Histochemical Studies in Atherogenesis

Human Cerebral Arteries

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The histology and histochemistry of cerebral arteries have been subject to only a few investigations. Duff, Forbus, Glynn, and Carmichael have stressed the importance of the integrity of the internal elastic membrane. Wolkoff studied the relationship between the elastic elements and ground substance components in cerebral arteries. He noted that lipid was occasionally seen between the reduplications of the internal elastic membrane. Binswanger and Schaxiel studied cerebral arteries of infants, of individuals 30 to 40 years of age, and 60 to 70 years of age. Changes in these arteries with aging were reported. Hackel has also reported on changes in cerebral arteries with aging. However, both these reports gave only a superficial survey of the structure of the cerebral arteries at different ages and contained nothing concerning the relation among the acid mucopolysaccharides and/or lipid, and/or elastic tissue.

The relation between acid mucopolysaccharides and lipids in the early pathology of coronary and aortic atherosclerosis has long been controversial. The lack of good methods for study of these components has caused much of the controversy of past years.

New histochemical techniques are now available to study the relationships between lipid and acid mucopolysaccharides, lipid and elastic elements, and acid mucopolysaccharides in tissue. These techniques have been used in an earlier study of the relationships of these elements in human aortas of various age groups. It is the purpose of this paper to extend this study to human cerebral arteries from various age groups.

Methods

Human cerebral arteries were obtained fresh at autopsy from 75 individuals ranging from fetuses to 70 years of age. This included 25 arteries from gestational to 6 years of age, 20 arteries from 7 to 15 years, and 30 adult arteries. Individual lesions were graded for 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 one-plus. Both zero grade and one-plus tissues were taken from the basilar artery, circle of Willis, vertebral artery and internal carotid artery at their bifurcation with the circle of Willis.

The very fragile cerebral arteries in the fetuses and infants were readily processed by the method of Zugibe et al. as follows: A drop of saline was placed on a small piece of liver. The tip of the piece of artery was picked up with a pair of fine iris scissors and the section allowed to float in the saline on the surface of the liver until the artery flattened out. The liver area beyond the artery was trimmed with a single-edge razor blade, and the artery plus liver section was freeze-dried. This method resulted in well-flattened sections in which the artery layers were clearly demarcated and the liver was used as a guide in locating the artery sections as well as a control in determining whether various stains were working. All tissues were freeze-dried and carbowax-embedded according to the method of Zugibe et al.

A series of six adjacent sections were cut at 6 μ and stained as follows: sections 1 and 2 with hematoxylin and eosin and aldehyde fuchsin-chlorantine fast red (elastic and collagen stains),
Figures 1 through 5
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Figure 6
Basilar artery of a 30-year-old male. Note the accumulation of acid mucopolysaccharides in the proximal media below the internal elastic membrane (arrows). Alcian blue, X 400.

respectively;11 section 3 with oil red 0-carbowax 400 for lipid;12,* section 4 with alcian blue-oil red 0 combination technique13 for acid mucopolysaccharides and lipid; section 5 with alcian blue for acid mucopolysaccharides;14 and section 6 with alcian blue after digestion with testicular hyaluronidase (Worthington). This enzyme (1,000 T.R.U./100 cc.) was buffered at pH 6.0 according to Meyer,15 and the sections were incubated at 37 C. for 18 to 24 hours. Sections of umbilical cord digested with each batch of tissue sections served as an index to enzyme activity. These digestion experiments were carried out in an attempt to learn something about the chemical nature of the polysaccharide present. A series of serial sections were cut at 1 and 2 μ and stained

*The use of the word lipid herein refers to those substances that stain with oil red 0 and are soluble in fat solvents, while the word lipoid refers to oil red 0 positive substances that are insoluble in fat solvents.

†The specificity of the alcian blue method, described by Zugibe et al.,13 for all of the acid mucopolysaccharides in human arteries was verified by in vitro testing of various acid mucopolysaccharides, neutral mucopolysaccharides, proteins, RNA and DNA,14 and by digesting sections with mixtures of enzyme extracts from bacteria adapted to various acid mucopolysaccharides.15

Figure 7
Basilar artery of a 30-year-old male cut adjacent to figure 6. This was pretreated with testicular hyaluronidase. Most of the mucopolysaccharide staining still remains. Alcian blue, X 400.

