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Alterations of glomerular basement membrane relevant to haematuria

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Summary. To elucidate the morphological basis of glomerular haematuria, morphometric analysis of the glomerular basement membrane (GBM) and lamina densa (LD) was performed on silver impregnated samples for electron microscopy. The cases studied consisted of 3 groups: group A, normal controls, being from donors for kidney transplantation; group B, haematuric; and group C, non-haematuric cases with isolated proteinuria. Qualitative analysis revealed that gap formation, splitting, segmental and diffuse thinning of the GBM occur preferentially in haematuric cases. The morphometry of the GBM and LD yielded increased mean values of the GBM and of LD thickness in groups B and C. The coefficient of variation (CV, SD/mean) for the GBM and LD, however, was the highest in group B among the 3 groups, suggesting the most irregular GBM and LD in group B. In addition, CV was significantly higher in cases with splitting, segmental attenuation and gap of the GBM than cases without. The findings suggest that the irregularity of the GBM rather than its mean thickness is clearly associated with splitting and ultimately with haematuria via the gaps produced.

Key words: Haematuria – Glomerular basement membrane – Lamina densa – Gap formation – Silver impregnation

Introduction

Theoretically, haematuria of glomerular origin occurs through the passage of erythrocytes via glomerular capillary walls. However, light microscopic examination reveals only a minor abnormality or, if anything, slight mesangial proliferation in patients with isolated haematuria. Even by electron microscopy, evidence which displays a leak of erythrocytes from the capillary lumen directly into the urinary space has been scarce (Mouradian et al. 1975; Lin J-T et al. 1983; Bohle et al. 1984). Some investigators have suggested that discontinuity of the glomerular basement membrane (GBM) and/or thinning thereof might be responsible for haematuria (Rogers et al. 1973; Yoshikawa et al. 1984; Bohle et al. 1985; Terasaki et al. 1986). To our knowledge, however, little information is available concerning the quantitative evaluation of the GBM in adults (Dische et al. 1985; Coleman et al. 1986), although some data suggest the importance of the above mentioned GBM lesions regardless of hereditary or immunological causes. In this study we performed a morphometric analysis of the GBM in kidneys of patients with non-familial isolated haematuria and negative immunohistology.

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Materials and methods

Renal specimens obtained from 39 patients were used for the study. The sex, mean ages and major clinical findings of the patients at the time of renal biopsy are listed in Table 1.

The sample for group A were randomly selected from the kidneys of donors for kidney transplantation, who had not shown any renal disorder. Light microscopic findings appeared normal. Group B consisted of patients with persistent haematuria of more than 15–30 RBC/high power field, proteinuria of less than 0.5 g/day and normal creatinine clearance. No haematuric trait was noticed among the blood-related family members. Patients with extraglomerular haematuria or with systemic diseases were excluded. Group C, defined as the non-haematuric group, comprised those cases with less than 5 RBC/high power field, but with various amounts of proteinuria. It therefore included the patients with asymptomatic proteinuria and minimal change type nephrotic syndrome. Patients with systemic diseases were also excluded. Morphologically, groups B and C showed either minor abnormality or mild me-

Table 1. Major clinical findings

Groups	Age (yr)	Sex (M/F)	History of gross haematuria (% of patients)	History of nephrotic syndrome (% of patients)	Duration of the illness (months)	Urinalysis at biopsy	
						Haematuria (numbers/HPF)	Proteinuria (g/day)
A ($n=11$)	45.5 ± 14.8[a]	4/ 7[c]	0	0	–	0	0
B ($n=18$)	20.8 ± 6.2[b]	5/13[d]	61	0	29.4 ± 26.4	>15–30	0.18–0.19
C ($n=10$)	25.8 ± 12.8	8/ 2	0	60	56.1 ± 70.7	<5	5.09 ± 8.93

M: male. F: female. HPF: high power field. means $\pm$ SD

[a] $p<0.01$ vs. group B and group C. [b] n.s. vs. group C
[c] n.s. vs. group B and group C. [d] $p<0.05$ vs. group C

sangial proliferative glomerulonephritis. No crescent formation was noted in any of the cases. Immunofluorescence observation was not done in group A or in some cases of groups B and C. The cases presenting urinalysis compatible with group B, were however excluded when immunofluorescence provided the diagnosis of IgA nephropathy. The cases in which immunofluorescence was not done did not display electron dense deposit suggestive of immune complex by electron microscopy. The mean age was higher in the order of groups A, C and B (Table 1). No difference in the age distribution was noted between groups B and C (Table 1, student's *t*-test). The difference in the sex ratio was found only between the groups B and C (Chi-square test).

