The Effect of Agents Interfering with Soft Tissue Calcification and Cell Proliferation on Calcific Fibrous-Fatty Plaques in Rabbits

DIETER M. KRAMSCH AND CATHERINE T. CHAN

SUMMARY We tested agents that might suppress the formation of fibrous plaques which are the significant lesions in human atherosclerosis. Six groups of rabbits were studied for 8 weeks: one group was fed a control diet and five groups received an atherogenic diet known to produce accumulations of connective tissue in arterial lesions. One group received only the atherogenic diet and the remaining four groups were treated simultaneously with the calcification inhibitor, ethane hydroxy diphosphonate (EHDP), at two dosage levels, as well as the antimitotic agent, N-desacetyl-N-methylcolchicine (Colcemid), or a combination of EHDP and Colcemid, respectively. None of the drugs reduced to normal the high serum cholesterol levels induced by the diet, but all reduced the elevated serum calcium concentrations occurring in untreated rabbits on the atherogenic diet. The lesions in the untreated group included marked accumulations of intra- and extracellular lipids (chiefly cholesterol) and calcium, as well as of glycosaminoglycans, collagen, and elastin. Aortic "elastin" from these atherosclerotic rabbits had an increased content of polar amino acids, calcium, and cholesterol. Treatment with EHDP or Colcemid alone markedly inhibited these atherosclerotic processes, whereas combination therapy with both drugs completely suppressed all aspects of atherogenesis except for a few superficial lipid deposits close to the vessel lumen. The prevention of excessive deposition of arterial calcium by EHDP and also by Colcemid may play an important role in the antiatherogenic effect of these drugs.

THE LIFE-THREATENING lesion in human atherosclerosis appears to be the fibrous atheromatous plaque which often is calcified. It generally is agreed that hypercholesterolemia is one of the most potent atherogenic stimuli. Unfortunately, the National Coronary Drug Project has shown that, of the commonly employed antilipemic drugs, none halts the progression of advanced disease after treatment for many years. It is common knowledge that the arterial system of individuals of middle age and above in our society almost always shows various degrees of fibrous atherosclerosis that may lead to serious sequelae—even in individuals with serum cholesterol levels not considered to be elevated (<250 mg/100 ml). On the other hand, studies in less developed countries have shown that populations consuming little or no cholesterol and animal fats have low serum cholesterol levels (<150 mg/100 ml) and rarely develop clinically significant atherosclerosis. It is uncertain whether the large-scale National Primary Prevention Trials that are currently underway and that use antilipemic dietary and/or drug treatment will be able to lower serum cholesterol to safe levels in a significant number of persons in our society. Therefore, we believe it to be reasonable to search for drugs that may inhibit atherogenesis without significantly altering the serum cholesterol.

Several such studies already have been performed in experimental animals, using agents that inhibit arterial uptake of calcium. In the present study we treated rabbits on an atherogenic diet with 2 dose levels of the disodium salt of ethane-1,1-diphosphonic acid (EHDP) which inhibits the deposition of calcium in soft tissues. We also treated rabbits on the same diet with the antimitotic agent, N-desacetyl-N-methylcolchicine (Colcemid; synonym, Demecolcine). Because we also wanted to test the effects of these drugs on the connective tissue components of plaques, we chose as the atherogenic diet the slightly modified fibrogenic diet described by Kritchkevsky et al.

Methods

Forty-eight New Zealand White rabbits weighing 2.2-2.7 kg were divided randomly into six groups of eight rabbits each. One group received the control diet of Purina Rabbit Chow (normal controls). The other five groups received an atherogenic diet consisting of 80 g of the basic chow with 8% peanut oil and 2% cholesterol (by weight) mixed into the chow. One of these groups was fed only the atherogenic diet (atherosclerotic control group), whereas the remaining four groups were treated simultaneously with the following drugs administered orally (per kg body weight per day): 20 mg EHDP (active substance of the hexahydrate), 40 mg EHDP, 0.06 mg Colcemid, and 20 mg EHDP + 0.06 mg Colcemid, respectively. Two animals died prematurely, one control rabbit of an inner ear infection and one rabbit on the atherogenic diet without drugs of obstructive liver disease. Both were

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* Kindly provided as the hexahydrate by the Monsanto Co., St. Louis, Missouri.
† Purchased as the repeatedly recrystallized anhydrate from the Ciba-Geigy Corp., Summit, New Jersey.
replaced by other rabbits. All rabbits that completed the 8-week study period consumed their respective diets well and were killed at the end of the study by decapitation and exsanguination.

