Original Article

Ultrastructural study of the undulated epithelial side of glomerular basement membrane in membranous nephropathy；clinicopathological study of 20 patients

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Abstract

Introduction：Membranous nephropathy（MN）is a common cause of nephrotic syndrome in adults and is characterized by electron-dense deposits（EDD）in the glomerular basement membrane（GBM）. On the other hand, undulations, which are formed on the epithelial side of the GBM irrelevant to EDD, are observed in some cases；these findings were not considered in the Ehrenreich and Churg（EC）classification in MN.

Methods：Twenty adult patients with MN formed long segments of undulations on the epithelial side of the GBM without reactions to subepithelial EDD by EM were enrolled. We compared the clinicopathological findings of these cases with 20 control cases of MN patients who did not have this undulation. These two patient groups were matched for gender, age, and class of disease stage in the EC classification of MN.

Results：In the study group, the lamina densa thickened in many areas in comparison with the control group（962.4 ± 309.1 vs. 604.0 ± 296.5 nm；p = 0.001）. In addition, the number of C3c positive cases were less in the immunofluorescence（IF）study,（n = 4 vs. n = 12；p = 0.009）, and serum complement levels tended to be higher than those in the control group.

Conclusion：In our study, the pathogenesis of GBM undulation was not determined. Nevertheless, these results indicate that diverse immune responses, in addition to the complement system, may be involved differently from usual cases of MN.

（Keywords：Complement, electron micrograph, glomerular basement membrane, membranous nephropathy, renal biopsy）

Introduction

Membranous nephropathy（MN）is a common cause of nephrotic syndrome in adults[1]. The main pathological feature of MN by electron microscopy（EM）is the presence of subepithelial electron-dense deposits（EDD）and the subsequent growth of lamina densa of the glomerular basement membrane（GBM）around the deposits. This leads to thickening of the GBM and the appearance of spikes and stipplings on sections stained with periodic acid methenamine-silver（PAM）. The characteristic immunofluorescence（IF）finding is peripheral granular staining along the GBM for immunoglobulin（Ig）G and C3, occasionally for IgM and IgA, and rarely for C1q and C4[1-3]. In light microscopy（LM）, the lamina densa is seen as a spike with PAM staining. The Ehrenreich and Churg（EC）classification, which has four major stages, has been defined primarily based on histological and ultrastructual features[4].

The EM features of MN often show a complicated expansion of the epithelial side of the GBM. These features are difficult to observe using LM and are not considered

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in the EC classification. Because morphological changes in MN are also observed in the podocytes, we thought that the complicated expansion and changes in the GBM would be distinct and characteristic findings of glomerulopathy associated with injury to the podocytes. This undulation of the GBM can appear to be nonspecific if it only involves occasional and short stretches of the GBM. However, in MN, we found that the epithelial side of GBM with undulations demonstrated long segments that did not have reactions to the subepithelial EDD, and surmised that these findings may be a reaction of the GBM to the podocytes. In Alport syndrome (AS), complications on the epithelial side of the GBM is an important characteristic[5]. In AS, these features can be seen in long sections and have been described as a "wavy and irregular margin" on the epithelial side near the pedicles of the podocytes[6,7]. This ultrastructural feature is similar to the undulation of the GBM in MN.

In the present study, we focused on the characteristic feature of undulation by EM and investigated the clinicopathological significance of this finding among MN patients.

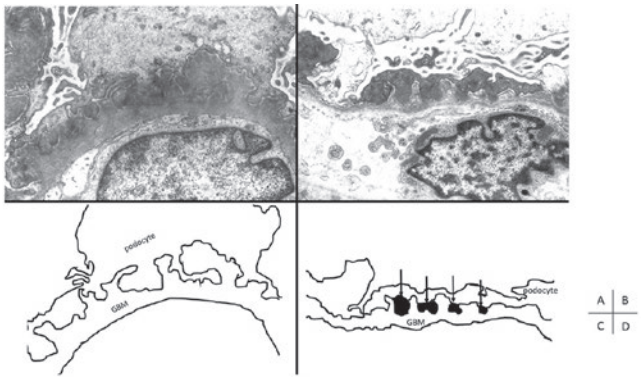

Fig. 1
Electron micrograph of the undulation group (A) and control group (B). The glomerular capillary loop showed undulated epithelial side without reactions to the subepithelial EDD compared with the control group. C and D are schematic diagram of electron micrograph, C corresponds to A, and D corresponds to B. Subepithelial EDD were seen in control group (B) and are indicated as an arrow (D).

