Glomerular Localization of Thrombomodulin in Human Glomerulonephritis

ヒト糸球体腎炎におけるThrombomodulin (TM) の
発現について

水谷 大裕
# 論文目録

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### 主論文
**題目**
Glomerular Localization of Thrombomodulin in Human Glomerulonephritis
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（同上）
Glomerular Localization of Thrombomodulin in Human Glomerulonephritis

ヒト系球体腎炎におけるThrombomodulin（TM）の発現について

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Running Title
Thrombomodulin expression in glomerulonephritis
ABSTRACT

BACKGROUND: Thrombomodulin (TM), a glycoprotein expressed on the surface of endothelial cells, transforms protein C into a potent anticoagulant by binding thrombin. TM may be an important regulator of intraglomerular coagulation because functional TM activity was demonstrated in glomeruli isolated from normal human and rat kidneys. The role of TM in glomerulonephritis is unknown.

EXPERIMENTAL DESIGN: Sections from four normal human kidneys and from kidneys of 75 patients with various forms of glomerulonephritis were studied by light and transmission electron microscopy, and by light and electron immunohistochemical techniques using polyclonal antibodies to recombinant human TM, and monoclonal antibodies to the membrane attack complex of the complement system (MAC). The expression of TM was graded from 0 to 4 according to the intensity and extent of the distribution, and the results were compared with the clinicopathological findings.

RESULTS: In normal glomeruli and in glomeruli with minimal abnormalities a small amount of TM was localized at the vascular pole only (grade 0-1). In membrano-proliferative glomerulonephritis (MPGN) and lupus glomerulonephritis (LN) the amount of TM found on the plasma membrane of endothelial cells was significantly increased (grade 2-4). The expression of TM was directly correlated with proteinuria (p<0.001), glomerular hypercellularity (p<0.01), and number of subendothelial immune deposits (p<0.01). In contrast, in other forms of glomerular diseases TM was not increased and no correlation was found with the clinicopathological parameters.

CONCLUSIONS: In MPGN and in LN the amount of TM expressed by the plasma membranes of glomerular endothelial cells is increased, and this finding is a marker of disease activity. The significance of an increased expression of an endothelial anticoagulant glycoprotein in diseases characterized by pathologic intraglomerular coagulation is unknown, and requires further studies.

Keywords
#1 Thrombomodulin, #2 glomerulonephritis, #3 systemic lupus erythematosus
#4 membrano-proliferative glomerulonephritis, #5 endothelial injury
Introduction

Thrombomodulin (TM) is a thrombin receptor of endothelial cells that regulates intravascular coagulation (1, 2). TM forms a 1:1 complex with thrombin, and this complex converts protein C into activated protein C, which functions as a potent anticoagulant by the proteolytic degradation of Factor Va (3, 4) and Factor VIIIa (5). Upon binding to thrombin, TM also induces the conformational change of thrombin and inhibits the procoagulant activity of thrombin (6, 7). TM shows remarkable anticoagulant functions "in vivo" as well as "in vitro" (8, 9, 10). Kumada et al. (10) reported that recombinant human TM decreased the mortality of mice after thrombin-induced embolization, whereas TM antibody increased the mortality with sublethal dose of thrombin.

TM is mainly localized on the surface of endothelial cells (11), and is also present in platelets (12), megakaryocytes (13), and monocytes/macrophages (14). Recently, TM was detected in normal human plasma (15), and was found increased in the plasma of patients with a variety of diseases (16, 17, 18, 19). Thus, it was suggested that the plasma level of TM may be a marker of endothelial injury.

In the kidney, TM is localized in the endothelial cells of large vessels and of peritubular capillaries. In glomeruli only a minimal amount of TM is detected at the vascular pole. Jiang He et al. (20) recently demonstrated functional activity of TM in glomeruli isolated from normal human and rat kidney. Thus, TM might function as a regulator of intraglomerular coagulation. In the present paper we report the results of a study designed to investigate the role of TM in human glomerulonephritis.

