The Evolution of Early Fibromuscular Lesions
Hemodynamically Induced in the Dog Renal Artery

I. Light and Transmission Electron Microscopy

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SUMMARY In view of the important roles of arterial intimal fibromuscular lesions as precursors of atherosclerotic plaque and occlusive lesions in arterial reconstructions, a model has been developed for the rapid hemodynamic induction of these lesions by anastomosis of the dog right renal artery to the inferior vena cava. Light and transmission electron microscopic observations were made on the arterial shunt after periods of rapid flow ranging from 10 minutes to 2 hours to identify initial factor(s) and evolutionary mechanisms in the etiology of the lesions. The sequence of events included aberrations in ruthenium red staining of the endothelial laminal membrane at 10 minutes, multilayered thickening of the subendothelial basement membrane (BM) at 15 minutes, and initial reorientation and migration of smooth muscle cells (SMC) into the intima along with the appearance of areas of degeneration of the internal elastic lamina (IEL) at 30 minutes. The endothelial cells were still intact in some areas overlying the SMC migration and IEL degeneration, but they were separating from the surface in other such areas. As subendothelium became exposed, some platelet adherence was noted. By 2 hours, the entire wall reaction was fully developed. Initial observations indicate that in the evolution of this hemodynamically induced lesion visible alteration in the endothelial cells is not prerequisite to degeneration of the underlying IEL and reorientation and migration of medial SMC.

THE INTIMAL fibromuscular lesion, comprising modified smooth muscle cells (SMC) in a collagenous and elastic matrix, is considered a probable precursor of the more complex "compound" atherosclerotic plaque. Furthermore, intimal and neointimal fibromuscular lesions, occurring as early as 3 months postoperatively, account for one-third to one-half of all late failures of arterial reconstruction whether performed by bypass or endarterectomy techniques. A variety of hypotheses has been suggested to explain the etiology of intimal fibromuscular lesions; many of these theories propose a role for hemodynamic factors even though the exact mechanism of action is not agreed upon. In addition, the role of hemodynamic factors in the formation of human atherosclerotic plaques has been deduced from autopsy observations of the distribution of these lesions in the arterial vasculature. In the early 1960's, Texon and co-workers demonstrated that alteration of the configuration of arteries and the resultant changes in flow produced intimal fibrous plaques at characteristic but not entirely predictable sites within periods of 12-18 months. Recently, it has been shown that these lesions can be produced even more quickly and at more predictable sites (unpublished observations). Of a number of models tested, anastomosis of the distal end of the transected right renal artery to the side of the inferior vena cava proved most suitable for detailed study. In this model intimal fibromuscular lesions were first observed 3 months after creation of the anastomosis, and, as the observation period was gradually shortened, fully developed lesions were recognized as early as 2 days postanastomosis.

Since it was not clear from the above studies what the initial events leading to the fully developed fibromuscular intimal plaque were, and since it appeared that the lesions might have started forming almost as soon as the anastomosis was completed, observations in the present study were started at time zero and continued at longer intervals until typical small fibromuscular plaques were recognizable. The goals of this study were the morphological identification of the initial factor or factors in the pathogenesis of the hemodynamically induced intimal fibromuscular lesion and elucidation of the mechanisms involved in its early evolution.

Methods

EXPERIMENTAL MODEL

Eight adult, male, mongrel dogs, approximately 15 kg in weight and fed a regular kennel diet, were used. They were anesthetized by intravenous injection of sodium pentobarbital (Nembutal) (30 mg/kg), and laparotomy was performed on each dog. The distal third of the right renal artery was dissected free and detached from the right kidney, which then was removed. The right renal artery, measuring approximately 3.5 x 0.4 cm, was anastomosed end-to-side to the vena cava. The proximal two-thirds of the right renal artery remained untouched, as did the entire left renal artery, which served as a control. In two
dogs, blood was allowed to flow through the shunt for 10 minutes, in one dog for 15 minutes, in one dog for 30 minutes, in one dog for 1 hour, and in three dogs for 2 hours. All eight of the experimental dogs included in this study exhibited prominent thrills at the sites of the anastomoses throughout the shunting periods, and light microscopic examination showed all renal arteries to be free of any thrombus large enough to have significantly affected the flow. The pressure in the right renal artery was measured directly at the end of the shunting period and was normal in all dogs.