Methods

Patients and specimens

This study was based on the renal histological records (1995-2011) of 3230 patients from Jichi Medical University Hospital and its affiliated hospitals. Renal biopsies were performed in all patients after obtaining informed consent. From the 277 cases that were diagnosed with MN based on pathological findings, 20 adult cases (7.2%) that demonstrated long segments of undulation of the epithelial side of the GBM and no reaction to the subepithelial EDD using EM were enrolled as the undulation group. This finding of undulation is different from the findings in EC stage II or III in which the epithelial side of GBM expands as a response to podocytes rather than subepithelial EDDs (Fig. 1). Patients with secondary MN associated with collagen disease-related conditions, such as clinical or serological SLE, were excluded regardless of the MN being idiopathic or secondary. As a control group, 20 patients that were matched for age (years), gender, and class of disease stage of MN based on EC findings, were enrolled in the study.

Clinical evaluation

The clinical data were collected from all patients immediately or within three months before renal biopsy. We compared the values of age (years), gender, blood urea nitrogen (BUN, normal range 8–20 mg/dL), serum creatinine (Cr, normal range 0.38–0.90 mg/dL), Cr clearance (Ccr, mL/min), estimated glomerular filtration rate (eGFR, mL/min/1.73 m^2), C3 (normal range 86–160 mg/dL), C4 (normal range 17–45 mg/dL), urinary occult blood, and daily urinary protein excretion content (g/day) of the patients with those in the control group. eGFR was calculated using the following formula : eGFR (male) = 194 × $Cr^{-1.094}$ × $Age^{-0.287}$ and eGFR (female) = eGFR (male) × 0.739. This equation is a Japanese coefficient of the modified Isotope Dilution Mass Spectrometry－Modification of Diet in Renal Disease (IDMS–MDRD) Study[8]. These parameters were chosen because they were sufficient for statistical evaluations. This study was approved by the ethics review board of the Jichi Medical University Hospital (A13-85).

Histopathological study

To prepare for LM evaluation, the specimens were fixed in 15% formalin, embedded in paraffin, cut into 3-μm sections, and stained with hematoxylin–eosin (HE), periodic acid-Schiff (PAS), periodic acid methenamine-silver and Masson trichrome stain (PAM-MT), and Elastica van Gieson (EVG) stains. Also, LM findings documented the diagnosis and their pathological descriptions were conferred.

Immunofluorescence examination

To prepare the specimens for immunofluorescence (IF) study, fresh frozen tissue sections were cut into 3-μm sections on a cryostat at －25℃. After being fixed in acetone and embedded in OCT compound, the specimens were evaluated using an immunofluorescence microscope (Leica, Germany) that was equipped with optical excitation and barrier filters for fluorescein isothiocyanate (FITC). For direct immunofluorescence, the sections were stained with polyclonal goat anti-human antibodies against IgG (MBL, Nagoya, Japan) at a dilution of 1 : 200 ; polyclonal rabbit anti-human antibodies against IgM,

IgA, and C1q at a dilution of 1 : 20 ; and polyclonal rabbit anti-human antibodies against C3c, C4, and fibrinogen (DacoCytomation, Copenhagen, Denmark) at a dilution of 1 : 40. These staining results were evaluated by direct IF method using an FITC-conjugated antibody using internal controls existing in the tubular epithelium, tubular cast and blood vessel. A positive control and a negative control were omitted for each run. The intensity of IF staining was graded on a scale of 0 (negative), ± (mildly positive), 1+ (moderately positive), or 2+ (strongly positive). For the statistical analysis, the values of 0 and ± were classified as negative, and 1+ and 2+ were classified as positive.

