The role of fibrin deposition in diabetic glomerulosclerosis: a light, electron and immunofluorescence microscopy study

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SYNOPSIS   The possible role of fibrin deposition in the development of diabetic glomerulosclerosis has been investigated by light, immunofluorescence, and electron microscopy examination of renal tissue obtained by percutaneous biopsy from seven diabetic patients having minimal clinical evidence of renal involvement and at necropsy on nine diabetic patients. Although the biopsy specimens showed only early to moderate diffuse glomerulosclerosis without either nodular or exudative lesions, approximately 70% of glomeruli examined showed specific fluorescence for fibrinogen in a discontinuous linear pattern along the capillary basement membrane and diffusely in the mesangium. Moreover the immunofluorescence findings correlated well with the distribution patterns of material thought to be fibrin both in the light microscopy studies, by virtue of its staining properties, and by electron microscopy, because of periodicity, texture, and electron density. It is suggested that electron-dense granular deposits seen on electron microscopy may represent intermediate compounds in the fibrinogen to fibrin conversion, and that endothelial and mesangial trapping of fibrinogen or other macromolecules may initiate or accelerate mesangial enlargement, nodule formation, and irregularity of the basement membrane.

There is much evidence to suggest that the microvasculature in diabetic subjects is hyperpermeable to certain plasma constituents (Lendrum, 1963; Davies, Woolf, and Carstairs, 1966; Berkman and Rifkin, 1966; Fisher, Perez-Stable, Amidi, Sarver, and Danowski, 1967; Larsson, 1967; Balodimos, Chlouverskis, Gleason, Jarrett, Kahn, Keen, and Soeldner, 1971). Although the nature and onset of the early structural changes in the glomerular capillaries remain controversial, it is possible that altered permeability may precede the lesions seen on light and electron microscopy (Ditzel and Schwartz, 1967; Trap-Jensen, Alport, Garcia del Rio, and Lassen, 1967). Other investigations suggest that a hypercoagulable state, manifest by increased platelet adhesiveness (Shaw, Pegrum, Wolff, and Ashton, 1967; Abastado, Doumenc, Prost, Bara, Samama, Dérot, and Bousser, 1968), elevated plasma fibrinogen levels (Egeberg, 1963; Valdorf-Hansen, 1967), and diminished fibrinolytic activity (Fearnley, Chakrabarti, and Avis, 1963; Cash and McGill, 1969; Sharma and Malhotra, 1969) may develop in some diabetic patients.

In the light of these two major aspects, and in view of the increasing importance attached to fibrinogen and related products in human and experimental glomerulopathies (McCluskey, 1969; Clarkson, MacDonald, Petrie, Cash, and Robson, 1971), the possible role of fibrin deposition in the development of diabetic glomerulosclerosis was investigated by light, immunofluorescence, and electron microscopy examination of renal tissue.

Materials and Methods

Renal tissue was obtained by percutaneous biopsy using a Franklin-modified Vim-Silverman needle from seven diabetic patients having minimal clinical evidence of renal involvement and at necropsy on nine diabetic patients within 24 hours of death (Table I). Control tissue was obtained from necropsies on six non-diabetic patients who had shown no clinical evidence of renal or metabolic disease.

BIOPSY TISSUE
Two cores of tissue were obtained from each patient, one providing material for electron microscopy and

Case	Age	Sex	Duration of Diabetes & Treatment	Endogenous Creatinine Clearnance (ml/min)	Urinary Protein for 24 Hours	Blood Pressure (mm Hg)
Biopsies						
1	45	M	5 yr Tolbutamide	110	500 mg	140/100
2	50	M	14 yr Insulin	85	700 mg	130/90
3	31	M	17 yr Insulin	95	300 mg	140/90
4	49	M	5 yr Insulin	97	650 mg	130/80
5	48	M	12 yr Insulin	124	250 mg	135/90
6	66	M	?2 mth Diet	78	600 mg	125/80
7	55	M	19 yr Insulin	66	550 mg	60/90
Necropsies						
8	67	M	5 yr Insulin	Data not available	Data not available	180/110
9	69	F	26 yr Insulin	Data not available	Data not available	150/100
10	63	F	7 yr Insulin	Data not available	Data not available	140/100
11	63	F	24 yr Insulin	34	2 g	180/120
12	74	F	21 yr Insulin	Data not available	Data not available	160/100
13	52	F	9 yr Insulin	Data not available	Data not available	130/90
14	72	F	11 yr Insulin	Data not available	Data not available	160/100
15	69	M	2 yr Diet	Data not available	Data not available	150/100
16	79	F	41 yr Insulin	Data not available	Data not available	140/90

Table I *Clinical data*

for thick-section studies by light microscopy using modified haematoxylin and eosin, MSB, and Picro-Mallory stains, the other for immunofluorescence studies. Blocks for electron microscopic examination were fixed in Palade's 1% buffered osmic acid, dehydrated in alcohol and embedded in Araldite. Using the method of Mayor, Hampton, and Rosario (1961) for de-Aralditing, 0·5-1·5μ thickness sections were cut for light microscopy also. For electron microscopy ultrathin sections were cut on a Porter-Blum MT2 ultramicrotome, stained with uranyl acetate and Reynold's lead citrate, and viewed in an AEI EM6 electron microscope.

