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Ultrastructure of Aortic Lesions in Restricted-Ovulator Chickens

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ABSTRACT

Aortas from normal roosters, normal layers and hereditary restricted-ovulator hens (nonlayers) were examined electron-microscopically and biochemically. In accordance with an abnormal increase in plasma lipid levels, lipid-rich aortic lesions were more frequently observed in these nonlayers than in the layers and roosters.

The three types of lipid-containing cells observed in these experimental animals originated from smooth muscle cells, fibroblast-like cells or macrophages. The malonaldehyde content was remarkably high in the plasma and aortic tissue of the nonlayers. Degenerate cells without stainable lipid, characterized by cytolysis and pyknotic nuclei, were frequently observed in the abdominal aortas of the nonlayers. These findings suggest that oxidized lipids, as well as hyperlipidmedia, may be responsible for the development of atherosclerosis in these nonlayers.

Key words: Ultrastructure. Aortic atherosclerosis. Oxidized lipid. Nonlayers.

INTRODUCTION

Various avian species have proven to be useful experimental animal models for the induction of atherosclerosis. Of these, pigeons,[10] turkeys[19] and chickens[17] are most extensively studied. The avian aortic sturucture is morphologically unique as reported by

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several authors.[17)25)]

A mutant strain of DeKalb white leghorn restricted-ovulator hens (nonlayer) are known to develop hyperlipidemia.[12)3)] MITCHEL *et al.*[15)] reported that the hepatic β-hydroxy-β-menthyl glutaryl (HGM)-CoA reductase activity of these nonlayers is several times lower than that of the layers. However, the mechanism of atherogenesis in the nonlayers has not been fully ellucidated. In our previous report, we suggested that the frequent occurence of cell degeneration with[illegible]t stainable lipid in the coronary arterial lesions of the nonlayers, which may have been related to the presence of some angiotoxin, is one of the major anatomical features in the development of coronary atherosclerosis.[21)] In the present study, we conducted ultrastructural survey of the aortic lesions in the rooster, layers and nonlayers of this mutant strain in order to understand the atherogenesis of the nonlayers.

MATERIALS AND METHODS

Six roosters, six layers and six nonlayers, all from the mutant DeKalb strain of white leghorns, one year of age, were used in this study. The chickens were housed in individual cages and fed with a commercial mash ad libitum throughout the experiment. The chicken mash contained 2% crude fat and a trace amount of cholesterol. The nonlayers in this study laid no eggs at all. When this experiment was terminated, the chickens were decapitated at which time blood was collected and stabilized with heparin so that the plasma could easily be obtained.

Plasma total cholesterol concentrations were enzymatically determined by the method of ALLAIN *et al.*.[1)] Triglyceride concentrations in the plasma were determined according to the FOSTER and SUNN method.[6)] Plasma phospholipid concentrations were determined by measuring the phosphorous content in the plasma lipid extracts according to the method of ENG and NOBLE.[4)] Liver total cholesterol, triglyceride, and phospholipid concentrations were determined from aliquots of lipid extracts by the method of FOLCH *et al.*.[5)] Fifty microliters of concentrated FOLCH extract were used in the FOSTER and DUNN procedure for the determination of liver triglyceride concentrations; and 0.2ml of evaporated FOLCH extract was used to determine the phospholipid concentrations; and liver cholesterol concentrations were measured from evaporated FOLCH extracts by a modified procedure of GLICK *et al.*.[9)] Determination of plasma malondialdehyde levels was accomplished according to the method of YAGI[27)] on the same day the blood was collected. Total lipid of the abdominal aorta was extracted by the method of FOLCH *et al.*.[5)] Segments of the abdominal aorta were uniformly collected for tissue malondialdehyde determination. Peroxidation standard, made according to the method of TROMBLY and TAPPEL[25)] were run and located by exposure to UV light. Fluorescence of the tissue extracts was determined at 435 nm emission

350 nm excitation.