The kidney tissues were cut into 1 mm^3 pieces and fixed in 0.1 M phosphate buffer (pH 7.4) containing 2% glutaraldehyde, at 4° C for 2 h with subsequent postosmication in 1.0% osmium tetraoxide resolved in the same buffer for an additional 1 h. Dehydration was carried out in a graded alcohol series. The samples were finally embedded in Quetol-812 (Nissin EM Co., Ltd., Tokyo, Japan). Ultrathin silver sections approximately 0.08 μm were prepared in an LKB 8800 Ultratome ultramicrotome (LKB, Bromma, Sweden). The ultrathin sections were then subjected to conventional double staining and also to silver impregnation by the modified method of Movat et al. (1961). Morphometry was performed on the silver-impregnated samples. Photographs were taken at $\times$1100 magnification with an LEM-2000 electron microscope (Akashi beam technology, Tokyo, Japan) and were enlarged up to a magnification of $\times$11000. Polystyrene latex (∅ 2.020 μm Nissin EM Co., Ltd., Tokyo, Japan) was used for morphometric calibration. The boundary between the peripheral and the mesangial GBM was defined by the first endothelial pore after a mesangial region. The widths of the GBM and lamina densa (LD) were measured on the spots where the plane of the GBM was perpendicular to that of the section. Actually, the spots for measurements were selected on the basis that the epithelial and the endothelial cytoplasmic membranes were clearly visible, and that the endothelial pores were not circular (Osawa et al. 1966). The measurement was performed at all sites of the peripheral GBM which satisfied the above mentioned criteria. The thickness of the GBM was expressed as the shortest distance between the epithelial and the endothelial plasma membranes; and that of LD as the width of the silver-impregnated zone in between. The width was measured at 0.5 μm intervals where available, using a computerized image analyser (Videoplan, Kontron, Munich, FRG). In each individual case, one to three glomeruli were subjected to the morphometry. The mean widths of the GBM and LD were calculated out of 175 to 808 measurements in each individual case, and from these values the mean $\pm$ SD in each group was obtained. To quantify the irregularity of the GBM or LD, the coefficient of variations (CV, SD/mean) was also calculated in each individual in terms of the GBM and LD thickness. An attempt was also made to quantify the density of the GBM-gap using 30 consecutive ultrathin sections (0.08 μm in thickness). Series of the 30 consecutive sections were selected, which provided the views where the planes of the GBM were perpendicular to the planes of the sections according to the above mentioned cirteria. Then the $D=B/A$ was calculated in each of the available cases, where D stands for the maximum number of gap per surface area of the GBM, and B for the number of the gaps found in each series of the sections. A, the surface area of the GBM, was obtained by multiplying the circumference (μm) of the glomerular capillary loop by 30×0.08, where 30 connotes the number of consecutive sections and 0.08, thickness (μm) of each section. The size of the GBM-gap was evaluated as summation of the area of the rectangle by multiplying the distance of the GBM-gap by 0.08 μm (thickness of section).

For statistical analysis, the unpaired student's *t*-test, Mann-Whitney's U-test and Chi-square test were used, when appropriate. All *p* values of less than 0.05 were considered as being statistically significant.

Results

Electron microscopic observations disclose the thickening as well as the thinning of the GBM, solely in group B (Table 2). The alterations take both the diffuse and segmental form (Fig. 1). On some occasions, the changes extend to the whole capillary loop, on others there are attentuations or even discontinuities (gap) restricted to very small areas of the GBM (Fig. 2A, 2B). No correlation is noted between the gap and the thickness of the GBM, that is, some of the ruptured ends of the GBM are thick while the others are thinned. The thickness of the GBM of the whole length of disrupted capillary loop is also variable, with striking irregularity. The gap space of the GBM is sealed with endothelial and epithelial cells in direct contact with each other (Fig. 2C), or with amorphous material between the cells. There is no monocyte or polymorphonucleocyte infiltration adjacent to the altered GBM.

