Antiatherosclerotic Action of Elastase
—With Special Reference to Its Effect on Elastic Fibers—

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Summary

In order to determine the antiatherosclerotic action of elastase, the aortas from four groups of rats, group N fed a normal diet, group D fed an atherogenic diet with vitamin D₂, group N+E fed a normal diet with elastase, and group D+E fed an atherogenic diet and vitamin D₂ treated with elastase, were studied histologically after 3, 6, 9, and 12 weeks of feeding.

Group D displayed remarkable atheromatous changes, but the changes were insignificant in group D+E. The histopathological findings in group D consisted of marked rarefaction, fat deposition, and atheroma in the subendothelial tissue and tunica intima; van Gieson’s staining revealed a conspicuous decrease in elastic fibers; the remaining elastic fibers had such abnormalities as a tortuous course, bends, and fissures. In group D+E, on the other hand, there were few foam cells in the subendothelial tissue and tunica intima and much less marked rarefaction of interstices and fat deposition. Further, the arrangement of elastic fibers was not very disorderly, and the decreases in elastic fibers and their fissures were moderate. In sharp contrast to group D, which showed marked calcium deposition, group D+E was found to have low calcium deposition. No macroscopic and histopathologic abnormalities were found in either group N or N+E.

Elastase exhibited an inhibitory effect on experimental atherosclerosis in rats and the ability to regulate the degradation and biosynthesis of elastin, resulting in the regeneration of elastic fibers.

I. Introduction

Atherosclerosis, the most frequent of all sclerotic disturbances in the aorta, begins with the formation of fatty streaks. Fatty streaks in turn arise from a cell reaction (intimal edematous reaction) that is induced in the subintimal region of the artery when the tunica intima of the arterial wall is permeated by plasma protein. Fatty streaks give rise to intimal thickening and modified smooth muscle cell proliferation, which in turn develops into diffuse intimal thickening. As a result, collagen and elastic fibers proliferate. In the end, the fatty streaks are covered by modified smooth muscle cells and intercellular fibers, and lipid deposited on the covering forms fibrous plaques. The formation of fibrous plaques is followed by fragmentation and breakdown of con-

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nective tissue, especially intimamedia elastic fibers. These sequential events are generally believed to constitute the process of development of typical atherosclerosis. As can be inferred from this description of atherosclerosis, the fibrous plaque, which is also called an atherosclerotic plaque, provides an important clue to the judgement of the atherosclerotic condition of arterial wall tissue.

In atherosclerosis, the arterial tissue levels of elastic fibers and elastin are decreased, and elastin is altered qualitatively. Further, the level of elastin in the arterial wall becomes reduced with increasing age. When all these findings are coupled together, it can readily be deduced that elastin plays an important physiological role in the development and progress of atherosclerosis. However, much less information is available on elastin than on collagen fibers.

In 1950 Baló and Banga reported for the first time that elastase extracted from pancreas degraded elastin. Since then, some reports dealing with the antiatherosclerotic action of elastase have appeared, but they only discuss the effect of elastase on blood levels of cholesterol and lipoproteins and its inhibitory effect on the formation of atherosclerotic plaques. Little information has been obtained concerning the fragmentation of elastic fibers and the reparatory or inhibitory action of elastase on elastic fiber fragmentation, especially the role of elastase relative to the degradation and biosynthesis of elastin.

The work presented here is an investigation undertaken to determine the inhibitory effect of elastase on the fragmentation of elastic fibers and its antiatherosclerotic action in rats fed with 2% cholesterol and intravenously administered with vitamin D₃ in a dose of 350,000 IU/kg for 4 days to induce experimental atherosclerosis.

II. Materials and Methods

1. Animals

SD-SLC male rats (body weight, 210 g) at 8 weeks of age were used in this experiment.

2. Methods

The rats were divided into four groups of 10 each: group D received an atherogenic diet (containing 2% cholesterol, 0.5% cholic acid, 10% lard, 5% cane sugar, 0.2% methylthiouracil, and 82.3% basal diet) for 6 weeks, 35,000 units/kg body weight of vitamin D₃ for 4 days, and oral olive oil solution (0.5 ml/100 g body weight) as well; group N received a normal diet; group N+Ela received a normal diet, 450 ELU/kg/day of elastase (Elaszym® of Eisai Co.), and a daily injection of 450 ELU/kg/day of elastase into the psoas muscle; and group D+Ela received the atherogenic diet with vitamin D₃ for 4 days and elastase as above. The observation period was 12 weeks.