In Vivo Studies

Blood samples were drawn from an ear vein after an 18-hour fast, once before starting the experiment and then at 2-week intervals. Total serum cholesterol was analyzed by the method of Abell et al., and total calcium was determined by atomic absorption spectrophotometry according to the method of Parker, and inorganic phosphorus was measured by the method of Chen et al. Prior to the determination of calcium and phosphorus, the serum samples were refrigerated overnight and then centrifuged at 3000 g for 30 minutes to remove the chylomicrons which dilute the serum and mask its actual content of calcium and inorganic phosphorus in animals on the atherogenic diet.

Postmortem Studies

Morphological Studies

At autopsy, the entire aorta, the proximal portions of the pulmonary arteries, and the heart, lungs, kidneys, adrenals, liver, and spleen were removed. The aortas were opened longitudinally along the midthoracic line and cleaned of grossly adherent adventitial tissue. All organs (except the pulmonary artery) were weighed after they had been cleaned of blood by brief immersion in normal saline and rapid blotting with dry gauze. Their weight was expressed as wet weight per kg body weight of the rabbit at the time at which it was killed. The aortas then were cut into two longitudinal halves of about equal size which again were weighed separately. From the aortas of each group representing the most common gross intimal characteristics of that group (i.e., at least five aortas of comparable appearance), two aortic halves were fixed in buffered formalin and stained with Sudan IV. After staining, the aortic halves were covered with sheets of clear plastic, their contours traced, and the stained lipid lesions filled in with black ink. The total surface area of the aortic halves, as well as the total surface area of the stained lipid lesions, was measured by planimetry from the tracings. Small representative cross-sections from plaque areas and adjacent normal areas of thoracic and abdominal aortas, as well as from comparable aortic areas of normal control rabbits, were taken from one unfixed half of each aorta for histological analyses. The weight of the histological samples then was subtracted from the total wet weight of the aortic half from which they had been removed. Similar horizontal sections from plaque and normal areas were taken from the pulmonary arteries. Cross-sections of whole hearts about midway between the A-V groove and apex, as well as tissue blocks of lung, kidney, and adrenals, were taken for histological study. All histological specimens were fixed in 10% buffered formalin containing 0.5% trichloroacetic acid to better prevent extraction of soluble proteins, including lipoproteins, during the initial phase of fixation. The fixed segments then were embedded in Carbowax according to the method of Jones and Goodale, cut to 4-μm thickness, and stained with Verhoeff’s-Van Gieson stain for connective tissue, hematoxylin-Oil Red O for lipids, periodic acid-Schiff (PAS)-alcian blue and toluidine blue for glycosaminoglycans and with alizarin red and the Yasue technique for calcium.

Biochemical Studies

Eight unfixed aortic halves from each group were used to determine total and esterified cholesterol, collagen, calcium, and nonlipid phosphorus. The six other unfixed aortic halves from each group were used separately to determine elastin content as well as the amino acid, cholesterol, calcium, and phosphorus composition of the elastin. The formalin-fixed and Sudan IV-stained aortic halves were not used for biochemical analysis because such pretreatment led to inaccurate results. The intima-media of all unfixed aortic halves was stripped from the adventitia from the luminal side according to the method of Wolinsky and Daly. The tissues designated for the determination of aortic components other than elastin were delipidated with chloroform-methanol 2:1 (vol/vol), and the total and ester cholesterol contents were determined from the lipid extracts. The dry weight of the delipidated intima-media of one aortic half from each rabbit was determined. Small samples of well-mixed tissue then were ashed and analyzed for the aortic content of calcium by atomic absorption spectrophotometry, and for phosphorus content. For collagen determination, the main portion of the aortic tissues was extracted with 0.1 N NaOH for 50 minutes. The samples then were centrifuged and the supernatant fluids (containing the alkali hydrolysates of the aortic collagen) were hydrolyzed further with 6 N HCl. The hydroxyproline content of the acid hydrolysate was determined according to the method of Bergman and Loxley, using a factor of 7.46 for the conversion of the hydroxyproline values to collagen.

