Can von Willebrand factor, platelet-endothelial cell adhesion molecule-1 and thrombomodulin be used as alternative markers of endothelial cell injury in human glomerulonephritis?

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Abstract

Purpose: **There is growing evidence that endothelial cells (EC) are active participants of an inflammatory process in glomeruli.**

Material and methods: **We compared the glomerular expression of three EC-coupled molecules, i.e. platelet-endothelial cell adhesion molecule-1 (PECAM-1 or CD31), von Willebrand factor (vWF) and thrombomodulin (TM) in 60 patients with glomerulonephritis (GN) and five normal kidneys (NK). The alkaline phosphatase anti-alkaline phosphatase method was used to examine the expression of these proteins in the biopsy specimens.**

Results: **In NK, the expression of CD31 and vWF comprised the whole glomerular network. In contrast, the expression of TM was much lower and localized mainly to EC at the vascular pole and adjacent areas. In GN, the glomerular staining for CD31 and vWF was significantly reduced. A fall in the expression of both these EC antigens was more pronounced in proliferative forms of GN (PGN) than in non-proliferative GN (NPGN) (CD31: NPGN vs. PGN, $p < 0.02$; vWF: NPGN vs. PGN, $p < 0.05$). In addition, a linear relationship between the expression of CD31 and vWF was found in GN ($r = 0.8$, $p < 0.001$). Conversely to CD31 and vWF, a marked increase in glomerular reactivity for TM was observed in all the patients with GN (GN: 2.12 ± 0.32, NK: 0.95 ± 0.05, $p < 0.02$). However, the highest expression of TM was found in membranoproliferative GN and lupus GN.**

Conclusions: **Our results suggest that CD31 and vWF may be used as markers of glomerular EC loss during GN, whereas TM staining seems to reflect EC activation in response to circulating and/or released in situ procoagulant factors.**

Key words: von Willebrand factor, platelet-endothelial cell adhesion molecule-1, thrombomodulin, glomerulonephritis.

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Received 14.06.2004 Accepted 27.06.2004

Introduction

Injury of the glomerular capillary network with endothelial cells' (EC) damage has been shown to play an important role in the progression of glomerulonephritis (GN) [1]. Shimizu et al. have demonstrated a marked loss of EC in glomeruli in experimental rapid progressive GN, using an antibody to thrombomodulin (TM) as the marker of EC [2]. The same findings were observed in rats with anti-Thy 1.1 GN when an antibody to platelet-endothelial cell adhesion molecule-1 (PECAM-1 or CD31) was applied to stain EC [3,4]. The authors of the above studies suggest that damage to the glomerular EC with the following incomplete repair is an important factor in the appearance and progression of the glomerular sclerosis [2-4].

Others and we have previously demonstrated the loss of glomerular EC also in various morphological forms of human GN with CD31 used as the marker of EC [5-7]. Results concerning the expression of TM in human GN are, however, inconsistent. Increased expression of TM has predominantly been shown in glomeruli of patients with lupus GN (LGN) [8,9]. In this context, Frijns et al. have observed significantly higher plasma levels of soluble TM and von Willebrand factor (vWF) in patients with a history of lupus glomerulonephritis LGN than in systemic lupus erythematosus (SLE) patients without GN [10]. The authors have suggested that the increase in these EC markers reflects a state of persistent EC activation specifically localized in the kidneys. Woywodt et al. [11] used antibodies against vWF

and CD31 in order to isolate EC from peripheral blood of patients with ANCA-associated vasculitis and came to the same conclusion. In primary GN, the highest expression of TM, compared to other forms of glomerular diseases, has been observed in membranoproliferative GN (MPGN) [8]. On the other hand, higher intraglomerular staining for TM has been reported in the remission phase of focal-segmental glomerulosclerosis (FSGS) than in the nephrotic stage of this disease [12].