Experimental Design

Sections obtained from normal human kidneys and from kidneys of patients with various forms of glomerulonephritis were studied by immunofluorescence microscopy using polyclonal antibodies to human recombinant TM, and monoclonal antibodies to the membrane attack complex of the complement system (MAC). The glomerular fluorescence was graded from 0 to 4 according to intensity and extent. Glomerular cellularity was assessed by counting the average number of nuclei in glomerular sections. Glomerular immune deposits were quantitated by electron microscopy. In lupus glomerulonephritis (LN) the "activity index" (21) was also estimated. Clinical data were obtained at the time of renal biopsy. The relationship between glomerular deposits of TM and clinico-pathological findings was statistically analyzed.
Results and Discussion

Specificity of TM antibodies

The reactivity of the polyclonal antibodies to human TM with sections of normal kidney was completely abolished by preincubation of the antibodies (5 μg of rabbit IgG) with 32 ng of recombinant human TM. Furthermore, normal rabbit serum did not react with sections of normal kidney or kidney from patient with LN (Fig.1-f).

Distribution of TM in normal human kidneys and kidneys of patients with minimal glomerular abnormalities

In normal kidneys and in kidneys of patients with minimal glomerular abnormalities TM was localized in the endothelium of small arteries, veins and peritubular capillaries. In glomerular small deposits of TM were detected at the vascular pole only (Fig. 2). This segmental pattern of distribution is similar to that observed in glomeruli of rabbits and rats (11). Besides, in rabbits and rats TM is also localized in many other vascular districts, including the heart and the lung (11).

Distribution of TM in Normal and Diseased Glomeruli

The histological diagnosis, sex and age of the patients are listed in Table 1. Fig.3 represents the grade of TM expression in glomeruli of normal and pathologic kidneys. The amount of TM was significantly increased in glomeruli of patients with membranoproliferative glomerulonephritis (MPGN) and with LN as compared to normal glomeruli (p<0.05), whereas there were no significative modifications in other diseases. Glomerular hypercoagulability is a common characteristic of patients with thrombotic microangiopathy and of patients with MPGN and LN. We did not find, however, an increased expression of TM in patients with thrombotic microangiopathy. The more severe damage occurring in the endothelia of these patients (22), as compared to patients with MPGN and LN, might explain their failure to overexpress TM.

In order to establish the precise localization of TM in glomerular structures studies by double immunofluorescence light microscopy and by immuno-electron microscopy were performed. By light microscopy granular deposit of human IgG were found in the capillary walls and in the mesangium. In contrast, TM was distributed in a linear capillary pattern (Fig.4) that, by immuno-electron microscopy, corresponded to diffuse deposits of reaction product in the plasma membrane of glomerular endothelial cells (Figs.5 and 6).

Both intact and degraded forms of TM are present in normal human plasma (15). High levels of plasma TM are detected in thrombotic thrombocytopenic purpura (17), diabetes mellitus (16, 23), adult respiratory distress syndrome and disseminated intravascular coagulation syndrome (18). It is suggested that in these diseases the plasmatic level of TM may be a marker of endothelial injury. Besides, we have observed that in the rabbit divalent goat anti-rabbit TM antibodies, injected intravenously, can induce shedding of TM and TM immune complexes in the circulation (Yuzawa Y et al., unpublished observation). Taken together these findings indicate that in pathologic conditions TM can be released in the circulation. Therefore, the increase of TM in glomeruli of patients with MPGN or LN could result from passive trapping of circulating TM or TM immune complexes, from an upregulation of glomerular endothelial synthesis and/or from a decreased turnover of TM. The three mechanisms are not mutually exclusive. The validation of the first hypothesis would require a systematic study of TM or TM immune complexes in the sera of the patients, and these studies were not performed. However, the finding that TM was localized in the plasma membrane of glomerular endothelial cells, and not in the immune deposits, is in agreement with the interpretation that in MPGN and in LN the increase of glomerular TM was due to an upregulation of the local synthesis and/or to a decrease turnover.

The cytokines that govern TM metabolism are only partially known, and the information mainly derives from studies performed “in vitro”. Endotoxin (24), tumor necrosis factor (25,26,27), and interleukin-1 (28) downregulate TM expression. In contrast, homocysteine (29,30), forskolin and analogues of cyclic-AMP (31, 32) and phorbol myristate acetate (PMA) may upregulate TM expression. TM decreases in
endothelial cells exposed to PMA for short periods of time (33), but increases after long incubation since PMA can stimulate the synthesis of TM both at protein and mRNA levels (34,35).