The nondelipidated minced intima-media of the aortic halves designated for the isolation of the elastin fractions were extracted with 0.1 N NaOH, according to a modification of the method of Lansing et al., as previously described. As shown previously, this treatment also removes virtually all glycosaminoglycans from the elastin. The isolated elastin fraction was delipidated, desiccated, and its dry weight determined. The total and ester cholesterol content were determined from the lipid solutions and the calcium and phosphorus content from small samples of the dry elastin preparation. The methods used for the analyses of cholesterol, calcium, and phosphorus were the same as those described above for similar analyses of whole aortic tissue. The protein content of the elastin fraction was measured from another small sample by the method of Kjeldahl method, or a modification of this method, by converting the protein nitrogen of the hydrolysate into ammonia and determining the ammonia content with an ammonia-specific electrode (Orion Research) on an expanded pH meter according to the method of Bremner and Tabatabai. The modified Kjeldahl method abbreviates the procedure considerably, the results were in
close agreement with those of the original method. The remaining elastin preparation was further hydrolyzed in a vacuum oven with 6 N HCl at 110°C for 24 hours and analyzed for its amino acid composition by an amino acid autoanalyzer as previously described.15

The extraction procedure with hot alkali was controlled for possible binding of tissue lipids to the elastin during the extraction. In three separate studies, in duplicate, 10 ml of hypercholesterolemic rabbit serum containing 358.5 mg cholesterol were added to delipidated minced tissues of normal and atherosclerotic rabbit aortas before the hot alkali extraction. After extraction, less than 2% of the added cholesterol was associated with the elastin preparations of normal and atherosclerotic aortas. Similar control studies have been performed15 on elastin preparations from normal and atherosclerotic human intima.

The dry weight of the total aortic intima-media of each rabbit was calculated by using the dry weight of the intima-media from one aortic half and the wet weights of both aortic halves as reference points, after taking into account the small tissue portions removed for histology. The total aortic content of calcium and phosphorus was calculated in a similar manner and then subtracted from the dry weight of intima-media of whole aorta. All figures referring to aortic components are expressed in absolute amounts per intima-media of whole aorta per kg body weight. The division of data on aortic components by the body weight of the rabbit was necessary because the weights of the rabbits within each group, including the control group, showed great variations. The significance of the data was determined by statistical analysis of unpaired data.

**Results**

**In Vivo Studies**

All rabbits gained weight, with the average gain in the groups varying between 469 and 512 g. The weight increases among the groups did not differ significantly. All rabbits receiving only the atherogenic diet developed marked arcus senilis, many marked abdominal xanthomata, and hair loss. These changes were only moderate in both EHDP groups, slight in the Colcemid group, and absent in rabbits treated with both EHDP and Colcemid. The mean values for serum constituents are summarized in Table 1. The mean total serum cholesterol levels rose in all rabbits on the atherogenic diet with or without drugs to levels above 3000 mg/100 ml within 2 weeks and

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Total cholesterol</th>
<th>Total calcium</th>
<th>Inorganic phosphorus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>82 ± 23</td>
<td>13.7 ± 0.3</td>
<td>6.0 ± 1.4</td>
</tr>
<tr>
<td>Atherogenic diet without drugs</td>
<td>3788 ± 676</td>
<td>16.2 ± 0.6†</td>
<td>5.6 ± 1.2</td>
</tr>
<tr>
<td>Atherogenic diet + EHDP (20 mg/kg B.W.)</td>
<td>3409 ± 994</td>
<td>13.4 ± 0.5</td>
<td>5.7 ± 0.9</td>
</tr>
<tr>
<td>Atherogenic diet + EHDP (40 mg/kg B.W.)</td>
<td>3427 ± 765</td>
<td>14.0 ± 0.6</td>
<td>6.2 ± 1.3</td>
</tr>
<tr>
<td>Atherogenic diet + Colcemid (0.06 mg/kg B.W.)</td>
<td>3841 ± 801</td>
<td>13.2 ± 0.7</td>
<td>6.1 ± 0.7</td>
</tr>
<tr>
<td>Atherogenic diet + EHDP (20 mg) and Colcemid (0.06 mg/kg B.W.)</td>
<td>3535 ± 938</td>
<td>13.8 ± 0.4</td>
<td>5.8 ± 0.7</td>
</tr>
</tbody>
</table>

