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CAVEOLIN-1 *IN SITU* EXPRESSION IN GLOMERULAR AND PERITUBULAR CAPILLARIES AS A MARKER OF PROGRESSION AND SEVERITY OF RENAL THROMBOTIC MICROANGIOPATHY.

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ABSTRACT

Background. Thrombotic microangiopathy (TMA) is a severe and potentially life-threatening condition inducing severe endothelial injury in many organs, particularly native and transplanted kidneys. Current pathological studies of our group have identified the use of Caveolin-1 immunohistochemistry (IHC) as a potential marker of endothelial damage and progression grade of TMA. The aim of the present work was to evaluate Caveolin-1 as a marker of severity in TMA kidney disease, according to the ultrastructural progression of the disease as transmission electron microscopy (TEM).

Materials and Methods. Twenty-nine patients (17 native and 12 transplanted) were retrospectively selected, biopsied for suspected or histologically confirmed TMA. TEM was performed in all cases, and an ultrastructural score of TMA-related glomerular disease was assessed (from 0 to 3+). IHC analysis for Caveolin-1 was automatically performed.

Results. The mean percentage of Cav1-positive glomerular capillaries was 53.2±40.6% and 28.0±42.8% in the active TMA versus previous TMA cases (p=0.085), considering both native and transplanted kidneys. The presence of a progressive disease correlated with diffuse Cav1 immunoreactivity (p=0.031), and TEM score correlated with glomerular Cav1 positivity, progressively increases from 22.5% of Score 0 group to 95.5% of Score 3 group (p=0.036).

Discussion. Cav1 proved to be a very useful marker of early endothelial damage in course of TMA on both native and transplanted kidneys, to be considered in routine practice. A diffuse glomerular Cav1 immunoreactivity correlates with the severity of the thrombotic disease and it can make its appearance very early, even before ultrastructurally-evident endothelial damage.

Keywords (up to 4): Caveolin-1; immunohistochemistry; kidney pathology; thrombotic microangiopathy; transmission electron microscopy.

Competing Interests:

The authors declare no conflicts of interest.

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INTRODUCTION

Thrombotic microangiopathy (TMA) comprises a complex set of life-threatening diseases inducing severe endothelial injury in many organs of the body and particularly kidneys[1]. Given TMA's varying forms and degrees of severity, diagnosis presents a relevant clinical problem for physicians. The major common denominator that groups these sets of diseases together is damage to endothelial cells that –in the kidney– affects glomeruli, arteries, and arterioles[1]. Damage of endothelial cells of capillaries and small arteries caused by TMA results in platelet aggregation and fibrin deposition within vessels. As erythrocytes travel through damaged vessels, they fracture and spill their contents into the bloodstream, leading to further obstruction and reduced blood flow. This condition is diagnosed as microangiopathic hemolytic anemia (MAHA)[2, 3]. Characteristically, MAHA causes hemolytic anemia and thrombocytopenia (low blood platelet count and decreased clotting ability). This combination of phenomena of MAHA and thrombocytopenia make up the primary diagnostic criteria of TMA[2, 3]. Ensuing complications are serious and can include multiorgan involvement, malignant hypertension, and kidney injury. In most cases, causes of TMA are multifactorial and span genetic mutations, autoantibodies, infection, or hereditary causes. As a result, TMA is commonly diagnosed using a combination of clinical findings, laboratory testing, and pathology[4, 5]. The histopathological definition of acute TMA requires the presence of thrombi in blood vessels, including arterioles, interlobular and arcuate arteries, hilum or glomerular capillaries and, more rarely, peritubular capillaries (sickle-cell syndrome). Nevertheless, in clinical practice many cases show indirect signs of TMA, characterized by endothelial swelling and activation, apparent thickening of basement membrane (due to subendothelial expansion), and mesangiolysis[5, 6]. From an ultrastructural point of view, features of TMA include swelling of glomerular endothelial cells with loss of fenestrae, followed by minimal to marked expansion of subendothelial space, entrapment of schistocytes, and cell debris and fibrin tactoids in subendothelium.

