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Focal segmental glomerulosclerosis plays a major role in the progression of IgA nephropathy. I. Immunohistochemical studies

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IgA nephropathy (IgAN) often shows lesions morphologically identical with those of focal segmental glomerulosclerosis (FSGS). In order to determine the possible role of FSGS in IgAN lesions, we measured glomerular capsular adhesions, often the first step toward FSGS, in biopsies from 127 patients with IgAN, 100 with lupus nephritis, and 26 with primary FSGS. Capsular adhesions with no lesions in the underlying tuft, consistent with podocyte abnormality or loss, were found regularly in FSGS and IgAN, but infrequently in lupus. Fifteen biopsies of patients with IgAN were studied immunohistochemically using markers for podocytes, Bowman's parietal epithelial cells, proliferating cells, and macrophages. Cytokeratins CK-8 and C2562 differentiated normal podocytes (negative) from parietal epithelial cells (variably positive). There was focal loss of the podocyte markers synaptopodin, glomerular epithelial protein 1 (GLEPP-1), nephrin, and vascular endothelial growth factor (VEGF), particularly at sites of capsular adhesions in otherwise histologically normal glomeruli. Cells displaying the parietal epithelial cell markers PAX2 (paired box gene 2) and the cytokeratins were also positive for the proliferating cell marker, proliferating cell nuclear antigen. These cells gathered at sites of adhesion, and in response to active lesions in the tuft, grew inward along the adhesion onto the tuft, forming a monolayer positive for parietal markers and the podocyte marker Wilms tumor protein-1 (WT-1). These cells deposited a layer of collagen over the sclerosing tuft. Thus, all biopsies of patients with IgAN had changes basically identical to those classically described in FSGS. Hence, our study strongly suggests that podocytopathy of a type similar to that in primary FSGS occurs frequently in IgAN.

Kidney International (2011) **79,** 635–642; doi:10.1038/ki.2010.466; published online 15 December 2010

KEYWORDS: focal segmental glomerulosclerosis; IgA nephropathy; podocyte

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Received 9 April 2010; revised 27 August 2010; accepted 28 September 2010; published online 15 December 2010

Two sets of factors have converged in recent times, pointing to the need for a study of the role of FSGS in IgA nephropathy (IgAN). First, there are a number of studies describing lesions of focal segmental glomerulosclerosis (FSGS) in IgAN,[1–3] including post-transplant IgAN.[4,5] Haas[6] included FSGS as one of the classes in his classification of IgAN, although this term has been superseded in the new Oxford classification[7,8] by the term segmental glomerulosclerosis, which subsumes not only lesions resembling FSGS but other segmental scars, including capsular adhesions, under this rubric. Furthermore, we found in preliminary analysis of the series of IgAN patients to be reported here that roughly half had clearcut lesions either of FSGS with hyalinosis or collapsing glomerulopathy. We were also struck, as others have been,[7–9] by the frequency of capsular adhesions in IgAN, a feature found nearly universally in FSGS.

Second, a series of exciting studies has appeared recently demonstrating deleterious effects of IgA on glomerular podocytes. Aggregated IgA1 from patients with IgAN inhibits nephrin expression in podocytes,[10,11] mediated through platelet-derived growth factor,[12] and appears to produce podocyte apoptosis[13] as well as reduced adhesive capacity.[14] *In vitro*, not only nephrin but also podocin and synaptopodin are reduced,[13,15] likely mediated through tumor necrosis factor-α.[15] It has also been shown, as indicated in one study using B-cell lymphoma 2, a proto-oncogene found in podocytes, that downregulation of podocyte activity is associated with poor prognosis in IgAN.[16]

For these reasons we undertook a study of IgAN biopsies to examine the possible role of FSGS in IgAN. The study is divided into two parts. The present article deals primarily with immunohistochemical studies of podocyte and glomerular parietal epithelial cell (PEC) markers, with attention to capsular adhesions also. A companion article[17] deals with standard light microscopic studies of IgAN, particularly the parallels with FSGS, and correlates the findings with clinical and follow-up data.

RESULTS

Glomerular capsular adhesions

To begin to understand the possible role of capsular adhesions in IgAN, we performed a comparative study of

Table 1 | Comparative study of glomerular capsular adhesions in IgAN, SLE, and primary FSGS

	SLE			IgAN			FSGS	
Adhesions	100 biopsies			128 biopsies	26 biopsies			
Percent of biopsies	No.	%	P^a	No.	%	P^a	No.	%
Percent with adhesions	82	82%	NS	92	71.9%	0.002	26	100%
Percent with adhesions but without underlying lesions	8	8%	0.0000	53	41.4%	0.006	18	69.2%
Percent with underlying active lesions	49	49%	0.0000	21	16.4%	0.01	10	38.5%
Percent with underlying necrotizing lesions	9	9%	0.003	1	0.8%	NS	0	0%
Percent with underlying scarring lesions	59	59%	NS	77	60.5%	NS	16	64%