Although PECAM-1, vWF and TM can serve as EC markers, their localization in the cell [13,14] and function [13, 15-17] differ from each other. PECAM-1 is one of the adhesion molecules of 130 kDa and belongs to the immunoglobulin (Ig) superfamily. It is present in thrombocytes, lymphocytes, neutrophils, and EC, in which it concentrates at the intercellular borders of adjacent cells. Its redistribution depends on cytokines, such as INF-γ and TNF-α [14]. Its ligands include homophilic interactions with itself, $\alpha v\beta 3$ integrins and CD38 [18]. It has been implicated in various biological functions such as modulation of integrin-mediated cell adhesion, angiogenesis, apoptosis, cell migration, negative regulation of immune signaling, autoimmunity, macrophage phagocytosis, IgE-mediated anaphylaxis, and thrombosis [18]. CD31 plays an important role in transendothelial migration of leukocytes without influence on adhesion of these cells to endothelium [15].

TM is a thrombin receptor present on the surface of the EC in arteries, veins, capillaries, lymphatic vessels, platelets, and placental syncytiotrophoblast cells. It is considered to be the main factor taking part in the control of thrombogenesis [16]. TM acts as a cofactor for the activation of plasma protein C. EC membrane-bound TM forms a high-affinity complex with thrombin, and inhibits thrombin interaction with fibrinogen and protease-activated receptor. TM-thrombin complex is also a potent activator of protein C that enhances thrombin-dependent protein C activation [17]. TM-thrombin complex activates also latent plasma carboxypeptidase B, which removes carboxy-terminal arginine and lysine residues from fibrin. These residues are important for the sequestration of fibrynolytic enzymes on the fibrin matrix, and their removal renders the clot more resistant to lysis [19]. In vitro, exposure of EC to neutrophils or hydrogen peroxide promotes the release of TM. Soluble TM consists of fragments of various molecular weights, which are most likely either proteolytically degraded from cellular TM or derived from the injured EC [20,21].

Another marker of EC is vWF [22]. It is a multimeric plasma glycoprotein with the molecular weight of 270 kDa. This glycoprotein mediates platelet adhesion to injured vessel walls and serves as a carrier and stabilizer for coagulation factor VIII, as well as it has functional binding domains to platelet glycoprotein Ib, glycoprotein IIb/IIIa, collagen and heparin. It is present in EC, platelets, and megakaryocytes, as well as in numbers of tumors including hemangiomas, hemangiosarcomas and Kaposi's sarcomas [22].

In the light of the above data, it seemed reasonable to address the question whether all these three EC markers can alternatively be used for EC visualization in an inflamed glomerulus. Our results show a variable loss of the glomerular staining for CD31 and vWF in renal specimens with GN. On the other hand, an increased immunoreactivity for TM is observed in GN.

Material and methods

Sixty patients with biopsy-proven GN were enrolled into the study. The morphological diagnosis was based on conventional light microscope and immunopathological examinations. Biopsy material remaining after the latter investigation was used in this study. Fourty-five patients with primary GN had proliferative forms of GN (PGN). Among them, 41 had mesangial proliferative GN (MesPGN) while membranoproliferative GN (MPGN) was diagnosed in four. In the group with non-proliferative glomerulopathies (NPGN), seven patients had idiopathic membranous GN (IMGN) and four had focal-segmental glomerulosclerosis (FSGS). Out of four patients with lupus nephritis (LGN), three were categorized as PGN (1 patient, class III; 2 patients, class IV) and one patient having class V of glomerular lesions was classified to the NPGN group. Clinical and morphological data of patients are presented in *Tab 1*.

The expression of TM, CD31 and vWF was examined by immunohistochemistry on acetone-fixed sections using the three-step alkaline phosphatase – anti-alkaline phosphatase (APAAP) method as previously described [7]. Monoclonal antibodies against TM (Dako Corporation, Glostrup, Denmark), and CD31 (Novocastra, Newcastle, UK) were used, whereas a plyclonal antibody to vWF (Novocastra) was applied. The expression of CD31 was examined in 46 cases, that of vWF in 37 subjects and TM in 38 patients. The expression of all these molecules was examined in 19 patients (MesPGN, 14; MPGN, 2; IMGN, 2; LGN, 1), that of CD31 and vWF in 25 subjects (MesPGN, 17; MPGN, 2; IMGN, 4; FSGS, 1; LGN, 1), TM and CD31 in 29 patients (MesPGN, 20; MPGN, 4; IMGN, 2; FSGS, 1; LGN, 2), and TM together with vWF in 26 individuals (MesPGN, 19; MPGN, 2; IMGN, 2; FSGS, 1; LGN, 2). Normal-appearing kidney tissue (n = 5) surrounding the removed tumor served as controls.