* Milligrams/100 ml; results are expressed as mean ± sd. B.W. = body weight.
† P < 0.01 compared to control.
remained at that level throughout the study, with the cholesterol values among all cholesterol-fed groups being not significantly different. The mean serum calcium rose significantly in all rabbits on the atherogenic diet without drugs and reached hypercalcemic peak levels during mid-experiment. In contrast, all drug-treated animals had normal serum calcium levels at all times; the serum inorganic phosphorus remained unchanged in all groups.

**Postmortem Studies**

**Morphological Results**

**Macroscopic Findings.** The average weight/kg body weight of the kidneys and hearts was comparable in all groups. Although all kidneys appeared to be normal, the hearts of all but one of the rabbits on the atherogenic diet without drugs showed gross atherosclerosis. The left coronary arteries were most frequently involved (left anterior descending in nine, circumflex in seven); involvement of the right coronary arteries occurred in only three rabbits. The extent of the grossly visible disease ranged from about 1 cm of the length of the artery to the entire length, including even the branch vessels. One of the six rabbits with maximal involvement of the left anterior descending and circumflex coronary arteries appeared to have survived a myocardial infarction as evidenced by a 1- × 1-cm scar at the lateral aspect of the left ventricle. In all drug-treated groups, gross coronary atherosclerosis was rare and, if present, consisted of a few small visible dots of lipid infiltration. In the group treated with both drugs, gross coronary atherosclerosis was absent. The weight of the aortas was comparable within each group if expressed per kg body weight. However, there was a highly significant increase \( (P < 0.01) \) in mean aortic weight from 84.4 mg/kg body weight in the control group to 123.2 mg/kg in the untreated group on the atherogenic diet. The increase in mean aortic weight was markedly smaller; 108.0 mg/kg or 108.8 mg/kg in both EHDP groups and 106.4 mg/kg in the Colcemid-treated group. However, the increases still were statistically significant \( (P < 0.05) \). The group treated with EHDP and Colcemid in combination had a mean aortic weight of 89.2 mg/kg which was not significantly different from that of the control group. Figure 1 shows the outlines of representative aortic surface area involvement with lipid-staining lesions for each atherogenic group. EHDP reduced the sudanophilic aortic areas markedly to about the same extent at both dosage levels (from an average of 65% to an average of 18% and 15%, respectively); Colcemid resulted in even greater reductions (average 7%), whereas the combination therapy suppressed gross atherosclerosis, leaving only minimal lesions (average < 3%). Liver, adrenals, lungs, and spleen showed marked enlargement in all untreated rabbits on the atherogenic diet. This presumably was caused in large part by grossly visible infiltration with fatty material. The average weights of these organs were increased about 2-

![Figure 1](http://circres.ahajournals.org/)

**Histological cross-section of thoracic aorta of a control rabbit showing a thin intima including an intact internal elastica (top black line) overlying an orderly structured normal media. Verhoeff’s-Van Gieson. Scale is 100 μm.**

![Figure 2](http://circres.ahajournals.org/)

**Histological cross-section of thoracic aorta of a control rabbit showing a thin intima including an intact internal elastica (top black line) overlying an orderly structured normal media. Verhoeff’s-Van Gieson. Scale is 100 μm.**
sclerosis of the same type as in the aorta. Such regular

Microscopic Findings. Figure 2 shows the micrograph of a cross-section of thoracic aorta from a normal control rabbit. The intima consists of one layer of cells on an intact internal elastica which is followed in an orderly fashion by layer upon layer of intact medial elastic laminae alternating with layers of unaltered medial smooth muscle cells.

Figure 3, A-C, shows serial microscopic sections through one of the characteristic fibrous-fatty aortic plaques elicited in rabbits fed the atherogenic diet without drugs. The intima was raised considerably by a marked increase in cellularity, and there was a striking accumulation of collagen in the intima and subintimal media (Fig. 3A); the internal elastica and many of the subintimal medial elastic laminae were severely deranged and fragmented. There was heavy deposition of calcium (Fig. 3B) and of lipid (Fig. 3C) on the damaged elastica. The increased intimal cells also contained large deposits of Oil Red O-stainable lipids (Fig. 3C). However, only minor deposits of stainable calcium were seen between calcified elastic laminae, including those overlying cells (Fig. 3B). In addition, PAS-alcian blue staining revealed moderate accumulations of glycosaminoglycans on the altered elastica and over accumulated collagen.