After freezing, the second core of tissue for immunofluorescence studies was sectioned at 2 to 3μ and fixed in 95% ethanol for 10 minutes. If not immediately required, the frozen sections were stored at −20°C. Frozen sections were stained with FITC conjugated antihuman fibrinogen serum after the method of Nairn (1969).

NECROPSY TISSUE
Small blocks of cortical tissue were fixed in formol-corrosive for two to 10 hours, dehydrated in alcohol, and embedded in Araldite for thick section studies by light microscopy. Tissue was taken for immunofluorescence investigation also and treated as described above.

Results

LIGHT MICROSCOPY
The general morphological features observed in glomeruli were in agreement with those recorded in the literature and referred to as diabetic glomerulo-sclerosis (Ireland, 1971). All biopsy specimens

showed early to moderate diffuse glomerulosclerosis but no nodular or exudative lesions were evident. In the necropsy tissue, cases 9, 15, and 16 showed, in addition to moderate arteriosclerosis and diffuse glomerular lesions, nodular changes and extensive exudative lesions of the 'fibrin cap' and 'capsular drop' type. The remaining cases showed only moderate diffuse glomerulosclerosis with the exception of case 10 which had arteriosclerosis in addition.

In de-Araldited sections, though staining was often faint, differentiation of the lesions and structural relationships in the glomerular capillaries was facilitated. Patchy areas staining as fibrin were
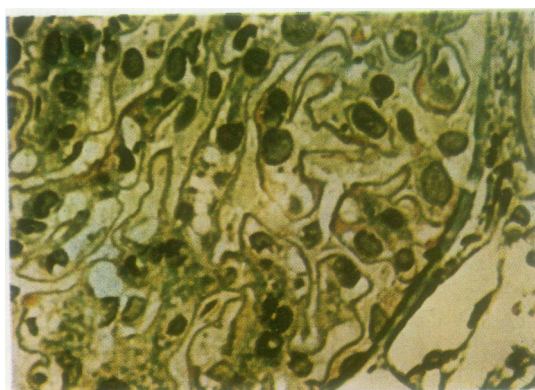

Fig. 1 *Case 1   The biopsy tissue was embedded in Araldite, the section de-Araldited with sodium methoxide and stained by the MSB method. This part of a glomerulus shows red staining, corresponding to fibrin, scattered irregularly along capillary walls and also in mesangial regions.* × 800
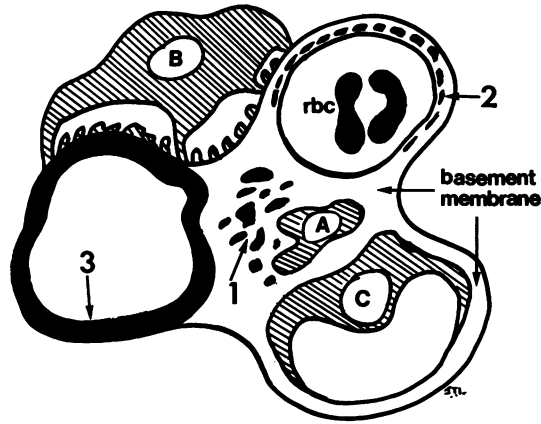
frequently found within mesangial regions and also in a fine, discontinuous linear pattern along the inner aspect of the capillary walls (Fig. 1). Often these areas of positive staining seemed unrelated to any local glomerular damage, and on occasion represented the only lesion evident apart from an unequivocal increase in mesangial mass.

FLUORESCENCE MICROSCOPY

In each case approximately 10 glomeruli were examined. Grading of the intensity of fluorescence was on an arbitrary semiquantitative three-point scale (0, +, ++). The distribution of fluorescence was recorded as granular, beaded, or diffuse (Fig. 2). Approximately 70% of all glomeruli showed positive staining; the degree of immunofluorescence in different glomeruli varied quantitatively as summarized in Table II. Specific fluorescence was found most constantly in a beaded pattern along the capillary wall or in the mesangial regions (Fig. 3).

Fig. 2 *Diagram of glomerular capillary lobule showing patterns of distribution of fibrin.*
1 Granular fibrin scattered within mesangium.
2 Beaded in discontinuous linear distribution either subendothelial or within basement membrane.
3 Diffuse: uniform throughout capillary wall.
A Mesangial cell; B Epithelial cell attached by foot processes; C Endothelial cell within capillary lumen.

ELECTRON MICROSCOPY

In every biopsy thickening of the glomerular capillary basement membrane was the most prominent change seen (Fig. 4). Thus the mean basement membrane thickness in individual patients ranged from 2750 A° to 8000 A°, whereas in non-diabetic

Case	Fluorescence	Mesangial Region	Capillary Wall	Nodule	Exudative Lesion	Arteriole
Biopsies						
1	I	++	++	0	0	0
	P	Patchy-granular	Beaded			Diffuse
3	I	++	++	0	0	0
	P	Patchy-granular	Beaded			
5	I	++	++	0	0	0
	P	Patchy-granular	Beaded			
6	I	++	++	0	0	0
	P	Patchy-granular	Beaded			
7	I	++	++	0	0	0
	P	Patchy-granular	Beaded			
Necropsies						
8	I	++	+	0	+	0
	P	Patchy-granular	Beaded		Diffuse	
9	I	++	++	++	0	+
	P	Patchy-granular	Beaded	Diffuse		Diffuse
10	I	++	++	0 to ++	++	0
	P	Patchy-granular	Beaded	Diffuse	Diffuse	
11	I	+	+	0	+	++
	P	Granular	Beaded		Diffuse	Diffuse
12	I	++	+	0	++	0
	P	Granular	Beaded		Diffuse	
13	I	+	+	0	0	0
	P	Patchy-granular	Beaded			
14	I	+	+	0	+	0
	P	Patchy-granular	Beaded		Diffuse	
15	I	+	++	0 to ++	++	+
	P	Granular	Diffuse	Diffuse	Diffuse	Diffuse
16	I	++	++	0 to ++	++	++
	P	Patchy-granular	Beaded	Diffuse	Diffuse	Diffuse