Silver impregnation provides simpler and more

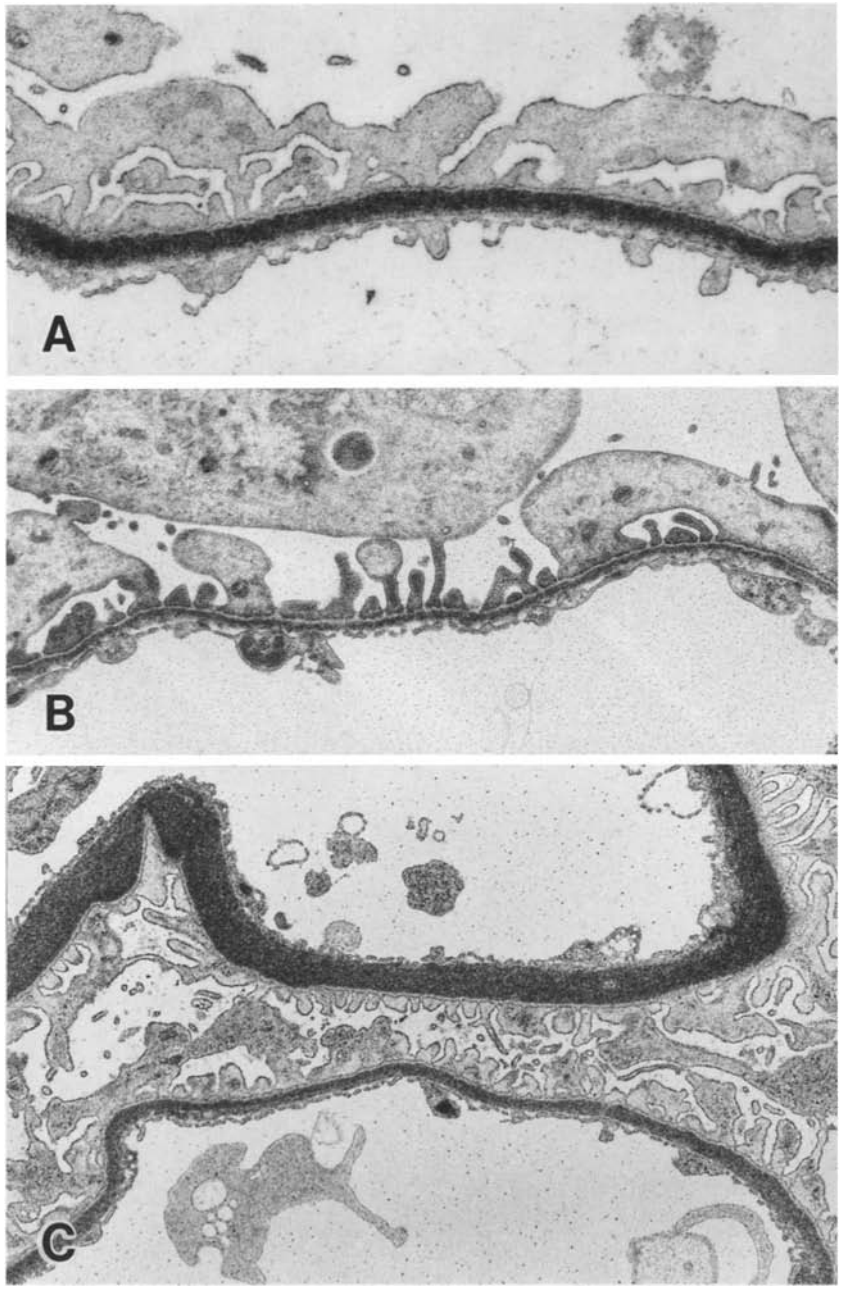

Fig. 1 A–C. Electron micrograph for a case in group A showing **A** the GBM with normal thickness, ×13000 silver-impregnation (PASM); **B** Electron micrograph for case 1 in group B. Note diffuse thinning of the GBM extending to the whole capillary loop, ×13000 (PASM); **C** A glomerulus from case 6 in group B. The thickening and thinning of LD coexist in the same glomerulus. × 13000 (PASM)