Figure 4, A and B, shows serial sections through a typical aortic plaque of a rabbit receiving the atherogenic diet and treated with EHDP (20 mg/kg body weight). The cellularity of the intima was only minimally increased, with the small number of intimal cells present being mainly of the foam cell type (Fig. 4A) and containing Oil Red O-stainable lipids. There was no accumulation of collagen and glycosaminoglycans, but the internal and subintimal elastic laminae still were somewhat deranged and often stained mildly for lipids. However, there was no detectable calcification of the elastica (Fig. 4B). The higher dose of EHDP did not appear to have an additional effect, since the lesions seen were similar in size and characteristics to those in aortas of rabbits treated with the lower dose. The aortic lesions observed in Colcemid-treated rabbits were very similar to those of rabbits treated with EHDP.

Figure 5 shows a representative section through one of the few aortic plaques seen in rabbits receiving the atherogenic diet and treated with a combination of EHDP and Colcemid. The structure of the vessel was essentially normal, with the exception of a small superficial layer of lipid-containing cells overlying an unbroken internal elastica.

In untreated rabbits receiving the atherogenic diet, the common pulmonary artery and the most proximal portions of its main two branches regularly showed severe atherosclerosis of the same type as in the aorta. Such regular involvement of the pulmonary arteries does not occur in all species with induced atherosclerosis and is rare in the human disease. The medium and smaller sized arteries of all other organs tested (heart, lungs, liver, kidneys, adrenals, spleen) showed histological involvement with less regularity and variable degrees of severity. Any of the drug treatments tested resulted in complete suppression of atherosclerosis in the major pulmonary arteries but not in the arteries to other organs; in the latter, only combination treatment with EHDP and Colcemid had that effect.

Biochemical Results

The biochemical changes observed in intima-media from whole aortas were in agreement with the structural alterations. Table 2 summarizes the changes in the content of aortic components for each group. As compared to control animals, rabbits given the atherogenic diet without drugs showed large increases in aortic collagen, "elastin," free and ester cholesterol, calcium, and nonlipid phosphorus. In rabbits treated with EHDP in addition to the atherogenic diet, these aortic components were reduced markedly but, except for phosphorus, were elevated significantly. Both dose levels of EHDP had comparable effects. When Colcemid was given, only the aortic "elastin," cholesterol, and calcium remained moderately elevated, whereas the collagen and phosphorus content was normal. However, when rabbits receiving the atherogenic diet were treated with both EHDP (lower dose) and Colcemid in combination, the accumulations of those aortic components were prevented completely, with the exception of the cholesterol content, which still was slightly above normal.

Table 3 shows the constituents of the isolated elastin preparation of aorta. In rabbits fed the atherogenic diet without drugs there also were large increases in the elastin cholesterol, calcium, and phosphorus content. When the rabbits were treated with both dosage levels of EHDP or Colcemid alone, there was a marked and comparable reduction of all elastin constituents, although not to normal. In contrast, combined treatment with EHDP and Colcemid resulted in complete suppression of these elastin changes. Similarly, there was a marked increase in polar amino acids in the isolated elastin preparation from the aortas of untreated rabbits on the atherogenic diet which was not prevented by treatment with EHDP or Colcemid alone. However, treatment with both drugs resulted in complete inhibition of the changes in amino acids, with the elastin from treated rabbits on the atherogenic diet having an amino acid composition comparable to that of normal aortic elastin from the control animals.