For morphological examination, the aortas were uniformly divided into the ascending, distal thoracic and abdominal segments. These cross sectioned specimens were fixed in phosphate-buffered, 3% glutaraldehyde (pH 7.4), postfixed in phosphate-buffered 1% osmium tetroxide (pH 7.4), serially dehydrated in ethanol and embedded in EM bed 812 epoxy resin. Thick sections were stained with alkaline toluidine blue and used for histological examination. The magnitude of intimal thickening in the abdominal aorta was measured using an ocular micrometer as described in our previous study.[24] Ultrathin sections were made with glass knives, stained with uranyl acetate and lead citrate and examined with a Hitachi HU-12 electron microscope. Three epoxy resin-embedded tissue blocks of the abdominal aorta per bird were examined for comparisons of the frequency of degenerate cells without stainable lipid. Cells were counted routinely at a magnifications of 5000. Higher magnifications were used for examination of details.

RESULTS

Plasma and liver lipid profiles are presented in Table 1. The nonlayers had the highest values in the all fractions of plasma lipid assayed, which included total cholesterol, triglyceride and phospholipid. The liver of the nonlayers contained higher levels of

Table 1. Lipid profiles of plasma and liver

	Plasma (mg/100ml)			Liver (mg/g wet tissue)		
	TC	TG	PL	TC	TG	PL
Rooster	71.7± 9.6	35.7± 9.7	157.9± 14.1	3.4±0.7	7.5± 3.6	25.8±2.0
Layer	96.3± 40.2	1563.1±1268.4	606.1±231.2	3.4±0.5	18.2± 14.2	29.2±1.4
Nonlayer	1075.0±131.7	10693.2±2245.1	5216.0±1888.2	3.8±0.6	188.1±102.3	27.9±2.0

Abbreviations: TC=Total cholesterol, TG=triglycerides, PL=phospholipid.
Date are expressed as mean ± standard deviation.

Table 2. Peroxide lipid concentration in plasma and abdominal aorta.

	n mol MDA per ml plasma	Fluorescensce units per g wet weight tissue	Total lipid extracted (mg) per g wet weight tissue
Rooster	1.8±0.7	44.2±21.8	40.9± 9.8
Layer	4.8±1.5	68.6± 4.2	65.2± 5.3
Nonlayer	39.5±3.6	129.0±41.5	126.2±32.1

MDA=malondialdehyde
Date are presented as mean ± standard deviation.

triglyceride than those of the layers and rooster. The peroxide lipid concentrations of the plasma and abdominal aortas are shown in Table 2. The malondialdehyde concentrations of the plasma and abdominal aortas were highest in the nonlayers and lowest in the roosters (t-test, $p<0.05$).

The degree of intimal thickening of the abdominal aorta was 9.3 ± 1.0 (10^{-2} mm) in the roosters, 17.9 ± 0.8 (10^{-2} mm) in the layers and 48.6 ± 1.3 (10^{-2} mm) in the nonlayers (Date are expressed as mean±standard deviation).

ELECTRON MICROSCOPIC OBSERVATION

The majority of cellular components in the intimal zone of the ascending aortas in the roosters consisted of fibroblat-like cells. These cells were characterized by long cytoplasmic processes, an absence of basement membrane, sparse myofilaments, and abundant organelles such as Golgi vesicles, lysosomes and endoplasmic reticulum (Fig. 1). The medial zone of the ascending aortas usually consisted of bundles of elastic fiber, and alternating layers of smooth muscle cells and fibroblast-like cells.

The ascending aortas of the layers occasionally contained small numbers of cytoplasmic lipid droplets, with or without retained electron density, in the endothelial cells. The intima, which contained such endothelial cells, was slightly thickened, displaying an increase in the amounts of glycosaminoglycans and elastic fibers. Stellate shaped lipid-containing cells with finger-like cytoplasmic extensions were present in the middle layer of the media (Fig. 2). Some of these lipid-containing cells had incomplete basement membranes and small numbers of pinocytotic vesicles. Most of these lipid-containing cells, however, did not have the characteristic features of the smooth muscle cells. An increase in collagen fibers, a small amount of extracellular lipid granules, and glycosaminoglycans accompanied the lipid-containing cells.