distinct visualization of LD. Generally, the changes of LD are in accord with those of the GBM, but occasionally LD thickness is disproportionate to that of the GBM with resultant larger variations in LD/GBM. Splitting of LD is found most frequently in group B. In contrast with Alport's syndrome, however, the lesions are not so conspicuous. The lamination of LD is not found in any of the subjects examined. In two cases of group B, the ruptured ends of LD are split, a finding which suggests that splitting may lead to gap formation, although the possibility that splitting is the sequel of the rupture cannot be discarded. In group C splitting is also noted, but with much lower incidence, and none is noted in group A.

The mean thicknesses of the GBM, LD and LD/GBM are larger in the order of groups C, B and A (Table 2). The CV, a parameter of the irregularity of the GBM and LD thickness, is most striking in group B (Fig. 3). The CV for LD and the GBM thickness is significantly higher in cases with splitting, focal thinning and gap formation than in those without (Fig. 4). The findings suggest that an irregularity in the thickness of the GBM

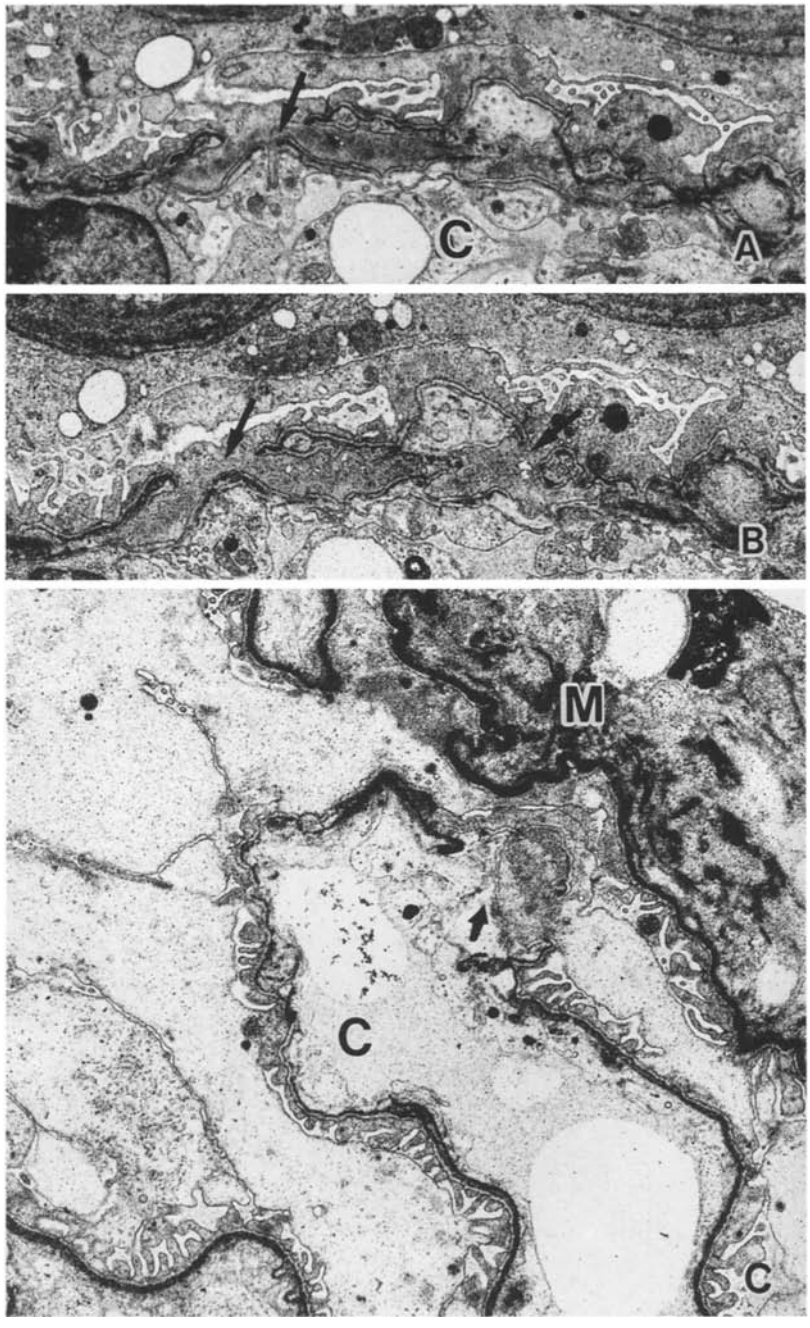

Fig. 2. Gaps of the GBM in glomeruli from group B. (*M*), mesangial; (*C*), capillary. **A** Case 3. Splitting and discontinuity of LD. Note epithelial cell intercalating between the split LD (*arrow*), which is argiophilic. **B** The glomerulus obtained in the section consecutive to that of **A**, showing the intercalation of epithelial cells (*arrow*) between the disrupted ends of the GBM. ×13000 (PASM). **C** Case 2. There is splitting at both ends of ruptured LD. The ruptured space is sealed with swollen epithelial and endothelial cells in contact with each other (*arrow*). Note also there are splitting and attenuation in other portions of the GBM. ×7000 (PASM)

and LD may be associated with gap formation and ultimately with haematuria. It should be noted that the decrease of the mean thickness per se does not simply imply the cause of gap formation.

The values of D obtained are between 1.14 and 8.33/1000 μm^2. It is therefore suggested that in our cases with GBM-gaps, the gaps are distributed with the densities of less than 1.14 to 8.33 per 1000 μm^2 surface area of the GBM. The approximate sizes of the gap are within the range of 0.024 to 0.904 μm^2.

Discussion

The ultrastructural features found in the present investigation can be traced back to the descriptions of glomeruli in Alport's syndrome in 1972 (Hinglais et al. 1972), which include the widening of the GBM associated with reticulation, splitting and lamination. Although the concept of familial haematuria had been already put forward as a clinical entity distinct from Alport's syndrome in 1966 (McConville et al.), it was not until 1973 (Rogers