One of the broader classifications used to distinguish types of TMA is the idea of *de novo* vs recurrent TMA after renal transplantation[7]. *De novo* patients are diagnosed with TMA for the first time after completing successful transplant whereas recurrent TMA patients experience a return of symptoms post-transplant. Prognosis for *de novo* post-transplant TMA has been reported to be quite poor; based on the United States Renal Data System (USRDS) records, Reynolds et al. reported a patient mortality rate of 50% after three years of diagnosis[8]. Patients' positive response to therapies and outcomes largely depends on the rapid and accurate identification of the presence of TMA[4]. Additional diagnostics procedures related to disease pathology could help guide the treatment decisions of physicians and allow them to better diagnose and discern the varying types of TMA.

Caveolin-1 (Cav1) is a structural protein that is widely expressed in terminally differentiated cells including endothelial cells, epithelial cells, and fibroblasts[9]. In prior studies, the prognostic capability of Cav1 was demonstrated in cancer-associated fibroblasts (CAFs) and cancer cells in stage I lung adenocarcinoma[9]. The study concludes that positive expression of Cav1 is an important predictor of disease recurrence and poor outcome for the patient; showing significant correlation with shorter overall survival time, shorter disease-free survival time, vascular invasion, and cancer stage[9]. Another instance displaying the potential of Cav1 was in the analysis of the immunohistochemical expression of Cav1 in kidney biopsies and their association with antibody-mediated injury[10–12]. Through evaluation of Cav1 as a prognostic marker, the study found that positive Cav1 expression is positively associated with microvascular inflammation and antibody-mediated rejection[10, 11].

Current pathological studies of our group have identified the use of Cav1 immunohistochemistry (IHC) in kidney biopsies from patients as a potential marker of endothelial damage and progression grade of TMA. Using a combination of histopathological analysis, IHC and ultrastructural diagnosis with transmission electron microscopy (TEM), semiquantitative analysis of Cav1 was performed to evaluate its use as a marker of severity in kidney disease related to TMA, according to the ultrastructural progression of the disease.

MATERIALS AND METHODS

Selected patients and histopathological analysis

Twenty-nine patients were retrospectively selected in two years. Inclusion criteria were: kidney biopsy with availability of both histopathological and transmission electron microscopy (TEM) analyses, suspected or histologically confirmed TMA.

The patients were 14 males and 15 females, mean age at biopsy 55.58±13.09 years (range 29-76 years). Twelve (41.4%) patients were transplanted, for end-stage kidney disease of unknown etiology in 8 cases, diabetes in one case, focal segmental glomerulosclerosis in one case, autoimmune/rheumatoid kidney disease in one case, and polycystic kidney disease in one case. Seventeen (58.6%) patients had native kidneys and received a biopsy with the following indications: autoimmune/rheumatoid kidney disease in 6 cases, IgA-related disease in 2 cases, infective kidney disease in 2 cases, suspect chemotherapy-associated kidney disease in 2 cases, suspect pregnancy-associated kidney disease in one case, suspect kidney disease in one heart transplant patient, suspect

kidney disease without known etiology in 3 cases. The occurrence of a clinically progressive or chronic disease was collected as a variable in the native cases. The administration of Eculizumab before biopsy (due to the clinical suspect of TMA) was considered as well.

The histopathological features of the selected cases were blindly revised by two pathologists on the slide stained with Periodic Acid-Schiff, and the following variables were considered for the purposes of the study: number of glomeruli, presence of fibrin deposits and/or evident thrombi in glomerular capillaries, presence of glomerular basal membrane (GBM) duplication or double contours, presence of capillaritis, and occurrence of GBM thickening with mesangial expansion and fibrin deposits.

Transmission electron microscopy

Electron microscopy was performed on 2.5% cacodylate buffered glutaraldehyde-fixed kidney biopsies. Samples were post-fixed in 1% buffered osmium tetroxide, dehydrated in graded ethanol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and then examined in a CM 100 Philips Transmission Electron Microscope (Thermo Fisher Scientific, Waltham, Massachusetts, USA) equipped with a Gatan camera.

