Biochemical Observations of Human Atheromatosis
Analysis of Aortic Intima

By Nancy L. Noble, Ph.D., Robert J. Boucek, M.D., and Kung-Ying Tang Kao, M.D., Ph.D.

Atherosclerosis develops chiefly in the intima of the artery. Biochemical changes associated with atheromatosis were studied serially in human aortic intima by a gross division of the intima into normal tissue, early and advanced atheroma. Earliest alterations observed are increases in collagen concentration and in binding of hexosamine with scleroprotein. Elevation of lipids occurs only after development of the atherosclerotic lesion. There appear to be more biochemical alterations in female intima with atheromatosis and age than in male tissue. Calcium concentration does not increase with age in normal intimal tissue.

The approach to the study of atheromatosis has been through gross and histopathologic observations, by histochemical studies, and by determinations of biochemical constituents of aortas. However, no attempt has been made to study in a serial manner the biochemical changes produced by atheromatosis as it develops in the human aorta.

The effects of aging on an artery have been investigated in a sketchy manner. To be certain, important observations have been made on the histopathology of aging and, more recently, Lansing and co-workers have analyzed the media of the human aorta as it is influenced by aging. However, too frequently the attrition to the artery by aging is confused with the disease process of atherosclerosis. Pathologists in general and many research workers in the field make a distinction between arteriosclerosis and atherosclerosis, but most clinicians continue to refer to any arterial change as arteriosclerosis.

Atherosclerosis occurs chiefly in the intima of the artery. In this study therefore, the intimas of human aortas were stripped from the underlying media and subjected to chemical analyses. The following report quantitates a number of biochemical events that occur in the normal intima and in the early and advanced atheromatous areas. When possible, these 3 areas were obtained from the same aorta for study and comparison.

Methods

Human aortas were obtained at necropsy from Negro and white individuals dying without antecedent illnesses of a protracted nature. Aortas from 37 males (age range from stillborn-term-infant to 94 years) and 23 females (31 to 94 years) were used in this investigation. Upon removal, the aorta was immediately frozen and kept at -20 C. For the isolation of the intima the aorta was thawed with the aid of a dissecting glass, fine forceps, and an ophthalmic spatula, the cleavage plane between the intima and media was located. Usually this was more readily accomplished in the aortas of the males and at the level of the lower thoracic or abdominal areas. The intima of the ascending aorta was more difficult to isolate.

Gross division of each intima into normal tissue and early and advanced atherosclerotic tissue was made when possible. Early areas consisted of thickened, white-yellow areas located usually at the site of the origin of an intercostal artery or at bifurcation of arteries. Yellow-golden areas were separated from the media when possible and classified as advanced atheroma. Normal intima or intima containing an early atheromatous lesion was readily identified and separated. In the advanced atheromatous area, an anatomic separation of intima and media was difficult, since the atherosclerotic lesion encompassed both regions of the artery completely or partially.

Thus, from each aorta at least 1 and usually 2 or 3 areas were obtained for comparative studies. After identification and isolation of the areas, the resected intima was washed by spraying with physiologic saline, blotted, and refrozen until analyzed.

The frozen tissue was crushed and then homogenized in physiologic saline as previously described, so that a saline-soluble fraction and a saline-insoluble fraction, the scleroprotein portion, were isolated.
Male

<table>
<thead>
<tr>
<th>Sex Tissue area</th>
<th>Hexosamine</th>
<th>Total lipid</th>
<th>Total cholesterol</th>
<th>Cholesterol Total Lipid × 100</th>
<th>Other lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline-Soluble</td>
<td>Saline-Insoluble</td>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female Normal (N)</td>
<td>0.066 ±0.017*</td>
<td>0.064 ±0.016</td>
<td>0.178</td>
<td>0.061</td>
<td>32.23</td>
</tr>
<tr>
<td></td>
<td>(6)</td>
<td>(6)</td>
<td>(7)</td>
<td>(7)</td>
<td>(7)</td>
</tr>
<tr>
<td>Early Athero-</td>
<td>0.064 ±0.015</td>
<td>0.0632</td>
<td>0.154</td>
<td>0.073</td>
<td>9.05</td>
</tr>
<tr>
<td>mat (E)</td>
<td>(7)</td>
<td>(7)</td>
<td>(8)</td>
<td>(8)</td>
<td>(8)</td>
</tr>
<tr>
<td>Advanced Ath-</td>
<td>0.066 ±0.016</td>
<td>0.066</td>
<td>0.186</td>
<td>0.055</td>
<td>3.99</td>
</tr>
<tr>
<td>eromata (A)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
<td>(10)</td>
</tr>
</tbody>
</table>