Regarding CD31 and vWF, evaluation of the glomerular expression of these EC markers was performed using a following grading scale: grade 3, reflecting the staining intensity (I) in the normal kidney; grade 2, defined as a moderate decrease in the staining; and grade 1, defined as a severe decrease in the staining intensity. With respect to TM, a scale in an opposite direction was used, beginning with the estimation of the staining intensity in normal glomeruli at 1 point and increasing upward to 3 points in specimens with GN. Based on the above grading scales, the mean glomerular expression scores (GES) were calculated for each molecule, according to the formula: $GES = I_{(1)}/100\% + ... I_{(N)}/100\%/N$, where N was the number of glomeruli in the renal specimen. The non-paired Mann-Whitney U test was used to test differences between patients with PGN and NPGN. Finally, a linear regression analysis between the expression CD31, vWF and TM was performed, respectively. The P values less than 0.05 were considered significant.

Results

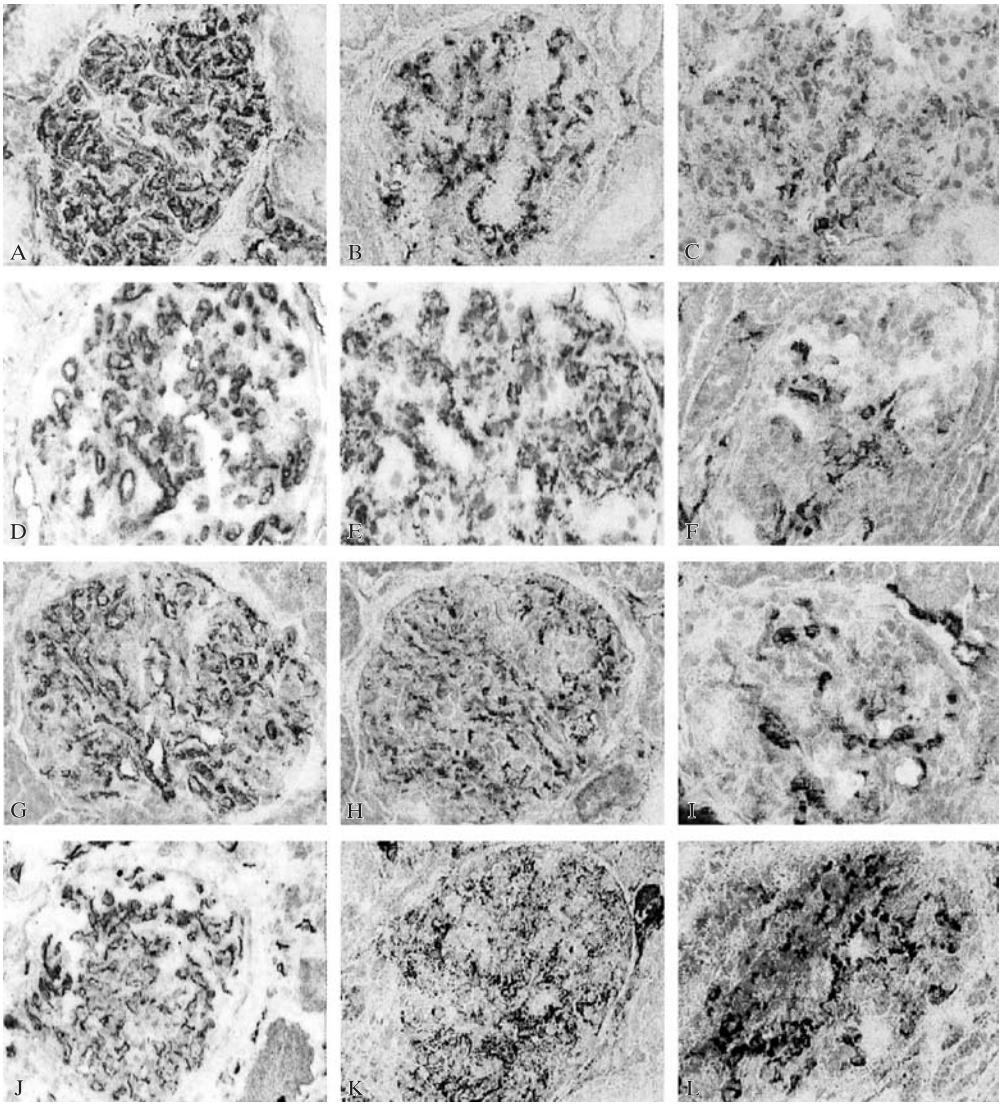
In the normal kidney, the expression of CD31 and vWF comprised the whole glomerular network, although some differ-