CONTROLS

The untouched left renal artery of each of the experimental dogs served as a control for the methods of fixation and tissue preparation employed. As a control for the effects of the surgical procedure itself, an additional dog was prepared, and the right renal artery was fixed immediately after the standard anastomosis to the vena cava had been completed and before any blood flowed through the shunt. In another dog a check was made on the rate of flow as the critical factor in the production of lesions. The right renal artery was clamped and manipulated in situ, but no anastomosis was made. After 30 minutes, the average time required for performing an anastomosis to the vena cava, the clamp was removed, and blood flow through the right renal artery was restored for 2 hours.

TISSUE PREPARATION

Fixation was obtained by perfusion in situ of the abdominal aorta, renal arteries, and inferior vena cava with 1 liter of fresh, cold 3% glutaraldehyde in Sörensen’s buffer (0.1 M, pH 7.3). The perfusion pressure was maintained at about 150 mm Hg to minimize folding of the arterial wall. The full details of this method have been described.10

After perfusion fixation the proximal thirds of the right and left renal arteries were dissected free and cut transversely into segments. The greatest distance between successive sampling sites was 2 mm. Specimens were screened by light microscopy and, where justified, corresponding pieces of tissue were postfixed in 1% OsO₄, dehydrated in acetone, and embedded in Epon; 1-µm sections were cut for orientation and stained with a polychrome stain.11 An LKB Ultratome III and a diamond knife were used to cut thin sections which were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a Siemens Elmiskop 1 electron microscope. In the two 10-minute dogs some of the tissue was stained with ruthenium red by adding 0.1% ruthenium red to the OsO₄ solution. In these cases cacodylic acid buffer (0.2 M, pH 7.3) was used throughout fixation and tissue preparation in place of phosphate buffer which interferes with the ruthenium red reaction.12

Results

CONTROLS

With rare exception the proximal third of the control left renal artery of each experimental dog looked normal by light and electron microscopy19 (Fig. 1). In two dogs isolated areas of thickening or multilayering of the subendothelial basement membrane (BM) were encountered. Only once a small intimal fibromuscular lesion was found in the proximal control left renal artery. The alteration encountered in this control artery may have been due to changes in left renal artery flow resulting from removal of the right kidney and the creation of a high flow arteriovenous fistula on the right side of the systemic circulation.

The proximal thirds of the right and left renal arteries in both the separately prepared control dogs appeared normal by light and electron microscopic examination.

EXPERIMENTAL

An earlier study of this model19 had shown the typical patterns and locations of intimal fibromuscular lesions. Characteristically, these lesions occur near the origin of the right renal artery just proximal and distal to the right renal artery ostium and at the site of anastomosis of the right renal artery to the inferior vena cava. The proximal renal artery was the preferred site for the study of fine structure because this segment of the vessel had not been touched until after fixation.

The morphology of the proximal 6 mm of the right renal artery varied slightly with the location of the sampling site as well as with the duration of the experiment. This small range of variation probably is attributable to local differences too small to measure or control in the type and velocity of flow through the proximal part of the shunt. Consistent observation of certain repeated phenomena, however, made possible the reconstruction of the probable sequence of events in the arterial wall.

After 10 minutes of shunting the arterial intima appeared normal in most places. In general, ruthenium red stained the outer surface of the endothelial luminal membrane in a characteristic fashion,14 as well as the outer surface of nearby red blood cells and platelets (Fig. 2). The ruthenium red-stainable surface material extended into the endothelial surface vesicles and was found within some cytoplasmic vesicles and sacculles which had no apparent connection with the cell surface in the plane of section (Fig. 3). In a few places, the ruthenium red staining of the luminal membrane was quite varied in density and thickness (Fig. 4).

