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Chronological Effects of Atherogenic Diets on the Aorta, Liver and Spleen of Rabbits

To investigate the temporal progression of atherogenesis on the aorta and involvement of the monocyte-macrophage system in the liver and spleen, we fed 74 rabbits with high fat (14 or 7 gm%) and cholesterol (2 and 1%) diets for 4 to over 24 weeks. Using both light and electron microscopies, we found that the fibro-fatty areas on the luminal surface of aortas was spread over along the feeding time dependently. The fat deposits also in the liver and spleen worsened depending on the time of feeding the atherogenic diets. Not only monocyte-derived foam cells, but also parenchymatous cells in the liver and spleen involved become fat-laden cells. According to these results, we propose that there are three stages: 1) the primary seeding, 2) the intermediate maturing and 3) the advanced periods. These periods may play very important roles in designing the management and treatment of atherosclerotic patients.

Key Words: *Dietary Fats; Cholesterol, Dietary; Diet, Atherogenic; Macrophage; Foam Cells; Hepato-Splenic Involvement; Three Stages of Atherosclerotic Development*

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INTRODUCTION

In the literature, there are a large number of biochemical studies that link dietary fats with regulation of plasma cholesterol levels as a risk factor of atherosclerosis development in animal models (1-3). Furthermore, it has been known experimentally that only one shot of atherogenic diets can not produce aherosclerotic plaques, and it takes some time of feeding for least 4 to 12 weeks depending on the species and diet formulas to produce the plaques (4, 5). We investigated dietary effects of high fat contents in the chow fed to white rabbits to induce atherosclerosis (6). From a series of studies using high fat and 2% or 1% cholesterol diets, we observed not only development of aortic lesions, but also involvement of the monocyte-macrophage system with fatty infiltrations in the liver and spleen (7). However, there is yet no information which describes hepato-splenic involvement in atherogenesis, and it is monocyte-macrophages which are considered to be one of the major culprits in altering the intimal surface of the aorta vulnerable to induce atherosclerotic plaques (8-10). As our previous studies were limited to only 4 weeks of feeding with 2% cholesterol and 14 g% fat diets (6), we were interested in if hepato-splenic lesions vary along with the advancement of atherosclerotic plaques or stay the same with progress of atherogenesis on the aortic endothelium for over 6 months of feeding with the same diets.

In this study, our objectives were to observe: 1) how fatty streaks of aortic walls develop temporally, 2) how hepatic structures are involved, 3) how spleen can contribute in terms of monocytes infiltration during feeding the animals with both high fat and cholesterol-diets.

We report the existence of three temporal stages for experimental rabbits to develop atherosclerosis on the lumenal surface of aorta. They are also in correlation with different stages of hepato-splenic involvements simultaneously.

MATERIALS AND METHODS

We randomly chose 94 white rabbits with body weights around 1.0 kg. As soon as possible from groups of the same litters to form 20 controls and 74 subject animals. The total number of rabbits are listed in Table 1, which also shows feeding methods and the time schedules to sacrifice the animals. The high fat diet contained 14% fat, 9% protein and 66% carbohydrate with 1.7% Na^+, and 0.036% Ca^{2+} (the rest of chow was vegetable fibers).

After feeding at a certain period in the schedule, the animals were lightly anesthetized using intraperitoneal injection of ketamine and the abdominal cavity was open

Table 1. Number of experimental animals in each group during different feeding periods

Diets \ Periods	0	4 weeks	6 weeks	12 weeks	24 weeks and over
14 g% fat		12			12
7 g% fat			6	12	6
Control	10				
2% cholesterol		6			
1% cholesterol			4	6	4
Control	10				
Total	20	18	10	18	28

to reveal the liver and spleen, and then the aorta was cut open to search for intimal elevations both of fatty and fibrotic plaques. Unclear or invisible lesions were stained with either Sudan III or Oil-red-O to confirm that the elevated lesions were fibro-fatty plaques. Pieces of the liver, spleen and aorta were fixed in 2.5% glutaraldehyde or 10% buffered formaldehyde solutions for histological sections. The microtome-cut thin sections were stained with hematoxylin and eosin (H&E). Mason's and/or Gomori trichrome, aldehyde fucshin and Van Gieson's connective tissue stains to observe the condition of elastin and collagen fibers in the aortic wall and small vessels in the parenchyma of both spleen and liver. The histological sections were observed with Olympus light microscope at magnifications ranging from ×40 to ×200.

