

## Preventive effects of elaszym on malondialdehyde-induced arterial lesions in chickens

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**SUMMARY :** Thirty-nine hens, 3 weeks of age, were divided into 6 groups with different treatment as follows ; group A with corn oil injection, group B with corn oil injection and cholesterol feeding, group C with injection of a malondialdehyde solution, group D with injection of malondialdehyde solution and administration of elaszym, group E with injection of malondialdehyde solution and cholesterol feeding and group F with injection of malondialdehyde solution, cholesterol feeding and administration of elaszym. Corn oil and 50% malondialdehyde in corn oil were subcutaneously injected at respective doses of 0.5 and 1ml per kg body weight 12 times for 4 weeks. A diet containing 1% cholesterol without supplementary fat was fed *ad libitum*. Elaszym was orally administered daily at a dose of 1,500 EU per Kg body weight.

A slight increase of plasma cholesterol was seen in group B. A small number of degenerate cells were seen in groups A and B. The plasma malondialdehyde levels were markedly increased in groups C and E. The result of malondialdehyde injection was a significant increase in the number of degenerate cells without stainable lipid in the abdominal aorta from group C. The feeding of a cholesterol-containing diet in combination with malondialdehyde injection produced numerous degenerate cells with or without stainable lipid in the abdominal aorta from group E. The administration of elaszym decreased the tissue level of malondialdehyde and the frequency of degenerate cells with or without stainable lipid in the abdominal aortas from groups D and F.

### INTRODUCTION

An earlier study by GLAVIND *et al.* (5) indicated that the human atherosclerotic aorta contains more oxidized lipid than the normal aorta. Cultler and Schneider (4) reported that a subcutaneous injection of linoleate hydro-

peroxide results in increased incidence of myocardial fibrosis and aortic lesions in rabbits. We previously reported that the two factors of hyperlipidemia and peroxidation may be related (13). Endothelial cells in tissue culture are also injured and killed by low density lipoprotein which contains peroxidation products (6). Thus lipid peroxidation products

have been shown to have an atherogenic effect in experimental animals and humans. However, little information is available concerning the ultrastructure of arterial injury by malondialdehyde, which is thought to be a product of the peroxidation of unsaturated fatty acid.

BALO and BANGA (2) reported that persons suffering from atherosclerosis had less elastase activity in the pancreas than did healthy individuals. There have been many reports on the possible anti-atherosclerotic action of elastase in humans (10, 21) and animals (7, 12, 14, 16). The present experiment was undertaken to answer the following two questions: a) How does malondialdehyde cause ultrastructural changes in the arterial wall? b) Does elastase have a preventive effect on injury to the arterial wall?

## MATERIALS AND METHODS

A total of thirty-nine female chickens were used in this study. As shown in Table 1, the chickens were randomly divided into 6 groups, which received the following treatment for 4 weeks: group A, corn oil injection; group B, feeding of cholesterol-containing diet; group C, injection of malondialdehyde solution; group D, injection of malondialdehyde solution and administration of elaszym; group E, injection of malondialdehyde solution and feeding of a cholesterol-containing diet; group F, injection of malondialdehyde solution, feeding of a cholesterol-containing diet and administration of elastase. A commercial chicken mash was used as the basal diet (Kyoei

Co Ltd.). A 1% cholesterol-containing diet was fed ad libitum without supplementing additional fat. Corn oil was applied in order to slow down the absorption rate of malondialdehyde acetal and to avoid sudden death of the chickens after the malondialdehyde injection. For injection of malondialdehyde acetal, a 50% solution of malondialdehyde acetal (1, 1, 3, 3, -tetraethoxypropane, Sigma Chemical Co., T 9889) in corn oil was prepared and subcutaneously injected at a dose of one ml per kg body weight 12 times during the whole experimental period. Elastase (Elaszym, Eisai Co. Ltd., Japan) was administered orally every day at a dose of 1500 EL units per kg body weight.

For biochemical analysis, blood was collected by decapitation and then stabilized with heparin. Plasma cholesterol levels were determined by the method of ALLAIN *et al.* (1). Serum lipid peroxidation was determined by the YAGI method of fluorometric assay (20). For determining tissue malondialdehyde, the whole aorta was collected. Peroxidation standards, synthesized by the method of TROMBLY and TAPPEL (19), were run by thin layer chromatography and visualized by UV light. Fluorescence of the tissue extracts was determined at 435 nm emission and 350 nm excitation.