Male

<table>
<thead>
<tr>
<th>Sex Tissue area</th>
<th>Hexosamine</th>
<th>Total lipid</th>
<th>Total cholesterol</th>
<th>Cholesterol Total Lipid × 100</th>
<th>Other lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline-Soluble</td>
<td>Saline-Insoluble</td>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (N)</td>
<td>0.067 ±0.025</td>
<td>0.0829</td>
<td>0.224</td>
<td>0.067</td>
<td>23.45</td>
</tr>
<tr>
<td></td>
<td>(10)</td>
<td>(3)</td>
<td>(4)</td>
<td>(6)</td>
<td>(6)</td>
</tr>
<tr>
<td>Early Athero-</td>
<td>0.078 ±0.024</td>
<td>0.033</td>
<td>0.138</td>
<td>0.075</td>
<td>14.52</td>
</tr>
<tr>
<td>mat (E)</td>
<td>(9)</td>
<td>(9)</td>
<td>(9)</td>
<td>(9)</td>
<td>(9)</td>
</tr>
<tr>
<td>Advanced Ath-</td>
<td>0.048 ±0.016</td>
<td>0.0536</td>
<td>0.194</td>
<td>0.086</td>
<td>9.16</td>
</tr>
<tr>
<td>eromata (A)</td>
<td>(15)</td>
<td>(13)</td>
<td>(13)</td>
<td>(10)</td>
<td>(10)</td>
</tr>
</tbody>
</table>

* Standard deviation of mean.
† Number of determinations.

In the determination of the hexosamine concentration in the aortic intima, both the saline-soluble and the bound saline-insoluble forms were determined. The spheroprotein of the intimal tissue was incubated with testicular hyaluronidase (300 viscosity units)* for 3 days under toluene at 37.5 C. in acetate buffer pH 5.2, containing 0.15 M sodium chloride. Total hexosamine was determined by a modification of the method of Elson and Morgan" on the saline-soluble fraction, the supernatant and the residue from the hyaluronidase incubation. The total hexosamine value was obtained by adding the concentrations for the 3 component fractions.

Samples of intimal tissue were extracted with hot 3:1 alcohol-ether, and total cholesterol* and total lipid were determined on the extract. The analysis for total lipid was made gravimetrically or turbidimetrically by a modification of the turbidimetric method for serum." Total protein was determined on the saline-soluble and saline-insoluble fractions of proline, glycine, and proline, and for total protein the tissue, and analyses for the amino acids, hydroxyproline were made on the dried fat-free tissue by procedures previously described.

The calcium concentration of the areas of intimal tissue was determined on the hydrochloric acid hydrolysate of the fat-free tissue after ashing and precipitation as the oxalate by modifications of the methods of Biedermann and Schwarzenbach* and of Grette.* Results for each area of aortic intimal tissue were pooled for each sex, regardless of race, and are expressed on the basis of protein concentration.