Abbreviations: FSGS, focal segmental glomerulosclerosis; IgAN, IgA nephropathy; NS, not significant; SLE, systemic lupus erythematosus.
[a]Calculated using χ^2 tests.

our cases of IgAN with biopsies of 100 patients with systemic lupus erythematosus (SLE), representing an immune complex-mediated process, and of 26 cases of primary FSGS (Table 1). Capsular adhesions are extremely frequent in all three conditions as a result of active/proliferative and sclerosing lesions. However, one aspect in which they differ markedly is the presence of adhesions without evident underlying lesions in the tuft (Figure 1a). Such lesions are infrequent in SLE (8% of cases) and quite common in FSGS (69.2%), with IgAN intermediate between the two at 41.4% of cases. The presence of adhesions without underlying lesions suggests that in some fashion podocytes (or their absence) may be responsible for the adhesion.

Immunohistochemistry in IgAN

Podocyte markers. All save one of the podocyte markers used in this study, including synaptopodin, glomerular epithelial protein 1 (GLEPP-1), nephrin, vascular endothelial growth factor (VEGF), and Wilms tumor protein-1 (WT-1), show similar features (the exception is WT-1, described separately below). It is therefore simplest to discuss them together, with comments on minor differences. In normal glomeruli, there is uniform positivity for podocyte markers along all capillary loops, synaptopodin and nephrin having a striated appearance along loops cut on the bias, corresponding to the distribution of slit diaphragms and pedicels (Supplementary Figure S1 online). Staining of rare cells along Bowman's capsule is seen, particularly for GLEPP-1 (Figure 1b and e) and synaptopodin (Supplementary Figure S7 online), representing parietal podocytes.[18] There may be isolated diminution or loss of staining in capillary loops, particularly on the periphery of the tuft in histologically normal glomeruli (Figure 1b and Supplementary Figure S2 online). Small capillary adhesions to Bowman's capsule may occur without loss of staining (Supplementary Figure S4 online), but larger adhesions are invariably attended by complete loss of podocyte staining (Figure 1c and Supplementary Figure S5 online). In glomeruli with capsular adhesions and proliferative lesions, podocyte staining may stop abruptly at points where PECs extend down onto the surface of the glomerulus (Figure 1c and d). Staining tends to be preserved more frequently over patent capillary loops than over areas of endocapillary proliferation, but in advanced lesions, it is absent from the center of the glomerulus, despite some still-patent capillaries (Figure 1e). In the majority of instances, active proliferative or necrotic lesions within the tuft are overlain by cells staining negatively for all four podocyte markers (Figure 1c and d and Supplementary Figures S3, S7 and S8 online). On occasion, one may see active lesions in which residual synaptopodin-positive or GLEPP-1-positive cells overlie endocapillary lesions within the tuft and are in turn overlain by negatively staining cells (Supplementary Figures S6 online and S10 online). (In other studies using double-marking techniques, similar overlying cells in FSGS have been shown to be PECs.[19]) In advanced lesions, only vestiges of former podocyte staining remain and the scarred flocculus is covered by a monolayer of 'cobblestone' cells staining negatively for all of the podocyte markers overlying a band of newly laid down collagen (Figure 1e and Supplementary Figure S9 online). (These cobblestone cells stain as PECs—see below.) In advanced lesions one may also see rounded-up cells staining for podocyte markers (Supplementary Figures S3 online and S9 online) lying free in Bowman's space, as well as occasional positive cells along Bowman's capsule. Finally, some glomeruli show signs of simple ischemia, without loss of podocyte markers.

Paired box gene 2 (PAX2). PAX2 marks PECs, with uniform distribution along the capsule of normal portions of glomeruli (Supplementary Figure S11 online). There is proliferation adjacent to capsular adhesions with minor underlying proliferative lesions (Figure 1f and Supplementary Figure S11 online). More extensive proliferation along the adhesions covers the adjacent glomerular tuft in glomeruli with more extensive tuft lesions (Figure 1g). Usually, a connection with capsular PECs can be traced or readily envisioned, but rarely a positive cell will be found in the center of the flocculus without evident connection to the capsule (Figure 1g). The rounded-up cells in Bowman's space are negative for PAX2 (Figure 1e).