In the ascending aortas of the nonlayers, there were numerous lipid-containing cells present in both the intimal and medial zones. Intimal thickening was frequently seen and lipid deposition was greater in the fibroblast-like cells than in the smooth muscle cells. Various size of cholesterol clefts were present in the inner medial stroma. Myelin figures, small cholesterin crystals, and clusters of electron-dense particles were also present in the cytoplasm of the lipid-containing cells (Fig. 3-A). The stroma had abundant extracellular lipid granules, collagen fibers, small pieces of elastic fibers, and amorphous materials.

The pre-existing elastic fibers in the inner media were smaller and more fragmented than those of the layers.

In the distal thoracic aortas of the rooster, tiny focal lesions from smooth muscle cells proliferation were present. These lesions, which were nost prominent in the nonlayers,

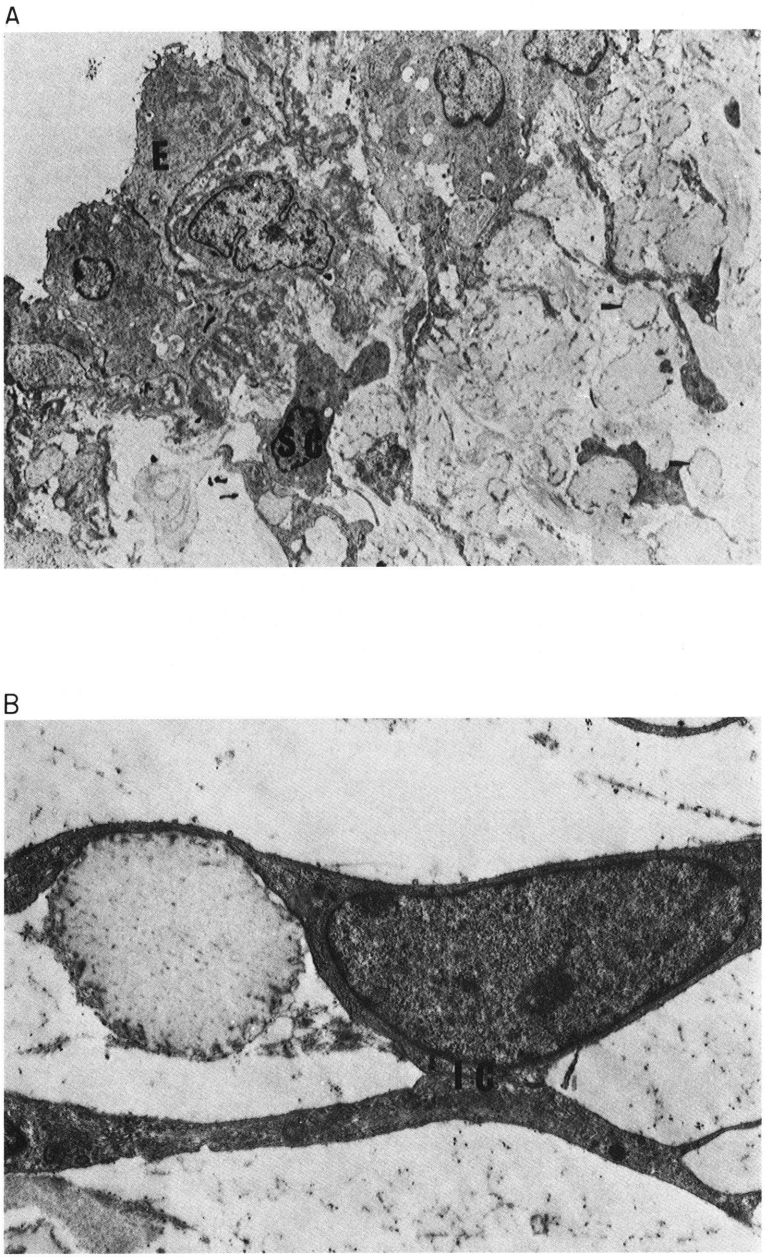

Fig. 1. Ascending aorta from a rooster.
A. Stellate fibroblast-like cells (SC) and spherical elastic fibers (arrow) are shown in the subendothelial areas (×3,700).
E: endothelial cell.
B. Higher magnification of fibroblast-like cells with long cytoplasmic projections (×12,600).
IC: intercellular junction.

had numerous electron-dense stromal particles and deeper deposits of extra- and intracellular lipids. Irregularly shaped lipid-containing cells were observed in the half of the media (Fig. 3-B). Small pieces of elastic fibers were scattered throughout the stroma, often adjacent to the smooth muscle cells.