**Results**

The saline-soluble, saline-insoluble, and total hexosamine concentrations in the 3 human intimal areas are given in table 1. Since the hexosamine concentration of the supernatant from the hyaluronidase incubation was apparently constant, the values for this hyaluronidase-susceptible fraction and the residue from

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* Alidase (a brand of hyaluronidase) was graciously supplied by G. D. Searle & Co.
the incubation were combined and termed "saline-insoluble" hexosamine. In the pooled and meaned data of table 1 there are only 2 significant differences among the saline-soluble, saline-insoluble, and total concentrations of hexosamine in the 3 intimal areas of either sex. The early atheromatous lesion of the female aortic intima has significantly greater concentrations of saline-insoluble and total hexosamine than a comparable area of the male (p < 0.05 and p = 0.02 respectively). However, if the different areas from the aorta of a single individual are compared, there is an apparent decrease in the saline-soluble form of hexosamine, a rise in the saline-insoluble hexosamine, and an increase in the total concentration with atheromatosis (table 2). In view of the increase noted in total hexosamine concentration with the concomitant decrease in saline-soluble hexosamine, the relative rise in the saline-insoluble hexosamine is greater than it would appear to be on an absolute basis. These changes occur in both female and male intimal tissue.

The results of the lipid analyses are presented in table 1. Both total cholesterol and total lipid are significantly elevated in the advanced atheromatous areas of female aortic intimas when compared with either normal tissue or early atheroma. The only lipid component of the male tissue that is elevated in the atheroma is cholesterol. In the atheromatous areas of the male and female intima, the percentage of total lipid that is cholesterol is significantly greater than in the grossly normal area. Furthermore, when the cholesterol concentration was subtracted from the total lipid value so that the concentration of other lipids, presumably phospholipid and neutral fat, was obtained and correlated with degree of atherosclerosis, a significant rise in lipids other than cholesterol was found only in the tissue of the female.

There are no significant differences in the total protein concentrations of the saline-soluble and saline-insoluble fractions or in the total value for the 3 tissue areas of either sex (table 3). However, the concentrations of hydroxyproline and glycine, characteristic amino acids of collagen, increase with atheromatosis. Hydroxyproline is significantly elevated in the early and advanced lesions of female tissue and in the advanced atheroma of the male, and glycine, in the early atheroma of the female and in the advanced lesion of the male.

The only significant change in calcium concentration with atheromatosis is an elevation in the advanced atheroma of the male. This alteration is not apparent in the female tissue. In correlative studies of biochemical changes in the different areas of human aortic intima with age, very few significant alterations were observed. Normal intimal tissue from a young person is generally similar in biochemical composition to that from an elderly person. The only significant variations with age in normal tissue are a positive correlation for Gm. per cent cholesterol (r = 0.69, p < 0.05) and a negative correlation for glycine (r = -0.67, p < 0.05) in the male. A general biochemical similarity, regardless of age, also appears to exist between the tissues representing the 2 stages of atherosclerotic lesions. In the atherosclerotic tissue, however, there seems to be more correlation between biochemical composition and age than in normal tissues. In the advanced atheroma, Gm. per cent total lipid (r = 0.67, p < 0.05), hydroxyproline (r = 0.80, p < 0.01), glycine (r = 0.79, p < 0.02) and calcium (r = 0.97, p < 0.02) increase with age in the female while proline increases with age in the male (r = 0.64, p < 0.02). The only significant change in the early atherosclerotic area with age occurs in female tissue, i.e., total lipid (r = 0.72, p < 0.05).

A comparison of the absolute biochemical composition of the 3 tissue areas of the male and female aortas indicates that intimal tissue

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**Table 2.** Connective Tissue—Human Aortic Intima, Variations in Hexosamine Concentration with Atheromatosis

<table>
<thead>
<tr>
<th>Sex</th>
<th>Saline-Soluble</th>
<th>Saline-Insoluble</th>
<th>Total</th>
<th>Saline-Insoluble/Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decreased</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Increased</td>
<td>3</td>
<td>6</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decreased</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Increased</td>
<td>3</td>
<td>7</td>
<td>7</td>
<td>9</td>
</tr>
</tbody>
</table>
of the male and female is not significantly different. As stated above, the early lesion of the female intima has a greater concentration of both saline-insoluble \((p < 0.05)\) and total \((p = 0.02)\) hexosamine than the same area of the male. Proline concentration is greater in the early atheroma of the male \((p < 0.05)\) than in the comparable area of the female.