WT-1. WT-1 marks normal podocytes and also stains capsular epithelium sparsely, presumably parietal podocytes. In histologically normal glomeruli there may be segments of the glomerulus from which podocyte staining seems to have disappeared. WT-1-positive cells proliferate adjacent to capsular adhesions (Supplementary Figure S13 online). WT-1 cells are more extensive over more extensive tuft lesions (Figure 1h), penetrating into the tuft. In advanced lesions there is a continuous layer of positive cells extending

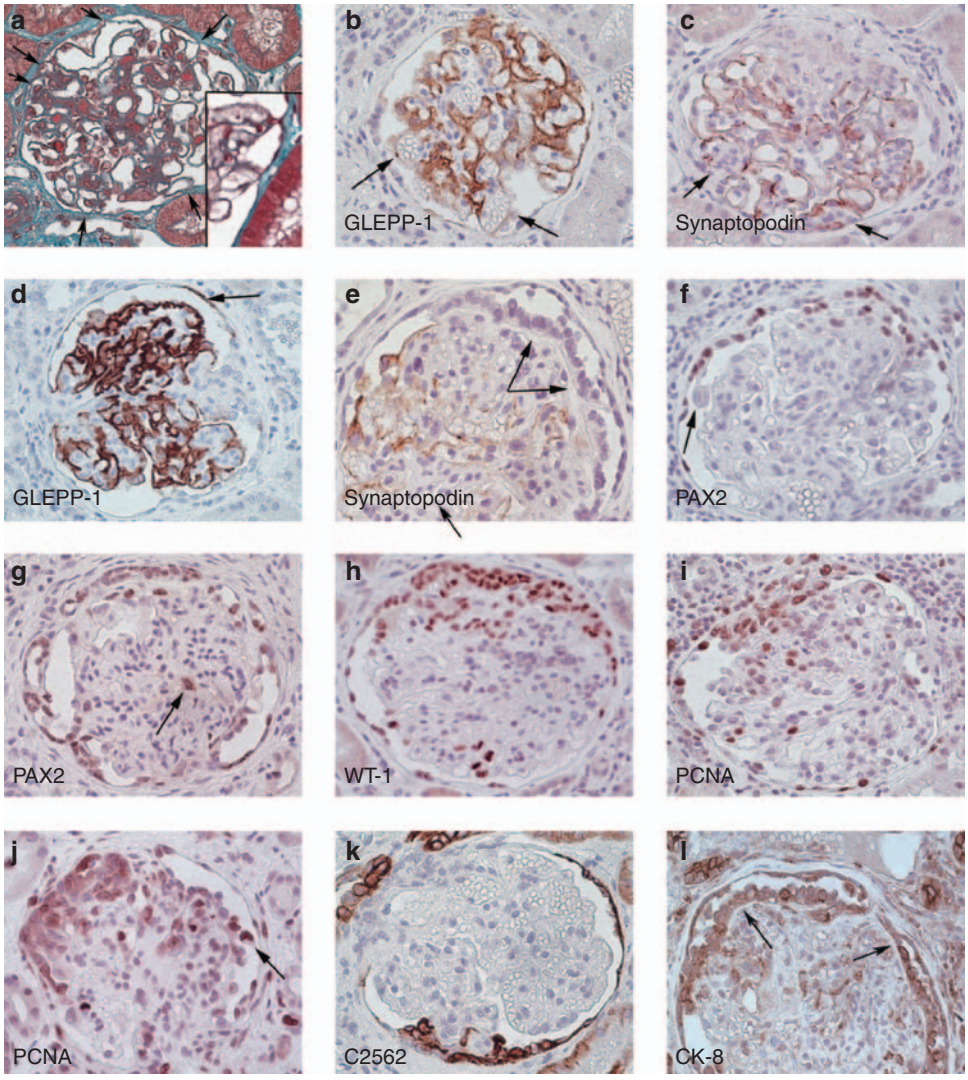

Figure 1 | Immunohistochemical studies showing similarities between IgAN and primary FSGS. (**a**) Capsular adhesions in IgA nephropathy (IgAN). There are numerous capsular adhesions (arrows) without apparent lesions in the underlying tuft. Mesangial deposits in this instance are easily recognized by their red staining. (Inset) Frequently, such adhesions appear to exert traction on Bowman's capsule. Original magnifications: (Masson's trichrome (MT)) × 350, (inset) × 650. (**b**) Focal loss of podocyte staining. Several capillaries (arrows) show loss of staining, including one with an early adhesion, but without evident lesions in the underlying tuft. Some cells lining Bowman's capsule are also positive. Glomerular epithelial protein 1 (GLEPP-1); original magnification × 400. (**c**) Capsular adhesions. Early adhesions may show only diminution of staining, but larger ones (arrows) show complete loss of synaptopodin staining. Podocyte staining may stop abruptly at sites of proliferative lesions (top). Synaptopodin; original magnification × 350. (**d**) Loss of podocyte staining over active lesions. Podocyte staining stops abruptly at the point where proliferative cells coming in from the capsule reach the tuft. A parietal podocyte (arrow) is positive. GLEPP-1; original magnification × 350. (**e**) Advanced lesion with 'cobblestone' epithelium. Monolayer of negatively staining 'cobblestone' epithelium overlies line of new collagenous matrix (arrows) surrounding advanced lesion. Synaptopodin staining has disappeared from the center of lesion, but a synaptopodin-positive cell lies free in Bowman's space. Synaptopodin; original magnification × 350. (**f**) Parietal epithelial cell (PEC) positivity at capsular adhesions. Paired box gene 2 (PAX2)-positive cells are sparsely aligned along Bowman's capsule in the lower, normal part of the glomerulus. On top, numerous PAX2-positive cells line the capsule and overlie proliferative lesion in flocculus. A round cell in Bowman's space (arrow) is PAX2 negative. PAX2; original magnification × 350. (**g**) PAX2 positivity in advanced lesion. PAX2-positive cells pass from capsule onto glomerulus forming pseudotubules. Most positive cells can be traced back to the capsule, but rare cells may be found in the interior (arrow) without evident connection to capsule. PAX2; original magnification × 350. (**h**) Wilms' tumor protein-1 (WT-1) positivity in active lesion. In the normal (lower) portion of the glomerulus, podocytes and occasional PECs are positive. On top, extensive WT-1 positivity is seen along capsule and over tuft lesion. WT-1; original magnification × 350. (**i**) Proliferation of proliferating cell nuclear antigen (PCNA)-positive cells. Numerous positive