In the abdominal aortas of the layers, intimal cellular proliferation, often several layers thick, was more frequent than in those of the roosters (Fig. 4). These proliferated intimal cells were identified as smooth muscle cells because of their closely packed myofilaments, fusiform densities, peripheral cytoplasmic vesicles and conspicuous basement membranes. Unclassified cells were also observed among these slender smooth musle cells. The internal elastic lamina was usually continuously banded, and fenestrae were rarely observed. Closely packed layers of medial smooth muscle cells, rich in compact myofilaments, contained few lipid droplets in their cytoplasm.

The nonlayers exhibited lipid-rich abdominal aortic lesions including numerous lipid-containing cells (Fig. 5), degenerate cell without stainable lipid (Fig. 6) and aggregates of cellular debris. Most of these lipid-containing cells in the deep intima had incomplete basement membranes, a few pinocytotic vesicles, and myofilaments (Fig. 5). Degenerate cells without stainable lipid were characterized by lucent cytoplasm and/or pyknotic nuclei, which have been described in our previous study (TODA, 1980).

The frequency of degenerate cells without stainable lipid in the abdominal aorta was 17/1551 (1.1%) in the roosters, 27/1150 (2.3%) in the layers and 118/1731 (6.8%) in

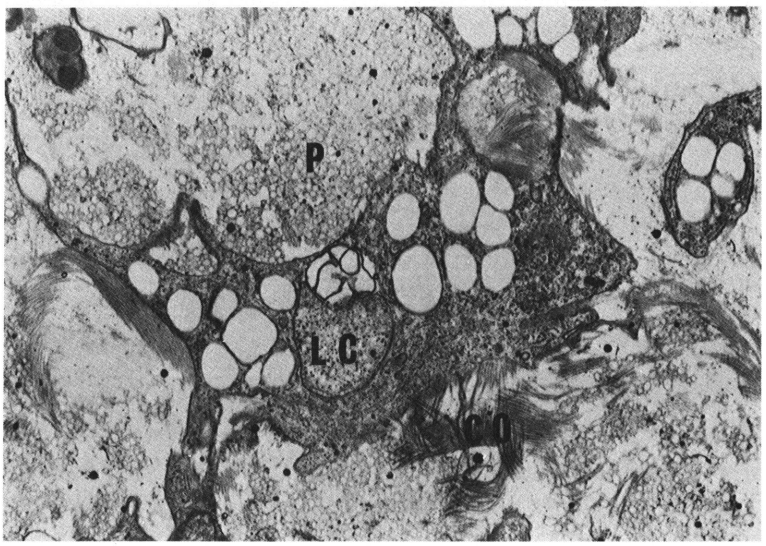

Fig. 2. Stellate lipid-containing cell (LC) in the media of the ascending aorta from a layer.
Note the masses of electron-lucent particles (P), probably lipoproteins, and the bundles of collagen fibers (CO) in the stroma (×9,600).