Table 1. **Clinical and morphological data of patients at the time of renal biopsy**

Morphology	No of Patients	Age (yr) (Mean ± SD)	Serum Creatinine (μmol/l) (Mean ± SD)	Urinary Protein (g/24 h) (Mean ± SD)
		Primary GN		
Proliferative GN				
MesPGN	41	31.4 ± 15.9	81.4 ± 19.2	2.2 ± 1.3
MPGN	4	52.0 ± 1.0	99.2 ± 18.1	3.7 ± 0.3
Non-proliferative GN				
FSGS	4	24.1 ± 14.1	97.4 ± 20.3	3.4 ± 0.8
IMGN	7	37.3 ± 12.7	82.6 ± 18.4	3.2 ± 1.4
		Lupus nephritis		
Class III	1	29	97.24	3.4
Class IV	2	37.5 ± 17.7	165.7 ± 102.4	3.2 ± 0.7
Class V	1	31	79.6	2.5

Abbreviations are: PGN, proliferative glomerulonephritis; MesPGN, mesangial proliferative GN; MPGN, membranoproliferative GN; NPGN, non-proliferative GN; IMGN, idiopathic membranous GN; FSGS, focal-segmental glomerulosclerosis

Figure 1. **A comparison between glomerular expression of CD31 (A, D, G, J), von Willebrand factor (B, E, H, K), and thrombomodulin (C, F, I, L), respectively, in the normal kidney (A, B, C), idiopathic membranous nephropathy (D, E, F), mesangial proliferative GN in the course of IgA GN (G, H, I), and class IV lupus nephritis (J, K, L)**



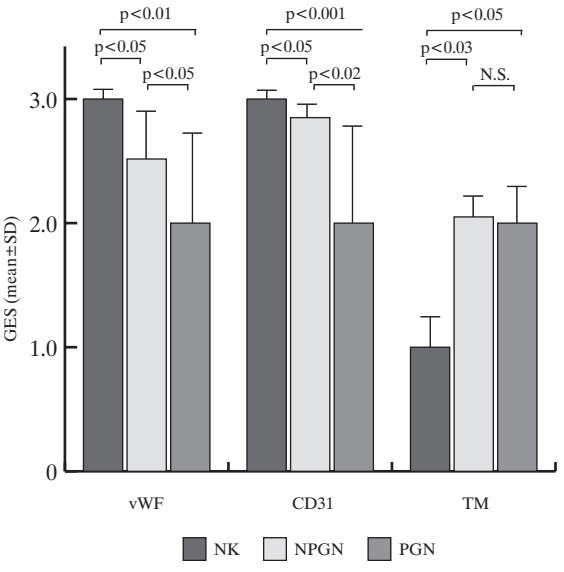
ences in the staining patterns could be noticed (*Fig. 1A* and *1B*). In contrast, small amounts of TM localized to the vascular poles of normal glomeruli (*Fig. 1C*).

In GN, some reduction in the expression of CD31 and vWF was observed in biopsies with NPGN (*Fig. 1D* and *1E*). It was related with the appearance of sclerotic alterations in glomeruli. On the other hand, a variable decrease in the expression of both these EC markers, dependent on the degree of the proliferative response of glomerular cells, was noted in PGN. Thus, the immunoreactivity for CD31 and vWF was mildly reduced in IgA-GN with moderate mesangial cell proliferation (*Fig. 1G* and *1H*), but markedly diminished in biopsies with severe proliferative lesions in MPGN and class IV lupus nephritis (*Fig. 1J* and *Fig. 1K*). In particular, the lowest values of GES for CD31 and vWF were observed in MPGN (CD31: 1.43 ± 0.64, $p < 0.02$ vs. IMGN and $p < 0.01$ vs. the whole NPGN group; vWF: 1.2 ± 0.0).