cells, some definitely PECs and others presumptively PECs, overlie an area of glomerular necrosis. PCNA; original magnification × 300. (**j**) Advanced lesion. Proliferated PCNA-positive cells overlying an active lesion (upper left) are positive. Some 'cobblestone' cells (right) are positive, and others are not. A PCNA-positive round cell (arrow) lies free in Bowman's space. PCNA; original magnification × 350. (**k**) Cytokeratin-positive cells at site of adhesion. Cells behaving as PECs proliferate at the site of adhesion. C2562; original magnification × 350. (**l**) Advanced lesion. PECs and cobblestone cells show strong positivity. Arrows indicate layer of collagenous matrix overlying advanced lesion. CK-8; original magnification × 350.

from the capsule over the damaged tuft (Supplementary Figure S12 online), largely coextensive with PAX2 staining, with both marking cells aligned along the bands of new collagen overlying the damaged tuft (Supplementary Figures S12 online, S13 online), in the instance of WT-1 on both sides of the layer. Occasional round cells staining positively for WT-1 may be seen free in Bowman's space.

Proliferating cell nuclear antigen (PCNA). In normal glomeruli, modest numbers of intraluminal cells (some definitely endothelial), mesangial cells, and PECs are positive. No definite podocyte staining is seen at any point in the evolution of lesions. In glomeruli with segmental cellular lesions, PECs lining the capsule show marked proliferation, continuous with numerous cells, also presumptively PECs, overlying the affected area (Figure 1i). In advanced lesions, PECs and cobblestone cells overlying the tuft are widely but not invariably positive (Figure 1j). Occasional round cells lying free in Bowman's space also stain (Figure 1j).

Cytokeratins (CK-8 and C2562). CK-8 and C2562, which recognizes nine different cytokeratins including CK-8, were used in the confirmation of identification of PECs. Normal podocytes are negative for both. When positive, both CK-8 and C2562 identified PECs, either in their normal capsular position or behaving as PECs as shown by PAX2 positivity in adjacent sections. CK-8 staining was more extensive than C2562, which in normal glomeruli showed only focal positivity, primarily in cells near the hilar pole. Neither antiserum stained normal or reactive PECs universally, even in normal controls, and hence only positive staining was helpful in identification. With that proviso, we found frequent accumulations of CK-8- and C2562-positive cells around the affected tuft at adhesion sites (Figure 1k and Supplementary Figure S14 online). The cobblestone, pseudotubular arrangements around advanced lesions could be markedly positive (Figure 1l), but other advanced lesions were only modestly positive, with some cells staining and others not (Supplementary Figure S15 online).

CD68. CD68-positive cells with morphologic features of PECs may be recognized in early lesions, with occasional positive cells free in Bowman's space (Supplementary Figure S16 online). Rarely is there positive staining underlying epithelial cells, but there is no staining of podocytes in their normal positions. Similarly, in advanced lesions, there is only isolated staining in the capsular and cobblestone layers (Supplementary Figure S16 online).