Electron Microscopic Examination

For the EM study, after immersing the tissues from the kidney biopsies in 2.5% glutaraldehyde and 2% osmium tetraoxide, the specimens were embedded in Epon and divided into ultrathin sections. These sections were stained with uranyl and lead citrate ; images were evaluated and captured using an electron microscope (HITACHI H7500, Hitachi, Ibaraki, Japan). The findings were primarily investigated using photographs prepared from features (17–68 features per patient) that were captured at the range from 1,000× to 15,000× magnification.

With respect to EM, we evaluated the length of the GBM that contained epithelial cell infolding, the thickness of the GBM (Fig. 2) and the lamina densa, the status of EDD, and the reticular degeneration of the lamina densa. Reticular degeneration was a feature of the lamellation of the lamina densa. We measured the thickest overall part of the lamina densa (1 site) as well as the thickness of the lamina densa (3 sites) that was beneath the area with the deposit or the area of the highest density, and the thickness of the lamina densa (3 sites) below the podocytic infolded area (Fig. 2). These values were measured using negative films, then the values were divided by the rate of magnification to give 1,000,000 folds.

Statistical Analysis

The results were analyzed using the IBM SPSS Statistics 21 and were expressed as the median (interquartile range : IR) and as the mean ± standard deviation (S.D.). The analyses of the continuous variables (BUN, Cr, Ccr, eGFR, daily urinary protein excretion, urinary occult blood, serum C3, serum C4, and EM measurements) were performed with the equal variance t-test or Mann–Whitney U-test, and the categorical variables (IF results) were analyzed by Fisher's exact test. $P < 0.05$ was considered statistically significant.

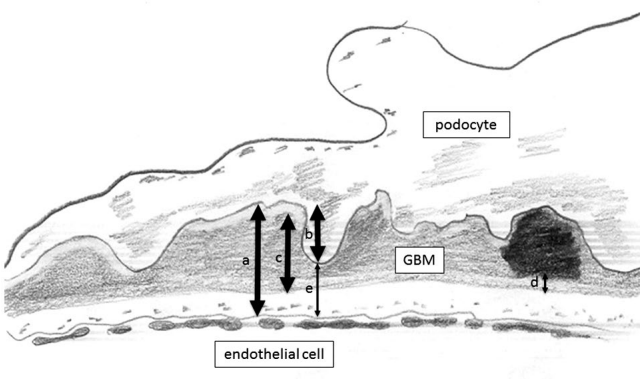

Fig. 2

Measurement sites in electron micrograph

The thickest overall part of the GBM (double arrow a) that contained epithelial cell infolding (double arrow b). The thickest overall part of the lamina densa (double arrow c). The thickness of the lamina densa that was beneath the area with the deposit or the area of the highest density (double arrow d). The thickness of the lamina densa below the podocytic infolded area (double arrow e).

Results

Patients

The clinicopathological characteristics of the 20 patients enrolled in this study are summarized in Table 1. The median age of the 20 patients was 66 years, ranging from 34 to 84 years. There were 17 males and 3 females. The characteristics of the study group and control group are shown in Table 2. For patients whose disease stage extended across two disease stages in the EC classification, we used the predominant disease stage determined from the LM, IF, and EM findings. Five (25%) patients were stage 1, 11 (55%) were stage 2, three (15%) were stage 3, and one (5%) was stage 4, as shown in Table 3.

Clinical findings

Laboratory examinations revealed proteinuria in all patients. With regard to the complement titer, 19 patients out of 20 patients were examined for serum C3 and C4. At the time of the renal biopsy, eight patients and eight controls had at least 3.5 g/day of proteinuria. Urinary occult blood that was graded at least 2+ was found in 14 (70%) patients, and the median value was 2.0 (interquatile range, 1.0 to 2.0). The renal function values were : BUN : 16.5 ± 8.1 mg/dL, Cr : 0.90 ± 0.57 mg/dL, Ccr : 95.4 ± 41.6 mL/min, and eGFR : 102.8 ± 50.9 mL/min/1.73m^2 ; these values were within the normal range. The mean value for serum C3 was 126.6 ± 18.5 mg/dL, and the mean value for serum C4 was 35.8 ± 0.4 mg/dL ; both were within normal limits.