For electron microscopic examination, abdominal aortas were collected and immersed in a cacodylate-buffered 2% glutaraldehyde solution (pH 7.4). They were then cross-sectioned into at least three small pieces. These specimens were post-fixed in cacodylate-buffered 1% osmium tetroxide, serially dehydrated in

Table 1. Summary of experimental design

Group	Number of chickens	Treatments			
		CO	CH	MDA	ELA
A	6	+	-	-	-
B	6	+	+	-	-
C	7	+	-	+	-
D	7	+	-	+	+
E	6	+	+	+	-
F	7	+	+	+	+

CO : injection of corn oil

CH : feeding of 1% cholesterol-containing diet

MDA : injection of malondialdehyde

ELA : administration of elaszym

increasing concentration of alcohol and embedded in spur resin. Semithin sections were cut with a MT2C ultramicrotome and stained with alkaline toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a JEM-2000EX electron microscope. Three spur resin-embedded tissue blocks from each chicken were examined for quantitative comparison of the frequencies of degenerate cells with or without stainable lipid. Cell counts were made routinely at a magnification of  $\times 6000$ , and higher magnifications were used for examination of details.

## RESULTS

**Biochemical analysis :** Figure 1 presents the plasma levels of total cholesterol. Increases in the plasma cholesterol levels were slight in groups B and E, which had been fed a cholesterol-containing diet. A slight reduction of the plasma cholesterol was seen in group F, which was treated by oral administration of elastase. The subcutaneous injection of malondialdehyde (groups C and E) resulted in an increased concentration in the level of plasma malondialdehyde which was twice as high as that of group A (Fig. 2). A moderate reduction in the plasma malondialdehyde level was seen in groups D and F. As shown in Figure 3, malondialdehyde accumulation was more marked in arterial tissue than in plasma since injected peroxidized are rapidly metabolized (4). Elastase administration also lowered

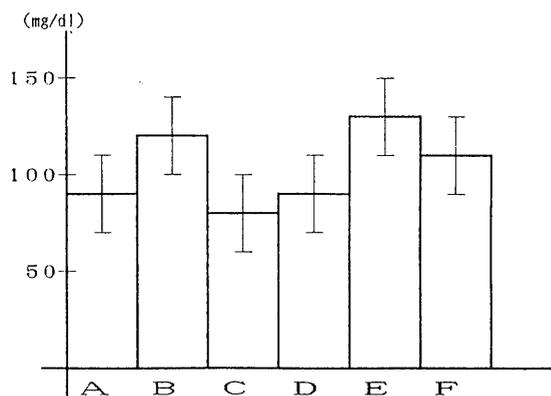


Fig. 1. Plasma total cholesterol levels in each experimental group.

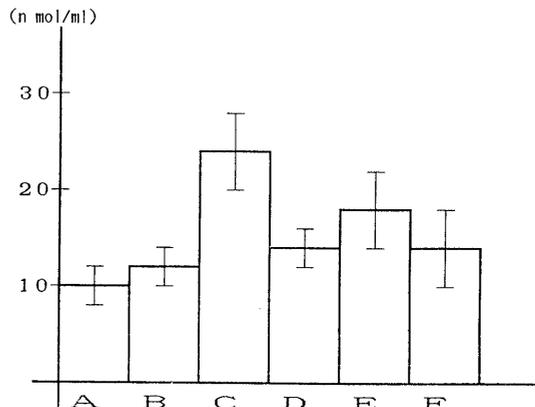


Fig. 2. Plasma level of malondialdehyde in each experimental group.

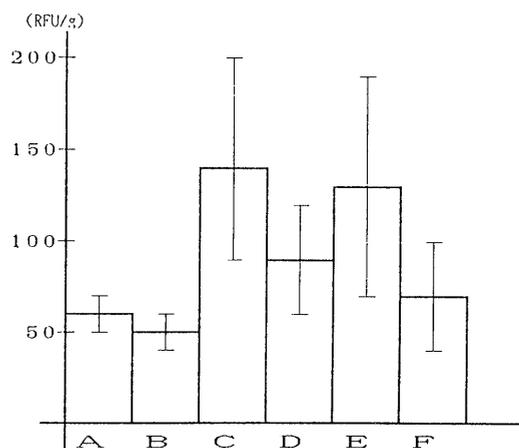


Fig. 3. Concentration of malondialdehyde in aortic tissue in each experimental group.

the malondialdehyde concentration in aortic tissues (groups D and F).