The possibility that the increase of TM detected in glomeruli of patients with MPGN and with LN may be - at least in part - the consequence of increased endothelial surface should also be entertained because swelling and vesiculation of endothelial cells lining subendothelial immune deposits was detected by electron microscopy in specimens obtained from these patients (Fig. 7).

Correlation between TM expression and clinico-pathological findings

There was no correlation between glomerular expression of TM and the age and kidney function (BUN, Cr) of the patients (Table 2). The plasmatic level of TM was increased in patients with chronic renal failure because of decrease of TM clearance by this organ (18). This finding indirectly supports the interpretation that the increase of TM in glomeruli is not induced by trapping of circulating TM but rather by "de novo" synthesis and/or a decreased turnover of TM in endothelial cells.

Statistically significant correlations were noted between glomerular TM expression and total protein, serum albumin, and urinary excretion only in patients with MPGN and LN (r = 0.440, p < 0.05; r = 0.507, p < 0.005; r = 0.568, p < 0.001, respectively), but not in patients with other forms of glomerulonephritis (Table 2). In addition, TM expression was not correlated with serum complement levels (C3, C4, and CH50).

The expression of TM in glomeruli was compared with the presence and the distribution of immune deposits, as detected by electron microscopy, with the deposition of MAC, and with glomerular cellularity. There was a direct correlation between degree of TM expression and subendothelial (r = 0.499, p < 0.01), but not subepithelial (r = 0.059, NS) and mesangial (r = 0.269, NS), immune deposits (Table 3). Patients with grade 2-3 subendothelial immune deposits showed a degree of correlation higher than that of patients with grade 0-1 (p < 0.01) (Fig. 8). In contrast, in patients with membranous glomerulonephritis or IgA glomerulonephritis, the expression of TM was not directly correlated with the subepithelial or mesangial immune deposits, respectively (Fig. 3). These observations are in agreement with the hypothesis that subendothelial immune deposits, directly, or by chemotactic attraction of inflammatory cells, may contribute to stimulate the endothelial synthesis of TM or to inhibit its turnover. Consistent with this interpretation is the observation that in MPGN and LN there was a direct correlation between expression of TM, glomerular cellularity, and "activity index" (Figs. 9, 10). In IgA glomerulonephritis, membranous glomerulonephritis, MPGN and LN MAC had the same distribution of immunoglobulins in glomerular deposits. There was no correlation between TM expression and MAC deposition.

In conclusion, the results indicate that increase of glomerular TM is a characteristic finding of MPGN and LN. Subendothelial immune deposits might contribute to stimulate the synthesis or to inhibit the turnover of TM in glomerular endothelial cells. The increase of glomerular TM in diseases characterized by glomerular deposits of fibrinogen/fibrin (36) is surprising, and its significance is unknown. Functional study of glomerular TM and measurement of TM mRNA in various stages of disease should contribute to explain this finding.
Methods

Kidney Specimens

Fragments of normal human kidneys were obtained from patients that were nephrectomized for a renal tumor. The normality of the tissue used for our study was established by the histologic examination. Fragments of renal cortical tissue from patients with glomerulonephritis were obtained by transcutaneous needle biopsy performed for diagnostic purposes. The pathologic specimens included: 9 with minimal glomerular abnormality, 12 with focal glomerulosclerosis, 9 with IgA glomerulonephritis, 14 with idiopathic membranous glomerulonephritis, 12 with MPGN, 5 with benign glomerulosclerosis, 6 with diabetic glomerulosclerosis, 4 with thrombotic microangiopathy, 5 with periarteritis nodosa, 19 with LN and 5 with renal amyloidosis. The patients with thrombotic microangiopathy included 3 patients with hemolytic uremic syndrome and one patient with thrombotic thrombocytopenic purpura. The histopathological diagnosis was based on the morphological criteria recommended by the WHO (37). Specimens diagnosed as LN were obtained from patients who fulfilled the 1982 revised criteria for the diagnosis of systemic lupus erythematosus according to the American Rheumatism Association (38). The classification of LN was based on the WHO morphologic classification (39). The "activity index" was estimated as the total scores of active lesions such as glomerular hypercellularity, leukocyte exudation, Karyorrhexis/hemorrhage, cellular crescents and hyaline deposits. The severity and extent of the lesions were graded as: 0, absent; 1, mild; 2, moderate; 3, severe as previously described (21). The kidney specimens were processed for light and electron microscopy and for immunohistochemistry.

