Histochemical Studies in Atherogenesis: Human Aortas
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There have been many conflicting reports concerning the relationships of lipid, acid muco-
polysaccharides, and elastic changes in the early pathology of atherosclerosis. Various
new histochemical methods have been applied to grossly normal and early lesions of
aortas of 50 individuals ranging from fetuses to 40 years of age with a view to illuminat-
ing these problems. The following paper presents our results of these studies.

ALTHOUGH many investigations have been reported both in human1-4 and in the ex-
perimental animal,5-9 the relation between acid mucopolysaccharides and lipids in the
early pathology of atherosclerosis is still an area of controversy. This has been partly due
to the inadequate technics for the demonstration of these components. New carbowax em-
bedding technics which retain lipid in statu nascenti have permitted the observation of
both lipid and acid mucopolysaccharide in a single tissue section.10-12 In addition, thin
serial sections of uniform thickness can be routinely prepared enabling adjacent sections
to be readily studied for other components. It is the purpose of this paper to report our
observations on the relationships between lipid and acid mucopolysaccharides, lipid and elas-
tic elements, and acid mucopolysaccharides and elastic elements in human aortas from
various age groups, utilizing these new technics.

Methods

Human aortas were obtained fresh at autopsy from individuals in the following age groups:
gestational, first year, 1 to 10 years, 11 to 20 years, 21 to 30 years, and 31 to 40 years. Individual
lesions were graded as to their severity of atherosclerosis as follows: zero, grossly normal areas;
one-plus, fatty streaking and slight elevation of the intima; and two-plus, all lesions more severe
than this. Arch, thoracic and abdominal areas containing both zero grade and one-plus tissue were
taken for examination. These were freeze-dried and carbowax-embedded according to the method
of Zugibe et al.12

A series of six adjacent sections, 6 micra thick, were prepared and stained as follows: sections 1
and 2 with hematoxylin and eosin and aldehyde fuchsin-chlorantine fast red (elastic and collagen
stains), respectively;12 section 3 by oil red O-carbowax "400";11 section 4 by alcian blue-oil red O
combination technic;10 and section 6 by alcian blue after digestion with testicular hyaluronidase.
Sections of umbilical cord were digested with each batch of aorta sections as an index to en-
zyme activity. These digestion experiments were carried out in an attempt to learn something about the
nature of the polysaccharide present.

Results

Gestational Group

Grade zero. Fraying, fragmentation and re-
duplication of the internal elastic membrane
was present in most of the cases studied (fig.
1). The degree of such change ranged from
slight to marked. The acid mucopolysac-
charide appeared concentrated in the proxi-
mal and medial aspects of the internal elastic
membrane and between the reduplicated
areas. Lipid was frequently present as fine
droplets in the intima, but there existed no
apparent correlation between the acid mucopo-
lysaccharide and lipid staining intensity
(fig. 5a). Frequently, in the same tissue sec-
tion, areas of minimal acid mucopolysac-
charide staining would contain appreciable
lipid, while areas of maximal acid mucopoly-
saccharide staining would contain little or
no lipid. Further, no correlation existed be-
tween acid mucopolysaccharide staining inten-
sity and the sites of internal elastic mem-
brane changes (fig. 2). In general, the acid mucopolysaccharide staining pattern extended throughout the entire length of the section without an increase in staining intensity in the areas of elastic change. Actually, in some sections the converse was true, i.e., in areas with an intact internal elastic membrane there was an increase in acid mucopolysaccharide staining intensity. In this group, digestion with testicular hyaluronidase decreased the acid mucopolysaccharide intensity considerably.