We compared the clinical data between the undulation group and the control group (Table 3). The mean value for serum C3 in the undulation group tended to be higher than the control group ($p = 0.06$) and the mean serum C4 level

Table 1. Patient profiles：Undulation group（20 cases）

Case	Age	sex	BUN (mg/dL)	Cr (mg/dL)	Ccr (mL/min)	eGFR (mL/min/1.73m^2)	Proteinuria (g/day)	Occult blood	Serum C3 (mg/dL)	Serum C4 (mg/dL)	IgG	IgA	IgM	C3c	EC class*
1	52	M	17	0.6	110	132.2	2.008	3	101	58	No sample				2
2	56	M	15	0.6	80.9	130.7	0.545	0	96	22	2	2	3	1	3
3	84	M	19	0.9	46	75.4	3.6	3	144	35	No sample				1
4	72	M	40.9	2.73	20.7	21.6	18	3	No sample		2	2	1	1	1
5	34	F	13	0.43	137	158.5	1.746	2	156	50	3	2	0	0	1
6	53	M	16	0.98	65.75	75	8.032	1	138	44	2	0	0	1	2
7	70	M	37	1.95	29	32.1	3.2	2	130	28	0	2	1	2	2
8	64	M	13	0.71	164	104.8	6	2	151	47	2	0	0	1	2
9	77	M	14	0.58	147.32	127.4	1.6	2	136	24	1	2	1	0	3
10	61	M	13	0.9	121	80.5	3.1	0	122	35	3	0	0	2	2
11	68	F	11	0.44	105	137.5	5.3	2	140	47	3	0	1	0	1
12	60	M	13	0.81	101.6	91.2	8.47	1	133	28	3	3	1	0	2
13	68	M	11	1.4	55.1	47.3	0.12	0	106	19	2	0	1	0	3
14	66	M	12	0.34	54.33	243.5	0.375	3	127	28	2	0	1	2	2
15	66	M	19	1.19	81.7	57.4	0.594	1	126	32	2	1	1	0	2
16	68	M	15	0.98	135	71.3	6.945	2	152	37	2	0	0	0	2
17	60	M	13	0.83	74.9	88.6	2.961	2	116	33	2	0	1	0	2
18	66	M	14	0.71	151	104.1	4.5	2	124	46	3	0	0	0	1
19	75	F	9	0.39	116.9	151.1	7.2	2	109	35	2	0	0	2	2
20	67	M	15	0.6	110.2	126	1.7	2	98	33	2	0	0	0	4

BUN, blood urea nitrogen；Cr, serum creatinine；Ccr, Cr clearance; eGFR, estimated glomerular filtration rate
*Ehrenreich and Churg classification

Table 2. Characteristics of the study population

	undulation	control	*P* value
Age（years）	66（34–84）	64（27–80）	-
Gender, men/women	17/3	17/3	-
Blood urea nitrogen（mg/dL）	16.5 ± 8.1	16.6 ± 6.1	0.9
Serum creatinine（mg/dL）	0.90 ± 0.57	0.94 ± 0.40	0.8
Ccr（mL/min）	95.4 ± 41.6	91.8 ± 53.2	0.8
eGFR（mL/min/1.73m^2）	102.8 ± 50.9	87.0 ± 35.8	0.3
Proteinuria（g/day）	4.3 ± 4.2	4.2 ± 3.2	0.9
Occult blood	2.0（1.0-2.0）	2.0（1.3-3.8）	0.2
Serum C3（mg/dL）	126.6 ± 18.5	113.3 ± 23.4	0.06
Serum C4（mg/dL）	35.8 ± 10.4	29.2 ± 7.8	0.03

Values represent mean ± S.D. or as median and interquartile range.
Ccr, creatinine clearance；eGFR, estimated glomerular filtration rate

Table 3. Number of the patients in each Ehrenreich and Churg classification

Stage	n	Males	Age（years）	Age of control
1	5	3	66（34-84）	65（27-80）
2	11	10	64（52-75）	62（49-78）
3	3	3	66（56-77）	66（57-74）
4	1	1	67	68
Total	20	17	66（34-84）	64（27-80）
			median（range）	