Table II *Intensity and pattern of fluorescence*[1]
[1]Grading of fluorescent intensity was on an arbitrary semiquantitative scale of 0, +, and ++.
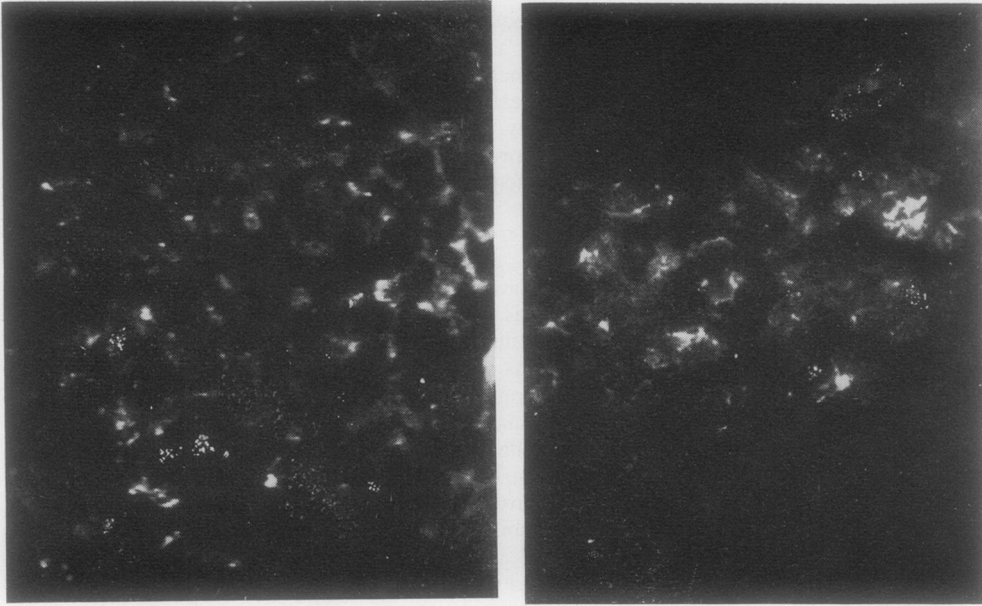[2]I = intensity; P = pattern.

Fig. 3   Cases 3 and 6: composite photograph of two glomeruli treated with FITC conjugated antifibrinogen serum. The left shows mainly a beaded pattern of specific fluorescence scattered irregularly along the capillary walls. The right shows specific fluorescence confined mostly to mesangial areas. These two patterns of fluorescence correspond to fibrin (or FDP) deposition predominantly in capillary walls (basement membrane) and in mesangial regions respectively. × 200

Fig. 4   Case 5: Low-power electron micrograph showing uniform thickening of glomerular capillary basement membrane. The thickening is mostly regular and homogeneous in character except where the basement membrane encroaches on mesangial areas (top centre and bottom right). The mesangial areas all show slight increase in mesangial matrix. × 5000
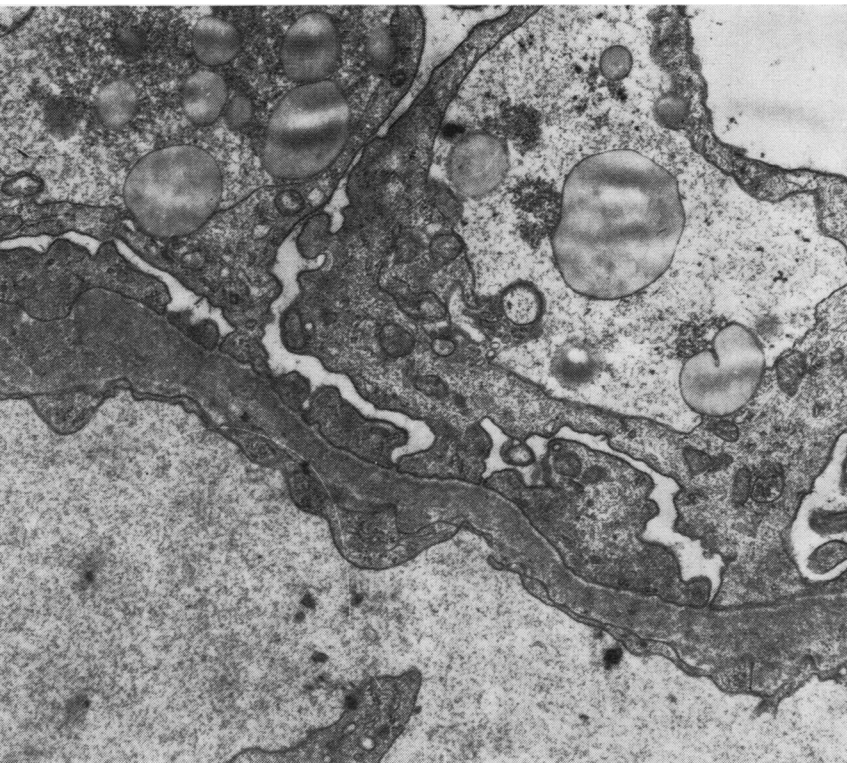
Fig. 5 *Case 6: in contrast to the previous figure this shows a predominantly focal and irregular type of basement membrane thickening especially on the endothelial surface. The epithelial cytoplasm (above) is healthy and contains many trapped protein aggregates.*
× *14 000*