Discussion

None of the drugs tested lowered the massively elevated serum cholesterol in rabbits fed the atherogenic diet; yet they all suppressed atherogenesis substantially with the combination therapy of EHDP and Colcemid, virtually halting all atherosclerotic processes. As recently summarized by Ross and Glomset, the main processes leading to plaque formation appear to be: (1) increased
FIGURE 3  A–C: Serial histological sections of a characteristics aortic plaque of a rabbit fed the atherogenic diet without drug treatment. Approximately the top half of the micrographs represents the markedly raised intima; the bottom half, the subintimal media. A: Verhoeff's-Van Gieson, showing that the raising of the intima was due to an increased cellularity interspersed with presumably collagenous loose connective tissue fibers (light grey); the increased intimal cells appeared to be smooth muscle type cells as well as "foam cells" of unidentifiable origin. In the center and the bottom of the micrograph, deranged and fragmented intimo-medial elastica (black) and considerable deposition of dense collagen fibers (dark grey) are seen, as well as some necrotic subintimal foam cells (right below center). B: Yasue's calcium stain—light green, showing dense deposition of calcium (black) mainly on the fragmented intimo-medial plaque elastica. C: Hematoxylin-Oil Red O, showing lipid deposition (black) in intimal and subintimal cells and on the deranged plaque elastica. Scales are 100 μm.
FIGURE 4  A and B: Serial sections through a typical aortic plaque of a rabbit fed atherogenic diet and treated simultaneously with 20 mg EHDP/kg body weight. Rabbits treated with 40 mg EHDP/kg body weight showed similar suppression of lesions. A: Verhoeff’s-Van Gieson, showing suppression of collagen accumulation, marked inhibition in the increase of intimal cellularity and of elastica derangement. B: Yasue’s-light green, showing absence of calcification. Scales are 100 \mu m.
permeability of arterial endothelium to macromolecules such as lipoproteins; (2) migration of smooth muscle cells (SMCs) from the media into the intima; (3) proliferation of these cells by mitosis; (4) secretion by the increased intimal SMCs of excessive amounts of collagen, elastin, and glycosaminoglycans; as well as (5) endocytosis (phagocytosis) of lipids and/or lipoproteins by intimal SMCs.

According to Marx,21 the cells appear to require for these cellular functions an intact system of contractile microfilaments, microtubules, and energy. Microfilaments

TABLE 2 Components of Aortic Intima-Media of Control Rabbits and Rabbits Fed the Atherogenic Diet with and without Drugs*

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Collagen</th>
<th>“Elastin”</th>
<th>Total cholesterol</th>
<th>Calcium</th>
<th>Nonlipid phosphorus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>4.7 ± 0.6</td>
<td>25.1 ± 1.5</td>
<td>0.3 ± 0.1</td>
<td>0.01 ± 0.007</td>
<td>0.07 ± 0.03</td>
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<tr>
<td>Atherogenic diet without drugs</td>
<td>7.5 ± 0.9†</td>
<td>37.1 ± 1.7†</td>
<td>2.8 ± 0.5†</td>
<td>0.05 ± 0.012†</td>
<td>0.14 ± 0.03†</td>
</tr>
<tr>
<td>Atherogenic diet + EHDP (20 mg/kg B.W.)</td>
<td>5.8 ± 0.7‡</td>
<td>32.8 ± 1.4†</td>
<td>1.1 ± 0.3†</td>
<td>0.02 ± 0.015‡</td>
<td>0.11 ± 0.05</td>
</tr>
<tr>
<td>Atherogenic diet + EHDP (40 mg/kg B.W.)</td>
<td>6.0 ± 1.1‡</td>
<td>31.5 ± 1.6†</td>
<td>1.3 ± 0.5†</td>
<td>0.03 ± 0.018‡</td>
<td>0.11 ± 0.06</td>
</tr>
<tr>
<td>Atherogenic diet + Colcemid (0.06 mg/kg B.W.)</td>
<td>5.1 ± 0.6</td>
<td>29.9 ± 1.6‡</td>
<td>1.7 ± 0.7†</td>
<td>0.02 ± 0.003‡</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>Atherogenic diet + EHDP (20 mg) and Colcemid (0.06 mg/kg B.W.)</td>
<td>4.9 ± 0.5</td>
<td>25.2 ± 1.6</td>
<td>0.6 ± 0.3‡</td>
<td>0.01 ± 0.005</td>
<td>0.08 ± 0.04</td>
</tr>
</tbody>
</table>