For transmission electron microscopy (TEM), we fixed the specimens in 2.5% glutaraldehyde buffered with 0.1 M phosphate solution (pH 7.2), and rinsed 3 times with the same phosphate buffered solution. Then they were treated with 1% OsO_4, washed with distilled water 3 times. The specimens were progressively dehydrated through stronger concentrations of ethyl alcohol from 50% to 98% and 100% propylene oxide. After embedding in Epon 812, and stained with uranyl acetate and lead citrate, the specimens were observed with Hitachi TEM apparatus (model No. H7100), with magnifications up to ×4,000.

RESULTS

Of the total 94 experimental rabbits presented in Table 1, 72 aortas (97%) showed marked fibro-fatty streaks except for 20 controls. Only 2 rabbits (9%), which were fed for only 4 weeks from the 14 g% fat group, showed weak reactions for fat streak on the endothelial surface of the aorta. After 6 weeks and thereafter, 100% of the rabbits fed with both fat or cholesterol diets had remarkable atherosclerotic plaques on the luminal surface of aortas. The edge between elevated plaques and the healthy-looking endothelium was cut to show any initial stages of atherogenesis in the aortic wall. Fig. 1 is the result of histological observations obtained from one of them. The observations represent elastolytic like pictures of the intimal elastin layers after 6 weeks on 2% cholesterol or 12 weeks on 7 g% fat diet. In this picture, the number of medial laminar unit (MLU) of central part is reduced from 28 to 24 or 20 and fragmentation of the elastic layers can be observed. A few places in the subendothelial space appeared to be empty, possibly occupied by fat deposits or foam cells. Also, there is a large gap due to accumulation of collagen fibers between IEL and the subendothelial space.

Tracing of the fatty streaks are obvious which are redrawn in Fig. 2 from the aortas that represent different periods of feeding from A, B, to C. Period 'A' is obtained from the 4-week group (2% cholesterol or 14 g% fat) and 6-week group (1% cholesterol or 7 g%

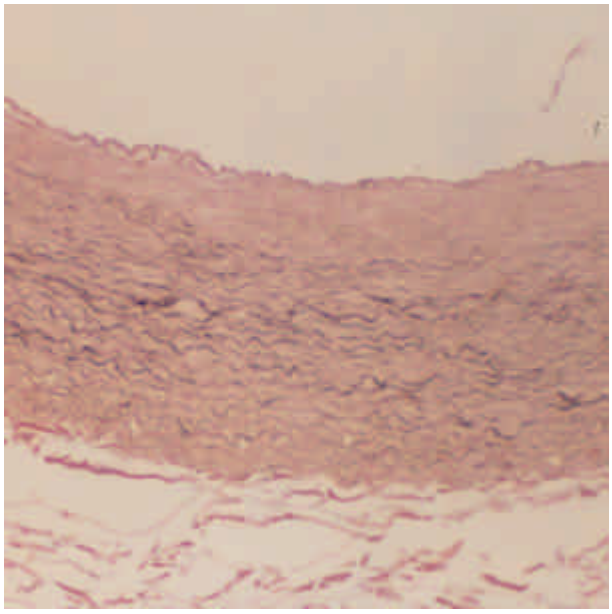

Fig. 1. A cross section of an aortic wall taken from a rabbit after 6 weeks on a 2% cholesterol or 14 g% fat diet. The section was stained with aldehyde fucshin (×200). The edge between elevated plaques and the healthy-looking endothelium was cut to show any initial stages of atherogenesis in the aortic wall.