DISCUSSION

Interpretation of immunohistochemical study results

Overall, the pattern is one of progressive loss of the podocyte markers, synaptopodin, GLEPP-1, nephrin, and VEGF, beginning very early in isolated capillary loops and at sites of adhesion, before there are recognizable signs of lesions in the underlying flocculus. There is near-total loss of podocyte markers over more advanced lesions, as for example in 'cobblestone' and pseudotubule regions. In contrast, PAX2, a marker of PECs, shows progressive increase, from early positivity at sites of adhesion to more diffuse positivity over active tuft lesions. There is continuity between the positive cells directly overlying the tuft lesion and capsular cells, suggesting they are all of PEC origin. The pattern of proliferating cell nuclear antigen positivity parallels that of PAX2, although not all cells stain. Cytokeratin CK-8 and C2562 positivity, although more variable, parallels that of PAX2 as well. WT-1 shows behavior intermediate between standard podocyte markers and PEC markers, with definite loss of podocyte marking at sites of adhesion, but progressively increasing positivity with advancing glomerular lesions, paralleling that of PAX2. The nature of the WT-1-positive cells in the areas of cellular proliferation may be questioned. The fact that WT-1 staining is largely coextensive with PAX2 and that staining for podocyte markers in these areas is negative, suggests that here WT-1 is marking primarily cells acting as PECs, but double-staining techniques would be required to completely rule out a role for altered podocytes.

Immunohistochemical profile of glomerular lesions in FSGS

Early immunohistochemical studies of FSGS documented loss of podocytic markers such as synaptopodin, podocalyxin, and GLEPP-1 over the affected areas of the tuft.[20] These changes were attributed to podocyte dysregulation. Bariéty *et al.*[21–23] confirmed these findings, adding to them several important observations. First, the so-called 'cobblestone' epithelium overlying areas of advanced tuft lesions was negative for podocyte markers. Second, they identified rounded macrophage-like cells lying free in Bowman's space, having podocytic markers identifying their podocytic origin. These cells also acquired macrophagic epitopes, and could later be found in the tubular lumens. Third, they demonstrated in elegant fashion that there was transdifferentiation of cells, identifying cells expressing simultaneously podocyte markers and macrophagic markers or cytokeratins.[22] They also felt that this transdifferentiation could be regarded as a form of podocyte dysregulation. Another early study[24] described increase of PAX2 and cytokeratin over cellular lesions and in cobblestone epithelium, attributing these findings to re-expression of PAX2 in podocytes.

These observations have stood up with time, but their interpretation has changed away from the notion of podocyte dysregulation. The emergence of reliable markers for PECs, such as PAX2[19,25] and CK-8,[25] has permitted the recognition that many of the cells covering the glomerular tuft are not podocytes, but rather PECs. Dijkman *et al.*,[19,25] using PAX2, CK-8, and podocyte markers, together with painstaking serial-section reconstruction of glomeruli in FSGS and collapsing glomerulopathy, demonstrated that the anomalous cells overlying the tuft are in fact PECs that have grown in along capsular adhesions to cover the tuft, surrounding areas of floccular damage and forming the flattened cobblestone layer seen in late lesions. A crucial aspect of their reconstructions is that adhesions and PEC ingrowth can invariably be found if one searches hard enough. This observation effectively rules out the notion of podocyte

phenotypic changes or dysregulation as an explanation for the lesions in FSGS. Furthermore, they demonstrated that the layer of new collagen typically laid down over the sclerosing lesions in FSGS is identical with the collagen of Bowman's capsule that is known to be elaborated by PECs.[19] Later experimental studies using genetically labeled PECs confirmed their role in crescentic glomerulonephritis and collapsing glomerulopathy, to the near exclusion of podocytes.[26]

Thus, the pathogenesis of FSGS now seems clearly related to loss of podocyte markers on the one hand and aggression of the tuft by PECs on the other. (These observations may be further refined in the future, with the recent identification of renal progenitor cells lying along Bowman's capsule with the ability to transform into podocytes or tubular cells depending on their position,[27,28] and may possibly have a role in repair and regeneration of podocytes.)

Immunohistochemical observations in IgAN

Our observations in IgAN regarded as a podocytopathy are, with only minor exceptions (see below), completely consistent with current views on FSGS. (In addition, because our series contained a number of early/mild cases, we have been able to make some observations regarding IgAN that seem likely to apply to FSGS as well, although they have not been commented upon specifically in the literature on FSGS.)