In contrast to the decreasing trend in the expression of CD31 and vWF, an increased glomerular reactivity for TM was a general feature of GN (*Fig. 1F*, *1I*, *1L*). However, the highest values of GES for TM were obtained in MPGN and LGN (2.36 ± 0.46 for both groups of patients).

Results of a semiquantitative evaluation of the glomerular expression of CD31, vWF and TM in NPGN and PGN, in comparison to the normal kidney, are shown in *Fig. 2*. As demonstrated in *Fig. 3*, a linear correlation between the glomerular expression of CD31 and vWF could be stated in the whole population of patients examined. No relationship between the glomerular expression of CD31 and TM, as well as vWF and TM could be observed in GN.

Figure 2. **Evaluation of glomerular expression of von Willebrand factor (vWF), CD31, and thrombomodulin (TM) in normal kidneys (NK), non-proliferative GN (NPGN), and proliferative GN (PGN). GES: mean glomerular expression score**



Discussion

Our study showed marked differences between the glomerular expression of TM, CD31 and vWF in the normal kidney. The expression of CD31 and vWF comprised the whole glomerular network, whereas the immunoreactivity for TM localized only to the vascular poles of the glomeruli. With respect to the distribution of CD31 in the normal glomeruli, Wada et al. have reported similar results in the rat kidney [3] and Sivridis et al. in the human kidney [6]. Also regarding the pattern of reactivity for TM in the normal kidney, previous results of Mizutani et al. [8] and Tomura et al. [9], are in line with these presented in this study.

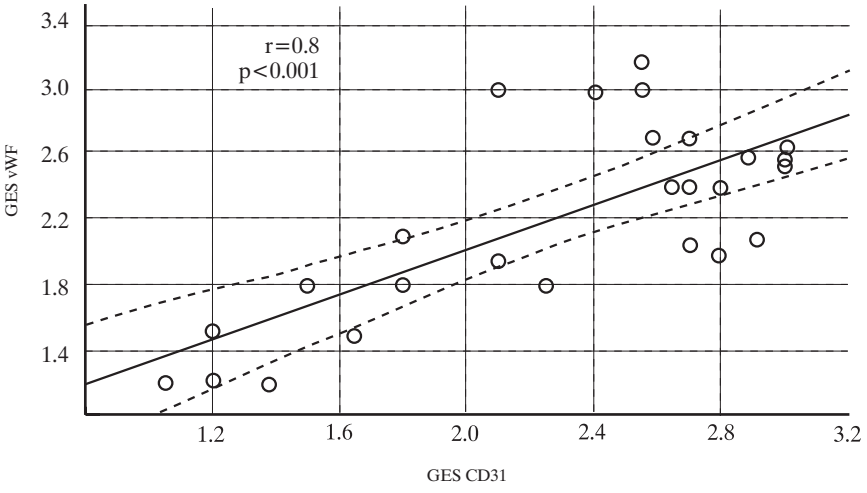
Not only did the expression of the examined proteins differ with respect of their pattern of reactivity in the normal kidney, but also in terms of their expression in different types of GN. Most of our patients presented features of MesPGN. Previously, we have observed a variable loss of CD31 staining in the course of IgA nephropathy [7]. This study demonstrates that the deepest fall in the expression of CD31 is found in MPGN, where mesangial cell proliferation largely exceeds that observed in MesPGN. Thus, in PGN, the reduction in the expression of CD31 seemed to be dependent on the extent of proliferative response of mesangial cells and subsequent destruction of the glomerular architecture.

Experimental studies on acute models of GN in which PECAM-1 has been used as the marker of EC show that these cells have a great regenerative capacity [3,4]. However, in models of progressive glomerular injury, loss of PECAM-1 staining signified impairment of capillary regeneration and preceded the development of glomerulosclerosis [3,4]. The expression of CD31 could particularly not be found in areas occupied by macrophages [4]. Since PECAM-1 has been shown to regulate the transmigration of neutrophils and monocytes across EC monolayer [15,23], loss of its expression may favor the leukocyte accumulation inside the glomerulus, which is the classical feature of the proliferative forms of GN [24,25].