Light microscopy and transmission electron microscopy

For light microscopy, kidney tissue was fixed in 10% buffered formalin overnight, dehydrated, and embedded in paraffin. Two μm thick sections were stained by hematoxylin and eosin (HE), periodic acid-Schiff (PAS), and silver methenamine. For electron microscopy, kidney tissue was fixed in 2% glutaraldehyde, and embedded in Epon 812. Ultrathin sections were studied with a JEOL 100CX electron microscopy. (JEOL 100CX, JEOL Co., Tokyo, Japan)

Monoclonal Antibodies

MAC was detected with a mouse monoclonal antibody against neoantigen of polymerized C9 (anti-TCCs antibody), kindly provided by Dr. A. F. Michael (University of Minnesota, Minneapolis).

Polyclonal Antibodies

Fluorescein isothiocyanate (FITC)-labeled IgG fractions of goat anti-mouse IgG antiserum, and of goat anti-rabbit IgG antiserum were purchased from Cappel Laboratories (West Chester, Pennsylvania) and preabsorbed with normal human serum to avoid cross-reaction with human tissues. Rhodamine-labeled IgG fractions of goat anti-human IgG antiserum was purchased from Cappel Laboratories. The characterization and specificity of IgG fractions of rabbit anti-recombinant human TM antiserum (40, 41) and of goat anti-rabbit TM antiserum (42) were described previously.

Immunofluorescence microscopy

For immunofluorescence microscopy kidney specimens were placed in OCT compound (Miles, Elkhart, IN), and snap-frozen in liquid nitrogen. Two μm frozen sections cut with a cryostat were fixed in cold acetone for 10 minutes according to DeBault's method (11). Indirect immunofluorescence staining was performed according to the method previously described (43). For the staining of human TM and MAC, the sections were immersed in 0.01 M phosphate buffered saline (PBS) containing 1% normal rabbit or normal goat serum for 20 minutes to avoid non-
specific binding of the secondary antibodies, especially through Fc receptors of the glomerulus. After washing with PBS, the sections were incubated at room temperature for 20 minutes with an optimal concentration of the first antibodies. The sections were washed three times with PBS and then were incubated with FITC-labeled secondary antibodies. After the final wash in PBS, all the sections were mounted with medium containing p-phenylendiamine (44) and examined with an Olympus BH-2 epifluorescence microscope.

In order to compare the glomerular co-localization of human TM and human IgG in MPGN and LN, the sections were incubated first with rabbit anti-human TM followed by FITC-labeled goat anti-rabbit IgG, and then incubated with rhodamine-labeled goat anti-human IgG.

**Immunoelectron microscopy**

Horseradish peroxidase (HPO)-conjugated, affinity purified IgG fraction of a goat anti-rabbit IgG serum was purchased from Cappel Laboratories. Small fragment of normal human kidney and some diseased kidneys were fixed with the periodate-lysine paraformaldehyde fixatives (45) for four hours at 4°C. The tissue was washed in PBS, pH 7.4, containing 10% sucrose for one day with several changes followed by the final wash with PBS containing 20% sucrose and 7% glycerol for one hour. The tissue was then snap-frozen in liquid nitrogen. Immunoperoxidase staining was performed according to the method previously described (46). Seven to eight µm thick sections were cut in a cryostat and mounted on egg albumin-coated slides, incubated with 0.05% sodium borohydride in PBS for 40 minutes at 4°C. For detection of human TM the sections were first incubated with normal rabbit or goat serum diluted 1:20 in PBS for 30 minutes, then with rabbit anti-human TM antibodies overnight at 4°C followed by the HPO-labeled IgG fraction of a goat anti-rabbit IgG serum diluted in PBS for two hours at room temperature. After fixation with 2.5% glutaraldehyde for 30 minutes at 4°C, sections were incubated for 20 minutes with 0.025% diaminobenzidine (Sigma) in 0.05 M Tris-HCl buffer, pH 7.8 and then for 2.5-5 minutes with the same solution containing 0.005% H2O2 at room temperature (47,48). The sections were postfixed in 2% osmium tetroxide, dehydrated in alcohol and flat embedded in Epon 812. Ultrathin sections were studied with a JEOL 100CX electron microscopy.