* Absolute amounts in mg/whole aorta per kg body weight (B.W.); results are expressed as mean ± s.d.
† P < 0.01 compared to control.
‡ P < 0.05 compared to control.
may serve as the connecting links between cell membrane components, including receptors, and microtubules. Microtubules are involved in cell division and cell motility. They direct the vesicular transport of cell membrane-bound extracellular material into the cells (endocytosis) as well as the secretion of intracellular products such as proteins, including, at least in part, the secretion into the extracellular space of procollagen22 and, possibly, of tropoelastin. The energy for these cellular processes presumably is provided by high-energy phosphates such as ATP and GTP. It is possible that the marked increase in total calcium in the serum and of calcium and phosphorus in atherosclerotic aortas of rabbits that did not receive drug treatment could have provided an increased availability of phosphorus for an enhanced rephosphorylation of deoxyribonucleotides in cells preparing for mitosis.

In any type of muscle cell, including SMCs, the relaxation-contraction cycle is dependent on the myoplasmic concentration of Ca$^{2+}$.\textsuperscript{23} with increasing amounts of Ca$^{2+}$ leading to increasing contraction of the cellular contractile proteins. Calcium ions also have been implicated as intracellular messengers in the regulation of other important cellular functions such as chemotactical movement\textsuperscript{24} and the secretion of proteins.\textsuperscript{25}

Of the drugs used in the present study, the beneficial actions of Colcemid may be explained in part by its ability to disrupt microtubules. As discussed by Yin et al.,\textsuperscript{26} Colcemid, like colchicine and vinblastine, binds specifically to the tubulin subunits and thereby disrupts microtubular assembly. This in turn appears to suppress, at least in part, excessive proliferation and mobility of cells as well as to inhibit enhanced endocytosis and secretion of connective tissue proteins and, possibly, also of glycosaminoglycans. The normalization of the elevated serum calcium and the lowering of the arterial calcium content by Colcemid presumably also inhibited some of these cellular processes. In addition, it suppressed deposition of calcium minerals in atherosclerotic lesions, especially on the intimo-media lamina.

The antiatherosclerotic actions of EHDP are more difficult to understand, with the exception of its inhibition of arterial calcification. EHDP has a well-known inhibitory effect on calcium mineral deposition,\textsuperscript{6} including that in arteries, and suppresses arterial calcification as well as lesion formation in vitamin D-cholesterol-induced atherosclerosis.\textsuperscript{27} However, Guilland et al.\textsuperscript{28} have shown that it also can inhibit calcium ion transport through a cellular membrane. Whether this diphosphonate in vivo has an inhibiting effect on the energy metabolism of cells and other cellular functions requiring Ca$^{2+}$ is not known, although the considerable reduction of the elevated total aortic calcium in EHDP-treated rabbits on the atherogenic diet suggests this possibility.

Treatment with Colcemid or EHDP alone partially suppressed atherogenesis, with both dose levels of EHDP being about equally effective. When given together, their effect appeared to be additive and resulted in complete inhibition of arterial accumulations of calcium, collagen, elastin, and glycosaminoglycans as well as in the prevention of abnormalities of elastin.

Elastin preparations isolated from atherosclerotic lesions by hot alkali extraction invariably have an increased content of polar amino acids,\textsuperscript{16,29} which appears to be prerequisite for an increased binding of cholesterol to the normal elastin protein.\textsuperscript{30} Keeley and Partridge\textsuperscript{29} have suggested that the abnormal amino acid composition of lesion “elastin” might be due to a binding of other proteins by calcium bridges to the original elastin in atherosclerosis. As discussed by Yin and Blumenthal,\textsuperscript{28} a number of workers have shown that the arterial elastica appears to be the principal target tissue for calcium mineral deposition in atherosclerosis and aging. Calcification of arterial elastica in preference to collagen also has