Compared to MesPGN and MPGN, reduction in the glomerular expression of CD31 in our patient population with NPGN, i.e. in IMGN and FSGS, was relatively low. These results are in disagreement with that presented recently by Sivridis et al. [6]. In the above study, complete loss of the glomerular expression of PECAM-1 was observed in 9 out of 15 cases with IMGN. It may be supposed that more advanced sclerotic lesions in glomeruli were responsible for the discrepancy between the results of both studies. Since ten of these patients had higher values of serum creatinine concentration than our subjects with IMGN, such an explanation seems to be reasonable [6].

In our study, the glomerular expression of CD31 was related with that of vWF. Although vWF has bee used as a marker of EC in chronic allograft nephropathy [26], its glomerular expression in GN has not been demonstrated until now. As in the case of CD31, the more pronounced the distortion of glomerular architecture, the lower expression of vWF was observed. Recently, increased urinary excretion of vWF, including its functionally active form, has been reported in patients with active lupus nephritis [27]. Intriguingly, the highest levels of urinary vWF were found in patients with rapid progressive forms of LGN, who presented with the same range of proteinuria as patients with nephrotic syndrome without rapid progressive renal fail-

Figure 3. **Results of the linear regression analysis between the glomerular expression of CD31 and von Willebrand factor (vWF) in the whole population of patients examined. GES: mean glomerular expression score**



ure. In this context, increased levels of vWF have also been found in sera of patients with history of LGN [10]. The authors suggested that both increased serum concentration and urinary excretion of vWF in LGN might be a marker of local renal EC damage, which reflected the severity of immune inflammation and intravascular coagulation [10,27]. Taking into account our results and the fact that vWF plays a pivotal role in platelet adhesion and aggregation and acts as a ligand protein to support endothelial cell adhesion [22], this supposition sounds as a plausible hypothesis. However, larger population of patients with MPGN and, particularly, with LGN should be examined to draw final conclusions.

In contrast to CD31 and vWF, our study showed an increased glomerular reactivity for TM in patients with GN. Intriguingly, the glomerular expression of TM did not statistically differ between patients with NPGN and PGN. These results do not correspond with previously reported studies on the expression of TM in GN [8,9]. Although a moderate staining for TM has been observed in IMGN and minimal change disease, a significant increase in its expression has primarily been observed in idiopathic MPGN and LGN [8,9]. In the study of Mizutani et al., a direct correlation between the degree of TM expression and amounts of subendothelial immune deposits in LGN and MPGN has been found. In contrast, the expression of TM was not related with the presence of subepithelial or mesangial immune deposits in IMGN and IgA nephropathy, respectively [8]. Though, Tomura et al. have noticed no apparent differences in the intensity and distribution of TM staining among the different morphological forms of LGN [9]. Furthermore, the appearance of TM staining in the remission phase of FSGS has been found in another study. The authors of this study has linked the emergence of TM staining with the recovery from EC damage and suggested TM involvement in the repair process in GN [12]. The latter assumption might be strengthen by the fact that intravenous administration of recombinant human soluble TM to the rats with thrombotic rapid progressive GN had not only anti-thrombotic effects, but also attenuated leukocyte infiltration into the glomerulus [28].

In the light of these data, TM appears to be not only an anti-thrombotic but also anti-inflammatory molecule. The emergence of its staining in GN could be interpreted as an EC trial to maintain the local homeostasis and prevent the progression of glomerular lesions. In the latter context, the association of the highest expression of TM and the lowest one of CD31, observed in our patients with MPGN and LGN, might reflect maximal EC activation, which is, however, insufficient under condition of continuing EC injury.

Conclusions

Our results suggest that the reduction in the glomerular expression of CD31 and vWF in GN seems to reflect EC loss as a result of the inflammatory process inside the glomerulus. On the other hand, increased expression of TM points out to the active participation of glomerular EC in mechanisms aimed at the preservation of the glomerular architecture.

Acknowledgements

We are grateful to Prof. Wiesława Salwa-Żukowska and Dr Aldona Woźniak from the Department of Clinical Pathology, and to the team of Prof. Jan Żeromski from the Department of Clinical Immunology, University of Medical Sciences in Poznan for the morphological and immunopathological evaluation of renal specimens from the examined patients.

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