**Discussion**

The genesis of human atheromatosis is most elusive, since it appears to be related to few significant biochemical changes in aortic intimal tissue. A graphic summary of these alterations is given in figure 1, in which the relative mean concentrations of total lipid, cholesterol, total protein, 3 amino acids, and total hexosamine of normal intima, early and advanced atheroma are drawn to scale. It is apparent that the bulk of the tissue solids is protein \((15 \text{ Gm. per 100 Gm. wet weight})\) while the total lipid concentration is about one fifth this value \((3 \text{ Gm. per cent})\) in the normal intimal area.

Total carbohydrate concentration was not determined. However, the mean total hexosamine remains unchanged with the development of atheromatosis. The component parts of the total hexosamine, the saline-soluble, and the saline-insoluble, likewise are not altered in mean value.

The concentrations of total protein are relatively constant in the 3 areas of human aortic intima. Yet a real change occurs in the nature of the protein as the atheromatous lesion progresses from the early to the advanced state. Hydroxyproline is significantly greater in the advanced atheroma than in normal tissue or in
The apparent change in the physical state of hexosamine may be related to the change observed in the nature of the protein as the atherosclerotic lesion progresses. The difference between the hydroxyproline concentrations of the normal tissue and the advanced atheromatous areas represents an increase in the collagen concentration of total protein from 28 to 46 per cent. A concomitant rise in "bound" and total mucopolysaccharide might be expected to accompany this increase in scleroprotein if mucopolysaccharides, in particular chondroitin sulfate, exist in combination with collagen as proposed by Bowers\textsuperscript{10}.\textsuperscript{11} and Hightberger and co-workers.\textsuperscript{12}

The development of a firmness and hardness of intimal tissue, which is grossly observed in the process of atherosclerosis, is probably related to the modification of the protein to a more "sclero" type. It also may result from a partial dehydration phenomenon or it may represent polymerization or copolymerization of the connective tissue protein.

The effect of age of the individual per se upon the biochemical characteristics of intimal tissue studied is almost negligible. However, it should be pointed out that the mean age of the group of individuals studied was high, i.e., 60 years, so that the data are weighted in the older age bracket. More changes in intimal tissue biochemistry with age may occur than are apparent in this study.

In spite of the high mean age of both the male and female group, it is of interest to note that the constituents of female intimal tissue appear to be more subject to alteration with age than those of male tissue. The first change observed in the development of atheromatosis also occurs in female tissue, i.e., significant increases in hydroxyproline and glycine concentrations in the early lesion. It would appear that female connective tissue is more responsive, but perhaps, the male tissue responded at an age earlier than was represented in our analyses. The relationship of such lability of intimal tissue, as observed in the female, to atherosclerotic lesions remains to be elucidated.

From the reported data it is apparent that total lipid and cholesterol concentrations do not necessarily parallel each other as the athero-

Fig. 1. Changes with atheromatosis in the connective tissue of the human aortic intima.

the early atheroma. Glycine concentration also increases with atheromatosis.

As expected, alterations in the lipid components of connective tissue were observed in atheromatous intimal tissue. In the ulcerative advanced atheroma, total lipid and total cholesterol are significantly elevated.

The earliest significant biochemical changes observed in atheromatosis, however, are the increases in hydroxyproline and glycine concentration in the female intimal tissue. No other biochemical component was found to be altered in the early atheroma of either sex. It is only after the development of the early lesion that lipid aberrances occur or that calcium concentration is significantly elevated.

When areas of the aortic intimal tissue from a single individual are compared, a greater amount of the total hexosamine appears to exist in a bound state in the atheromatous area than in the normal tissue. This alteration in the physical state of hexosamine from a mucopolysaccharide that is soluble in saline to one that is not may represent a significant and primary event in the ground substance or scleroprotein of intimal connective tissue in the genesis of atherosclerosis.
sclerotic lesion progresses from the early to the advanced state. Female connective tissue obtained from the human aorta has significant elevations of cholesterol and other lipids with atheromatosis. In the intimal tissue from the aortas of males, the cholesterol rise constitutes the only significant lipid elevation of an atheroma. Thus, it seems that a total lipid increase in the tissue is not an early or even an essential event in the development of an atheroma. It should also be noted that cholesterol accumulation in connective tissue occurs late in the process of atherosclerosis.