controls similarly measured the mean thickness ranged from 1400 A° to 2700 A° (Ireland, 1970). In addition to diffuse basement membrane thickening there was superimposed irregular and focal thickening of the membrane, but no subepithelial humps or other deposits of the immune complex type were evident (Fig. 5). In general mesangial regions were enlarged and contained nodules of hyaline basement membrane-like material. Frequently a dark fibrillary or granular material was seen subendothelially in a discontinuous linear pattern adjacent to, or within the basement membrane (Figs. 6, 7, and 8). This material often merged imperceptibly with the basement membrane proper (Figs. 9 and 10). Sometimes a similar material, associated with swelling and vacuolation of endothelial cells, was seen in the capillary lumen, or between layers of what was probably mesangial cytoplasm; the material was noted also between prolongations of endothelial cytoplasm (Figs. 11, 12, and 13). In three of the biopsies examined, this substance showed a mean periodicity of 190 A°. It was concluded that the granular material represented fibrin on account of its electron density, texture, position, occasional periodicity, and associated endothelial appearance. Frequently the material identified as fibrin was not associated with other major glomerular changes.

Discussion

The finding in diabetic glomeruli of specific fluorescence for fibrinogen in a discontinuous linear pattern along the capillary basement membrane and diffusely in the mesangium correlated well with the distribution patterns of material thought to be fibrin both in the light microscopy studies, by virtue of its staining properties, and by electron microscopy, because of periodicity, texture, and electron density.

Fibrin has rarely been described in diabetic glomeruli unless associated with severe structural changes in the capillaries (Salinas-Madrigal, Pirani, and Pollock, 1970). This may have been due to the relative inability of classical staining methods to show on light microscopy either 'young' or 'old' fibrin (Lendrum, Fraser, Slidders, and Henderson, 1962) and also because of the difficulty in interpreting very fine alterations on 2-3μ thick paraffin sections.

For the electron microscopist, fibrin has always been elusive, and only recently has it been acknowledged that banding, with a specific periodicity, is the exception rather than the rule (Prose, Lee, and Balk, 1965; Haust, Wyllie, and More, 1965). Also the coarse granular or fibrillar, electron-dense appearance once thought characteristic of fibrin is now regarded by many as representing one end of a scale
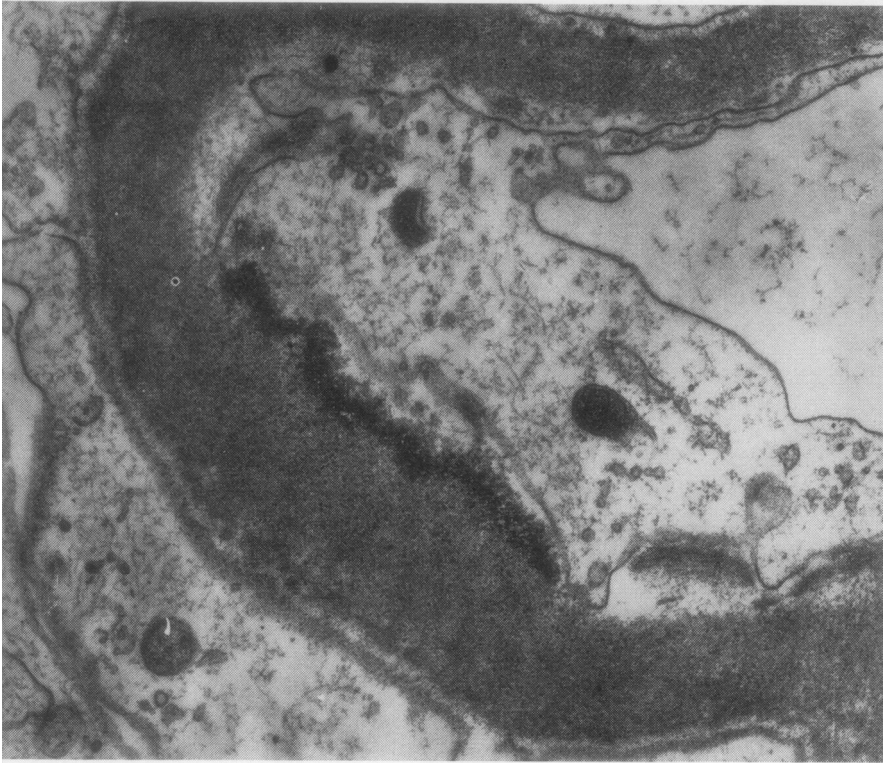
Fig. 6   *Case 2: note the elongated, subendothelial deposit of dark granular material lying in opposition to the basement membrane but sharply delineated from it.* × 28 000
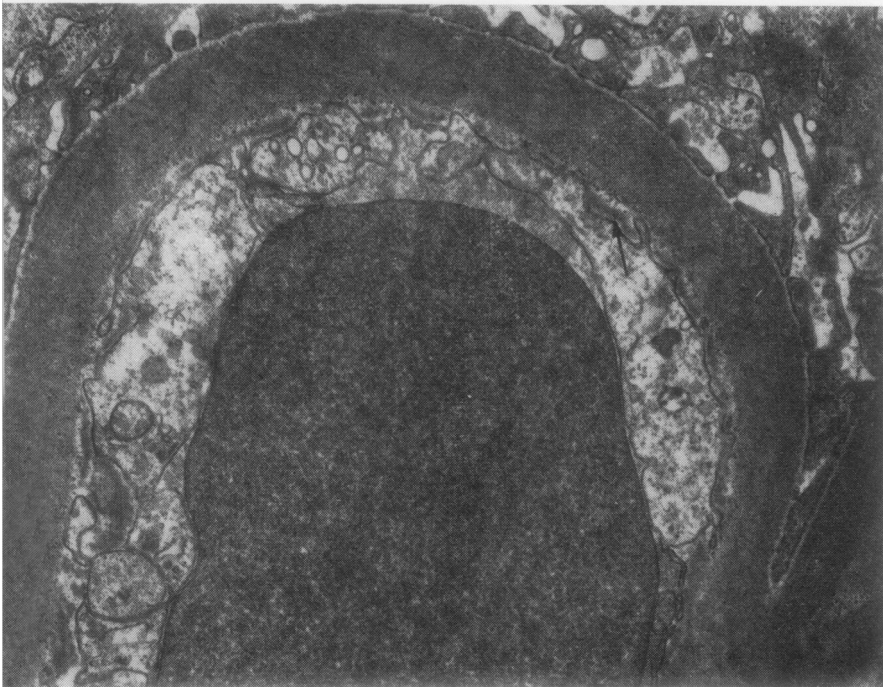

