Atherosclerosis in Rats Induced by Repeated Breedings, ACTH and Unilateral Nephrectomy

Acid Mucopolysaccharides, Fibroplasia, Elastosis and Other Changes in Early Lesions

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Repeatedly-bred rats of both sexes develop subintimal accumulations of acid mucopolysaccharides and fibroblastic proliferation followed by distortion and destruction of elastic lamellae. Fibrosis, endothelial hyperplasia, lipid deposition, calcification and atheromata are also observed. Changes in the elastic tissue occur independently, and later than the alterations of the ground substance and the fibroblasts. Calcification is found in more advanced lesions, showing an affinity for areas rich in mucopolysaccharides. Exogenous ACTH and unilateral nephrectomy augment early atherosclerotic changes associated with multiple breedings.

ONE of the initial alterations occurring in the development of atherosclerosis is manifested in the acid mucopolysaccharide content of the vascular ground substance. Moon and Rinehart, in investigations of human lesions, noted an early, marked accumulation of acid mucopolysaccharides in the intima, and emphasized that these lesions were not accompanied by deposition of lipid. Similar observations have been made in other species.

Wexler and Miller have described a severe fulminating arteriosclerosis in repeatedly-bred Sprague-Dawley rats which had undergone unilateral nephrectomy and had been treated with ACTH. A great variety of lesions were observed in both coronary and systemic vascular beds. The experimentally-produced coronary lesions were correlated with those found in the human disease. The lesions in all vessels were unusual in that they contained microscopic lipid droplets rather than the typical foam cell aggregates commonly seen in cholesterol-fed rabbits. In our experiments the serum cholesterol levels remained normal. Extensive morphological and chemical alterations of the connective tissue elements characterized both early and advanced lesions. The degrees of adrenal hypertrophy and thymic involution served as indices of the severity of the arteriopathy.

Rats after repeated breeding show a mild type of arteriosclerosis spontaneously with an incidence of 20 to 40 per cent. The combined treatment of unilateral nephrectomy and ACTH greatly intensifies the severity and incidence of the lesions. In order to ascertain the precise role of the repeated pregnancies in these rats we have conducted an investigation in which animals were bred at frequent intervals and in some instances treated with ACTH during the entire breeding period. We found that there was a definite relationship between the number of times the animal had borne litters and the severity of the arteriosclerosis produced by unilateral nephrectomy and administration of ACTH (unpublished results).

This study provided the opportunity to obtain many animals which had the very early form of atherosclerosis, thus making possible observations on the mucopolysaccharides in
the ground substance before extensive tissue alteration had set in.

Methods

Male (200) and female (200), Sprague-Dawley rats constituted the experimental group. The age of the control and experimental animals approximated 2 months. The animals were divided into 1 control group, consisting of 12 females and 12 males, and 4 experimental groups in which the members were paired for mating. The experimental animals were then bred repeatedly. The mating pairs were kept in separate cages. No periods of rest were allowed between successive pregnancies. The original pairing-off of mates was maintained throughout the experiment. The 4 experimental groups were subdivided as follows: (a) non-treated animals, (b) animals subjected to unilateral nephrectomy, (c) rats treated with ACTH, and (d) rats having both unilateral nephrectomy and ACTH treatment.

Animals subjected to unilateral nephrectomy were permitted 10 days for operative recovery and for the development of compensatory hypertrophy of the contralateral kidney before initiation of treatment with ACTH. On the day that males and females were paired the injections of ACTH were begun. The dose of ACTH (in gel) was 1/3 I. U./100 Gm. of body weight, subeutaneously, 3 times a week until the animal was sacrificed. Thereafter, the one-time breeders in Group I were under the influence of ACTH* for a minimum period of 54 days, i.e., the time required for mating, gestation, nursing, and a few days after weaning; whereas some animals, carried to the maximum number of breedings in Group V (5 breedings), were exposed to ACTH as long as 240 days. The one-time breeders were sacrificed after the final litter had been weaned. All rats were housed in air-conditioned quarters. They were fed Rockland Rat Chow ad libitum. No salt or fat was added to the diet.