Figures 1 through 5

(Fig. 1) Basilar artery of a three-year-old male. Note the accumulation of acid mucopolysaccharides beneath the internal refractile elastic membrane (arrows). There is no intima present. Alcian blue, X 250.

(Fig. 2) Cerebral arteries of a three-year-old male cut adjacent to figure 1. This section was treated with testicular hyaluronidase. The acid mucopolysaccharides noted in figure 1 have been completely eliminated. Alcian blue, X 250.

(Fig. 3) Basilar artery of a 12-year-old male. Note the reduplication of the internal elastic membrane (black band). There is no collagen present here. Aldehyde fuchsin, chlorantine fast red, X 250.

(Fig. 4) Basilar artery of a 12-year-old male cut adjacent to figure 3. Note the accumulation of acid mucopolysaccharides in the area between the reduplication of the internal elastic membrane. This material was completely digested after testicular hyaluronidase treatment. Alcian blue, X 250.

(Fig. 5) Basilar artery of a 65-year-old male. Note the extensive reduplication and fragmentation of the internal elastic membrane (IEM) and elastic elements (dark, fine lines). Aldehyde fuchsin, chlorantine fast red, X 400.

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with oil red 0-carbowax 400 in an attempt to determine the location of the lipid in relation to the elastic fiber. In addition, a series of paired sections were stained with oil red 0-carbowax 400 and oil red 0-carbowax 400 following acetone extraction, respectively, to determine if any materials other than extractable lipid were being stained by the lipid stains.

**Results**

**Fetuses, Infants, and Young Juveniles (to 7 Years of Age)**

In general, no evidence of pathological changes was observed. Fraying, fragmentation, and reduplication of the internal elastic membrane were rarely noted (fig. 1). Collagen material was seldom, if ever, present in the intima or area of internal elastic membrane. The acid mucopolysaccharides were usually concentrated on the medial aspects of the internal elastic membrane (fig. 1). In addition, the acid polysaccharides were frequently observed concentrated in patches in selected areas of the internal elastic membrane. Lipid material was rarely observed in the intima of this group. Digestion with testicular hyaluronidase abolished most, if not all, the mucopolysaccharide staining (fig. 2). In general, little or no correlation was observed between the acid mucopolysaccharides and any of the other substances studied.

**Older Juveniles (8 to 15 Years of Age)**

**Grade Zero**

The intima varied in thickness from section to section.
to section and within the same section. The endothelial layer lying on the internal elastic membrane was the usual pattern seen; however, patches of intimal thickening were seen in several sections (fig. 3). An increase in fibroblasts was present whenever there was intimal thickening. Occasionally, in some of the sections, reduplication of the internal elastic membrane was present (fig. 3). Lipid was rarely observed in the arteries of this age group, except for the occasional presence of lipid tinting in the internal elastic membrane. The acid mucopolysaccharides were accumulated in the intima and internal elastic membrane area of almost all vessels. Acid mucopolysaccharide accumulation was also noted between fragmented elastic fibers in the occasional vessel which showed focal reduplications of the internal elastic membrane (fig. 4).

Collagen was occasionally noted in localized areas of the intima and/or area of the internal elastic membrane. The acid mucopolysaccharides in this area of collagen deposition were resistant to hydrolysis by testicular hyaluronidase, whereas, in general, the acid mucopolysaccharides observed elsewhere in the vessels were hydrolyzed by testicular hyaluronidase. Increased numbers of fibroblasts were found in the area of collagen deposition. There was no correlation observed between the acid mucopolysaccharides and elastic changes and/or lipid.

Grade One-Plus

Lesions were very uncommon.

Adults (16 to 70 Years of Age)

Grade Zero

Reduplication of the internal elastic membrane was a common occurrence which increased with age (fig. 5). These changes were more frequently noted in the region where the intima was thickest. There was an accumulation of acid mucopolysaccharides in the intima and in the proximal media (fig. 6). The greater amount of acid-mucopolysaccharide material was resistant to the action of testicular hyaluronidase (fig. 7). Collagen was present in areas of the intima and proximal media, increasing in amount with age. Areas of accumulated acid mucopolysaccharides corresponded to these areas of collagen deposition (figs. 10, 11, 18, and 19).