First and foremost, as detailed in the companion article to this one,[17] we have identified examples of all of the major variants of FSGS as currently classified,[29] including hyalinosis lesions, collapsing glomerulopathy, and tip lesions. Second, in this study we have identified focal loss of podocyte markers (synaptopodin, GLEPP-1, nephrin, and VEGF) early on, along peripheral capillary loops and particularly at the sites of capsular adhesions, in the absence of overt lesions within the tuft. Importantly, this loss involves not only the immediate area of the adhesion, but for at least a short distance away. Loss of podocyte markers has been commented upon extensively in FSGS[19–23,25,30] and to a lesser extent in IgAN,[30–34] but focal early loss has not been specifically commented upon in light microscopic studies. However, focal loss of podocyte markers has been described by electron microscopy in a study utilizing immunogold-labeled nephrin in a variety of diseases including FSGS, showing that nephrin was absent from areas of foot process fusion and present in areas with preserved foot processes.[35] Loss of podocytes at points of adhesion has been extensively described in experimental FSGS,[36,37] but not specifically in humans, although various theories of pathogenesis presume its occurrence.[36,38,39] Third, we have observed early proliferation of cells staining for PAX2, cytokeratins, WT-1, and proliferating cell nuclear antigen at these points of adhesion, with extension of these cells along the adhesion onto the adjacent tuft. These findings parallel those of Dijkman *et al.*[19,25] who with their serial reconstructions in FSGS were able to demonstrate that there is always continuity with the capsular PECs. We have further shown that the epithelial cells overlying active lesions, the so-called 'cobblestone' epithelium, stain negatively for podocyte markers and positively for PAX2 as PECs, a finding entirely consistent with the studies of FSGS and collapsing glomerulopathy previously cited.[19–23,25] Finally, we have confirmed the presence of free round cells in Bowman's space marking both for podocyte and macrophagic markers, although on separate stains. Double-labeling studies would be required to confirm that these cells simultaneously express both podocyte and macrophagic markers as has been demonstrated in FSGS.[22,23]

One potential difference between our results and previous studies of FSGS is that we did not observe as widespread positivity for cytokeratin in cells behaving as PECs as described in the studies of Dijkman *et al.*[19,25] Our CK-8 and C2562 when positive did indeed identify cells behaving as PECs and doing all of the things that Dijkman *et al.* described, such as proliferating at sites of adhesions and forming 'cobblestone' monolayers over advanced lesions.[19,25] However, staining was not universal, even in normal glomeruli in our controls. It seems reasonable to posit that these differences reflect the different antisera used in the two studies.

Another minor difference is that we do not see quantities of cells with macrophagic epitopes covering the tuft, as described by Bariéty *et al.*[40] However, Dijkman *et al.*[19] specifically commented on their absence in their study of FSGS. The reason for this disparity is not evident, particularly as both groups studied FSGS recrudescent in transplanted kidneys.

Glomerular capsular adhesions

Many authors have commented on the prominence of capsular adhesions in IgAN,[7–9,41] so much so that they are included in the Oxford classification definition of segmental glomerulosclerosis,[7,8] but they have not previously been investigated systematically. As indicated in Table 1, in all three conditions studied, IgAN, SLE, and FSGS, adhesions were extremely common over areas of active glomerular lesions and scarring, as in other diseases. More importantly, however, in FSGS and IgAN they were frequently found over capillary loops without evidence of underlying lesions, suggesting an abnormality in the podocytes, rather than any underlying inflammatory or cicatricial lesion.

That these are real adhesions, and not simply an artifact of the biopsy procedure and processing, is attested to not only by their frequency compared with SLE biopsies, but also by the fact that in fortunate sections they can be seen to exert traction on the capsule (Figure 1a). They also create the pathway by which PECs penetrate onto the glomerular tuft and proliferate, parallel to that seen in FSGS.[19,25] These adhesions seem to provide the key to why IgAN so often behaves in a fashion parallel to FSGS.

Role of loss of podocytes in IgAN

Kriz and coworkers have developed a model of 'classical' FSGS, as summarized in the work of Kriz.[36] They demonstrated in a variety of animal models loss of podocytes with denuding of

the glomerular basement membrane and adherence to Bowman's capsule, which they regarded as the first 'committed step' in FSGS. Podocyte damage has also been shown to lead to capsular adhesions in experimental Masugi nephritis[42,43] and a model of glomerulosclerosis in which diphtheria toxin leads to destruction of podocytes in a dose-dependent fashion.[43] Our study has shown podocyte loss in IgAN in an elementary fashion, first by diminution and disappearance of podocyte staining, and second by the presence of free cells with podocyte markers in Bowman's space as has been previously reported in FSGS.[21,22] More quantitative approaches have been employed in IgAN, one by counting glomerular podocytes on biopsy, and the other by measuring cumulative excretion of podocytes in the urine,[44] both demonstrating that loss of podocytes is accompanied by disease progression. Yet, other studies have linked podocyte loss in IgAN to prognosis.[45]

Abnormal IgA1 as a pathogenetic factor in lesions of FSGS in IgAN

Although our immunohistochemistry results show strong parallels between IgAN and primary FSGS phenotypically, there are differences between the two. For example, one study has shown that in IgAN the extracellular domain of nephrin, a membrane protein, is virtually abolished whereas the intracellular domain remains intact, although in FSGS both domains seem intact.[31] This is consistent with the recent studies showing that aggregated IgA1 from IgAN patients reduces nephrin expression,[10,11] and IgA1 exerts other deleterious effects on podocytes as well.[13–15]