After 10 minutes clumps of a fuzzy, amorphous substance often adhered to the endothelial luminal membrane and in many places formed a connecting link between blood cells and the endothelial surface. This material could be demonstrated with uranyl acetate-lead citrate staining alone, but it was much more electron-opaque and obvious after staining with ruthenium red.

By 15 minutes the subendothelial BM appeared markedly replicated and consisted of up to 12 thin, interconnected layers each with a thickness of approximately 30 nm (Fig. 5). The greatest distance from the innermost layer to the outermost was approximately 1,500 nm, in contrast to the control renal artery subendothelial BM thickness of approximately 350 nm. In areas where there were occasional intimal smooth muscle cells (SMC) present, their basal laminas also appeared thicker than normal and, in places, were contiguous with the abluminal edge of the multilayered subendothelial BM (Fig. 6).
After 15–30 minutes of rapid flow the intima and inner media began to appear slightly edematous, with wide, clear, aifibrous areas between cells (Fig. 7). Many small cells with the fine structural characteristics of SMC\textsuperscript{16} began to appear in the intima and in gaps in the internal elastic lamina (IEL). These cells usually were surrounded by a thickened basal lamina and some displayed a prominent rough endoplasmic reticulum. The long axes of the SMC in the intima were parallel to the longitudinal axis of the vessel. The SMC in and near gaps in the IEL were small and their long axes appeared to be perpendicular to the luminal surface in both longitudinal and transverse sections.\textsuperscript{16} No sign of cell division was detected in these areas of the intima or subjacent inner media.

At about the time the small SMC began to appear, areas of degeneration were observed in the IEL (Fig. 8). This structure, which normally (Fig. 1) exhibits a fairly homogeneous appearance when embedded in Epon and stained with uranyl acetate and lead citrate, demonstrated broad areas of varying electron density (Fig. 9). Such a change in staining properties indicated a significant alteration in the molecular organization of the elastic protein resulting in metal iron-macromolecular interaction.\textsuperscript{18} Since these changes in staining properties were followed by fragmentation of the IEL, they are viewed as degenerative. Such degenerative changes in the IEL often were advanced and bits of unidentifiable electron-opaque debris could be seen in adjacent areas of the intima and inner media.
FIGURE 2  Electron micrograph of a canine right renal artery that has served as a high flow shunt for 10 minutes; stained with ruthenium red only. The ruthenium red complex has bound to the endothelial cell's (E) luminal surface and associated vesicles and to the surface of the red blood cell (RBC) and platelets (P) in the lumen (L). The other tissue remains unstained. IEL = internal elastic lamina; SMC = smooth muscle cell.

FIGURE 3  Higher magnification of section similar to above; stained with ruthenium red only. Ruthenium red complex is bound to the luminal surface (single large arrows), the surface of the caveolae intracellularis (double small arrows), and the inner surfaces of deeper vesicles and a saccule (S). L = lumen.
The endothelial layer overlying areas of degenerating and fragmenting IEL demonstrated various appearances from site to site. In some places the endothelial cells and interendothelial junctions looked relatively normal (Fig. 10) and no change in the fine structure of the endothelial lining could be detected by electron microscopic examination of sections cut at intervals of 5-10 μm for a distance of over 50 μm even though the underlying IEL obviously was degenerating in all the sections. In other areas endothelial cells overlying degenerating IEL showed luminal blebs to which platelets often adhered (Fig. 11).

After 30 minutes alterations were seen in most of the endothelial cells. At first these cells appeared to round up and separate from adjacent endothelial cells. The nucleus-cytoplasm ratio increased, cytoplasmic blebs appeared, and the preponderance of pinocytotic vesicles was seen on the luminal surface (Fig. 12). At a later stage the peripheral nuclear heterochromatin became thicker, the nuclear outline appeared folded, microfilaments and lipofuscin-like inclusions were much more prominent, and multilobular bodies became quite rare (Fig. 13). Finally, endothelial cells with a dark-staining, granular cytoplasm, dense nucleus, and perinuclear vacuoles were seen sloughing off the surface of the multilayered BM (Figs. 14 and 15). Neutrophils were occasionally seen on the vessel surface or in the inner media in regions where the endothelial cells were in the process of sloughing off (Fig. 16).