A most striking and consistent observation in the cerebral arteries of the adults was the presence of lipid intimately associated with the internal elastic membrane and reduplicated elements (figs. 8, 9, and 12 through 17). Serial sections cut obliquely through the internal elastic membrane revealed the lipid to be uniformly distributed throughout the fiber elements (fig. 16). The amount of this lipid appeared to increase with age. No correlation between the acid mucopolysaccharide and lipid was observed since areas were frequently seen with both acid mucopolysaccharide and lipid, with lipid but very minimal acid mucopolysaccharide, and with acid mucopolysaccharide but minimal or no lipid (figs. 13, 14, and 17).

Grade One-Plus

The changes were similar to those observed in the grade zero section with the exception that more lipid material was present, principally extracellularly, and the elastic changes were more extensive.

Discussion

The lack of relationship between acid mucopolysaccharides and lipids in respect to staining intensity and/or distribution in all of the groups agreed with our findings in human aortas. The major difference, however, between the two tissues was that lipid rarely appeared in the cerebral arteries below 15 years of age. In older juveniles and adults, a most striking and consistent observation was the presence of lipid intimately associated with the internal elastic membrane and other reduplicated elastic fibers even in the absence of gross lesions. The nature of this elastic-lipid relationship cannot be concluded from our observations. Further studies are presently under way to study the submicroscopic structure of these fibers. That this lipid was distributed within the visible elastic fiber and not oriented on the periphery was demonstrated by the 2-μ adjacent sec-
tions (figs. 8 and 9) and by the oblique section through the internal elastic membrane (fig. 16). As far as we know, this is the first time that these early lipid changes in the internal elastic membrane have been demonstrated. It is of considerable interest to note that Parker considers the initial elastic lesion in experimental atherosclerosis to be a rapid change in the internal molecular structure of the elastica interna probably due to the formation of a solid solution of lipid in the elastic protein. The conclusion that the oil red 0 positive material was lipid and not other lipid substances, such as ceroid, lipofuchsian pigment, Ciaccio's lipoids, unsaturated fats, etc., was confirmed by treating the sections with lipid solvents prior to staining. The oil red 0 positive material was completely extracted in every instance, indicating that the material was lipid. Comparative studies using the conventional alcohol and acetone solvents for the lipid stains failed to reveal early lipid changes within the internal elastic membrane or the presence of early extracellular lipid materials. It is obvious that the disagreement between various investigators as to whether lipid material is present early or as a later manifestation in atherosclerosis and the extent of lipid in the various stages of atherosclerosis is due to inadequate techniques for the demonstration of lipid. Lilley relates that the Herxheimer technique removed a considerable amount of fat and, in some cases, all of the fat present. Chiffelle and Putt have published an excellent discussion of the undesirable properties of the various lipid stain solvents. They point out the advantages of using propylene glycol and ethylene glycol as solvents for the azo and diazo "Sudan" dyes. Moreover, Zugibe, Fink, and Brown have reported the use of carbowax 400 as a solvent for oil red 0 and Sudan IV. This solvent fulfills all the requirements of an efficient solvent for lipid staining, as postulated by Chiffelle and Putt, and has additional advantages which make it superior to propylene glycol.

Fragmentation, fraying, and/or reduplication of the internal elastic membrane were rarely present in the fetuses, infants, and young juveniles. This is contrary to our findings in aortas, where these changes were present in all of the groups studied. Fragmentation, fraying, and/or reduplication of the internal elastic membrane were present in the older juveniles and adults. Duff relates that as long as the elastic membrane remains intact, atherosclerosis is rather sharply limited to the intimal layer and that breaks in the membrane are especially frequent between atherosclerotic lesions in the intima. He regards these breaks as areas where the atheromatous contents of the lesion could be squeezed out into the media, giving a peculiar "hour-glass" lesion. This is not always the case, because we have frequently noted advanced lipid-laden lesions containing multiple discontinuities in the elastic membrane, with no extrusion of lipid into the medial area. This suggests that there is, in addition, some type of medial change necessary for lipid infiltration. This concept is compatible with Carmichael's results from a study of aneurysms. He considers the basic lesion to be a developmental deficiency in the muscular media and states that a normal media or internal elastic membrane is sufficient to prevent aneurysm formation. Perhaps a similar situation exists in relation to atheroma formation in the cerebral arteries, i.e., changes in both the internal elastic membrane and media may be necessary for formation of the "hour-glass" design reported by Duff.