Fig. 7   *Case 7: this demonstrates the discontinuous linear pattern of deposits of material considered to be fibrin (or FDP) in a subendothelial location (arrow). Although this material merges with the basement membrane at the bottom right of the plate elsewhere it is of greater electron density than basement membrane.* × 10 500
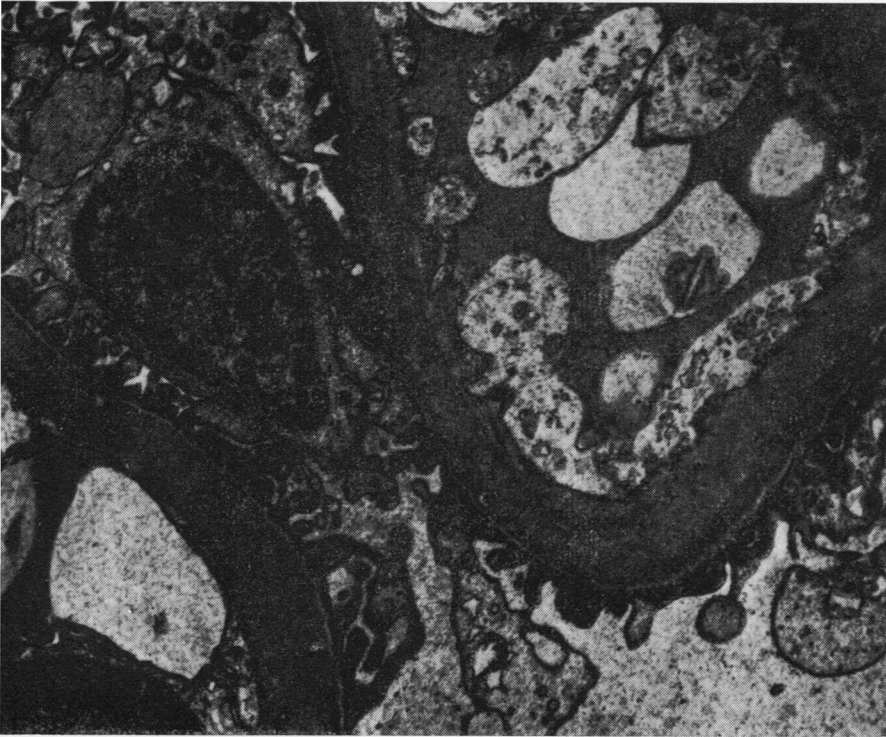
Fig. 8 *Case 1: within the diffusely thickened basement membrane some areas show a linear distribution of darker staining material (upper right). Material of similar electron density is seen in the lumen between projections of endothelial cytoplasm (lower right).* × 7000
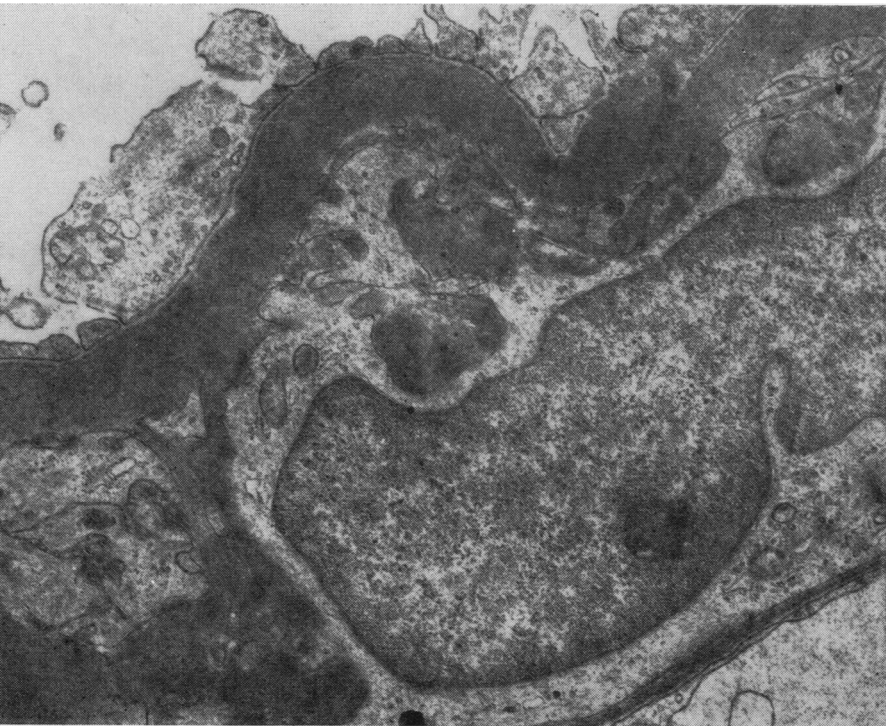