Areas of the arterial wall which already had been stripped of their endothelial lining by the rapid rate of flow displayed a highly edematous intima and inner media, intimal modified SMC, and extensive degeneration and fragmentation of the IEL. Occasional extravasated red blood cells and necrotic SMC also were seen in the intima and inner media in these areas (Fig. 17).

In some places where the subendothelium was exposed, platelets, most of which retained their granules, adhered (Fig. 18). This layer of platelets was interspersed with areas of bare subendothelium and only rarely were other platelets seen adhering to the base platelet layer. No thrombus formation was seen in the proximal right renal artery shunt in any of the dogs used.

Discussion
Since the general purpose of the present phase of our investigations was an attempt to determine the initiating events in the formation of fibromuscular intimal lesions, observations were discontinued as soon as it was obvious that all of the primary characteristics of this lesion noted at 2 days were apparent at 2 hours. The only significant difference between the plaques at 2 hours and at 2 days is
FIGURE 5  Electron micrograph of a canine right renal artery that has served as a high flow shunt for 15 minutes. The subendothelial basement membrane (BM) exhibits numerous anastomosing layers, and the basal lamina (BL) of the subendothelial smooth muscle cell (SMC) is also thickened. E = endothelium; L = lumen. Uranyl acetate and lead citrate stain.
FIGURE 6  Section similar to above except that the multilayered subendothelial basement membrane (BM) and smooth muscle cell basal lamina (BL) appear to merge. E = endothelium; L = lumen; SMC = smooth muscle cell. Uranyl acetate and lead citrate stain.
FIGURE 7 Photomicrograph of a canine right renal artery that has served as a high flow shunt for 2 hours. The left third of the section appears normal, but the right two-thirds demonstrates varying stages in the development of a hemodynamically induced fibromuscular intimal lesion. The endothelium is gone, the intima and inner media appear edematous, the darkly staining internal elastic lamina is broken, and small modified smooth muscle cells (arrows) are migrating into the intima. L = lumen. Polychrome stain.
FIGURE 8  Photomicrograph of a canine right renal artery that has served as a high flow rate shunt for 30 minutes. Throughout this entire sector of the wall the darkly staining internal elastic lamina exhibits degenerative foci and is fragmenting. An extreme example of such a degenerative focus is seen on the far right. A higher power electron micrograph of a degenerating segment of internal elastic lamina like that indicated by the arrow is shown in Figure 9. L = lumen. Polychrome stain.
FIGURE 9  Electron micrograph of a segment of degenerating internal elastic lamina like that shown in Figure 8. The stained elastic lamina shows a wide variety of electron densities over a relatively small area. In general the more electron-dense a region appears, the more advanced the stage of degeneration. Uranyl acetate and lead citrate stain.
Figure 10. Electron micrograph of a canine right renal artery that has served as a high flow shunt for 30 minutes. Although there is multilayering of the subendothelial basement membrane (BM), intimal edema, modification of a smooth muscle cell (SMC), and degeneration of the internal elastic lamina (DF), the overlying endothelium (E) looks relatively normal. DF = degenerative focus; L = lumen. Uranyl acetate and lead citrate stain.
Figure 11  Electron micrograph of a canine right renal artery that has served as a high flow shunt for 1 hour. A platelet (P) adheres to cytoplasmic protuberances on an otherwise normal endothelial cell (E). The subendothelial basement membrane (BM) is replicated and the internal elastic lamina (IEL) shows a degenerative focus (DF). L = lumen. Uranyl acetate and lead citrate stain.
Electron micrograph of a canine right renal artery that has served as a high flow shunt for 2 hours. An endothelial cell (E) with a large osmiophilic body is seen with its nucleus protruding into the lumen (L). The subendothelial basement membrane (BM) is multilayered and there are degenerative foci (DF) in the internal elastic lamina (IEL). Uranyl acetate and lead citrate stain.
FIGURE 13  Electron micrograph of a canine right renal artery that has served as a high flow shunt for 30 minutes. An endothelial cell (E) with a large, C-shaped, folded nucleus (N) containing thickened peripheral heterochromatin bulges into the lumen (L). To the right a portion of an adjacent endothelial cell (E*) contains a prominent group of microfilaments. The multilayered basement membrane (BM) is uncovered on the right. RBC = red blood cell; SMC = smooth muscle cell. Uranyl acetate and lead citrate stain.