The animals were autopsied immediately after decapitation. No anesthesia was used because of possible effects on the chemical analyses. Three segments from the arch, thoracic, and abdominal portions of the aorta were obtained. Each of the segments was then divided equally and fixed in 10 per cent buffered neutral formalin (Lillie). Tissues for histological study were embedded in paraffin and sectioned at 5 μ. Adjacent sections were then stained by the modified Hale’s stain for the demonstration of acid mucopolysaccharides, Alcian Blue and P.A.S. stain were not found to be related to morphologically altered fibroblasts. Vacuoles of various sizes were sometimes seen in areas of greatly swollen ground substance. No lesions of any kind were found in aortae taken from control animals.

With successive breedings, the number of areas showing fibroplasia and Hale-positive material increased, these 2 changes being closely related on a morphologic basis (figs. 2 and 3). The lesions tended to extend in the

*We assume that the ACTH was acting continuously because of the use of the long-acting gel preparation.
subintima around the lumen as well as into the subjacent media. Mild symmetric narrowing of the aorta was often observed. As the severity of the lesions intensified, collagen appeared in large amounts. Gross lesions were observable after the fourth breeding, especially in ACTH-treated female rats. Although male rats exhibited no lesions grossly, microscopic examination showed a surprising number of intimal atheromata that stained intensely with hematoxylin and Hale's stain. The luminal orientation of the atheromatous lesions was such as to preclude visual detection by external gross inspection. Lesions in the females invaded medial layers to a greater extent, causing obvious raised lesions visible on the external surface.

In all of the breeding groups, a radial alignment of the proliferating fibroblasts in plaques became more frequent with subsequent breedings, eventually culminating in the formation of a fibrous plaque with heavy accumulation of acid mucopolysaccharides at its base. Proliferating fibroblasts in these lesions were seen to contain acid mucopolysaccharide granules dispersed in their cytoplasm (fig. 4). Occasional giant cells were encountered in the lesions, close to the intimal surface. The degree of fibrosis within the plaques was qualitatively greater in animals receiving ACTH. It was less severe in non-treated breeders than in rats with unilateral nephrectomy which had been similarly bred. The combination of unilateral nephrectomy and ACTH-treatment produced intimal plaques of a very fibrous and relatively acellular nature.

Males suffered more severe involvement of the aorta than did the females of the corresponding groups, as evidenced by the greater incidence and severity of microscopic lesions. During the fourth and fifth breeding it was also clear that ACTH injections were associated with an intensification of the pre-existing atherosclerosis.

Elastic Tissue Alterations

Alterations in elastic lamellae of the subintima and media were consistently found. The abdominal aorta was the most severely affected in all cases. In areas of marked deposit of intercellular acid mucopolysaccharides, the distortion and fragmentation of the elastic lamellae appeared to be caused by the physical pressure of the pools of metachromatric substance (fig. 5). Delicate interlamellar fibrils became visible between the proliferating fibroblasts, the degree of interlamination increasing somewhat with the size of the plaque.

In the middle portion of the media, small vacuoles surrounded by a material which stained an intense red by the Hale technique were occasionally seen. In such areas the necrosis of elastic tissue was most severe (fig. 10).

Endothelial Cells

Changes in the endothelial cells could be correlated with the severity of the underlying intimal lesions. Very early, where mild to moderate subendothelial fibroblasts proliferation had occurred, the nucleus of the endothelial cell appeared increased in size, and also stained with undue intensity. Mild endothelial hyperplasia unattended by proliferation occurred at this time (fig. 1). In later breedings, endothelial cells were enlarged and their cytoplasm became greatly distended with mucopolysaccharide-staining granules. Cells were occasionally 4 to 5 times normal size (fig. 8). In the most advanced lesions, the nuclei were often irregularly oval and pyknotic, suggestive of degeneration (fig. 9).