Table 2. Renal biopsy findings

Group	Case	Light microscopy	Immunofluorescence						Electron microscopy				No.	LD thickness (nm) (mean ± SD)	GBM thickness (nm) (Mean ± SD)	LD/GBM (Mean ± SD)
			IgG	IgA	IgM	C1q	C3	C4	Splitting	Gap	Focal thinning	Dep.				
A	1–11	min	ND	ND	ND	ND	ND	ND	−	−	−	−	3546	228 ± 82	384 ± 82	0.59 ± 0.09
B	1	mp	−	−	−	ND	−	ND	+	−	−	−	280	160 ± 98	309 ± 117	0.50 ± 0.13
	2	mp	−	−	−	−	−	−	+	+	+	−	201	164 ± 45	332 ± 64	0.49 ± 0.09
	3	mp	−	−	−	ND	−	−	+	+	−	−	200	206 ± 56	368 ± 78	0.56 ± 0.08
	4	mp	no glomeruli						+	−	+	−	200	128 ± 28	243 ± 38	0.52 ± 0.08
	5	min	−	−	M+	ND	M+	ND	+	−	+	−	538	462 ± 151	605 ± 158	0.75 ± 0.07
	6	mp	−	−	M+	ND	−	ND	+	+	+	−	696	399 ± 155	556 ± 161	0.70 ± 0.10
	7	mp	−	−	−	ND	−	ND	+	−	+	−	808	334 ± 95	485 ± 106	0.68 ± 0.07
	8	mp	no glomeruli						−	−	+	−	207	285 ± 91	398 ± 94	0.71 ± 0.08
	9	mp	no glomeruli						−	−	−	−	604	260 ± 74	402 ± 88	0.64 ± 0.09
	10	mp	no glomeruli						+	−	+	−	211	297 ± 94	461 ± 110	0.64 ± 0.09
	11	min	−	−	−	−	−	−	+	−	+	−	502	324 ± 120	467 ± 127	0.68 ± 0.09
	12	mp	−	−	M+	ND	−	ND	+	+	+	−	352	104 ± 51	228 ± 62	0.44 ± 0.11
	13	mp	M+	−	M+	−	M+	ND	+	+	+	−	588	178 ± 58	323 ± 69	0.54 ± 0.10
	14	mp	−	−	−	ND	−	ND	+	−	+	−	216	142 ± 53	279 ± 66	0.50 ± 0.10
	15	mp	M+	−	M+	−	−	−	+	−	+	−	605	283 ± 96	449 ± 107	0.62 ± 0.10
	16	mp	−	−	M+	−	−	−	+	−	+	−	391	296 ± 83	428 ± 96	0.69 ± 0.07
	17	mp	−	−	−	−	−	−	+	+	+	−	729	258 ± 92	412 ± 96	0.61 ± 0.10
	18	mp	−	−	−	−	−	−	+	−	+	−	704	347 ± 97	508 ± 104	0.67 ± 0.07
												Total	8032	283 ± 136*	431 ± 146*	0.63 ± 0.12*
C	1	min	−	−	−	M+	ND	ND	+	−	+	−	366	311 ± 81	433 ± 89	0.71 ± 0.07