There was no association between lipid and these elastic changes because lipid was frequently observed in a fully intact internal elastic membrane. The acid polysaccharides were not associated with any of these elastic changes because these polysaccharides were frequently increased in areas devoid of elastic changes, and elastic changes were frequently present with no increase in acid polysaccharides. The pooling of acid polysaccharides cannot be due to fragmentation of the elastic fibers, as Taylor related in his studies of aortas, because fragmentation was rarely present in fetuses, infants, and young juveniles. The degree of elastic fragmentation in
the older juveniles and adults could not possibly account for the amount of acid polysaccharides. This is in agreement with the results of the work with *Lathyrus odoratus* by Churchill et al. and the results of our studies of the human aorta. The presence of acid mucopolysaccharides in the older juveniles and adults seems to be associated with the presence of coarse collagen, while no correlation was noted between these substances in fetuses and infants. In young juveniles between 7 and 13 years of age, there were areas containing acid mucopolysaccharides not associated with collagen, as well as areas associated with collagen. Baker has reported that changes due to aging in the media of cerebral arteries consist of the gradual loss of a few elastic fibers and an increase of collagen tissue at the expense of the medial muscle fibers. He further reports that there is a complete replacement of muscle by collagen at small cortical ramifications of the cerebral arteries.

The acid mucopolysaccharides of the fetuses and infants and the acid mucopolysaccharides of young juveniles not associated with collagen were hydrolyzed by testicular hyaluronidase. However, in the older juveniles and adults, the acid mucopolysaccharides were resistant to hydrolysis by testicular hyaluronidase. These findings are essentially in agreement with our previous findings in the aorta, with the exception that the onset of these changes in cerebral arteries occurred at a slightly later age. As stated in our previous paper, the acid mucopolysaccharides hydrolyzed by testicular hyaluronidase are probably hyaluronic acid and/or chondroitin sulfate A and/or C, while those not hydrolyzed by testicular hyaluronidase are probably chondroitin sulfate B and/or heparitin sulfate and/or heparin. Studies are under way in our laboratory to further characterize these acid polysaccharides.

The above observations support our previous hypothesis, based on studies in human aortas, that the initial lesion in atherosclerosis is lipid accumulation. A theory of atheroma formation which was postulated in our aorta studies may also be applied to the cerebral arteries.

**Summary**

Histochemical studies of grossly normal and early lesions of cerebral arteries of 75 individuals ranging from fetuses to 70 years of age were made, utilizing newer histochemical techniques. The following conclusions were made: There is no apparent relationship between acid mucopolysaccharides and lipids in respect to staining intensity and/or distribution in any of the age groups. In older juveniles and adults, lipid material was consistently observed intimately associated with the internal elastic membrane and other reduplicated elastic fibers, even in the absence of gross lesions. Fragmentation, fraying, and/or reduplication of the internal elastic membrane were absent in the fetuses, infants, and young juveniles. There was no apparent association between lipid and these elastic changes. In fetuses, infants, and young juveniles there was an accumulation of acid mucopolysaccharides hydrolyzable by testicular hyaluronidase, principally in the area of the proximal media, suggesting that the polysaccharide material was hyaluronic acid and/or chondroitin sulfate A and/or chondroitin sulfate C. In the adults, there was an accumulation in acid mucopolysaccharides resistant to hydrolysis by testicular hyaluronidase in the proximal media and in the intima. This accumulation in acid mucopolysaccharides corresponded to the areas of collagen increase. It was further suggested that these polysaccharides were chondroitin sulfate B and/or heparitin sulfate and/or heparin. The significance of these findings was discussed.

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