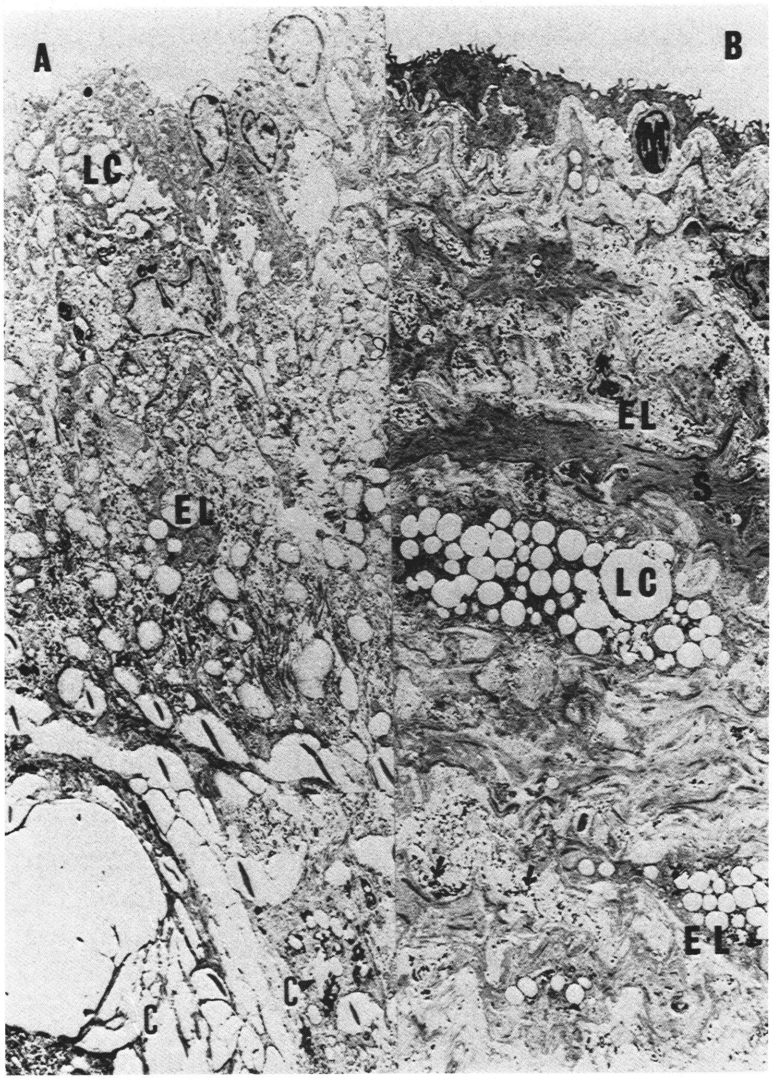

Fig. 3. Lipid-rich aortic lesions of a nonlayer.
A. Note lipid-containing cells (LC) and cholesterol cleft (C) in the ascending aorta. Elastic fibers (EL) are spherical (×3,400).
B. Lipid-containing cells (LC) and extracellular electron-dense lipid granules (arrow) are shown. Elastic fibers (EL) are long and slender in the distal thoracic aorta (×4,200).
M: monocyte-like cell.
S: smooth muscle cell.

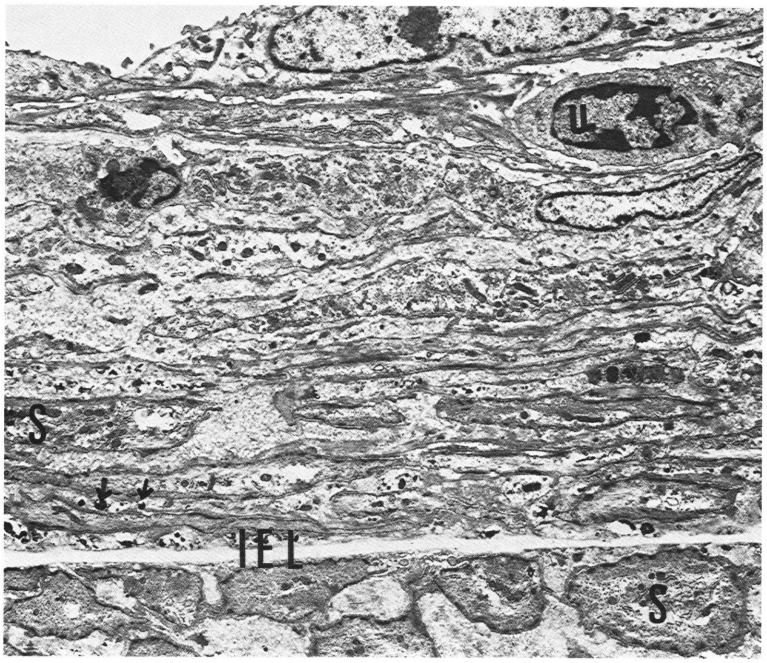

Fig. 4. Thickened intima of the abdominal aorta from a nonlayer.
An activated smooth muscle cell (S), an unclassified cell (U) and electron-dense particles (arrow) are present (×6,500).
IEL: internal elastic lamina.