Another interesting finding is the apparent difference between the relationship of calcium concentration of the intimal connective tissue and of the media elastic connective tissue to age. Lansing and co-workers\(^1\) reported a significant positive correlation between calcium concentration in the media of human aortas and advancing age. This could well be the origin of Monckeberg’s sclerosis, a process entirely different from atherosclerosis. Yet in the intima such a relationship does not exist. Only the female tissue of an advanced atheroma has a significant increase in calcium with age of the individual.

These findings seem to suggest that few biochemical changes occur in the early development of atheromatosis. Perhaps the physicochemical composition of the connective tissue of the intima is altered early and this may represent the initial event in the process of atherosclerosis.

**Summary**

The earliest biochemical alterations observed in the connective tissue of human intima with atheromatosis are an increase in the concentration of collagen and an increase in the binding of hexosamine with scleroprotein, i.e., collagen or elastin.

No significant elevation of lipids occurs in the early atheromatous areas of the intima.

Total lipid is significantly increased in the advanced atheromatous areas of the male and female intimal tissue. However, in the male, the increase in lipid is due only to a significantly elevated cholesterol concentration while both cholesterol and other lipids are significantly elevated in the advanced atheromatous areas of the female tissue.

The age of the patient does not significantly affect the biochemistry of the normal tissue, early or advanced atheromatous areas of the intima. Normal and atheromatous areas of the younger individuals are not generally different from similar areas obtained from older persons.

Calcium concentration does not rise significantly in the normal intimal tissue with age.

**Acknowledgment**

The authors gratefully acknowledge the continued support and interest of Dr. E. Sterling Nichol, Medical Director, Miami Heart Institute, the calcium determinations by Dr. David S. Howell, Assistant Professor of Medicine, and Miss Shirley Jean Wright, and the necropsy material obtained from Dr. Thomas M. Scotti, Associate Professor of Pathology, University of Miami School of Medicine.

**Summario in Interlingua**

Le prime alterationes biochimic observate in le histos conjunctive del intima human in casos de atheromatosis es un augmento del concentration de colageno e un augmento del ligation de hexosamina con scleroproteina, i.e. collageno o elastin.

Nule elevation significative de lipidos ocurreva in le prime areas atheromatose del intima.

Le nivellos de lipid total es elevate significativamente in avantiate areas atheromatose de histos intimal ab masculos e femininas. Tamen, in masculos le augmento lipidic reflete solmente un elevation significativ del concentration de cholesterol, durante que tanto le cholesterol como etiam le alte lipidos es elevate a grados significative in avantiate areas atheromatose in histos feminin.

Le etate del paciente exercise nule effecto significative super le biochimia del intima normal o de areas intimal con atheroma precoce o avantiate. Normal e atheromatose areas in juvane individuos non differe generalmente ab simile areas in personas de plus alte etates.

Le concentration de calcium in le intima normal non accresce significativamente con le avantiamento del etate del subjectos.
REFERENCES


This report is concerned with observations made on 78 patients with grade 3 and grade 4 hypertension according to the Keith, Wagner, and Barker classification and treated with the newer hypotensive agents. Fifty-six patients with grade 3 hypertension were treated from 4 to 31 months. Fifty-three of these patients had a significant drop in blood pressure and 34 showed improvement in the fundi. Forty-two patients of this group were alive at the time of the report. Twenty-two patients with grade 4 hypertension were treated. Sixteen of this group showed a significant drop in blood pressure. Thirteen of these patients survived the period of observation. The authors point out that carefully controlled dosage is necessary with these drugs, and this can be accomplished best by beginning treatment in the hospital and teaching patients to take and record their blood pressure at home.

Rosenbaum