3. Histological Examination

Rats from all groups were sacrificed 3, 6, 9, and 12 weeks after onset of the experiment. Their aortas and coronary arteries were fixed in formalin and stained with hematoxylin and eosin, van Gieson elastin stain, Sudan III fat stain, and AMPS.
4. Elastase Preparation

The elastase used in this experiment was prepared from pancreatin obtained through activation of pancreas, comprised of 234 amino acids and having 4 S-S bonds. Structurally, it bears a resemblance to trypsin and chymotrypsin; its N terminal also resembles that of trypsin and chymotrypsin. According to Hall’s analysis, elastase is composed of component E₁ having elastproteinase activity and component E₂ having elastmucase lipolytic lipase activity.

III. Gross and Histological Findings of Aortas

1. Groups N and N+Ela

Both the thoracic and abdominal aortas from groups N and N+Ela were unremarkable; their lumens had a smooth surface free of fatty streaks and atherosclerotic lesions. The results of staining with hematoxylin and eosin and van Gieson did not differ between group N and group N+Ela; elastic fibers that stained dark brown with van Gieson were present abundantly in the region from the intima to the media, and the course and arrangement of elastic fibers were normal (Fig. 1).

![Fig. 1. Effect of elastase on the course and arrangement of normal arterial elastic fibers ×100](image)

2. Groups D and D+Ela

The thoracic and abdominal aortas from groups D and D+Ela were found to have atherosclerotic lesions of differing process and varying degree, and there were obvious differences in vascular lesions revealed by autopsies performed in the same experimental stage. Specifically, there were atherosclerotic lesions in the aortic arches as well as in the thoracic and abdominal aortas from group D, whereas atherosclerotic lesions found
in group D+Ela were small in size, narrow, and showed a much lower degree of sclerosis.

Histological examination of aortas harvested from group D and stained with hematoxylin and eosin disclosed that interstitial rarefaction and remarkable lipid deposits were present in the region from the subendothelial layer to the tunica media and that elastic fibers staining with van Gieson were markedly decreased in number. Further, the remaining elastic fibers pursued an irregular, tortuous course and were bent and fragmented. Fine elastic fibers were strikingly sparse (Fig. 2).

In group D+Ela, on the other hand, staining with hematoxylin and eosin revealed the presence of foam cells in the region from the subendothelial layer to the tunica intima, but they were limited in number, and interstitial rarefaction was not as marked as in group D. The formation of fibrous plaques due to lipid deposition was also much less marked. The course of elastic fibers stained with van Gieson was not very tortuous, and their arrangement was not disorderly. At some sites not only had elastic fibers sustained little damage, but they had also maintained the normal structure seen in group N. As described above, the reduction in the number of elastic fibers was minimal, fine elastic fibers were retained, and elastic fiber fragmentation was slight in aortas (Fig. 3).

The changes described above can be considered to be lesions of relatively early sclerosis; for whereas in group D the atherosclerotic lesions stained indigo-blue, those of group D+Ela stained only light blue with AMPS. This finding suggests that the fragmentation of elastic fibers in lesions of experimental atherosclerosis induced with a high cholesterol diet may be inhibited or repaired with elastase.

In more advanced atherosclerotic lesions in group D calcification was remarkable, especially in the neighboring regions of the cardiac coronary arteries and heart muscle, while the changes in group D+Ela were slight\textsuperscript{19}. The findings obtained in group D+Ela


Table 1. Differences in arterial wall structure between treated and control groups.

<table>
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<tr>
<th>Foam cell</th>
<th>Fat</th>
<th>Elastic fibers</th>
<th>Fibrosis</th>
<th>Hyalinization</th>
<th>Calcification</th>
<th>AMPS</th>
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**Fig. 3.** Elastic fibers from group D+Ela (at week 6) x200

These effects of elastase were also recognized in the cardiac coronary arteries. The cardiac coronary arteries from rats maintained a cholesterol free normal diet showed no...
gross differences at all from those of rats treated with elastase. Histologically, there were no changes in arterial wall structure in rats of either group, and the elastic fibers of the internal elastic membrane in both groups were found to pursue a normal course and were not remarkable in any respect when stained with van Gieson.

In sharp contrast to the rats of groups N and N+Ela, those of group D were found to have foam cells and lipid deposits in the regions from the subendothelial layer to the tunica intima as seen in aortic lesions of atherosclerosis. Further, elastic fibers pursued a tortuous course and were markedly fragmented, and fine elastic fibers were sparse. In group D+Ela, on the other hand, all these changes were much less remarkable.