**First Year Group**

Grade zero. Fragmentation, fraying and reduplication of the internal elastic membrane was present to some degree in all of the cases studied. In a few, this extended to the elastic fibers in the proximal media. There was a concentration of acid mucopolysaccharides on both the intimal and medial aspects of the internal elastic membrane. This extended throughout the entire length of the section. In some sections, minimal amounts of lipid were present as fine droplets in macrophages and, also, as finely dispersed extracellular particles. One specimen from a female exhibited the same manifestations as seen in the aortas from the males. Areas with increased acid mucopolysaccharide and no lipid, increased acid mucopolysaccharide and increased lipid and/or increased acid mucopolysaccharide and minimal lipid in sections treated with the alcian blue-oil red O combination stain were seen. In general, the distribution of acid mucopolysaccharide and lipid and the relation of these substances to the internal elastic membrane was essentially the same as seen in the gestational group. Digestion with testicular hyaluronidase reduced to a considerable degree the alcian blue staining intensity.

**1-to-10 Year Group**

Grade zero. The intima varied in thickness from section to section and within the same section. This variation ranged from an endothelial layer lying on the internal elastic membrane to an intima about one-half the thickness of the media. The changes in the
internal elastic membrane were similar to but more pronounced than in the previous groups. No sex difference was observed. Fibroblastic proliferation was a consistent finding in the intima and proximal media. There was a slight increase in acid mucopolysaccharide staining intensity in these areas and some sections revealed this increase to be associated with either the intima or media. Collagen stains were negative in the areas where the acid mucopolysaccharide staining intensity was increased in all the individuals with the exception of a ten year old male where the converse was true. Some lipid was occasionally present in the intima in the form of fine droplets. The lipid was seen in both the macrophages and extracellular area. No correlation was observed between the lipid and acid mucopolysaccharide staining material present. That remaining appeared as tiny patches in the intima.

Grade one plus. The changes were similar to those observed in the grade zero section with the exception that more lipid was present and the elastic changes were slightly more extensive.

11-to-21 Year Group

Grade zero. There was extensive reduplication, fragmentation and fraying of the internal elastic membrane accompanied by fragmentation of elastic fibers in the proximal media. Lipid was present as fine droplets in the macrophages and extracellularly in the intima. In most cases there was slight fatty metamorphosis of the elastic elements and internal elastic membrane (fig. 5b). There appeared to be an increase in coarse collagen with some intermingled fine collagen fibers in the proximal media. This extended the entire length of the section, but was more marked in areas of fragmentation and reduplication of the internal elastic membrane. There was increased acid mucopolysaccharide staining in the areas of collagen increase (figs. 3, 4). Digestion with testicular hyaluronidase altered the acid mucopolysaccharide staining intensity to a negligible degree.

Grade one plus. The fragmented and frayed internal elastic membrane and other elastic elements were more advanced. Much lipid was present in the form of fine droplets in the macrophages and extracellularly, mainly...
at the site of the lesion. A consistent finding
was fatty metamorphosis of the internal elas-
tic membrane throughout the elastic elements
of the intima and proximal media (fig. 5c).
There was considerable fibroblastic prolifera-
tion in the intima and proximal media. Coarse
collagen was greatly increased in the region
of the proximal media along the entire length
of the section. An increase in acid mucopoly-
saccharide staining in the area corresponding
to the collagen increase was routinely ob-
served. Increased acid mucopolysaccharide
staining was seen in areas containing lipid as
well as in areas devoid of lipid (fig. 5d). Con-
versely, lipid staining was increased in areas
containing no acid mucopolysaccharide. Di-
gestion with testicular hyaluronidase pro-
duced little if any change in the intensity or
distribution of the acid mucopolysaccharide
stain.

20-to-30 Year Group

Grade zero. Fibroblasts were increased in
the internal elastic membrane and in the
proximal media. The intima was thickened
and contained many elastic elements. Frag-
mentation was quite extensive in the internal
esthetic membrane, elastic elements of the in-
tima and proximal media. Reduplications
were numerous and in many sections appeared
as web-like networks. Intracellular endothelial
lipid was occasionally present, mainly as fine
droplets. There was an increase in collagen
in the proximal media. This was predomi-
nantly the coarse type intermingled with
some fine collagen fibers. This extended
throughout the entire section. There was also
some collagen in the intima. This was almost
entirely aggregations of an amorphous nature.
There was no apparent correlation between
the presence of lipid and the acid mucopoly-
saccharide staining intensity (fig. 5c). Fur-
ther, there was no apparent correlation be-
tween lipid or acid mucopolysaccharide and
the areas of fragmentation, fraying and/or
reduplication of the elastic membrane. There
appeared to be some relationship between acid
mucopolysaccharide and collagen. Digestion
with testicular hyaluronidase did not affect
the acid mucopolysaccharide staining in the
intima. In other areas of the section only a
slight reduction of staining intensity was ob-
served.