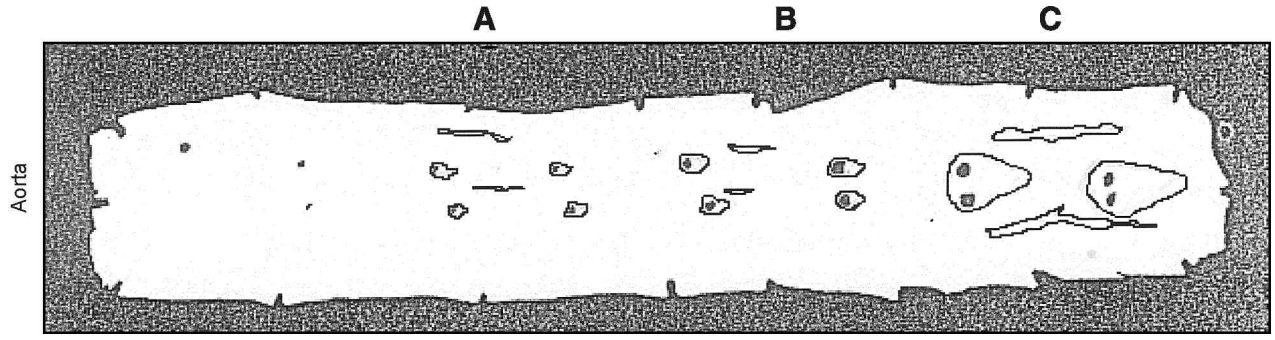

Fig. 2. A schematic diagram of atherosclerotic development on the lumenal surface of rabbit aorta, from A, B, to C. **A** is at the primary seeding period, **B** is at the intermediate and **C** at the advanced period. See text (×2.0).