Another finding requiring special mention is the fact that calcification of cardiac coronary arteries and heart muscle in group D was remarkable, but the degree of calcification in group D+Ela was very low.

The serum levels of cholesterol were determined concurrently with the histological study. In group D+Ela, the mean serum level of HDL-cholesterol was elevated, while in group D it was depressed. The mean serum level of cholesterol in group D+Ela was not depressed, but the ratio of total cholesterol to HDL-cholesterol was lower in group D+Ela than in group D. Under an electron microscope, many macrophages (foam cells) phagocytosing lipid appeared in group D around week 3. Cytoplasmic lipid was further increased at week 6. Fat appeared even in lysosomes and cell nuclei were pyknotic. At week 9, myelin appeared, and the sites corresponding to elastic fibers were hydropic and looked cloudy (Fig. 4). In group D+Ela, on the other hand, macrophages appeared at week 3, but vacuoles containing lipid were small in number. Unlike group D, there were mitochondria in the D+Ela cytoplasm, and, at week 6, mitochondria were much higher in number, while vacuoles containing lipid were reduced in number (Fig. 5). This fact sug-

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*Fig. 4. Macrophage from group D (at week 3)*

There are many cytoplasmic vacuoles. ×4500
Fig. 5. Group D+Ela (at week 6)
There are many mitochondria but not many vacuoles in cytoplasm. ×9000

gests that macrophages were activated by elastase so that they produced various kinds of enzymes. At week 9, there were no longer vacuoles in monocytes and macrophages and there rough-surfaced endoplasmic reticulum appeared in addition to mitochondria. In other words, biosynthesis of protein had apparently started by week 9. It is particularly of interest to note that at week 9 there were myofilaments in the cytoplasm of modified

Fig. 6. Group D+Ela (at week 9)
There are myofilaments in smooth muscle cells and granular matter and amorphous components around them. ×9000
smooth muscle cells. There was a lot of granular matter containing amorphous components around the cell. There were scattered microfibrils surrounding these amorphous components (Fig. 6). Group D+Ela was in sharp contrast to group D (Fig. 7) in that a great deal of granular matter appeared to cover the amorphous components at the sites where elastic fibers had presumably been, and microfibrils were present.

**Fig. 7.** Group D (at week 9)

There is no granular matter at the sites that correspond to elastic fibers. These sites appear edematous and opaque. ×12000

**Fig. 8.** Group D+Ela (at week 9)

At the sites that correspond to elastic fibers, a great deal of granular matter and amorphous components surrounded by microfibrils occur. ×12000
centering around the granular matter (Figs. 8 and 9). The inference can be made from these findings that in group D+Ela the macrophages were activated by elastase around week 6 and the monocytes and macrophages started producing not only enzymes but also protein at week 9. Further, the smooth muscle cell began to secrete amorphous components (proelastin). It can therefore be reasonably inferred that these events led to biosynthesis of elastin, ultimately setting the stage for regeneration of microfibrils (elastic fibers)\(^{13}\).

### IV. Discussion

1. **Compositional Differences between Arterial Wall Fibrous Plaque and Normal Elastic Fiber Elastin**

Typical aortic sclerosis caused by cholesterol is atherosclerosis with formation of characteristic fibrous plaque atheroma. Kramsch\(^{10,11}\) maintains that the development of atherosclerosis begins with the permeation of lipoprotein (LDL) into arterial elastin peptide. As a result, arterial elastin peptide changes to altered lipoprotein, giving rise to a change in the protein composition of arterial elastin.

According to Kramsch\(^{10,11}\), in the normal aortic intima the protein level is normally 986.9 mg/g elastin, while in the atherosclerotic plaque it is 777.2 mg/g elastin. The elastin-cholesterol level in the normal intima is 7.5 mg/g elastin, while in the atherosclerotic lesion it is 25 times as high as in the normal intima at 176.2 mg/g elastin. Free cholesterol is 30 times higher; phospholipid, 10 times higher; and neutral fat, 2 times higher than in the normal intima. Further, the amino acid composition of protein also differs from plaque elastin to normal aorta elastin; in the plaque lipid bound elastin,
the levels of polar amino acids such as aspartic acid, threonine, serine, glutamic acid, lysine, histidine, and arginine are elevated, while the levels of nonpolar amino acids such as isodesmosine, desmosine, and lysinonorleucine are remarkably depressed.