Lipids and Calcium

Lipophages were not observed in the early lesions, whereas occasional cells of this type could be found near the intimal surface of the more advanced lesions. Calcification could not be detected in early lesions of the intima or media. However, sections stained with hematoxylin and eosin occasionally showed moderately basophilic streaks on the intimal surface of the aorta, especially near its branches. Hale's method disclosed that these areas exhibited evidence of marked mucopolysaccharide changes. The areas which were consistently stained by Hale's method were
irregularly stained with hematoxylin.* Calcium salts may exist in higher concentrations in these doubly staining areas.

**Multiphasic Changes**

After 4 breedings, many of the animals receiving ACTH showed massive increases of acid mucopolysaccharide in the media and intima. Marked discontinuity of the medial architecture occurred in various sections of the aorta, especially in the aortic arch. In such sites, complete elastosis had occurred, associated with necrosis of the medial elements. In one animal, this degeneration had extended to the intimal surface of the aorta and a large aneurysmal outpocketing of the endothelial surface had occurred (fig. 6).

**Muscular Arteries**

A wide variety of lesions was found in this group of vessels. The most common feature was moderate to severe stenosis even with complete occlusion of many smaller arteries (figs. 11 and 12). The lumen was encroached upon by an intimal outgrowth containing marked deposits of acid mucopolysaccharide (fig. 7). The Hale positive material was never observed in the media and adventitia of these vessels. Occasionally, obliterative stenosis in the small muscular arteries was of a hyperplastic variety, showing some similarities to the cellular alterations noted in polyarteritis nodosa (P.A.N.).† Lesions of the type described above were more common in males.

**Discussion**

The earliest lesions observed in repeatedly-bred rats was the accumulation of acid mucopolysaccharide in close association with subintimal fibroblastic hypertrophy and hyperplasia. This atherosclerotic alteration which appeared inconstantly after the first breeding was consistently found in all animals after the second breeding. The increased amount of acid mucopolysaccharide may be the result of enhanced fibroblastic activity. This agrees in part with the detailed accounts by Moon* and Moon and Rinehart† in their studies of human coronary atherosclerosis.

The origin of intercellular acid mucopolysaccharides in mast cells has been suggested by Asboe-Hansen;17 on the other hand, the fibroblast is considered to be the source for this substance by Gersh and Catchpole.18 In our studies these cells were not encountered either in or near the atheroma. Acid mucopolysaccharide granules were often observed in fibroblasts or in areas of marked fibroblastic proliferation. Similar observations in fibroblasts of non-atherosclerotic tissues have been reported by others.18,19 Our results appear to agree with those of Taylor and Saunders20 who demonstrated that a correlation existed between the accumulation of intercellular acid mucopolysaccharides and active fibroblasts in granulation tissue.

Perhaps the acid mucopolysaccharides give rise to the collagen-like material found in the center of the fibrous plaques. Metachromasia or acid mucopolysaccharides (Hale positive material) in those instances was restricted to the base of the plaques where intense fibroblastic proliferation was found. Furthermore, the collagen-like material always appeared well after the initiation of the atherogenic process. These morphologic observations are consistent with the reported relationship of fibroblastic response and collagen formation following implantation of gel foam pledgets in rat skin20 and chemical assays in healing wounds in guinea pigs.21

Lipids appeared in the atheromata only in later stages of development; therefore, it does not appear likely that lipids are major factors in the initial stages of atherosclerosis in these animals, especially since normal serum cholesterol levels were maintained through this stage of development. Although only minute extracellular fatty droplets were present in the very early lesions, occasional foam cells

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*The inability of most metachromatic areas to be demonstrated by hematoxylin suggests a chemical alteration within the tissue which may govern the relative alkalinity and the subsequent tinctorial reactions.

