, it is notable that different vascular beds exhibit differential responses to calcium antagonists.

It has been reported that calcium antagonists retard the development of atherosclerosis in the aorta but not in the coronary arteries of cholesterol-fed rabbits. In that study, coronary lesions were graded according to a 0 to 3 scale of lesion severity. This limited scale may have been insensitive to small differences in lesion severity. In the present study, it was found that a grading scale of 1 to 5 was insensitive to small differences in lesion severity, particularly when the lesions were quite severe. Expansion of the grading scale to 1 to 10 permitted the identification of statistically significant differences missed by the 1 to 5 scale. The methodological limitations of the study were that the grading scale used to evaluate lesion severity was based only on lesion area and no consideration was given to differences among lesions with respect to cell type, intracellular versus extracellular lipid, or fibrous components. With the advent of computer-assisted morphometry systems designed for the quantification of atherosclerotic plaques and the development of antibodies to specific cell types and matrix components, more sophisticated studies should be feasible in the future.

There are many well-known criticisms of the cholesterol-fed rabbit as a model for the study of atherosclerosis, and two of these are particularly germane to this study. First, in contrast to humans, the large epicardial arteries of the rabbit are far less susceptible to lesion development than are the much smaller intramyocardial arteries. Second, established atherosclerotic lesions in humans contain less lipid and more fibrous tissue than in rabbits. Although the coronary lesions are quite different from those observed in humans, rabbit mitral valve lesions are remarkably similar. Despite the limitations of the animal model, the observation that LaCl3 retards the progression of established coronary atherosclerosis may have some clinical implications. It is notable that the effects of LaCl3 appear to be vessel wall-mediated and similar to what might be expected from maximal plasma lipid-lowering therapy. This underscores the potential therapeutic value of combining lipid-lowering drugs with antiatherogenic and antiatherosclerotic agents targeted directly at the vessel wall.

In conclusion, the data support the hypothesis that calcium antagonists can slow the progression of established atherosclerotic lesions. While the salutary effects were statistically significant, the clinical significance of small ameliorative changes in lesion severity remains to be established. The results of ongoing clinical trials with calcium antagonists such as nifedipine and nicardipine should help resolve this issue.

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