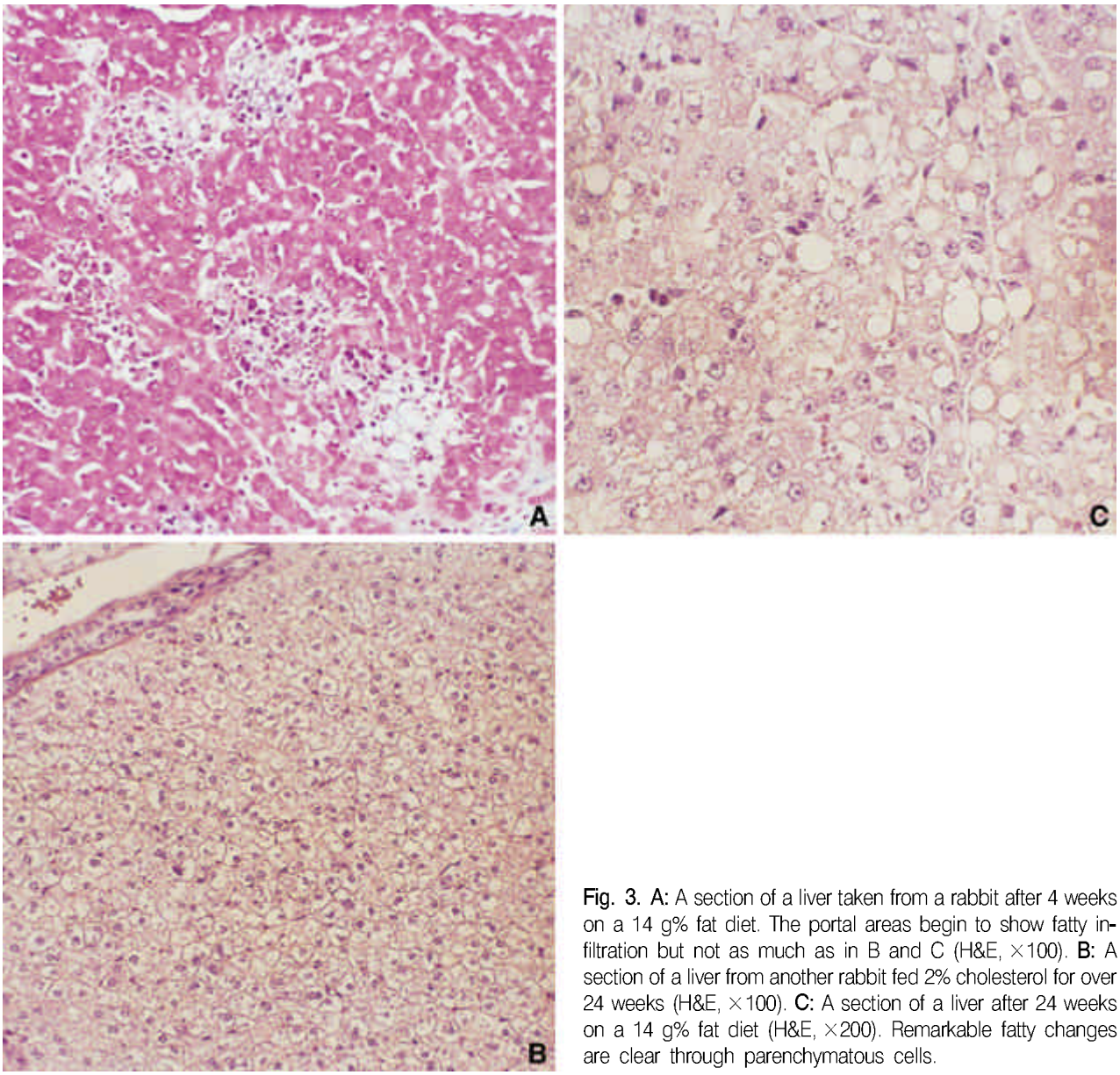

Fig. 3. **A:** A section of a liver taken from a rabbit after 4 weeks on a 14 g% fat diet. The portal areas begin to show fatty infiltration but not as much as in B and C (H&E, ×100). **B:** A section of a liver from another rabbit fed 2% cholesterol for over 24 weeks (H&E, ×100). **C:** A section of a liver after 24 weeks on a 14 g% fat diet (H&E, ×200). Remarkable fatty changes are clear through parenchymatous cells.

fat), while 'B' is from the 6-to-12-week group and 'C' is from the over-24-week group. Fatty infiltration began from the downstream edge of the arterial orifices, sometimes accompanied with fine streaks of fatty lesions. The

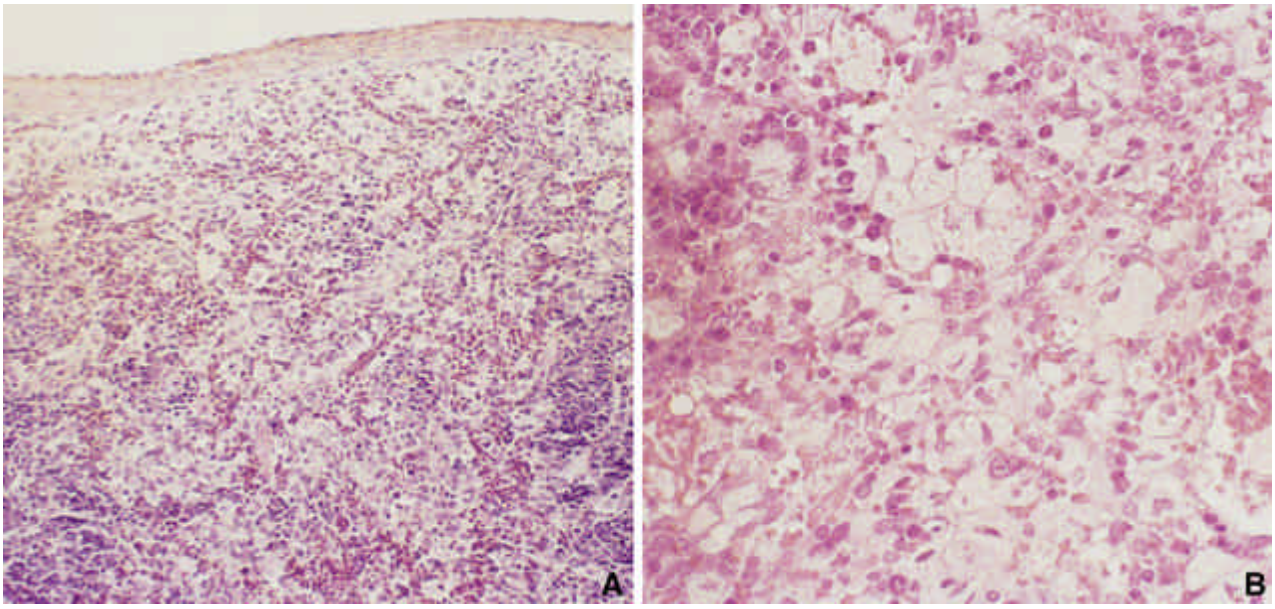

Fig. 4. Histological sections of spleens from rabbits fed with different schedules. **A:** Splenic red pulp is invaded by many foam cells at the 4th week of high fat or 2% cholesterol diets (H&E, ×40). **B:** A section of spleen taken from a rabbit fed with 14 g% fat meal for over 24 weeks shows almost the same degree of fatty changes as shown in the liver (Fig. 3C) (H&E, ×200).

first 4 weeks may be needed as a primary seeding stage for the atherosclerotic plaque formation. Then the fat deposits spread over the orifices and surround the whole areas (B). Eventually, the lesion fuse together, surrounding two orifices at the same time (C) at over 6 months of feeding.