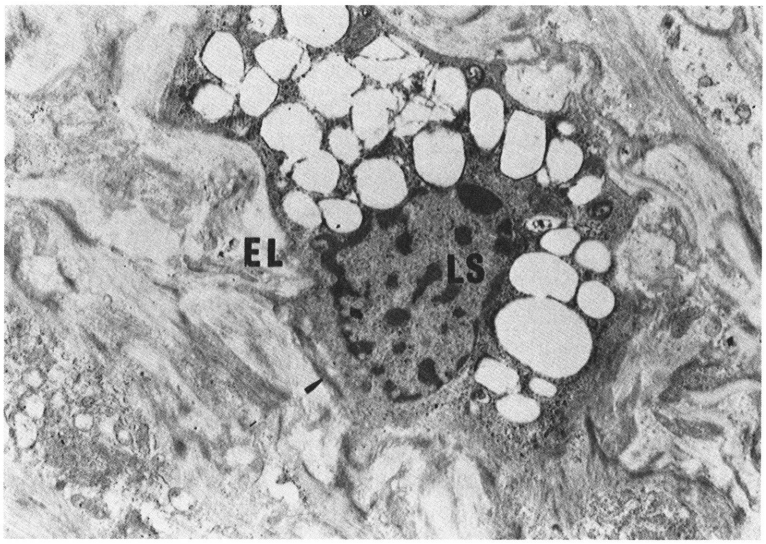

Fig. 5. Lipid-containing smooth muscle cell (LS) in the media of the abdominal aorta from a nonlayer.
Myofilaments with fusiform densities (arrow) and a basement membrane are visible in this lipid-containing cell (×8,300).
EL: elastic fibers.

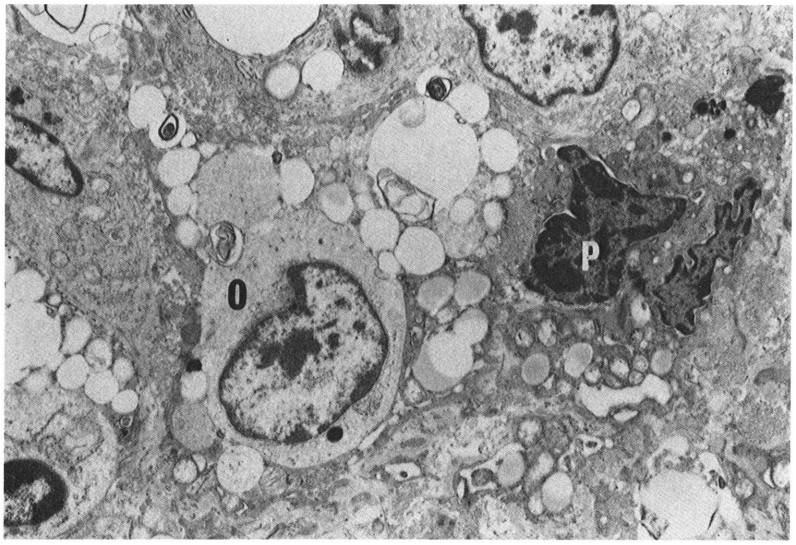

Fig. 6. Degenerate cells without stainable lipid in the abdominal aorta from a nonlayer.
Pyknosis of the nucleus (P) and opacity of the cytoplasm (O) are characteristic of these degenerate cells (×6,300).

the nonlayers. In accordance with the levels of lipid extracted and malondialdehyde in the plasma and aortic tissues, the nonlayers had significantly more detenerate cells without stainable lipid than the layers or the roosters (t-test, $p<0.05$).