	2	min	−	−	−	−	−	−	−	−	+	−	378	396 ± 103	523 ± 109	0.75 ± 0.06
	3	min	−	−	−	−	−	−	−	−	+	−	517	340 ± 96	472 ± 104	0.71 ± 0.07
	4	min	−	−	−	−	−	−	−	−	+	C+	183	431 ± 93	560 ± 105	0.78 ± 0.06
	5	min	−	−	M+	−	−	ND	−	−	−	−	175	384 ± 75	514 ± 83	0.74 ± 0.05
	6	min	−	−	C±	−	−	−	+	−	+	−	245	373 ± 94	506 ± 100	0.73 ± 0.07
	7	mp	M+	M+	M+	−	−	−	−	−	−	−	217	307 ± 45	447 ± 56	0.69 ± 0.07
	8	mp	no glomeruli						−	−	+	−	200	274 ± 61	412 ± 86	0.66 ± 0.07
	9	mp	M+	−	M+	−	−	−	−	−	−	−	212	241 ± 49	373 ± 60	0.65 ± 0.07
	10	min	−	−	M+	−	M+	M+	−	−	−	−	225	325 ± 74	484 ± 86	0.67 ± 0.07
												Total	2718	339 ± 97**	472 ± 104**	0.71 ± 0.07**

Dep.: deposit; min: minor abnormalities; mp: mild mesangial proliferative glomeluronephritis; ND: not done; M: mesangial; C: capillary; −: negative; +: positive; Intensity was graded from − to + + +; No.: numbers of measurements;

* $p < 0.001$ vs. group A and group C
** $p < 0.001$ vs. group A

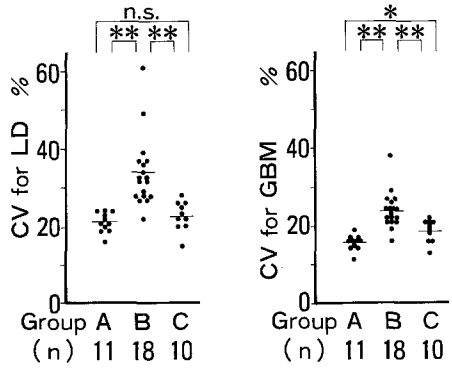

Fig. 3. Mean coefficient of variation (CV) for LD and for the GBM thickness. $*p<0.02$ $**p<0.002$ (Mann-Whitney's *U*-test) A: Group A. B: Group B. C: Group C

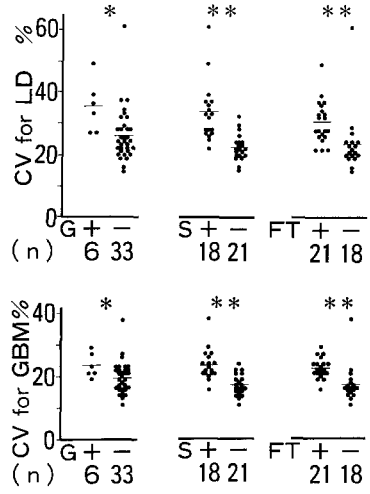

Fig. 4. Relationship between morphological changes in the GBM and the coefficient of variation (CV) for LD and the GBM thickness. CVs are depicted in terms of presence or absence of the ultrastructural lesions of the GBM and LD, i.e. gap formation, splitting and focal thinning. *G*, gap formation; *S*, splitting; *FT*, focal thinning; + presence of the lesion; − absence of the lesion, $*p<0.05$ $**p<0.001$