FIGURE 14  Electron micrograph of a canine right renal artery that has served as a high flow shunt for 30 minutes. A degenerate endothelial cell (E) with a very dense nucleus is in contact with the subendothelium at only one point in the plane of section. A multilayered basement membrane (BM) either exposed or in contact with platelets (P) is also shown. DF = degenerative focus in internal elastic lamina; L = lumen. Uranyl acetate and lead citrate stain.
FIGURE 15  Electron micrograph of a canine right renal artery that has served as a high flow shunt for 30 minutes. A degenerative endothelial cell (E) with a dense nucleus and without any connection to the subendothelium in the plane of section is shown near the center of the picture. This cell is surrounded by platelets (P). The surface of the debris-containing multilayered basement membrane (BM) is exposed. DF = degenerative focus in internal elastic lamina; L = lumen. Uranyl acetate and lead citrate stain.

in the larger number of cells which appears in the intima in the later lesions.

The validity of the experimental preparation used and the role of increased blood flow is attested to by the general failure of any of the control preparations to produce lesions. In addition, an experimental model of similar configuration, interposition of the carotid artery between the aorta and vena cava, produces lesions virtually identical to those reported here, although reaction to surgical trauma is superimposed in the carotid artery model. A significant advantage of the present model is that there is no surgical manipulation of the segment of artery studied, the proximal third of the right renal artery, until after this tissue is fixed in situ. Another major advantage of the present model is the rapidity with which lesions having the characteristics of chronic lesions develop.

Although flow and pressure measurements were performed on some dogs in this study, these measurements could not be made accurately without surgical manipulation of the tissue to be subjected to detailed histological study. Such measurements, therefore, were discontinued. A noninvasive technique in which sound recordings are made and flow is characterized by analysis of sound frequencies will be the subject of a later report.

The first observed effect of the shunt on the arterial wall was occasional aberrations in the ruthenium red staining of the endothelial luminal membrane (Fig. 4). In view of the fact that it has not yet been unequivocally determined whether the ruthenium red-osmium complex stains a surface mucopolysaccharide coat14-18 or fibrin precipitated on the endothelial surface,19 the present results are difficult to interpret and require further evaluation.

Replication of the subendothelial BM (Fig. 5) often has been cited as an indication of cellular injury and is seen in a wide variety of diseases.20-22 Although it has been suggested that such a multilayering implies the prior presence
of successive generations of cells, each in turn producing its own layer of BM, such a mechanism seems unlikely in this model since a subendothelial BM with as many as 11 interconnected layers appears during the first 15 minutes of shunting. Alternatively, one might assume that the multilayered BM is produced by new deposition of BM material by the same endothelial cells that supplied the original BM. It should be noted, however, that the endothelial cells overlying the rapidly laminated BM show no significant increase in rough endoplasmic reticulum or other intracellular machinery for protein production. Another possible explanation for the multilayering is that the subendothelial BM, seemingly a highly compact, dense structure, is affected by subendothelial edema so that it spreads into the subjacent connective tissue in an anastomosing pattern. Another hypothesis is that subendothelial BM is being made continually by the endothelial cells and any excess is removed by some unknown mechanism which has failed in this situation. A final possibility is that the BM is being fragmented by physical forces generated by the rapid, nonlaminar blood flow.