**Electron microscopic findings :** The abdominal aorta from all groups was a muscular type of artery with internal and external elastic laminae. These elastic laminae were frequently fenestrated and incomplete. Spontaneous plaques were occasionally seen and demarcated from the underlying media by the internal elastic lamina. These plaques were composed of more slender smooth muscle cells than those of the tunica media. They were arranged in parallel layers (Fig. 4). Plaque cells sometimes had distension of the endoplasmic reticulum. The basement membranes of the plaque smooth muscle cells occasionally showed irreg-

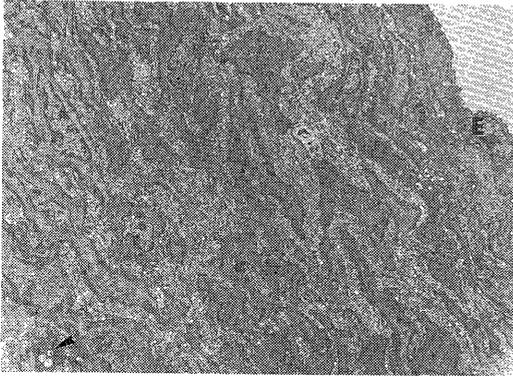


Fig. 4 Plaque of an abdominal aorta from group A. Note multilayered smooth muscle cells. A few small lipid droplets (arrow) are seen at the bottom of this picture. E : endothelial cell ( $\times 2,400$ ).

ular thickening. The media of the abdominal aorta contained smooth muscle cells and occasional elastic fibers. The adventitia was composed of packed collagen fibers, occasional slender elastic fibers, and stellate fibroblasts.

Abdominal aortas from group A sometimes showed cell degeneration both in the thickened intima and in the inner media. Small lipid vacuoles were occasionally observed in plaque smooth muscle cells. The plaque cells occasionally had irregularly thickened basement membranes and myelin figures (Fig.5). The tunica media of the abdominal aorta were



Fig. 5 Intimal smooth muscle cells of an abdominal aorta from group A. Irregular thickening of basement membrane (B), and myelin figure structure (M) are observed. Arrow indicates cell debris in the widened stroma ( $\times 6,900$ ).

almost intact.

Chickens of group B, which received corn oil injections and ad-libitum feedings of a cholesterol-containing diet, developed a slight increase in frequency of cell debris and lipid droplets compared to group A.

Numerous degenerate intimal cells, especially of smooth muscle cells, were found in the abdominal aorta from group C. These degenerate cells displayed marked vesiculation of organelles and partial coagulation necrosis (Fig. 6 and 7). Ultrastructural changes of abdominal aortas from group D were basically similar to those from group C.



Fig. 6. Degenerate smooth muscle cells of an abdominal aorta from group C. Both the endoplasmic reticulum (R) and the perinuclear cisternae (arrow) are remarkably dilated ( $\times 13,000$ ).

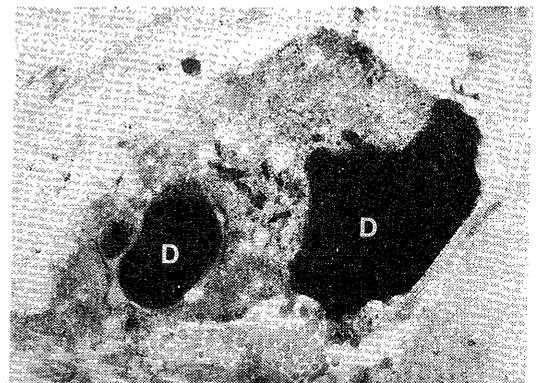


Fig. 7. Intimal smooth muscle cells with partial coagulation necrosis (D) in an abdominal aorta from group C. ( $\times 24,600$ ).

Group E exhibited the greatest variety of cellular changes of the abdominal aortas

among all the groups. Endothelial cells showed widening of intercellular junctions and vesiculation of cytoplasmic organelles. Frequent lipid vacuoles were also seen (Fig.8). Numerous intimal smooth muscle cells were involved in degeneration without stainable lipid. Degenerate cells without stainable lipid fundamentally consisted of both rarefaction and mummification types. The rarefied cells were characterized by lucent cytoplasm due to a decrease in myofilament density and vesiculation of cytoplasmic organelles. The mummification type of degenerate cells had an increased density in nuclei and/or cytoplasm. These degenerate cells sometimes contained lipid droplets (Fig. 9). In addition to degeneration without stainable lipid, lipid accumulation was more frequent in smooth muscle cells than in macrophages (Fig. 10). The smooth muscle cells of the inner media frequently displayed

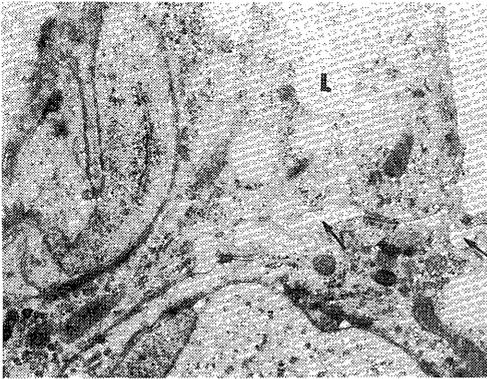


Fig. 8. Endothelial cells of an abdominal aorta from Group E. Several lipid droplets (L) are seen in endothelial cells of which the intercellular junction (arrow) is widened. ( $\times 19,700$ ).