Regarding the hepato-splenic lesions, progressively worsening pictures were observed from 4 weeks of feeding to 6-month experiments. Within 4 weeks of feeding, the liver and spleen started to show spotty yellowish speckles, and later patchy, fatty parts appeared on the surface and capsules of the organs. Under low-power light microscopy, only fat-laden monocytic macrophages appeared around portal areas at the 4th week, but later with feeding of 6 months, the lesions both in the liver and spleen also developed remarkably larger areas of fat deposits in the hepatic cords and parenchymatous cells.

Fig. 3 depicts the livers of rabbits fed from 4 weeks to 6 months. Hydropic changes together with many foam cells differentiated from the monocyte-macrophage systems can easily be noticed in these sections. At the last stages of feeding, the liver takes on a cobble stone appearance (Fig. 3B).

Histological sections of the spleens (Fig. 4) also showed many foam cells that seemed to be developed from histiocytes, monocytes and other white blood cells, starting from the red pulp and near the peripheral zone of white pulp. From observations of the present studies, it is not possible for us to discern the types of white blood cells which they originated from.

Fig. 5 show not only the distinct pictures of invasion of foam cells in the hepatic cord in both light microscopy and transmission electron microscopy, but also presence of fat vacuoles in the hepatic cord cells. Fig. 5A, magnified to ×2,000 using TEM (see methods), shows red blood cells on the right side of the picture; most of cells including hepatic cells contained fat vacuoles. Fig. 5B, which was taken at ×4,000 magnification shows round fat vacuoles of various sizes, which may give the impression of successive, and temporal fat ingestion pinocytosed by hepatic cells. In Fig. 5B, it is clear that the vacuoles numbered over 50, and the sizes are varied between 0.5 to 5 μM in diameters.

These histopathological sections clearly showed some degrees of progression from light lesions to worse lesions according to periods of feeding, which indicates there must be a dose-response relationship between the development of atherosclerosis and the quantity of fat or cholesterol fed to the rabbits. This can be interpreted as relation functions between the severity of atherosclerotic lesions and period of feeding, or in other words, the amount of fat or cholesterol ingested.

DISCUSSION

It may be impossible to trace the temporal development of atherosclerotic plaques in vivo, because we do not have the right tool to quantify the sequential areas of fibro-fatty lesions on the endothelial surfaces of aortas