Grade one-plus. The changes were similar
to but more extensive than those observed in
the grade zero sections. The internal elastic
membrane appeared more severely damaged
and much more lipid was present in the area
of the lesion. The lipid was mainly extrael-
cellular and extended from the internal elastic
membrane to the endothelium. Fatty meta-
morphosis of the internal elastic membrane
and elastic elements of the intima was present
in varying degrees (fig. 5f). The acid mucopoly-
saccharide staining intensity was greatly
increased in the proximal media and intimal
area of the lesion.

31-to-40 Year Group

The changes in both grades were similar to
but more extensive than those seen in the 21-
to-30 year group. The collagen and acid muco-
polysaccharide increases were more extensive,
and there was considerably more fatty meta-
morphosis of the internal elastic membrane
and elastic elements of the intima. No correla-
tion was observed between increased acid
mucopolysaccharide staining and lipid stain-
ing. Many sections showed an increase in acid
mucopolysaccharide staining in the proximal
media where the area was laden with lipid.
However, acid mucopolysaccharide staining
was as intense and extensive in areas of the
intima which contained little or no stainable
lipid.

Discussion

The random relationship between lipid and
increased acid mucopolysaccharide in all of
the groups indicates that there is no relation-
ship between acid mucopolysaccharide and
lipids in respect to staining intensity and/or
distribution in any of the groups.

These observations are not in agreement
with those of Rinehart and Greenberg. They
speculated that the acid mucopolysaccharides
have a special affinity for lipids and related
that the possibility existed that lipid may be
derived from the degeneration of acid mucop-
Figure 5a
Thoracic aorta of an 8 month male fetus. There is no correlation between the acid mucopolysaccharides and lipid staining intensity. Oil red 0, alcian blue, X 250.

Figure 5b
Thoracic aorta of an 11 year old male. Note the fatty changes in the internal elastic membrane and elastic elements. Oil red 0, X 400.

Figure 5c
Thoracic aorta of a 15 year old male. There is a fatty metamorphosis of the internal elastic membrane and other elastic elements of the intima and proximal media. Oil red 0, X 250.

Figure 5d
Thoracic aorta of an 11 year old male cut adjacent to Fig. 3. The acid mucopolysaccharides are increased in areas containing lipid as well as in areas devoid of lipid. Oil red 0, alcian blue X 100.

Figure 5e
Thoracic aorta of a 21 year old male. The acid mucopolysaccharides are about equal in intensity if not less in the areas of the focal lipid deposit as they are in the area devoid of lipid. Oil red 0, alcian blue, X 250.

Figure 5f
Thoracic aorta of a 21 year old male. Note the fatty changes in the internal elastic membrane and other intimal elastic elements. Oil red A, X 400.
polysaccharides. In a paper concerned with coronary arteries, Moon and Rinehart reported that in the early lesion no relation existed between the occasional presence of fine lipid droplets and increased amounts of mucoid ground substance and that in the moderately advanced lesion an increased affinity of an abnormal intercellular ground substance for lipid could be a cause of lipid deposition. Such situations require that lipid be concentrated in areas of increased acid mucopolysaccharides. This, however, is not the case since we often observed lipid in areas with no increase in mucopolysaccharide staining. Further, one would assume that the primary lipid deposition would be concentrated in the region of the medial portion of the intima because of the great increase in acid mucopolysaccharides which occurs there.