Fig. 9 *Case 1: this shows a mesangial area with nucleus to the right and basement membrane uppermost. Scattered throughout the mesangial area are dark granular fibrillar deposits with an electron density varying from black to the lighter grey characteristic of basement membrane proper. The electron-dense material within the mesangial area can be seen to merge imperceptibly with basement membrane proper at top right and to left of centre of plate.* × 5800
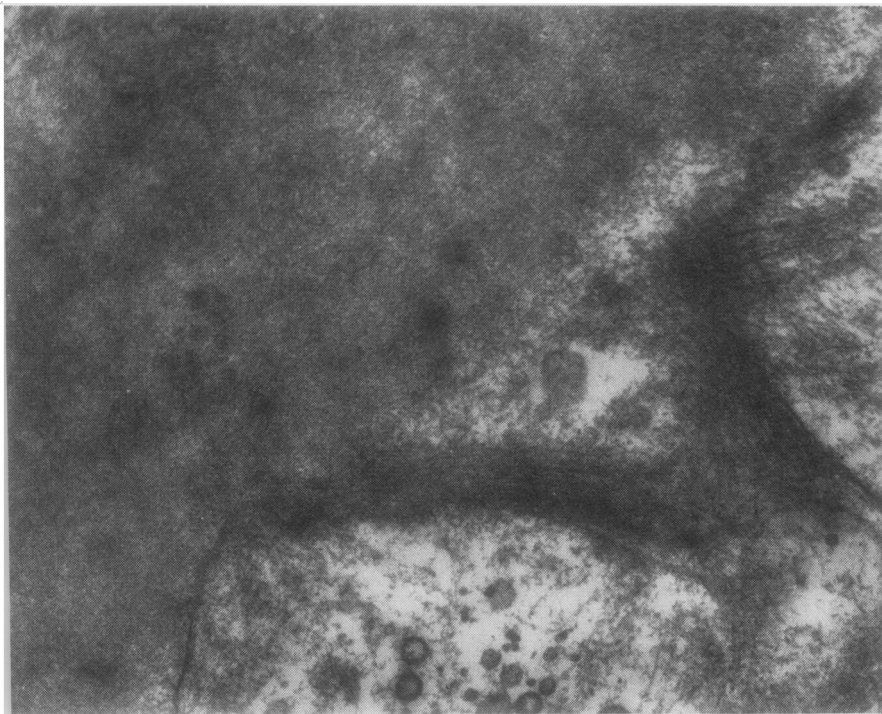
Fig. 10 *Case 4: this demonstrates intraluminal material with a distinctly fibrillar structure (lower part of plate), merging gradually with basement membrane (upper part of plate).* × 52 500
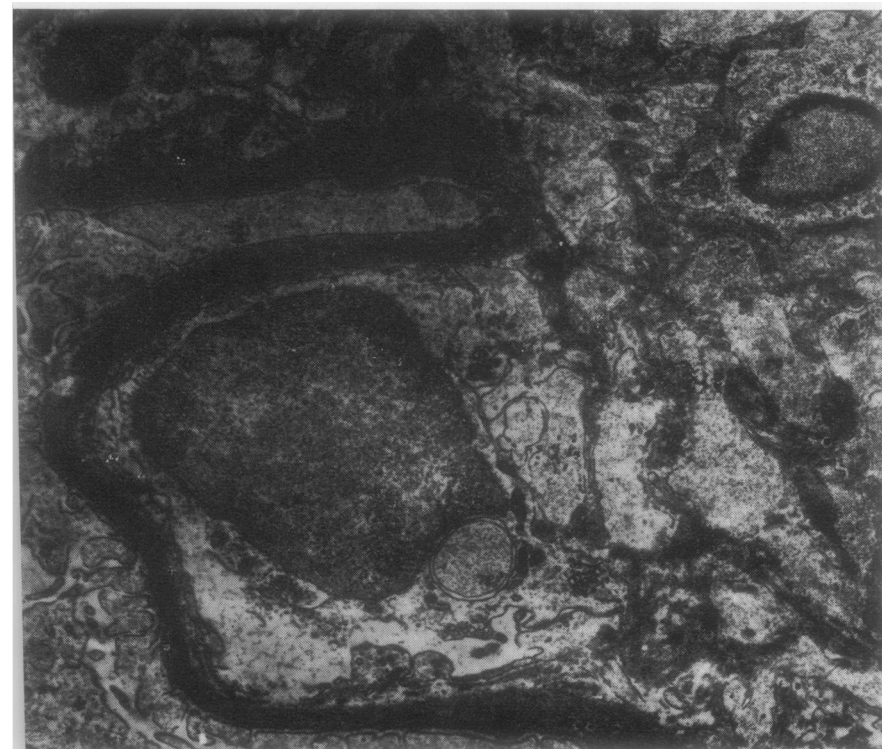