DISCUSSION

Abnormally high levels of plasma and liver lipids were observed in the nonlayers. Since the chicken diet used in this study contained only a small amount of fat and a trace of cholesterol, the extreme hyperlipidemia observed was produced endogenously due to increased liver synthesis of lipid. Abundant lipid accumulation in the arterial wall suggests that continuous inhibition of lipid-rich plasma components plays an important role in the atherogenesis of the nonlayers.[21]

Foam cells are generally believed to originate from macrophages[20] and smooth muscle cells[7] both in man and animals. In this study, two hypothetical, transitional forms of lipid-containing cells were present in the lipid-rich aortic lesions of the nonlayers, suggesting dural origins from medial smooth mascle cells and fibroblast-like cells. Lipid-containing macrophages, which penetrated the aortic endothelium in such lipid-rich lisions, suggested the existence of a third possible type of foam cells.[21]

Degenerate cells without stainable lipid frequently occur in the arterial wall under hypoxic conditions.[22] It has been reported that oxidized cholesterol, such as 25-hydrox

y-cholesterol[13] and 7-ketocholesterol[23] cause cell degeneration without stainable lipid. Furthermore, BROWN, *et al.*[2] reported that oxidized cholesterol enhanced cholesterol ester formation in human fibroblasts. On the other hand, GLAVIND, *et al.*[8] originally reported by using the thiobarbituric acid test, that atheromatous arteries contain more peroxide lipid than non-atheromatous arteries. These are the augmenting evidences indicating that peroxidation products from unsaturated fatty acids damage cell membranes[14] and the toxic effects of LDL to endothelial cells may be due to lipid peroxidation.[11] In this study, the peroxide lipid content and frequency of cell degeneration without stainable lipid were highest in the abdominal aorta of the nonlayers. Malondialdehyde acetal can specifically induce cell degeneration without stainable lipid in the chicken arteries. Furthermore, the administration of both malondialdehyde acetal and cholesterol produce lipid-containing cells in the chicken arteries (manuscript in preparation).

IMAI, *et al.*[14] indicated that arterial wall injuries beyond a certain degree would lead to the development of arteriosclerosis. Arterial wall injury is one of the major factors of atherogenesis[18] and has been the objective of a series of investigations concerning the development of arterial lesions.

REFERENCES

1) ALLAIN, C. C., POON, L., CHAN, S. H., RICHMOND, W. and FU, P.: Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20**: 470–475 (1974).
2) BROWN, M. S., DANA, S. E. and GOLDSTEIN, J. L.: Cholesterol ester formation in cultured human fibrablasts. *J. Biol. CHem.* **250**: 4025–4027 (1975).
3) CHO, B. H. S., LAWSON, L. D., TODA, T. and KUMMEROW, F. A.: Oxidation of fatty acid by heart mitochondria of chickens with endogenous hyperlipidemia. *Biochem. Med.* **31**: 347–351 (1984).
4) ENG, L. F. and NOBLE, E. P.: The maturation of rat brain myelin. *Lipid.* **3**: 157–162 (1968).
5) FOLCH, L., LEES, M. and SLOANE-STANLEY, G. H.: A simple method for isolation and purification of total lipid from animal tissues. *G. Biol. Chem.* **226**: 497–507 (1957).
6) FOSTER, L. B. and DUNN, R. T.: Stable reagents for determination of serum triglycerides by a colorimetric Hantzsch condensation method. *Clin. Chem.* **226**: 497–507 (1973).
7) GEER, J. C. and WEBSTER, W. S.: Morphology of mesenchymal elements of normal artery, fatty streaks and plaques. *Adv. Exp. Med. Biol.* **43**: 9–33 (1974).
8) GLAVIND, J., HARTMAN, S., CLEMMESEN, J., JESSEN, K. E. and DANN, H.: Studies on the role of lipidperoxides in human pathology. Part 2. The presence of peroxides lipids in the atherosclerotic aorta. *Acta. Pathol. Microbiol. Scand.* **30**: 1–6 (1952).
9) GLICK, D., FELL, B. F. and SJOLIN, K. E.: Spectrophotometric determination of nanogram amounts of total cholesterol in microgram quantities of tissue of microliter volumes of serum. *Analyt. Chem.* **36**: 1119–1121 (1964).
10) HAJJAR, D. P., WRIGHT, T. N. and SMITH, S. C.: Lipid accumulation and ultrastructural changes within the aortic wall during early spontaneous atherohenesis. *Amer. J. Patol.* **100**: 638–706 (1980).