Table 4. Number of positive cases

	undulation (n = 18)	control (n = 18)	*P* value*
IgG	16	18	0.2
IgA	6	2	0.1
IgM	1	0	0.5
C3c	4	12	0.009
C4	0	0	-
C1q	2	3	0.5
Fib	0	0	-

*Fisher's exact test

Table 5. Electron microscopic study

	undulation	control	*P*-value*
Infolding distance (nm)	516 ± 299.7	538.5 ± 224.4	0.795
Thickest of the GBM (nm)	1083 ± 426	1302 ± 416	0.108
Thickest of the lamina densa (nm)	962.4 ± 309.1	604.0 ± 296.5	0.001
Thickness of the lamina densa under the deposit (nm)	333.2 ± 99.6	226.5 ± 90.3	0.001
Thickness of the lamina densa under the podocytic infolding (nm)	254.0 ± 127.9	167.3 ± 87.1	0.017
Number of the cases observed electron dense deposit	8	20	0.000
Number of the cases observed reticular degeneration of the lamina densa	4	1	0.342

Values represent mean ± S.D.
*Student's t-test or Fisher's exact test.

in the study group was significantly higher than the control group ($p = 0.03$).

Morphological findings

With regards to LM and IF features, global spike formations in the GBM on the PAM-MT stain and granular deposits of IgG along the GBM were observed on IF in the undulation group (Fig 3).

Of the 18 cases which had IF materials, 16 (88.9%) cases were IgG positive (Fig 3), six (33.3%) were IgA positive, and one (11%) was IgM positive on IF examination. Four (22%) out of 18 cases were C3c positive, and this proportion was significantly less compared with the control group ($p = 0.009$) ; two (11%) were C1q positive, and none were C4 or fibrinogen positive (Table 4). Almost all the positive features displayed coarse granular GBM staining.

On EM evaluations, there was no significant difference in the GBM thickness and length of the infolding distance of the GBM between the two groups. However, in the undulation group, the EDDs were significantly decreased ($p = 0.000$) and the thickness of the lamina densa was significantly increased in many areas compared with the control group ($p < 0.05$). Reticular degeneration was observed in four patients in the undulation group and in one patient in the control group as a focal lesion. (Table 5).

Discussion

In this study, we investigated the clinicopathological characteristics of MN patients who had EM findings of undulation for long segments on the epithelial side of the GBM. Many cases in the undulation group had been diagnosed as stage II to III or stage I to II, according to the findings of epithelial deposit in the other area or new layer of GBM-like materials (observed using EM) and spike formation (observed using PAM-MT). The finding of undulation differs from typical EC stage II or III because the epithelial side of the GBM expands in response to podocytes rather than subepithelial EDD. In the undulation group, the lamina densa was thicker, the numbers of EDD were lower on EM, the complement deposits on IF were lower, and the serum complement levels were higher compared with the control group.

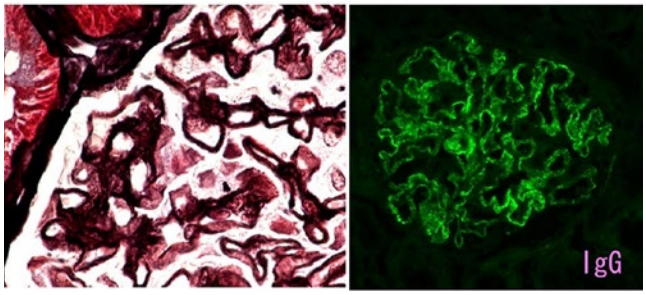

Fig. 3
Light microscopic features of the undulation group. PAM-MT stain shows diffuse spike formation, (a ×1000). Immunofluorescence study for IgG and diffuse granular deposit were strongly positive along the glomerular capillary wall (b). (case 20)