Electron microscopy analysis was focused on glomerular capillaries and TMA-related glomerular changes were scored as:

- *Score 0*: absence of florid TMA alterations; this group included cases with normal fenestrated endothelium, cases with segmental and focal glomerular endothelial cell activation without any change in the subendothelium, and cases with homogeneous thickening of GBM without evidence of lesion activity at the moment of the biopsy.
- *Score 1*: presence of mild and early florid TMA, described as predominant endothelial cell activation with loss of fenestrae and segmental, focal expansion of the subendothelial space by electron-lucent material.
- *Score 2*: presence of moderate florid TMA, described as global endothelial cell activation, loss of fenestrae, and circumferential expansion of the subendothelial space by electron-lucent material.
- *Score 3*: presence of severe and diffuse florid TMA, described as endothelial cell activation with cell detachment from the GBM, circumferential enlargement of the subendothelial space

containing entrapped schistocytes, cell debris, mesangial cell protrusions and / or fibrin deposits.

Immunohistochemistry for Caveolin-1

Immunohistochemistry (IHC) analysis for Caveolin-1 was performed on 3-μm-thick sections with the Caveolin clone E249 (AbCam, USA), using the Benchmark Ultra immunostainer (Ventana/ Roche) according to the following protocol: a) dewaxing; b) antigen retrieval in Cell Conditioning 1 for 24 min at 95°C; c) incubation with primary antibody (1:2000 dilution); d) development using the Optiview detection kit and (e) counterstaining in haematoxylin.

For each case, the following IHC variables were evaluated separately:

- Cav1 immunoreactivity in glomerular capillaries, assessed in focal (<5% positive glomeruli) and diffuse.
- Percentage of Cav1-positive glomeruli.
- Cav1 immunoreactivity in peritubular capillaries, assessed in focal (<5% positive areas) and diffuse.
- Number of Cav1-positive peritubular capillaries in the whole biopsy.

As a positive IHC control, a case of kidney transplant biopsy with a histologically proven acute cellular rejection was used: it shows diffuse immunoreactivity in all glomerular capillaries as well as in >90% of peritubular capillaries.

As negative IHC controls, 10 biopsies from 5 male and 10 female patients were selected, who received a definite histopathological diagnosis of minimal changes disease (MCD). Only two (20%) of these cases showed diffuse Cav1 immunoreactivity in glomerular capillaries, while the mean number of positive peritubular capillaries was 36.60±20.01.

Statistical analysis

All analyses were performed by means of the SPSS® software for Windows, ver. 20. Continuous variables were reported as means±standard deviations and ranges; discrete variables were reported as frequencies and percentages. The chi-square, Mann-Whitney and Kruskal-Wallis tests were applied. A *p* value <0.05 was considered significant to reject the null hypothesis.

RESULTS

Immunohistochemical Caveolin-1 expression and the clinical onset of thrombotic microangiopathy

The mean number of visible glomeruli in kidney biopsies was 7.21±3.86 (range 1-15). Fibrin deposits and/or evident thrombi in glomerular and peritubular capillaries were observed in 17 (58.6%) cases; GBM changes were present in 18 (62.1%) cases; capillaritis was present in 13 (44.8%). The overall picture was diagnostic for current TMA on biopsy in 10 (34.5%) cases.

At IHC, 15 (51.7%) cases showed diffuse positivity for Cav1 in the glomerular capillaries: in particular, the mean percentage of Cav1-positive glomeruli was 44.51±42.39% (range 0-100%). Fifteen (51.7%) cases showed diffuse positivity for Cav1 in peritubular capillaries: overall, a mean number of 40.24±31.37 positive peritubular capillaries per biopsy was counted (range 12-130).

A major diffusion of the Cav1 immunoreactivity in the glomerular capillaries was observed in the cases with active TMA (by definition, associated with the presence of fibrin/thrombi): 12 out of 19 (63.2%) active-TMA cases showed diffuse Cav1 positivity, versus 3 out of 10 (30.0%) cases with previous TMA at histology (p=0.095, chi-square test).