The reasons for the observed thickening of the basal
lamina around the subendothelial SMC which appeared simultaneously with the multilayering of the subendothelial BM are equally poorly understood. As was noted, this thickened basal lamina appears to merge in places with the multilayered BM (Fig. 6). Chemical differences between these two morphologically similar but distinguishable structures are not well characterized. 15

The origin of the increased numbers of small, modified SMC in the intima (Fig. 7) within 15 minutes after the institution of a rapid flow rate remains uncertain. The number of these cells seen oriented with their long axis perpendicular to the luminal surface in gaps in the IEL suggests that the cells migrate from the media into the intima.10 Those SMC present in the intima seem to be longitudinally oriented. The small sizes of most of the intimal SMC suggest that they are the product of recent cell division, but no sign of mitosis was detected among the medial SMC, nor was cell division ever observed among preexisting subendothelial SMC, a mechanism that has been proposed for the pathogenesis of human atherosclerotic plaque.56

It has been suggested by many investigators that the initiating cause of fibromuscular lesion formation in the arterial intima is endothelial damage and the resulting infiltration from the blood into the vascular wall of substances which are normally excluded.2 The finding in this study of many areas where a clearly damaged IEL was seen beneath an apparently normal endothelial layer (Fig. 10) which was observed to extend for a distance of at least 50 μm in the same sector of the wall requires a reappraisal of this concept. There are a number of possible explanations for the observations of areas of damaged IEL under an apparently normal endothelial layer: (1) The overlying endothelium had undergone some subtle damage which
did not result in morphological changes detectable by current techniques, or (2) damage to the IEL was caused by lateral diffusion of substances which had entered the wall at remote points of endothelial injury, or (3) the increased rate of flow damaged the IEL not indirectly as a result of increased endothelial permeability, but directly, perhaps by some vibrational or sonic effect. An attempt currently is underway to find an unequivocal means of damaging only the arterial endothelial lining in order to settle this issue.

The process of endothelial cell sloughing in the face of an increased blood flow begins with the apparent rounding up of the individual cells and their separation from adjacent endothelia (Figs. 12 and 13). This same response has also been observed in experiments involving other types of insult to the endothelial layer, such as the injection of cholesterol, angiotensin, epinephrine, or norepinephrine into the arterial lumen. At about the same time the endothelial cells round up, they show other little understood characteristic changes, such as the presence of pinocytotic vesicles almost exclusively on the luminal surface and decreased numbers of multitubular bodies in the peripheral portions of the cell. In only a few instances, however, was actual disintegration of the endothelial cell observed, and it appears that the endothelial cell usually sloughs off in a degenerate but intact form (Figs. 14 and 15). This finding is contrary to the light microscopic observations of Fry, who reported rupture of endothelial cells when shear stress in the dog thoracic aorta was increased by insertion of a channeled plug.

In those areas where the endothelial cells have been shed, platelets sometimes adhere to the exposed BM (Figs. 17 and 18), as has been observed in other experimental systems. The platelets are relatively few and rarely more than one cell layer thick. There usually are gaps of exposed BM in between these platelets, and the relative paucity of platelets probably is due to their inability to adhere firmly to the exposed subendothelium in the presence of a high rate of flow. Most of the platelets seen on an exposed BM surface retained their granules, and no thrombus formation was ever observed in the proximal third of the shunt.

The finding of rare, minute lesions in the contralateral renal artery of two of the dogs used in this experiment is...
difficult to explain. The distribution of these lesions is unlike that found in the right renal artery shunt, which characteristically shows ostial lesions extending from the aorta into the right renal artery for a variable distance. The rare left renal artery lesions are discrete, usually appear well beyond the origin of the vessel, and are minute compared to the lesions in the shunt. Although the dogs used were from random sources, what might be considered spontaneously occurring lesions have been encountered in no more than 2% of a total of over 100 canine renal arteries studied by our group. It is, therefore, suggested that the lesions seen in the left renal arteries examined in this experiment are flow-induced, although the precise mechanism involved is not clear. In view of the fact that the right kidney has been removed, however, the occurrence of lesions in the left renal artery is compatible with the basic hypothesis that aberrations of flow cause such lesions.

In conclusion, we suggest that the present study provides a useful, physiological model for the study of the early changes in the arterial wall associated with the development of hemodynamically induced intimal fibromuscular lesions. This model has demonstrated that an increased rate of blood flow can produce early intimal fibromuscular lesions within a surprisingly short period of time. In such a system, fragmentation of the IEL, alterations in intimal SMC, and intimal edema can occur even before endothelial damage is visible.

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