Rumpelt et al reported that GBM splitting may indeed occur in various glomerular disorders, such as mesangioproliferative glomerulonephritis (GN), membranoproliferative GN, membranous GN, focal glomerulosclerosis, transplant glomerulopathy, or obsolescent glomeruli[6]. On the other hand, it has been considered that long segmental undulation may specifically be found in AS. AS is a disease that results from a genetic abnormality that codes type IV collagen[5], which is a constituent of the GBM. The EM for AS shows undulation as well as reticular changes in the lamina densa. With regard to type IV collagen, anti-GBM GN (Goodpasture's syndrome) is a disease that is triggered by anti-GBM antibodies to the type IV collagen in the GBM[9]. However, undulation is not specific to anti-GBM GN. This suggests abnormalities in type IV collagen do not always lead to the formation of undulation and do not necessarily trigger the formation of undulation.

In 2008, Joh et al proposed podocytic infolding glomerulopathy (PIG)[10]. The diagnostic criteria were proposed as a glomerulopathy that demonstrated microspheres and/or microtubular structures associated with podocytic infolding into the GBM on EM. This lesion shows a non-argentaffin hole in the GBM with PAM staining and is similar to MN. As a new disease entity, this disease proposal is rapidly being leaded to a significant amount of research. In patients with PIG, spike formation of the GBM is not one of the criteria ; thus, almost all patients with MN are excluded from the diagnosis of PIG[10]. Masuda et al reported that primary MN showed podocytic infolding similar to PIG. Infolding epithelial cells within or around EDD had been observed in 77.8% of cases in primary MN. Furthermore, they found microstructures in 31.7% of cases in primary MN[11]. The images similar to undulation often appears in PIG. The mechanism of PIG formation might be related to the role of podocytes[11,15]. On the other hand, microstructures were rarely observed in the undulation group. Although these findings were intricately interrelated, the mechanism between PIG and undulation was thought to be different. As the numbers of investigated cases in both PIG and our study were small, further studies are needed to elucidate the mechanism underlying the formation of both PIG and undulation.

Complement activation may play a central role in the onset of MN. Complement findings on IF have been correlated with the prognosis of the patients with MN and with the amount of proteinuria[12-14]. C5b-9 (membrane attack complex : MAC), which is the end-product of complement, and C3 were found in the immune deposits of MN patients[13]. C5b-9 activated the podocytes causing the thickening of the GBM through the production of an extracellular matrix with substances such as type IV collagen. Activation of complement caused both immune deposition and GBM thickening. However, the undulation group demonstrated less immune deposits and GBM thickening. This indicated that other factors may be involved in the pathogenesis of the undulations. Although, it may be difficult to clarify the relationship between the immune deposits, the activation of complement, and the components of the lamina interna and lamina densa according to our findings, we hypothesized that type IV collagen played an important role in the formation of the undulations of the GBM[15-20].

In our study, the pathogenesis of GBM undulation was not determined. Nevertheless, these results indicated that diverse immune responses, in addition to the complement system, may be involved differently from usual cases of MN [12]. Further investigation focusing on GBM undulation will be necessary.

Acknowledgments

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Conflict of interest

All the authors have declared that no conflict of interest exists.

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膜性腎症症例の中で超微形態にて糸球体基底膜にうねりを示す20例の臨床病理学的検討

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要　　約

膜性腎症は成人ネフローゼ症候群の代表的な原因疾患であり，糸球体基底膜（GBM）領域にみられる高電子密度沈着物（EDD）が特徴的所見である。一方で，膜性腎症例においてEDDとは無関係にGBMのうねる構造（undulation）が時に観察される。膜性腎症の病期分類でもこの点について十分に検討されていない。我々はこの変化に注目し，陽性の20症例について，undulationを示さない膜性腎症20例を対照として比較検討した。undulation群では緻密層が有意に肥厚していた（962.4±309.1 vs. 604.0±296.5nm; p=0.001）。また，蛍光抗体ではC3cの糸球体沈着例が少なく（n=4 vs. n=12; p=0.009），血清補体値も保たれる傾向にあった。一般的に膜性腎症では補体の関与が報告されるが，undulation群では異なる機序を有することが示唆された。

（キーワード：補体，電子顕微鏡，腎糸球体基底膜，膜性腎症，腎生検）

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