et al.) that the ultrastructural abnormality of the GBM of this disease was shown to be similar to that in Alport's syndrome. However, a disparity of opinions exists as to the thickness of the GBM in this disease: some describe the thickening and the others the thinning (Rogers et al. 1973; Hill et al. 1974; Piel et al. 1982). Indeed, a quantitative analysis was made showing the mean thickness of the GBM by some investigators (Tina et al. 1982; Yoshikawa et al. 1984; Milanesi et al. 1984; Dische et al. 1985; Coleman et al. 1986), but their attention was not directed toward the irregularity of the GBM thickness as a probable morphological implication of haematuria.

Recently, Yum et al. (1983) have also proposed a concept under the name of Basement Membrane Nephropathy consisting of 3 subtypes: type 1, characterized by irregular and marked thickening of the GBM with severe structural distortion (compatible with Alport's syndrome): type 2 with diffuse attenuation of the GBM; and type 3 with variations in the GBM thickness. They, however, did not perform quantiative evaluation on the irregularity of the thickness in the GBM. Our cases do not include the haematuric trait, but, may have sporadic cases of familial haematuria. It is worth noting that Yum et al. have not recognized the familial (hereditary) implication as an important pathogenic factor for the GBM alteration. Apart from the standpoint of familial (hereditary) disorder, the GBM abnormalities mentioned so far have recently been the focus of attention in terms of the pathogenesis of hematuria.

Lin J-T et al. (1983) have suggested 4 factors which contribute to the generation of haematuria: the deforming force on the erythrocytes, their deformability, the gap in the GBM and its thickness. The latter two variables are important as responsible glomerular factors for haematuria. Gaps in the GBM have been known to occur in glomerular disorders other than hereditary disease.

IgA nephropathy is also widely known to accompany haematuria as a characteristic urinary symptom. Shigematsu et al. (1982) have emphasized the presence of local thinning or gaps in the peripheral GBM, despite the fact that the glomerular lesions are generally restricted to the mesangium in this disease. Bohle et al. (1984, 1985) have likewise described the cases of IgA nephropathy in which erythrocyte was detected at the site of the GBM rupture. Therefore regardless of the cause of the lesions (hereditary or immunological), it is likely that the gaps associated with abnormalities of the GBM substructure become a possible cause of haematuria. In our investigation, the mean thicknesses of the GBM and LD were most prominent in group C (non-haematuric). Group B (haematuric) also showed larger mean GBM and LD thicknesses than group A (normal control), suggesting that the simple evaluation of mean GBM (LD) thickness does not provide insights to the pathogenesis of haematuria. CV (SD/mean) for the GBM or for LD, however, was significantly higher in group B (Fig. 3), indicating irregular thickness of the GBM (LD) in this group. The values of CV were also shown to be related to splitting, focal thinning and gap formation of the GBM and LD (Fig. 4). Based on these findings, it is suggested that haematuria is caused by the leak of erythrocytes via the gaps of the GBM (LD) and that gap formation is linked with the local

attenuation and the irregular thickness of the GBM.

As suggested by Lin J-T et al., the deformed erythrocytes may possibly pass through the comparatively small space of disrupted GBM. In fact, some investigators (Mouradian et al. 1975; Bohle et al. 1984, 1985) have demonstrated direct morphological evidence that deformed erythrocytes are at the site of the GBM disruption. It is not clear, however, that ruptured sites sealed by epithelial and endothelial cells, demonstrated in the present study, represent the recovery stage or simply the earlier stage of further disruption.

It would be interesting to know the minimum number of gaps per surface area of the GBM and the minimal size of these gaps required to induce haematuria. We have attempted here, using consecutive ultrathin sections, to obtain tentative values. However, due to the technical limitations for this purpose in the use of transmission electron microscopy (TEM), the values herein obtained are not accurate enough. Scanning electron microscopy already used for qualitative study (Bonsib 1985) might provide a more useful strategy for this purpose rather than TEM.

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