The mean percentage of Cav1-positive glomerular capillaries was 53.2±40.6% and 28.0±42.8% in the active TMA versus previous TMA groups (*p*=0.085, Mann-Whitney test). Albeit this association did not reach statistical significance, the trend is very interesting also considering that no differences were observed between the transplanted and not transplanted groups of kidneys (*data not shown*).

In the group of the 17 native kidneys, the presence of a progressive disease correlated with diffuse Cav1 immunoreactivity at both intraglomerular (71.4% *versus* 20.0% positive cases, *p*=0.031) and peritubular (85.7% versus 20.0% positive cases, *p*=0.013) capillaries. The mean percentage of positive glomeruli was 64.4±44.7% in the progressive kidney diseases, and 24.2±40.5% in clinically "stable" diseases (*p*=0.042, Mann-Whitney test). The mean number of Cav1-positive peritubular capillaries was 53.0±25.2 and 31.2±34.9 in the two clinical groups (*p*=0.050).

Ultrastructural progression of thrombotic microangiopathy

In two cases, no glomeruli were found in the allocated samples for TEM, and the further analysis were carried out considering the remaining 27 patients.

According to the ultrastructural criteria listed above:

- Eleven (40.7%) cases were TMA Score 0; this group includes 3 cases without any sign of TMA at TEM (Figure 1a), 2 cases with very early signs of TMA, and 6 cases with signs of previous TMA (Figure 1e).
- Sixteen (59.3%) showed florid TMA lesions at TEM. In particular, 3 cases were Score 1 (Figure 1b), 9 cases were Score 2 (Figure 1c) and 4 cases were Score 3 (Figure 1d).

At IHC TEM classification correlated with the Cav1 positivity seen in the glomerular capillaries: the mean percentage of Cav1-positive glomerular capillaries progressively increases from 22.5% of Score 0 group to 32.5% of Score 1, 57.5% of Score 2 and 95.5% of Score 3 group (p=0.036, Kruskal-Wallis test; Figure 1f-j). It is noteworthy that breaking up the Score 0 group into the different TEM entities, they showed different Cav1 expression: indeed, the 3 cases with absence of florid TMA alterations showed 5.6% of positive glomerular capillaries (the lowest value of all), while the 6 cases with signs of previous TMA had 21.8% (comparable with Score 1 cases). The 2 cases with initial ultrastructural signs of TMA had 50% of mean positivity in the glomerular capillaries: despite the very limited numerosity, one could speculate that Cav1 immunoreactivity might make its appearance even before ultrastructural alterations in course of TMA.

DISCUSSION

TMA is a potentially life-threatening condition affecting both native and transplanted kidneys, characterized by intravascular fibrin deposition and endothelial damage of variable amount[1, 3, 5] The typical histopathological features can be missing on biopsies in cases of early or resolving TMAs, making difficult the morphological diagnosis; the ultrastructural analysis by TEM is very useful, but not always available. The main purpose of the present work was to assess the usefulness of Caveolin-1 (Cav1) immunohistochemical expression as a potential marker of endothelial damage and progression in course of TMA, on both native and transplanted patients.

Cav1 is the main component of caveolae: it is a 22 kDa protein, and the first of the members of the Caveolin family to be identified, and it plays a key role in the biogenesis of the caveola itself[13],

[14]. Caveolae are small (50–100 nm) invaginations of the plasma membrane of most cells, rich in glycosphingolipid and cholesterol[15, 16]. In the human kidney, Cav1 is described as constitutive of podocytes, mesangial cells, and arterial muscular cells[11, 12]. It has been recently described that Cav1 is fundamental for mesangial cells for the matrix production during renal fibrogenesis, in response to profibrotic and mechanical stimuli[15]. Endothelial cells of human kidneys are negative for Cav1, but its expression significantly increases in injured endothelial cells[11]. One of the most studied models of human kidney endothelial damage is antibody-mediated rejection[10, 11], and recently some authors suggested the use of Cav1 IHC together with C4d in the diagnosis of this condition, emphasizing how Cav1 is likely to be a good marker of endothelial damage independently from the complement activation[12].