As a result of this change in protein composition lipid is attracted toward the plaque elastin and deposited on the plaque elastin surface, followed by phagocytosis by macrophages. When the macrophage is destroyed, lipid is released into the spaces between cells, causing damage to the external elastic membrane of the media as well as the internal elastic membrane.

2. Antiatherosclerotic Action of Elastase

When the effect of antiatherosclerotic agents is to be determined in experimental atherosclerosis induced with a high cholesterol diet, their cholesterol-lowering effect is referred to as a parameter. In recent years increasing attention has been focused on the HDL-cholesterol lowering effect of antiatherosclerotic agents. Apart from the cholesterol-lowering effect of elastase, it is necessary to determine whether or not it affects the pathologic condition of the intima and media with fibrous plaques being formed, counteracts the mechanism by which lipids are deposited on the arterial wall, and helps maintain elastic fibers intact. There are reports that elastase lowers plasma levels of cholesterol in hypercholesterolic mouse, lowers liver tissue levels of cholesterol and triglycerides in hypercholesterolic rabbit, and increases lipoprotein lipase activity in human hyperlipidemia. It has also been reported that when elastase is administered to SHR maintained on a high cholesterol diet, intimal thickening, vascular necrosis and fibrous plaque formation are less marked than in untreated SHR. Further, there are also reports that the development of atherosclerosis and fatty liver in rabbits maintained on a high cholesterol diet is inhibited by elastase.

All these reports provide suggestive evidence that elastase inhibits the progress of atherosclerosis and promotes lipolipase activity in animals with experimental arteriosclerosis.

3. Effect of Elastase on Elastic Fibers

Baló's brilliant discovery in biochemistry established that elastase can degrade elastin in vitro. However, a search of the literature reveals few reports dealing with the effect of elastase on elastic fibers in experimental atherosclerosis. The sparsity of work done in this field may be due to the fact that lesions take a long time to develop in elastic fibers in experimental animals.

In rats with experimental atherosclerosis induced by an atherogenic diet and vitamin \( D_2 \), marked fragmentation, loss and tortuosity of elastic fibers were noted in a short time; rats given an atherogenic diet and vitamin \( D_2 \) plus elastase showed only mild alterations in elastic fibers. Furthermore, depending on the site, the elastic fibers were orderly and pursued a normal course. On the other hand, the fact that there were no differences in elastic fibers between elastase-treated and normal rats, may appear to be in conflict with the theory of Baló and Banga. Several assumptions could be made from these findings—(1) elastase does not act on normal elastin but on only degenerated
elastin, (2) after degradation of degenerated elastin, biosynthesis of elastin occurs so that the regeneration of elastic fibers is promoted. In our electron microscopic study it was revealed that, around week 9 of treatment with elastase, a great deal of granular matter appeared, enveloping amorphous components around modified smooth muscle cells as well as in the region of elastic fibers; microfibrils occurred around amorphous components. In this connection, it is suggested that following the dissolution of elastic fibers by elastase, the biosynthesis of elastin and the regeneration of elastic fibers may be promoted.

4. Mechanism of Degradation of Lipid Bound Elastin by Elastase

Kagan proposed an electrostatic charge hypothesis on the basis of his discovery that cations outnumbered anions in 1000 amino acid residues of normal elastin and that elastase, carrying itself a positive charge, would not easily react with normal elastin. In atherosclerosis, on the other hand, arterial elastin is lipid-bound and thus negatively charged. Accordingly, he proposed that lipid-bound elastin might easily combine with positively charged elastase, producing the degradation of elastin followed by biosynthesis of elastin, and promoting the regeneration of elastic fibers.

V. Conclusions

1) Elastase produced an inhibitory effect on the atherosclerosis of thoracic and abdominal aortas induced by oral administration of 350,000 IU/kg/day of vitamin D in rats that were maintained on an atherogenic diet (containing 2% cholesterol, 0.5% cholic acid, 10% lard, 5% cane sugar, 0.2% methylthiouracil, and 82.3% basal diet).

2) Elastase inhibited the splitting and diminution of elastic fibers in atherosclerotic lesions. No differences, however, were recognized in elastic fiber findings between normal rats and experimental ones that received elastase.

3) The mechanism of action of elastase suggested by the results of this investigation consists of selective degradation of lipid bound elastin. It appears to be by this mechanism of action that elastase promotes the biosynthesis of elastin and the regeneration of elastic fibers. In short, it may be said that elastase exhibits an antiatherosclerotic action through regulation of elastin metabolism.

References


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