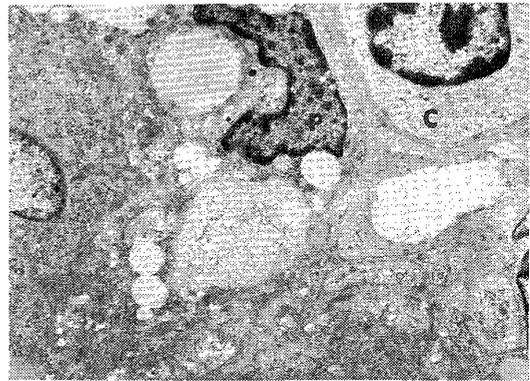


Fig. 9. Degenerate cells without stainable lipid of an abdominal aorta from Group E. Cytoplasm (C) is lucent and nuclei (P) are pycnotic. ( $\times 9,800$ ).

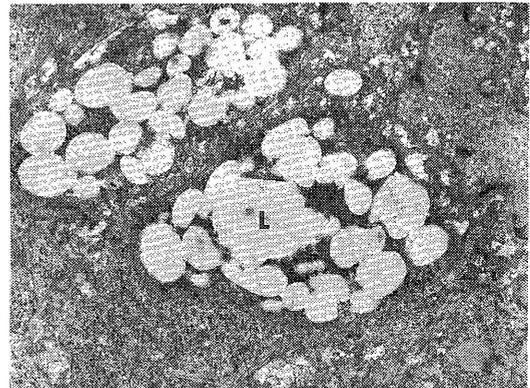


Fig. 10. Lipid-containing cells (L) in an abdominal aorta from group E ( $\times 9,800$ ).

degeneration with or without stainable lipid. Similar cellular changes were ultrastructurally seen in both groups E and F. The interstitium of abdominal plaque was widened and contained an aggregate of cell debris and round electron-dense particles probably of lipid

Table 2. Frequency of degenerate cells with or without lipid droplets in the abdominal aorta

Group	Degenerate cells without lipid droplets	Degenerate cells with lipid droplets	Total number of cells counted
A	32 ( 3.6)	6 (0.7)	891
B	38 ( 4.1)	11 (1.2)	935
C	165 (13.7)	10 (0.8)	1204
D	134 (11.8)	13 (1.1)	1139
E	103 (12.3)	30 (3.6)	837
F	109 ( 9.1)	20 (1.7)	1193

Parenthesis shows percentage of degenerate cells with or without lipid droplets

granules.

Tables 2 presents counts and computations of degenerate cells with or without stainable lipid in the thickened intima and inner media of the abdominal aorta from all experimental groups. Group C had the highest frequency of degenerate cells among all the groups.

Group E showed a slight decrease in the frequency of degenerate cells without stainable lipid and a significant increase of degenerate cells with stainable lipid ( $X^2$ -test,  $p < 0.05$ ). The administration of elastase more significantly lowered the frequency of degenerate cells with or without stainable lipid in group F than in group D.

## DISCUSSION

As recently proposed by Ross and Glomset (11), response to injury of the arterial wall has an important function in the initiation or development of atherosclerosis. In the present experiment, malondialdehyde injection produced frequent degeneration without stainable lipid, which indicates potent angiotoxic effects of malondialdehyde. This type of degenerate cell is reportedly induced as a result of aging (8), hypoxia (18), hemodynamic stress (15), and administration of oxidized sterol (17). In the view of LEBEDEVY *et al.* (9) peroxidation products of unsaturated fatty acid influence membrane transport of liposome and enhance cellular influx of cations such as calcium, these actions may be related to the formation of degenerate arterial cells without stainable lipid. Since supplementary fat was not added to the diet, no significant cellular changes were observed in cholesterol-fed chickens (group B).

The presence of foam cells in the intima, which derived from macrophages and smooth muscle cells, is known to be one of the characteristic features of atherosclerosis. BROWN *et al.* (3) reported that malondialdehyde-modified LDL is easily taken up by a non-regulated secondary receptor, resulting in the conversion of macrophages to foam cells. The smooth muscle type of lipidcontaining cell was also frequently seen in abdominal aortas from group E. These results suggest that smooth muscle cells activated by malondialdehyde may

absorb lipid and form foam cells.

Elastase has been reported to have a variety of antiatherosclerotic functions, including a hypolipidemic effect (12), lowering of hypertension (7), and inhibition of arterial calcium deposition (16). Our previous experiment has shown that elastase treatment results in a reduction in calcium and lipid accumulation in aortic tissue in hereditary non-laying hens with hyperlipidemia (14). In the present experiment, elastase significantly reduced the frequency of degenerate cells and lipidcontaining cells but not the plasma cholesterol level. Therefore, it seems that the anti-atherogenic function of elastase can be ascribed more to the inhibition of arterial calcium and lipid deposition than to lowering the plasma lipid level.

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