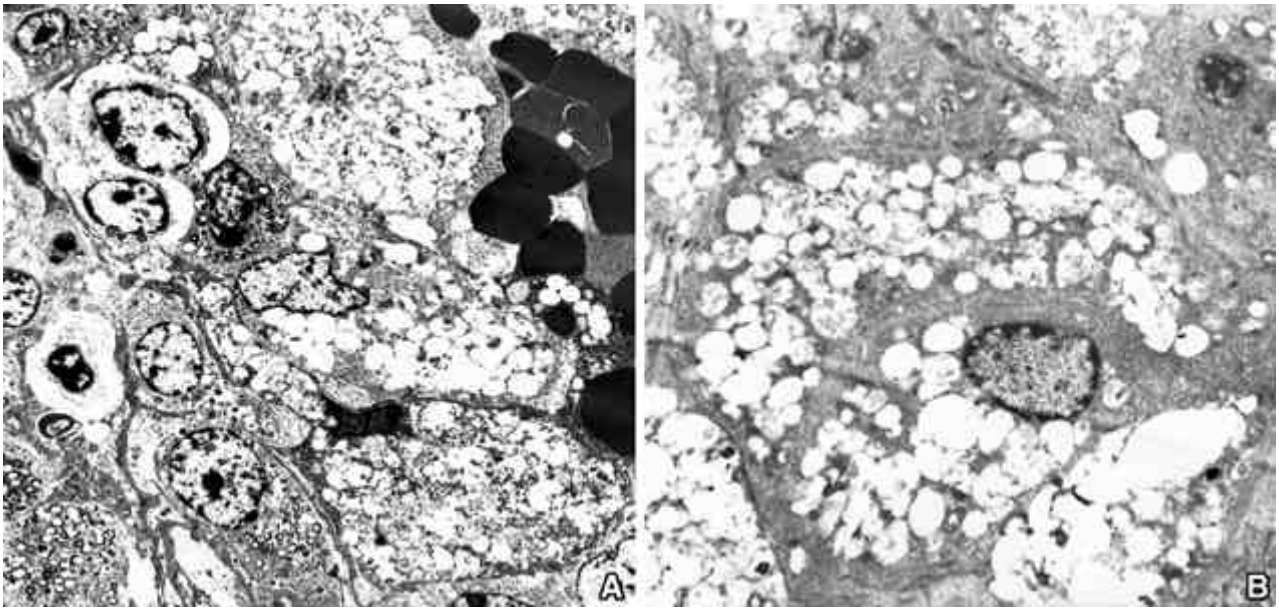

Fig. 5. A: A section of the liver from a rabbit which had been fed for over 6 months with 2% cholesterol diet was observed with TEM (see text), showing prominent fatty droplets in the cytoplasm of the hepatic cells (×2,000). B: The same section was magnified to ×4,000 to show clearly the state of fat vacuoles inside the cytoplasm of the liver cell.

at this time. Kratky et al. (11) fed 50 rabbits for over 6 to 10 months and tried to establish quantitatively the relationship between time and amount of lesions. Davies et al. (12) also studied the temporal sequence of G-protein expression in intimal hyperplasia. Yet their studies were also limited in explaining the continuity of atherogenetic changes. To elucidate pathophysiologically the periodic stages of atherogenesis, it is required to observe temporally the progress and regress of atherosclerotic procedures in vivo.

Though most investigators have been successful in inducing atherosclerotic plaques in animal studies (11, 13, 14), yet, sometimes, depending on the specific species, even on the same dietary regimes, they may not always show the classical atherosclerotic plaques. There can be many reasons why in individual animals the quantity or quality of stress they receive during the experiment may not be exact, thus different result for atherogenesis. Another reason may be that metabolic responses of individual animals fed with high fat diets or cholesterol-2 or 1% in the diets are not always the same as expected from feeding with atherogenic diets. There is not a standard method yet known to induce atherogenesis in animal experiments except the work of Kolodgie et al. (4).

In the present study, we attempted to establish periodical observations of atherogenesis, especially in experiments, using rabbits. When the rabbits were fed for 4 to 6 weeks, the body weights were under 1.0 kg, we assumed they did not yet reach puberty, which is equivalent to 15 years old or younger in human terms. If we extend the feeding period to over 6 months, the rabbits were all full-grown adults in human terms. Fig. 2 shows stage A, or feed for 4 weeks. We designate "A" as the primary seeding period when fatty streaks start to build up on the luminal surface of aortas. The next stage "B" between 4 weeks and 24 weeks, we propose as the intermediate and maturing period for atherogenesis, and the last stage "C" after 6 months of feeding is the advanced period. These stages are our own arbitrary designations, but except for in vitro, as in clinical situations, these designations can help investigators to observe patients or subject animals without open surgery. Another reason for the designation is to standardize the experimental methods, and depending on the designs and objectives of investigations, the researchers may choose the time periods of feeding. If anyone seeks to study the reversible progress of atherosclerosis, the primary seeding or intermediate stages are recommended over the advance stage.

Pleomorphic lesions, traditional fibro-fatty invasion of the intimal surfaces, appearing as atherosclerotic plaques are not necessarily the only pathological condition (15-19). Our observations revealed sometimes either fatty streaks only, or fibrous elevation of the intimal surface, especially at the downstream edge of the ostia of small arterial branches like intercostal and lumbar arteries.