Wang et al., working with the rabbit, concluded that an increase in acid mucopolysaccharides was the earliest change which occurred in atherosclerosis, with lipid deposition as a later manifestation accompanied by a decrease in acid mucopolysaccharides. In a subsequent paper, these workers reported that the decrease in acid mucopolysaccharides could have been due to a masking of these substances by the lipid. This was based on Craig's work with Gaucher's Disease where he reported that lipid in a tissue inhibited the colloidal iron reaction. Craig, however, worked with frozen section in which the lipid material was retained, while Wang used paraffin-embedded material from which a percentage was leached by subsequent treatment. Gore and Barr, working with cholesterol induced atherosclerosis in the rabbit, reported that augmented quantities of ground substances did not appear until atheroma had been established. The increase in ground substance was considered due to the reaction invoked by lipid deposition. Assuming the rabbit disease is similar to the human disease, our results are in complete disagreement with those of Gore and Barr.

The presence of fragmentation, fraying and/or reduplication of the internal elastic membrane in all of the fetuses and infants studied, indicated to us that if such changes were pathological and are the primary factors involved in the later development of atheroma, one would expect far more involvement of the aorta than is now reported. These results are in disagreement with Moon who considered fragmentation in these groups to be one of the earliest changes in atheroma formation. No definite correlation existed between acid mucopolysaccharide and the elastic changes. Accompanying the changes of the elastic tissue with age, there is a general increase in acid mucopolysaccharides. This increase, as evidenced by staining intensity, is not restricted to those areas immediately involved in elastic change, but is general throughout the entire section. This is not in agreement with Taylor's view that fragmentation of the elastic fibers may be primary, resulting in a pooling of the acid mucopolysaccharides.

The presence of increased collagen in the areas corresponding to those showing increased acid mucopolysaccharide in the juvenile and adult groups implied that a close correlation existed between the collagen and acid mucopolysaccharide. The resistance of this polysaccharide to testicular hyaluronidase indicated that it was probably chondroitin sulfate B and/or heparitin sulfate. Meyer et al. and Berenson have reported the presence in aorta of hyaluronic acid, chondroitin sulfate A and chondroitin sulfate B, and Meyer found that chondroitin sulfate B was the only component resistant to testicular hyaluronidase. According to Linker, heparitin sulfate accounts for about 10 per cent of the polysaccharide in the aorta and is re-
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Assistant to testicular hyaluronidase. The absence of collagen in the areas corresponding to the increased acid mucopolysaccharide of the first three groups and their susceptibility to testicular hyaluronidase indicated that hyaluronic acid and/or chondroitin sulfate A accounts for the increased acid mucopolysaccharide. It is suggested from our observations that the deposition of collagen is conceivably a reinforcement mechanism to strengthen the arterial wall in areas of fragmentation of the internal elastic membrane and elastic fibers of the proximal media. Our data will only permit speculation about the relationship between collagen formation and acid mucopolysaccharide concentration. The acid mucopolysaccharide of ground substance has been implicated with the formation of collagen by many investigators. Chondroitin sulfate B has been associated with the coarse type of collagen fibers in skin. Taylor and Saunders have reported that the metachromatic staining material is involved in the formation of reticulum and collagen in granulation tissue. The derivation of reticulum, collagen and elastic tissue from ground substance is discussed in Ham's Histology. Rinehart and Greenberg state that, "mucoid material is the mother substance which normally acts as an effective cement and from which collagen and elastic tissue fibers are maintained and formed."