Fig. 11 *Case 2: strands of granular-fibrillar material are seen in the capillary lumen and between projections of endothelial cytoplasm. The electron density of this material again varies from light grey, characteristic of basement membrane, to black. An endothelial nucleus is seen at bottom centre of photograph and scattered areas of endothelial cytoplasmic vacuolation are evident.* × 7000
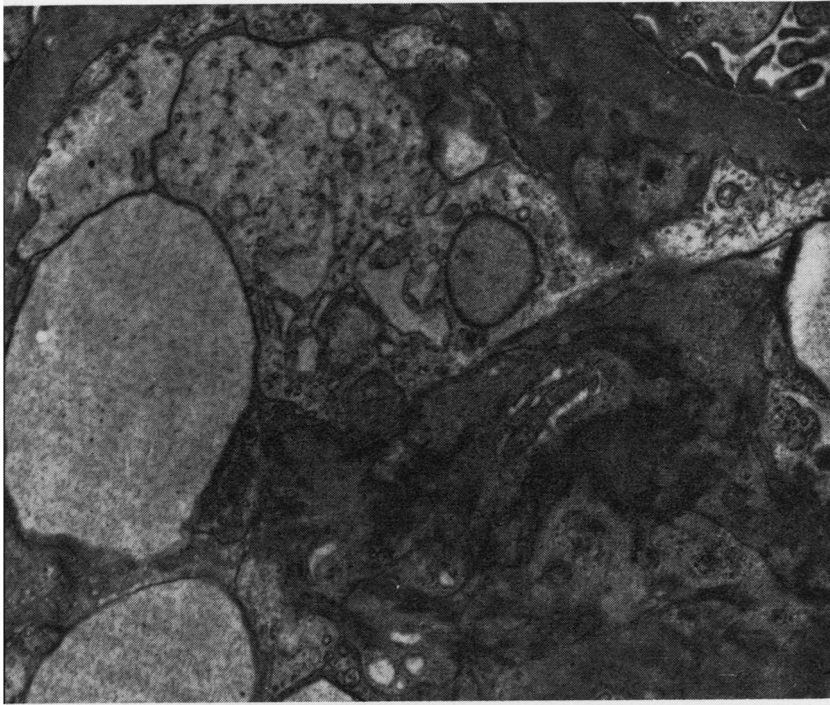
Fig. 12 *Case 3: much granular fibrillar material of varying electron density can be seen scattered throughout the mesangial region (right). There is gross vacuolation of endothelial and possibly mesangial cytoplasm. Linear deposits of dark granular material can be seen throughout the basement membrane (especially left), and some granular material in a subendothelial location is seen to merge gradually with basement membrane (top).* × 700
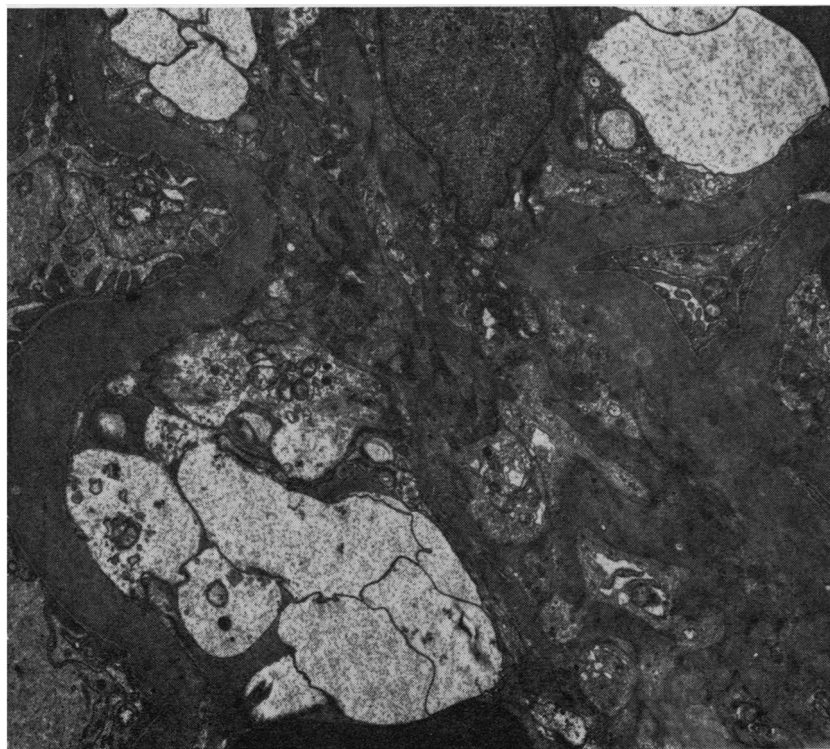