At present, the mechanism of fatty accumulation can be explained by two different views: 1) formation of foam cells derived from the monocyte-macrophage system (8-10) and 2) fatty infiltration directly into extracellular

matrix of vascular walls by diffusion due to changes of shear stress (20-25) under hypercholesterolemia. The monocyte-derived foam cells had been studied well, and due to atherogenic diets monocytopenia develops in the peripheral blood (7), while the membranes of monocytes in adipose tissue and spleen which strongly reveal PPAR-gamma (peroxisome proliferator-activated receptor-gamma) which stores fat droplets in the cytoplasm of cells (26). The finding of fat lesions in the liver and spleen is not surprising (27-28), if we consider the liver as the exclusive organ for lipid metabolism by which chylomicrons and VLDL (very low density lipoproteins) or LDL (loe density lipoproteins) can be converted to HDL (high density lipoproteins). It is arguable that plaques are formed after foam cells invade, elastases excreted, followed by elastin being destroyed, and the proliferation of vascular smooth muscle cells. However, according to our present results, we can not clarify the sequences developing in the process of dietary-induced atherogenesis in rabbits in terms of the above steps like the chicken and egg analogy.

During aging and senescence for over 6 months of feeding, the aorta becomes stiffer and its elasticity is reduced (29-31). This stiffness cause hypertension at the same time with improper function of the liver, and the unbalanced monocyte-macrophage system with hyperactive cytokines also lead to the inducement of atherosclerosis in the later stage of experiments, as reported in the rat model (32, 33). It is also possible that the aging process along with hypercholesterolemia may enhance atherogenesis under stress conditions.

As of yet it is very difficult to match the degree of atherosclerotic involvement in the aorta with the fatty lesions of hepato-splenic progress. But it is plausible that wax-wane progression and regress can occur at the same time both in the aorta, liver and spleen within the three different stages.

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REFERENCES

1. Brown MS, Goldstein JL. *Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. Annu Rev Biochem 1983; 52: 223-61.*
2. Reaven PD, Witztum JL. *Oxidized low density lipoproteins in atherogenesis: role of dietary modification. Annu Rev Nutr 1996; 16: 51-71.*
3. Kritchevsky D. *Nutritional factors. In: Gotto AM Jr, Smith LC, Allen B, eds. Atherosclerosis V. New York: Springer-Verlag, 1979; 351-4.*
4. Kolodgie FD, Katos AS Jr, Largis EE, Wrenn SM, Cornhill JF, Herderick EE, Lee SJ, Virmani R. *Hypercholesterolemia in the rabbit induced by feeding graded amounts of low-level cholesterol. Methodological considerations regarding individual variability in response to dietary cholesterol and development of lesion type. Arterioscler Thromb Vasc Biol 1996; 16: 1454-64.*
5. Inoue Y, Toda T, Igawa T, Tani T, Kimura Y. *The response of serum and hepatic lipids and the aortic wall to different levels of dietry cholesterol: a comparative study between hyperlipidemia-and-atherosclerosis-prone quail and commercially available quail. Tohuku J Exp Med 1995; 175: 1-13.*
6. Song SH, Min BI, Lee JH, Cho KS, Ro S, Cho YW. *Effects of high fat diet on pre-atherogenic changes in the aorta, liver and spleen and Baroreflex sensitivity. Korean J Stress Res 1999; 7: 31-9.*
7. Song SH, Min BI, Lee JH, Cho KS. *Involvement of the monocyte-macrophage system in atherogenesis of rabbits when fed with high fat diets. Kyung Hee Med J 1999; 15: 36-43.*
8. DiCorleto PE. *Cellular mechanisms of atherogenesis. Am J Hypertens 1993; 6(11 pt2): 314-8s.*
9. Gerrity RG. *The role of the monocyte in atherosclerosis: I. transition of blood-borne monocytes into foam cells in fatty lesions. Am J Pathol 1981; 103: 181-90.*
10. Gerrity RG. *The role of the monocyte in atherosclerosis: II. migration of foam cells from atherosclerotic lesions. Am J Pathol 1981; 103: 191-200.*
11. Kratky RG, Ivey J, Rogers KA, Daley S, Roach MR. *The distribution of fibro-fatty atherosclerotic lesions in the aortae of casein-and cholesterol-fed rabbits. Atherosclerosis 1993; 99: 121-31.*
12. Davies MG, Barber L, Dhanraj DN, Gettys TW, Ramkumar V, Hagen P-O. *The temporal sequence of G-protein expression in intimal hyperplasia. J Surg Res 1996; 63: 115-22.*
13. Bruel A, Oxlund H. *Changes in biochemical properties, composition of collagen and elastin, and advanced glycation endproducts of the rat aorta in relation to age. Atherosclerosis 1996; 127: 155-65.*
14. Navab M, Fogelman AM, Berliner JA, Territo MC, Demer LL, Frank JS, Watson AD, Edwards PA, Lusis AJ. *Pathogenesis of atherosclerosis. Am J Cardiol 1995; 76: 18-23C.*
15. Kruse R, Marten M, Yoshida K, Schmit A, Volker W, Buddecke E. *Cholesterol-dependent changes of glycosaminoglycan pattern in human aorta. Basic Res Cardiol 1996; 9: 344-52.*
16. Winlove CP, Parker KH, Avery NC, Bailey AJ. *Interactions of elastin and aorta with sugars in vitro and their effects on biochemical and physical properties. Diabetologia 1996; 39:*