This makes it seem likely that abnormal IgA1 is at the root of the pathogenesis of podocytopathy in IgAN. Should this be the case, abnormal IgA1 would join a long list of pathogenetic factors, including heritable mutations of podocyte-specic proteins, drug toxicity, infections, and adaptive responses to reduced functioning renal mass, capable of producing the morphologic picture of FSGS.[29,46,47]

In conclusion, this study concludes that the podocyte and PEC alterations seen in IgAN are basically identical with those seen in primary FSGS, and it relates them to progression of disease. Podocyte changes begin focally, not previously appreciated, and with progression of lesions, podocytes are progressively lost and replaced by PECs, giving a morphologic basis for earlier morphologic and clinical studies showing podocyte loss. These changes form a theoretical basis on which recent *in vitro* observations on the role of IgA1 in podocyte damage might be related to the lesions seen on biopsy.

Table 2 | Definitions of Oxford criteria for IgAN and of the Columbia classification of FSGS as applied to IgAN

Oxford criteria for IgAN Term	Definition
Mesangial hypercellularity	> 4 to 5 mesangial cells/mesangial area in >50% of glomeruli
Segmental glomerulosclerosis	Any amount of the tuft involved in sclerosis, but not involving the entire tuft or the presence of an adhesion, in 1 glomeruli
Endocapillary hypercellularity	Hypercellularity due to increased number of cells within glomerular capillary lumina causing narrowing of the lumina, segmental or diffuse in at least one glomerulus, graded as present/absent from biopsy.
Tubular atrophy/ interstitial fibrosis	Percentage of cortical area involved by tubular atrophy or interstitial fibrosis, whichever is greater T0, 0–25%; T1, 26–50%; and T2, >50%

Columbia classification of FSGS in the context of IgAN Variant	Inclusion criteria	Exclusion criteria
FSGS, NOS	At least 1 glomerulus with segmental increase in matrix obliterating the capillary lumina, or segmental glomerular capillary collapse, *associated with hyalinosis lesions and/or epithelial hypertrophy/hyperplasia.*[a]	Exclude perihilar, cellular, tip, and collapsing variants.
Perihilar variant	At least one glomerulus with perihilar hyalinosis, with or without sclerosis. Also, >50% of glomeruli with segmental lesions must have perihilar sclerosis and/or hyalinosis.	Exclude cellular, tip, and collapsing variants.
Cellular variant	At least one glomerulus with segmental endocapillary hypercellularity occluding lumina, with or without foam cells and karyorrhexis, *with hyperplasia of overlying epithelium.*[b]	Exclude tip and collapsing variants.
Tip variant	At least 1 segmental lesion involving the tip domain (outer 25% of tuft next to origin of proximal tubule). The tubular pole must be identified in the defining lesion. The lesion must have either an adhesion or confluence of podocytes with parietal or tubular cells at the tubular lumen or neck. Tip lesion may be cellular or sclerosing.	Exclude collapsing variant and any perihilar sclerosis.
Collapsing variant	At least 1 glomerulus with segmental or global collapse and overlying podocyte hypertrophy and hyperplasia.	None

Abbreviations: FSGS, focal segmental glomerulosclerosis; IgAN, IgA nephropathy; NOS, not otherwise specified.
[a]Added to exclude segmental scars of possible non-FSGS origin.
[b]Added to exclude endocapillary proliferation of possible non-FSGS origin.

PATIENTS AND METHODS

Patients

All the adult (>18 years) patients diagnosed with IgAN from January 2002 to January 2008 in the Pathology Department of Georges Pompidou European Hospital were enrolled in this study. These biopsies came from four different medical centers. The diagnosis was based on the presence of predominant IgA and C3 deposits in the mesangium. Patients with SLE, Henoch–Schönlein purpura, chronic liver disease, or human immunodeficiency virus infection were excluded, as well as patients whose renal biopsy specimen contained <8 glomeruli, leaving a total of 128 patients. A wide variety of clinical and laboratory data were gathered, as reported in the companion article[17], but are not relevant to the present study focusing on immunohistochemical studies.