### Table 3

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Total cholesterol</th>
<th>Calcium</th>
<th>Nonlipid phosphorus</th>
<th>Aspartic acid$^t$</th>
<th>Glutamic acid$^t$</th>
<th>Lysine$^t$</th>
<th>Arginine$^t$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>10.4 ± 2.3</td>
<td>0.2 ± 0.04</td>
<td>0.8 ± 0.07</td>
<td>4.3</td>
<td>16.3</td>
<td>4.1</td>
<td>4.0</td>
</tr>
<tr>
<td>Atherogenic diet without drugs</td>
<td>37.4 ± 3.5$§$</td>
<td>1.1 ± 0.30$§$</td>
<td>1.3 ± 0.40$§$</td>
<td>13.1</td>
<td>31.0</td>
<td>9.3</td>
<td>8.9</td>
</tr>
<tr>
<td>Atherogenic diet + EHDP (20 mg/kg B.W.)</td>
<td>15.7 ± 4.8$§$</td>
<td>0.3 ± 0.05$§$</td>
<td>1.1 ± 0.32$§$</td>
<td>8.2</td>
<td>23.3</td>
<td>8.3</td>
<td>7.9</td>
</tr>
<tr>
<td>Atherogenic diet + EHDP (40 mg/kg B.W.)</td>
<td>17.2 ± 6.3$§$</td>
<td>0.3 ± 0.06$§$</td>
<td>0.9 ± 0.14$§$</td>
<td>9.4</td>
<td>19.3</td>
<td>10.5</td>
<td>6.6</td>
</tr>
<tr>
<td>Atherogenic diet + Colcemid (0.06 mg/kg B.W.)</td>
<td>26.4 ± 9.8$§$</td>
<td>0.4 ± 0.16$§$</td>
<td>1.0 ± 0.31$§$</td>
<td>11.5</td>
<td>21.9</td>
<td>7.9</td>
<td>8.5</td>
</tr>
<tr>
<td>Atherogenic diet + EHDP (20 mg) and Colcemid (0.06 mg/kg B.W.)</td>
<td>8.2 ± 0.8</td>
<td>0.2 ± 0.03</td>
<td>0.8 ± 0.11</td>
<td>3.8</td>
<td>14.7</td>
<td>3.3</td>
<td>4.4</td>
</tr>
</tbody>
</table>

* Results in columns 2-4 are expressed as mean ± SD.
† Results in columns 5-8 are expressed as averages; three aortas per group.
§ The remaining elastin amino acids were not significantly different in all groups.
‡ P < 0.01 compared to control.
¶ P < 0.05 compared to control.
SUPPRESSION OF CALCIFIC FIBROUS-FATTY PLAQUES/Krams and Chan

been shown in vitro. The reduced availability of calcium and phosphorus in the arteries of drug-treated animals on the atherogenic diet may have been instrumental in the suppression of elastica calcification, including binding of other proteins and of increased amounts of cholesterol to the arterial elastin.

On the other hand, alkali-isolated newly synthesized elastin from the growing rat aorta, as well as from arterial smooth muscle cell culture, also shows increased amounts of acidic (polar) amino acids, indicating the presence of microfibrillar glycoproteins or other proteins. It is of interest that recent preliminary radiochemical studies in our laboratory with rabbits on the same atherogenic diet as in the present report have indicated an increased biosynthesis of elastin and collagen in atherosclerotic aortas in vivo; this increased connective tissue synthesis, or at least the secretion of the respective precursors, appear to be decreased by simultaneous treatment of the animals with Colcemid or EHDP. Inhibition of proline hydroxylation by EHDP has been shown in organ cultures of bone. As discussed by Grant and Prockop, unhydroxylated procollagen appears not to be extruded by the cells. Another explanation for the decrease in the aortic connective tissue content in treated rabbits may have been stimulation of its degradation. Colchicine has been shown to increase collagenase activity of synovial explants up to 10-fold. The closely related Colcemid may have had a similar effect.

The single common denominator of the lesion-inhibiting effect of both drugs presented in this report was the reduction of elevated serum and arterial calcium (and phosphorus) content in rabbits on the atherogenic diet. This suggests that deposition of calcium and/or calcium minerals may play an important part in atherogenesis beyond that of the well-known later calcification of plaques.

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References

20. Ross R, Glomset JA: The synthesis, or at least the secretion of the respective precursors, appear to be decreased by simultaneous treatment of the animals with Colcemid or EHDP. Inhibition of proline hydroxylation by EHDP has been shown in organ cultures of bone. As discussed by Grant and Prockop, unhydroxylated procollagen appears not to be extruded by the cells. Another explanation for the decrease in the aortic connective tissue content in treated rabbits may have been stimulation of its degradation. Colchicine has been shown to increase collagenase activity of synovial explants up to 10-fold. The closely related Colcemid may have had a similar effect.

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