1131-9.

17. Chen M, Mason RP, Tulenko TN. *Atherosclerosis alter the composition, structure and function of arterial smooth muscle cell plasma membranes. Biochim Biophys Acta 1995; 1272: 101-12.*
18. Avolio A. *Genetic and environmental factors in the function and structure of the arterial wall. Hypertension 1995; 26: 34-7.*
19. Mugge A, Brances RP, Boger RH, Dwenger A, Bode-Boger S, Kienke S, Frolich JC, Lichtlen PR. *Vascular release of superoxide radicals is enhanced in hypercholesterolemic rabbits. J Cardiovasc Pharmacol 1994; 24: 994-8.*
20. Thoumine O, Nerem RM, Girard PR. *Changes in organization and composition of the extracellular matrix underlying cultured endothelial cells exposed to laminar steady shear stress. Lab Invest 1995; 73: 565-76.*
21. Meyer G, Merval R, Tedgui A. *Effects of pressure-induced stretch and convection on low-density lipoprotein and albumin uptake in the rabbit aortic wall. Circ Res 1996; 79: 532-40*
22. Guyton JR, Bocan TM, Schifani TA. *Quantitative ultrastructural analysis of perifibrous lipid and its association with elastin in non-atherosclerotic human aorta. Arteriosclerosis 1985; 5: 644-52.*
23. Sarig S, Utian WH, Sheean LA, Gorodeski GI. *Distribution of unesterified cholesterol-containing particles in human atherosclerotic lesions. Am J Pathol 1995; 146: 139-47.*
24. Song SH, Roach MR. *Surface features of human aortic atherosclerosis as seen with scanning electron microscopy. Yonsei Med J 1998; 39: 430-8.*
25. Thubrikar MJ, Robicsek F. *Pressure-induced arterial wall stress and atherosclerosis. Ann Thorac Surg 1995; 59: 1594-603.*
26. Tontonoz P, Nagy L, Alvarez JG, Thomazy VA, Evans RM. *PPARγ promotes monocyte/macrophage differentiation and uptake of oxidized LDL. Cell 1998; 93: 241-52.*
27. Hahn SE, Goldberg DM. *Factors affecting the regulation of apo B secretion by liver cells. J Clin Lab Anal 1995; 9: 431-49.*
28. Wolle S, Via DP, Chan L, Cornicelli JA, Bisgaier CL. *Hepatic overexpression of bovine scavenger receptor type I in transgenic mice prevents diet-induced hyperbetalipoproteinemia. J Clin Invest 1995; 96: 260-72.*
29. Roach MR, Burton AC. *The reason for the shape of the distensibility curve of arteries. Can J Biochem Physiol 1957; 35: 681-90.*
30. Song SH, Roach MR. *The effects of aging and atherosclerosis on elastin of human aortas; quantitative analysis of elastin-content and SEM analysis of elastolysis. Korean J Physiol Pharmacol 1998; 2: 591-600.*
31. Sebkhi A, Weinberg PD. *Age-related variations in transports properties of the rabbit arterial wall near branches. Atherosclerosis 1994; 106: 1-8.*
32. Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL, Edwards PA, Watson AD, Lusis AJ. *Atherosclerosis: basic mechanism. Oxidation, inflamation, and genetics. Circulation 1995; 91: 2488-96.*
33. Ross R. *Cell biology of atherosclerosis. Annu Rev Physiol 1995; 57: 791-804.*