Renal histopathology

The renal biopsies were processed for light microscopy and direct immunofluorescence. Tissue for histology was fixed in acetic acid/formol/absolute alcohol fixative, paraffin embedded, and stained by standard techniques. Sections (6 μm) were stained for immunofluorescence study with fluorescein isothiocyanate-conjugated antibodies specific for human IgG, IgM, IgA, C1q, C3, κ and λ light chains, and fibrin (DAKO, Carpinteria, CA). All biopsy slides were re-reviewed by two senior pathologists (DN and GH) without knowledge of clinical outcomes. The biopsies were graded according to the Oxford classification of IgAN[7,8] (Table 2). Glomerular lesions were also evaluated in terms of possible lesions of FSGS, using the proposed 2004 classification of FSGS,[29] as adapted to the setting of IgAN (Table 2). The following types were distinguished: (1) FSGS, NOS (not otherwise specified); (2) FSGS, perihilar variant, with hyalinosis lesions at the glomerular hilus; (3) FSGS, cellular variant; (4) FSGS, tip lesion; and (5) FSGS, collapsing glomerulopathy. We also performed a comparative study of capsular adhesions in our IgAN cases, plus 100 biopsies of patients with severe lupus nephritis, from a previous study, and 26 cases of primary FSGS. For each biopsy, the different types of lesions in the underlying adherent lobule were noted (none; proliferative; necrotizing; scarring, with or without hyalinosis). Capsular adhesions were evaluated as follows: those points where adherence of the capillary to the capsule was accompanied by epithelial proliferation were considered definite adhesions, as were those where evident traction was exerted on the capsule. Other points of touching of the capillary to the capsule with evident open Bowman's space on either side were also considered positive. Where Bowman's space was obliterated, often relating to biopsy technique, this determination could not be made.

Immunohistochemical studies

Patient selection. A total of 15 biopsies were studied using immunohistochemistry. They were selected so as to display the entire spectrum of abnormalities on light microscopy, particularly cellular and collapsing variants, as well as cases with mild mesangial lesions with adhesions. They did not differ significantly from the overall group in terms of proteinuria or serum creatinine at diagnosis.

Immunohistochemistry markers

Podocytes were characterized using an anti-synaptopodin monoclonal antibody (mAb), clone G1D4 (Biogen and Biotechnik, Heidelberg, Germany), an anti-GLEPP-1 mAb, clone 5C11 (Biogenex, San Ramon, CA), an anti-human nephrin N-20 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), an anti-human VEGF-C mAb, clone C1 (Santa Cruz Biotechnology), and an anti-human WT-1 polyclonal antibody, C-19 (Santa Cruz Biotechnology) directed against the transcription factor WT-1 normally expressed in mature podocytes. PECs were characterized using an anti-human PAX2 polyclonal antibody (Zymed, San Francisco, CA), a transcription factor expressed in normal PECs and fetal immature podocytes, and lost in mature podocytes. Cytokeratins were labeled using CK-8 (Zymed, Carlsbad, CA) and C2562 mAb cocktail (Sigma Aldrich, Saint Quentin Fallavier, France) that was directed against nine cytokeratin types, including CK-8. Macrophagic epitopes were identified using an anti-CD68 mAb, clone PGM1 (Dakopatts, Glostrup, Denmark). Immunohistochemistry procedures were performed as described previously.[23] The different markers used are listed in Table 3.

Table 3 | Cellular markers used in this study

Podocyte plasma membrane proteins	
	Podocalyxin
	GLEPP-1
Slit-diaphragm-associated protein	
	Nephrin
Cytoskeleton-associated pedicel protein	
	Synaptopodin
Protein synthesized by podocyte	
	VEGF
Parietal epithelial cells	
	PAX2
Cytoskeleton intermediate filaments	
	Cytokeratins (CK-8, C2562, a mixture of 9 cytokeratins, including CK-8)[a]
Transcription factors	
	WT-1
Macrophage epitopes	
	CD68

Abbreviations: GLEPP-1, glomerular epithelial protein-1; PAX2, paired box gene 2; VEGF, vascular endothelial growth factor; WT-1, Wilms tumor protein-1.
[a]Variable staining of normal parietal epithelial cells (PECs); normal podocytes negative.

Statistical methods

Differences between percentages of capsular adhesions (Table 1) were evaluated using χ^2 tests. A value of $P<0.05$ was considered significant.

DISCLOSURE

All the authors declared no competing interest.

SUPPLEMENTARY MATERIAL

Figure S1. Normal glomerulus.
Figure S2. Early loss of podocyte staining.
Figure S3. Early loss of podocyte staining.
Figure S4. Focal capsular adhesion without loss of podocyte staining.
Figure S5. Capsular adhesions with loss of podocyte staining.
Figure S6. Negatively staining cell overlying synaptopodin positive podocytes.
Figure S7. Glomerulus with varied lesions.
Figure S8. Advanced lesion.
Figure S9. Advanced lesion stained for synaptopodin.
Figure S10. Advanced lesion stained for GLEPP-1.
Figure S11. Parietal epithelial cells (PECs).
Figure S12. WT-1 staining in active lesion.
Figure S13. WT-1 staining in advanced lesion.
Figure S14. Possible tip lesion.
Figure S15. Advanced lesion with variable CK-8 staining.
Figure S16. CD68 in PECs.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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