†The microscopic appearance of affected vessels resembled atherosclerosis, lacking the usual mural alterations and conspicuous periadventitial reaction of polyarteritis nodosa.
Figure 1
Aortic arch of young female breeder (non-treated) (Group I). Subintimal and medial fibroblastic hypertrophy and hyperplasia. Surrounding the cells are "halos" of acid mucopolysaccharide deposits. Note also the mild elastic distortion and appearance of interlamination between adjacent elastic fibers. The endothelium exhibits early hyperplasia and cytoplasmic metachromasia. Modified Hale's stain. X470.

Figure 2
A high-power view of aortic arch to show close morphologic association of increased intercellular acid mucopolysaccharides with adjacent fibroblasts. Modified Hale's stain. X970.

Figure 3
Aortic arch of a male breeder treated with ACTH and unilateral nephrectomy (Group II). Generalized fibroplasia and increased acid mucopolysaccharides of subintima and adjacent media. Endothelial hyperplasia has proceeded to layering and characterized by marked cytoplasmic vacuolation ("hydropic degeneration"). Modified Hale's stain. X470.

Figure 4
Aortic arch of unilaterally nephrectomized male (Group IV). Severe destruction of medial elastic tissue, and marked accumulation of intercellular acid mucopolysaccharides. Overlying this area is an intimal protrusion of cellular and unidentified debris. Modified Hale's stain. X100.

Figure 7
Medium artery near abdominal aorta of a non-treated male breeder (Group II). Note the marked endothelial hyperplasia and metachromasia. Many of the cells are pyknotic. The underlying internal elastic membrane is intact. The arterial lumen is almost completely occluded. Modified Hale's stain. X210.

Figure 8
Abdominal aorta of unilaterally nephrectomized male (Group IV). Peculiar giant endothelial cell overlying subintimal fibroplasia. Its cytoplasm is filled with coarse metachromatic granules, and its nucleus is somewhat vesicular. Modified Hale's stain. X970.

Figure 9
Abdominal aorta of a non-treated male breeder (Group II). Well-developed atheroma characterized by intense metachromasia and fibroplasia in central and basal portions of plaque. Marked irregular vacuolization at luminal side of atheroma. Normal underlying deeper layers of the media not shown. Modified Hale's stain. X210.

Figure 10
Aortic arch. Lesion frequently encountered characterized by intimal pools of acid mucopolysaccharide subjacent to central pools of red-staining material which may be glycoprotein, (pro-) collagen, fibrin, or other types of mucopolysaccharides. Modified Hale's stain. X210.

Figure 11
Medium-sized artery near abdominal aorta of unilaterally nephrectomized male rat (Group III). Marked intimal sclerosis with complete occlusion of lumen and revascularization. Much of the internal elastic membrane is destroyed, these areas being adjacent to sites of medial hyperplasia. H & E. X210.

Figure 12
Some artery as above stained with Hale's stain. Note that acid mucopolysaccharide accumulation is mild. The intercellular ground substance is apparently glycoprotein or (pro-) collagenous in nature. Modified Hale's stain. X210.
Early atherosclerotic changes in aortae of young repeatedly-bred rats

(See legends on opposite page)

*The color reproduction of the photomicrographs was provided through the courtesy of the Wm. S. Merrell Co., Cincinnati, Ohio.
appeared in the more advanced ones. It is difficult to believe that the fatty deposits encountered bear any relationship to the cholesterol-induced atherosclerosis of rabbits. The vacuolated endothelial cells encountered in the rat aorta suggest that degeneration of these cells may contribute lipid to the atheroma. Since cell degeneration is often accompanied by increased lipid content (among other substances), the phagocytosis of these lipids extruded upon disintegration during normal reparative processes might account for the occurrence of lipophages at, or just beneath, the surface of the atheroma.

The reasons for the accumulation of lipid in atheromata is still unclarified. Perhaps the accumulation of excess lipid in arterial tissue overcomes those metabolic processes concerned with the removal of fats, a possibility suggested by Shore et al. Faber believes that lipids have an affinity for regions in which increased amounts of acid mucopolysaccharides are manifest. However, this observation was not verified in our studies nor in investigations of rabbit atherogenesis. A more likely possibility in our experimental lesions is that plasma lipids tend to localize in areas of damaged tissue. This is suggested by appearance of lipids only where subintimal lesions were advanced, or where fibrosis was severe.