11) HESSLER, J. R., MOREL, D. W., LEWIS, L. J. and CHRISOLM, L. W.: Lipoprotein oxidation and lipoprotein-induced cytotoxicity. *Atherosclerosis.* 98: 161−172 (1974).

12) HO-K. J., LAWRENCE, W. D., LEWIS, L. A., LIU, L. B. and TAYLOR, C. B.: Hereditary hyperlipidemia in non-laying chickens. *Arch. Path.* **98**: 161−172 (1974).

13) IMAI, H., WERTHESSEN, N. T., TAYLOR, C. B. and LEE, K. T.: Angiotoxicity and arteriosclerosis due to contaminants of USP-grade cholesterol. *Arch. Path. Lab. Med.* **100**: 565−572 (1976).

14) IMAI, H., WERTHESSEN, N. T., SUBRAMAYAM, V., LEQUESNE, P. W., SOLOWAY,A. H. and KANISAWA, M.: Angiotoxicity of oxygenated sterols and possible precurssors. *Science.* **207**: 651−653 (1980).

15) JAIN, S. K.: The accumulation of malondialdehyde, a product of fatty accid peroxidation, can disturb aminophospholipid organization in the membrane bilayer of human erythrocytes. *J. Biol. Chem.* **259**: 3391−3394 (1984).

16) MITCHELL, A. D., CARLSON, S. E., MCGIBBON, W. H. and GOLDFARB, S.: Hepatic HMG-CoA reductase and 7-α-hydroxylase activities in normal and hyperlipidemic-restricted ovulator atherosclerosis-prone chickens before and after the commencement of egg laying. *Atherosclerosis.* **32**: 11−21 (1979).

17) MOSS, N. S. and BENDITT, E. P.: Spontaneous and experimentally induced arterial lesion. I. An ultrastructural survey of the normal chicken aorta. *Lab. Invest.* **22**: 166−183 (1970).

18) ROSS, R.: Atherosclerosis: A problem of the biology of arterial wall cells and their interactions with blood components. *Arteriselerosis.* 1: 293−311 (1983).

19) SIMPSON, C. F. and HARMS, R. H.: The nature of atherosclerotic plaque in turkeys. *J. Atheroscler. Res.* **8**: 143−153 (1968).

20) STARY, H. C.: Coronary artery fine structure in rhesus monkeys: The early atherosclerotic lesions and its progression. *Prim. Med.* **9**: 359−395 (1976).

21) TODA, T., LESZCZYNDKI, D. E., MCGIBBON, W. H. and KUMMEROW, F. A.: Coronary arterial lesions in sexually mature non-layers, layers, and rooster. *Virchows Arch A. Path. Anat and Histol.* **388**: 123−135 (1980).

22) TODA, T., LESZCZYNDKI, D. E. and KUMMEROW, F. A.: Degenerative changes in endothlial and smooth muscle cells from aging swine ductus arteriosus and venosus. *Amer. J. Anat.* **160**: 37−49 (1981 a).

23) TODA, T., LESZCZYNDKI, D. E. and KUMMEROW, F. A.: Angiotoxic effects of dietary 7-ketocholesterol in chick aorta. *Paroi Arterielle.* 7: 167−176 (1981).

24) TODA, T., MAHFOUZ, M. M. and KUMMEROW, F. A.: Influence of dietary fats on ultrastructure and fatty acid composition of swine arterial tissue. *Acta Pathol. Jpn.* **34**: 935−945 (1984).

25) TODA, T., TODA, Y. and KUMMEROW, F. A.: Electron microscopic comparison of the tunica media of the thoracic aorta between species. *Tohoku J. exp. Med.* **143**: 141−147 (1984).

26) TROMBLY, R. and TAPPEL, A. L.: Fractionation and analysis of fluorescent products of lipid peroxidation. *Lipid.* **10**: 441−447 (1975).

27) YAGI, K.: Assay for serum lipid peroxidation level and its clinical significance. In: Lipid peroxides in Biology and Medicine. (Yagi, Academic Press, INC. New York, 1982).