The presence of intimal lipid in grossly normal and one-plus sections in all of the groups studied suggests that fatty changes are the primary alteration in atherosclerosis. This is in accord with the most recent observations of Wissler et al. but in opposition to the results reported by several other workers. Dock concluded that areas of intimal thickening were predisposing to later atheroma formation. Similar conclusions were reached by Wilens who further stated that intimal thickening occurs early in life, is not a natural development, and is related to the same mechanism that eventually leads to atherosclerosis. If these conclusions are correct, it would be reasonable to assume that when lipid accumulation occurs it should be associated with intimal thickening or fibrous plaque formation. We have observed instances of this relationship, but in many instances lipid accumulation is in areas other than intimal thickening or fibrous plaque formation. These observations are in agreement with Moschowitz, who claims intimal thickening of fibrous plaque formation and atherosclerosis are different processes. He, however, concludes that atherosclerosis is not a primary disorder but, "this does not imply that lipid is deposited only in those areas" (areas of intimal thickening). Moon and Rinehart stated that fibrosis of the intima, increased acid mucopolysaccharide in the ground substance and fragmentation of the internal elastic membrane occur at the same time and are the earliest changes in atheroma formation. They further relate that lipid deposition occurs after these changes have taken place and is secondary in nature. Our observations of the well-defined fatty metamorphosis of the elastic elements in the intima and internal elastic membrane, as previously stated, indicate that these may be the primary alteration in atherosclerosis. This accumulation of fat with the elastic fibers does not seem to be a gross surface phenomenon, but rather a degenerative or infiltrative change. When thin (2 micra) serial sections of an area containing lipid and elastic changes are prepared, some of the fibers will be cut, and any lipid physiologically oriented on the surface of these fibers would be readily observed. None is found and most of the lipid in these preparations has been distributed within the area of the fiber. Such a situation seems to indicate that the lipid is produced within the fiber by focal degenerative changes or by infiltration and trapping of lipids probably because of a special affinity of altered elastic fibers for the lipids that permeate the arterial walls as related by Duff et al. These antecedent elastic changes due to some local factor such as infection, or a local metabolic defect alone or coupled with the collagen deposits (enhanced by early elastic fragmentation) may alter the permeability of

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the arterial wall at specific sites. This, combined with elevated circulating lipid, could cause a pooling of lipid in such areas and result in atheroma formation.

Summary

A critical study of grossly normal and early lesions of the aortas of 50 individuals from five-month gestational to 40 years of age, utilizing newer, histochemical and histological methods, resulted in the following conclusions:

There is no apparent relationship between acid polysaccharides and lipids in respect to staining intensity and/or distribution in any of the age groups.

Fragmentation, fraying and reduplication of the internal elastic membrane or any combination of these is a consistent finding in all of the groups. However, no definite correlation exists between acid polysaccharides and these changes in the fetuses and infants. What appeared to be a correlation in juveniles and adults was actually due to the deposition of supportive collagen and acid polysaccharides rather than the deposition of acid polysaccharides because of these changes.

The failure of testicular hyaluronidase to hydrolyze the major amount of the increased acid polysaccharides in the juveniles and adults suggests that this material is primarily chondroitin sulfate B and/or heparitin sulfate. It is further suggested that the elaboration of coarse collagen is associated with the presence of chondroitin sulfate B.

The presence of lipid in the grossly normal and one-plus sections of all the groups seems to implicate fatty changes as the primary alteration in atherogenesis. A theory of atheroma formation was postulated.

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Summario in Interlingua

Un studio critic de macroscopicamente normal aortas e de aortas con recente lesiones in 50 individuos de etates de inter le quinte mensu del gestation e 40 annos, utilisante moderne methodos histoehimic e histologie, resultera in le sequente conclusiones.

Il existe nulle apparente relation inter polysaccharidos acide e lipidos con respecto al intensitate de lor tincturation e/o lor distribution in omne del varie gruppos de etate.

Le fragmentation, grangiation, e reduplication del interne membranas elastic, o un combination de plures de iste phenomenos, es un occurrentia regular in omne le gruppos. Tamen, nulle definite correlation existe inter le polysaccharidos acide e iste alteraciones in fetos e infantes. Le apparente correlation in juveniles e adultos esseva de facto le consequentia do un depositio de collageno supportative e polysaccharidos acide e non le deposition de polysaccharidos acide a causa del mentionate alteraciones.

Le facto que hyaluronidase testicular non hydrolisa le plus grande parte del augmento de polysaccharidos acide in le juveniles e adultos suggere que iste materiale es primarimente sulfato de chondroitina B e/o sulfato de heparitin. Es suggerite in plus que le elaboration de collageno rude es associate con le presentia de sulfato de chondroitina B.

Le presentia de lipidio in sectiones macroscopicamente normal e in sectiones 1+ de omne le gruppos pare implicar alteraciones grasse como le alterationes primari in atherogenesis. Es postulate un theoria relative al formation de atheroma.

References

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