Certain effects of ACTH require comment. During initial breedings ACTH appeared to modify the developing atherosclerosis by reducing the severity of metachromasia in ACTH-treated animals. Increased fibrosis of the lesions might indicate that ACTH enhanced reparative processes in its early stages. As the number of breedings increased, however, ACTH administration was associated with augmented atherosclerosis. To date, we have been unable to describe any distinctive lesions attributable only to exogenous ACTH. The possibility exists that exogenous ACTH enhances the atherogenic process induced by successive breedings. Pregnancy and hyperadrenocortical activity are closely interrelated. It would be interesting to know whether the excess endogenous production of ACTH in pregnancy is responsible for the "spontaneous" atherosclerosis observed in young, repeatedly-bred rats. Support for this hypothesis is found in a previous report from this laboratory in which was shown that ACTH augmented pre-existing atherosclerosis in such subjects. Further, Gillman and Hathorn have also shown that repeated pregnancy will produce arteriosclerosis in the rat.

The similarity of atherosclerosis observed when only exogenous ACTH, or unilateral nephrectomy, or repeated breedings were the experimental variables, would seem to indicate that each of these mediates its action on the connective tissue components by a similar mechanism.

The marked difference in the severity of rat atherosclerosis in relation to sex is interesting from a general biological standpoint. This also the case in respect to the well-known dichotomy between the sexes in relation to coronary artery and other types of atherosclerosis. Atheromata in male rats grow inwardly toward the lumen; in the female rats they generally extend in the opposite direction, or tend to remain localized in subintimal areas. This may account for our earlier observations that gross appraisal of lesions indicated a marked sex difference, i.e., female rats only were reported as showing the atherosclerosis. The centrifugal outgrowth of lesions in females causes them to appear more severe grossly than the centripetal growth in males. One could speculate that female rats, although suffering greater connective tissue damage in the subintimal and medial areas, are in some way protected from the lethal effects of atherosclerosis. The male rats, showing lesions on a microscopic scale, develop myocardial infarction, age prematurely, and succumb at a more rapid rate than the females (unpublished observations). Therefore, this sex difference may be due to the degree and chronological appearance of intimal involvement.

It is possible that there may be a fundamental difference in the reaction of connective tissue of male and female rats to ACTH. It is
also conceivable that male and female sex hormones may condition the connective tissue elements of the arterial wall to respond differently to the effects of other hormones.

Summary
Early atherosclerotic lesions are described in aortas of repeatedly-bred rats of both sexes. Characteristically, subintimal accumulations of acid mucopolysaccharides and fibroblastic proliferation marked the onset of atherosclerosis, followed by distortion and destruction of elastic lamellae.

Several other pathologic alterations occurred later, notably fibrosis, endothelial hyperplasia, lipid deposition, and calcification. Elastic tissue changes occurred independently and usually secondary to alterations of the ground substance and fibroblasts. Calcification and lipid deposits are found in more advanced lesions, showing an affinity for areas rich in mucopolysaccharides. Some of these animals were subjected to unilateral nephrectomy, injections of ACTH, or combinations of both. Exogenous ACTH and unilateral nephrectomy augmented the early atherosclerotic lesions associated with multiple breedings.

Atheromata in the male rats progressed toward the lumen, whereas those in females tended to extend toward the adventitia. Despite the equal incidence of lesions in either sex, their severity as judged by distribution, mortality rate, and myocardial infarction was greater in the males. This sex difference is discussed in terms of a possible relationship between the arterial connective tissues and certain hormonal imbalances.

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Summario in Interlingua
Es describite precoce lesiones atherosclerotic constatate in le aortas de repetitemente copulate rattos de ambe sexos. Characteristicament, accumulaciones subintimal de mucopolysaccharides acide e proliferation fibroblastic marzavan le declaration del atherosclerosis, sequite per distortion e destruction del lamellae elastic.

References
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