In order to study Cav1 IHC expression in the different morphological manifestations of renal TMA, we used classic histology as well as TEM as the gold standard, defining an ultrastructural progression of TMA: by electron microscopy, we were able to identify the "florid" and active TMA lesions, characterized by progressive endothelial cell activation, with loss of fenestrae and expansion of the subendothelial space by electron-lucent material, up to endothelial detachment from the GBM and fibrin deposits. The first result of our study was that in "active" TMA (i.e. with fibrin deposition and/or endothelial damage on both histology and TEM) the Cav1-positive glomeruli were more than twice in percentage compared to the others. Of note, this was true in both native and transplanted kidneys: this seems to indicate that Cav1 is a marker of actual endothelial damage in course of TMA regardless of the etiology of the thrombotic disease. In native kidneys, the clinical severity and disease progression are directly correlated with immunoreactivity on both glomerular and intratubular capillaries. There were apparently no correlations between Cav1 immunoreactivity and therapy with Eculizumab (when given), as well as with the other clinical variables. Nevertheless, we think that this data needs to be further analyzed by a specific prospective study.

A second observation was that the percentage of Cav1-positive glomeruli progressively increased with the ultrastructural score of TMA activity (as defined in the Materials and Methods), from a 22.5% of the score 0 to the 95.5% of the score 3, meaning that in TMA cases with ultrastructural high activity, virtually all glomeruli showed Cav1 positivity. This is in line with the original reports on Cav1 expression in kidney with glomerulopathies[11], as well as transplanted kidneys[10], but with one further observation: the Score 0 cases were not all the same. Indeed, the Score 0 cases with no histological and ultrastructural features of active TMA had a very low glomerular Cav1 positivity (5.6%), while the cases consistent with initial phases of TMA had 50.0% positive glomeruli. These

cases are very few in our series, and surely deserve further analyses, but this data seems to suggest that, in the early phases of TMA, the endothelial activation leading to Cav1 overexpression precedes the morphological alterations.

For practical purposes, in line with our previous experience (*under revision*), we chose kidney biopsies with a diagnosis of MCD as controls, to be compared with our cases. We observed a variable amount of Cav1 positivity in peritubular capillaries, but in two cases in glomeruli as well. It was recently reported that the expression of endothelial activation markers such as Cav1 and thrombomodulin were increased during relapsing MCD[17], giving another proof of the concept that Cav1 expression is a very early circumstance, preceding the histological/ultrastructural endothelial damage. The meaning of Cav1 glomerular positivity in the evolution of MCD, e.g. towards a focal segmental glomerulosclerosis, should be the subject of future studies.

In conclusion, Cav1 is a very useful marker of early endothelial damage in course of TMA on both native and transplanted kidneys, to be considered in routine practice. A diffuse glomerular Cav1 immunoreactivity correlates with the severity of the thrombotic disease and it can make its appearance very early, even before ultrastructurally-evident endothelial damage.

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Figure Legend

Figure 1. Comparison between (a-e) ultrastructural score of thrombotic microangiopathy at transmission electron microscopy (TEM) and (f-j) glomerular Cav1 immunoreactivity. In Score 1 (b) glomerular basal membrane (GBM) is still intact; in Score 2 (c) initial GBM leakage is visible (arrows), while on the other side the GBM is intact; in Score 3 (d) the arrow indicates a schistocyte and the short arrow a platelet; in the last case, no active endothelial damage is visible but GBM is thickened. In Score 0 cases (f) no Cav1-positive glomerular capillaries are seen; immunoreactivity progressively increases from Score 1 to Score 3 (g,h and i); some positivity is seen in Score 0 cases with previous thrombotic microangiopathy (j).

L: capillary lumen; EC: endothelial cell; asterisk: GBM leakage.