**Absorption Test**

Aliquots of rabbit anti-human TM serum were absorbed with increasing concentrations of recombinant human TM. 1 µg/µl of rabbit anti-human TM serum diluted 1:30 with 0.05% Tween 20 in PBS was incubated with increasing concentrations of recombinant human TM, from 0.5 to 250 ng for 2 hrs at room temperature and processed for indirect immunofluorescence microscopy as described above.

**Evaluation of the immunofluorescence findings**

The immunofluorescence staining was graded without knowledge of the diagnosis. Glomerular TM was graded from 0 to 4 according to the intensity and extent of fluorescence as previously described (49): 0, negative; 1, segmental staining at the vascular pole; 2, segmental staining at the vascular pole with minimal peripheral capillary staining; 3, dense staining along the peripheral capillaries with mild to moderate intensity; 4, strong and diffuse staining along the peripheral capillaries (Fig.1-a,b,c,d,e: a and b are from normal glomeruli; c,d and e are from diseased glomeruli). The staining for MAC in glomeruli was graded according to the amount and extent as follows: (0: negative, 1: weak, 2: moderate, 3: strong). All biopsy specimens were examined by light and electron microscopy as well as by immunofluorescence microscopy. For immunofluorescence microscopy, only equatorially cut glomeruli were studied.

**Evaluation of the light and electron microscopic findings**

For the statistical analysis of glomerular cellularity 4 sections cut at 3 µm and
stained with PAS were used in each specimen. In each section the number of nuclei was counted in ten-twenty glomeruli with a diameter of about 200 μm (probable equatorial section (50)). Glomeruli with a diameter of less than 100 μm were excluded. The average number of cell per glomerulus was used for statistical analysis.

In each specimen obtained from patients with LN the "activity index" was estimated according to morphological criteria suggested by the WHO (21).

To assess the relationship between the foreign deposits seen by electron microscopy and the deposits of TM, the amount of foreign deposits in subepithelial, subendothelial and mesangial regions was graded from 0 to 3. Subepithelial deposits: 0, negative; 1, corresponding to stage I-II membranous glomerulonephritis; 2, corresponding to stage III membranous glomerulonephritis; 3, corresponding to stage IV membranous glomerulonephritis. Subendothelial deposits: 0, negative; 1, minimal amount of deposits without apparent thickening of the glomerular capillary walls; 2, moderate amount of deposits with thickening of the glomerular capillary walls; 3, large amount of deposits corresponding to wire loop lesions of LN. Mesangial deposits: 0, negative; 1, minimal; 2, moderate; 3, large.

**Evaluation of clinical activity**

At the time of renal biopsy the clinical data obtained from the patients included concentration of serum creatinine, blood urea nitrogen, total protein, serum albumin, complement level (C3, C4 and CH50) and daily urinary protein excretion.

**Statistical methods**

The results were expressed as mean ± standard error of the mean. Statistical comparison between the intensity of TM staining in normal and pathologic specimens was determined by Student's t-test for normally distributed data, and the Mann-Whitney rank sum test for non-parametric data. The relationship between the intensity of TM staining and clinico-pathological data was assessed by Spearman's correlation test. Values of p<0.05 were considered significantly different.