Fig. 13 *Case 1: three capillary lumina are seen enclosing a mesangial area and nucleus (nucleus top centre). In all, the endothelial cytoplasm is grossly vacuolated. One capillary lumen (bottom) contains much electron-dense, finely granulary material between projections of endothelial cytoplasm. The mesangial area in the centre of the photograph contains scattered granular deposits of varying electron density. Epithelial foot processes appear essentially normal.* × 4400

of appearances which this substance may assume. As Lendrum et al (1962) have shown on light microscopy using special stains, fibrin may go through a variety of appearances on electron microscopic examination, ranging from the dark, electron-dense nature of fresh fibrin to the lighter grey hyaline of older fibrin. Besides the difficulty of distinguishing fibrin from basement membrane-like hyaline, it is often impossible to distinguish the latter from basement membrane proper (Bencosme and Bergman, 1962). Stages of transition from a black, granular appearance through paler grey to typical hyaline basement membrane-like branches have been observed (Bloodworth, 1965a).

While fibrin having a periodicity of 200 to 240 A° has not, to our knowledge, been described in the early stages of diabetic glomerulosclerosis, some investigators have made reference to amorphous, electron-dense granular deposits within the peripheral capillary basement membrane or within the mesangial areas in glomeruli not severely affected by other morphological change (Suzuki, Churg, Grishman, Mautner, and Dachs, 1963; Rosenbaum, Kattine, and Gottsegen, 1963; Dachs, Churg, and Mautner, 1964). Similar changes have been described in those few instances where glomerular lesions convincingly similar to human diabetic glomerulosclerosis have been produced in animals (Østerby Hansen, Lundbaek, Olsen, and Orskov, 1967; Bloodworth, 1965b; Gibbs, Wilson, and Gifford, 1966).

Although fully polymerized fibrin is, except in the later stages, a rarity in human lesions those electron-dense granular deposits described above probably represent intermediate compounds in the fibrinogen to fibrin conversion; the fibrinogen-derived macromolecules now identified as fibrinogen degradation products (FDP). Their elusive nature possibly accounts for the rarity with which they have been identified on light and electron microscopy. Immunofluoresence microscopy with antifibrinogen serum is more highly specific, however, and gives a greater frequency of positive identification of FDP (Davies et al, 1966; Burkholder, 1965).

Until recently the concept of altered vascular permeability with early mural infiltration by mesometric substances, including fibrinogen, has received little support; Lendrum (1963), however, has consistently implicated fibrin in the pathogenesis of diabetic glomerulosclerosis. Whether deposition of fibrin or FDP in the diabetic glomerulus is of primary or secondary significance remains controversial. On the one hand it could be argued that such deposits are a secondary response to endothelial damage or represent the early stage of the exudative lesion. On the other hand, with more refined techniques for identifying fibrin and related products, our findings suggest that fibrin deposition might be a primary event in the pathogenesis of diabetic glomerulosclerosis. While homogeneous thickening of the capillary basement membrane distinguishes diabetes from other causes of glomerulosclerosis (MacDonald, 1966), whether the lesion arises in the peripheral capillary loops or in the mesangium is still disputed (Kimmelstiel, Osawa, and Beres, 1966; MacSween, 1971). Nevertheless, the metabolic defect leading to increased basement membrane thickness may simultaneously alter the membrane permeability; thus, the increased glycolyzation of hydroxylysine units in the peptide chains of basement membrane from the diabetic subject (Beisswenger and Spiro, 1970) may diminish the availability of the amino-acid to participate in cross linkages. This biochemical defect, together with the presence of bulky carbohydrate components on the peptide chains, may increase basement membrane permeability. Although there is little doubt that fibrin aggregates can cause severe glomerular damage (Vassalli, Simon, and Rouilles, 1963; Prose et al, 1965; Beller, Mitchell, and Gorstein, 1967), it may be that altered basement membrane permeability in diabetes mellitus together with a defect in fibrinolysis causes prolonged deposition of FDP in the glomerulus and ultimately stimulates structural changes whose nature depends on the pattern of distribution and rapidity of deposition of fibrinogen degradation products. One of our patients showed abnormal urinary FDP values in two of seven consecutive daily urine specimens. The other patients were not examined, however. Similar examination in other diabetic patients over a longer period of time has shown definitely increased urinary levels in some (Clarkson, 1971). Normal FDP levels, of course, are not inconsistent with slow or intermittent fibrin deposition or lysis. The local response to mesangial and endothelial trapping of fibrinogen or other macromolecules may play some part in mesangial enlargement and basement membrane irregularity besides initiating nodule development.

Despite attempts to explain the diabetic lesion on the basis of genetic, metabolic, immunological, endocrine or other factors, two significant aspects have never been adequately explained: first, the focal and peripheral lobular situation of the Kimmelstiel-Wilson nodule, and secondly the variability between accelerated microangiopathy in some diabetics and the relative freedom from such complications in others. Besides having relevance to nodule formation fibrin deposition and altered fibrinolysis may be relevant also to the variability in diabetic lesions. Important in this context may be the finding of Cash and McGill (1969) who isolated a subgroup of diabetic subjects who had an impaired fibrinolytic

response to stress. Possibly this finding indicates that some diabetic patients run a special risk of developing complications.

Certainly, diabetic small blood vessel disease cannot be composed into a straightforward picture. Whereas it would be unwise to stress any particular theory, however plausible, without considering the logical alternatives, it would seem that the focal structural and general metabolic relationships of fibrin deposition and fibrinolysis are worthy of further study in diabetes mellitus.

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