**Grants and Acknowledgements**

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Legends

Table 1: Study population
- Hist.Dx.: histological diagnosis, 1) NHuK: normal human kidney,
2) Minimal GA: minimal glomerular abnormality, 3) FGS: focal glomerulosclerosis,
4) IgA-GN: IgA glomerulonephritis, 5) MN: idiopathic membranous
glomerulonephritis, 6) Benign GS: benign glomerulosclerosis,
7) DM: diabetic glomerulosclerosis, 8) TMA: thrombotic microangiopathy,
9) PN: perianteritis nodosa, 10) Amy: renal amyloidosis
s.e.m.: standard error of the mean

Table 2: Correlation between glomerular expression of TM and clinical parameters
TP: total protein, Alb: serum albumin, Cr: creatinine, BUN: blood urea nitrogen
24H-UP: urinary protein excretion for 24 hours, NS: not significant
rs: r value of Spearman's correlation coefficient
1): all groups, 2): all groups except MPGN and LN
3): MPGN and LN

Figure 1: Grading of TM in normal glomeruli and in glomeruli of patients with
glomerulonephritis.
Grade 0, negative staining (a); Grade 1, segmental staining at the vascular pole
(b); Grade 2, segmental staining at the vascular pole with minimal peripheral
capillary staining (c); Grade 3, diffuse staining along the peripheral capillaries (d);
Grade 4, strong diffuse staining along glomerular capillary walls (e); Control
experiment using normal rabbit IgG in a section contiguous to that shown in panel e
(f); (a and b are from normal glomeruli: c,d,e and f are from diseased glomeruli).
x200

Figure 2: TM in normal renal tissue obtained from surgically resected kidneys.
TM was localized in the peritubular capillaries and in small arteries. Only minimal
deposits of TM were presented at the vascular pole of glomeruli. x100
G=glomerulus, The arrow indicates the vascular pole.

Figure 3: Glomerular expression of TM in normal and diseased kidneys.
The amount of TM was significantly increased in glomeruli of patients with MPGN
and with LN as compared to normal glomeruli (p<0.05), whereas there were no
significant modifications in other diseases.
NHuK: normal human kidney, Minimal GA: minimal glomerular abnormality,
FGS: focal glomerulosclerosis, IgA-GN: IgA glomerulonephritis, MN: idiopathic
membranous glomerulonephritis, Benign GS: benign glomerulosclerosis,
DM: diabetic glomerulosclerosis, TMA: thrombotic microangiopathy,
PN: perianteritis nodosa, Amy: renal amyloidosis
* : p<0.05, when compared to that of normal human kidneys, NS: not significant

Figure 4: Double immunofluorescence staining for TM (a,c) and human IgG (b,d) on a
section obtained from a LN patient whose TM staining was assessed as grade 3.
The arrows indicate the same capillary. The pattern of distribution of TM was linear;
in contrast IgG had a granular distribution along the glomerular capillary walls.
TM: thrombomodulin, HuG: human IgG, a,b, x200, c,d, x400

Figure 5: Immuno-electron micrograph showing the result of experiments designed to
localize TM in a glomerulus of a patient with LN. The reaction product is diffusely
localized at the endothelial surface of glomerular capillaries.
The asterisks indicate the glomerular capillary lumens. x5,000

Figure 6: High magnification immuno-electron micrograph.
TM is detected at the surface of endothelial cells (arrow heads). In contrast, the
deposits of foreign material(*) present in the glomerular basement membrane do not
contain TM.
CL: capillary lumen, x12,000
Figure 7: Electron micrograph of a glomerulus from a patient with LN. The endothelial cells covering subendothelial deposits (D) show swelling and vesiculation (asterisks). In contrast, the endothelial cells (arrow heads) present on normal segments of basement membrane show a normal appearance. x7,300

Figure 8: Correlation between glomerular expression of TM and subendothelial immune deposits in patients with MPGN and with LN. For the grading of subendothelial immune deposits see *Material and Methods*. *p<0.05*: when compared to the group-grade 0, NS; not significant

Figure 9: Correlation between glomerular expression of TM and glomerular cellularity in MPGN and LN

Figure 10: Correlation between glomerular expression of TM and "activity index" in patients with LN

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total 104 49/55 45.4±1.6
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Figure 8

Expression of Glomerular TM

Subendothelial Immune Deposits

Figure 9

Expression of Glomerular TM

Number of Cells Per Glomerular Cross Section

$r = 0.545$ (n = 24)

$p < 0.01$
Figure 10

Expression of Glomerular TM

Activity Index (21)

rs=0.658 (n=19)
p<0.001