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Posttransplantation Relapse of FSGS Is Characterized by Glomerular Epithelial Cell Transdifferentiation

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Abstract. This study examined six cases of idiopathic nephrotic syndrome with primary lesions of focal segmental glomerulosclerosis (FSGS) that relapsed after renal transplantation. The glomerular lesions comprised the cellular, the collapsing, and the scar variants of FSGS and showed shedding of large round cells into Bowman's space and within the tubular lumens. Immunohistochemistry and confocal laser microscopy carried out on kidneys with FSGS relapse disclosed several phenomena. (*1*) Some podocytes that expressed podocalyxin, synaptopodin, and glomerular epithelial protein-1 were detached from the tuft and were free in the urinary space. (*2*) In the cellular variant, most podocytes had lost podocyte-specific epitopes (podocalyxin, synaptopodin, glomerular epithelial protein-1, Wilm's tumor protein-1, complement receptor-1, and vimentin). In the scar variant, these podocyte markers were absent from cobblestone-like epithelial cells and from pseudotubules. (*3*) Podocytes had acquired expression of various cytokeratins (CK; identified by the AE1/AE3, C2562, CK22, and AEL-KS2 monoclonal antibodies) that were not found in the podocytes of control glomeruli. Parietal epithelial cells expressed AE1/AE3 CK that were faintly, if ever, found on the parietal epithelial cells of normal glomeruli. (*4*) Numerous cells located at the periphery of the tuft or free in Bowman's space and within tubular lumens expressed macrophagic epitopes (identified by PGM1 [CD68], HAM56, and 25F9 monoclonal antibodies). These macrophage-like cells expressed the activation epitopes HLA-DR and CD16. (*5*) A number of these cells coexpressed podocalyxin + AE1/AE3 CK, podocalyxin + CD68, and CD68 + AE1/AE3. These findings suggest that in primary FSGS relapsing on transplanted kidneys, some "dysregulated" podocytes, occasionally some parietal epithelial cells, and possibly some tubular epithelial cells undergo a process of transdifferentiation. This process of transdifferentiation was especially striking in podocytes that acquired macrophagic and CK epitopes that are absent from normal adult and fetal podocytes.

It has been well established that podocyte injury is the first event in the natural history of focal segmental glomerulosclerosis (FSGS) (1,2). Recent publications have shown that in collapsing glomerulopathy, podocytes are "dysregulated" (3,4) and that large round free cells that may be podocytes migrate into Bowman's space and tubular lumens (3,5–7).

Relapse of primary FSGS on transplanted kidneys presents the opportunity for one to study FSGS with the assurance of dealing with patients with the primary rather than secondary form of FSGS. It thus offers a privileged model for studying the early and later events that characterize this glomerulopathy, which has no equivalent in experimental animals (8). The present study was based on six cases of primary nephrotic FSGS that relapsed after renal transplantation and allowed immunohistochemical phenotypic identification of glomerular cells and of free migrating cells. Some were found to undergo phenotypic changes suggesting transdifferentiation (3,9–11), a phenomenon that has been defined as "a dynamic interplay between positive and negative regulatory molecules" in cells, such that their state of differentiation is characterized by a certain plasticity that may be molded by the environment (12).

Materials and Methods

Patients and Controls

Demographic and clinical characteristics of the six patients studied are detailed in Table 1. In total, 13 renal biopsies were performed, and 5 failing transplanted kidneys ultimately were removed surgically for loss of function after FSGS relapse. Standard microscopy, immunohistochemistry, and confocal laser microscopy were carried out on the renal biopsies and on blocks from the transplanted kidneys as detailed below.

Controls consisted of sound parts of three kidneys removed for renal carcinoma in patients with no proteinuria and of renal biopsies from three nephrotic patients with minimal change disease (MCD). One sample was a biopsy of the donor kidney in patient 1, minutes after unclamping the renal vessels. Three normal fetal kidneys (30 wk) were also used as controls, with glomerulogenesis spanning renal vesicle, S-shaped, capillary, and maturing stages.

Technical Methods

Standard Pathology. The renal samples were processed for light microscopy and immunofluorescence (IF) using standard techniques (3).

Immunohistochemistry. Podocyte phenotypes were characterized using an anti-human podocalyxin monoclonal antibody (mAb;

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Table 1. Main demographic and clinical features of six patients with primary nephrotic FSGS that relapsed after transplantation[a]

Patient No.	Gender	Age at Time of Biopsy	Ethnic Origin	Primary Renal Disease (Yr)	Year	Evolution	Indication for Renal Histology	Time between Tx and Histology	Serum Creatinine (mg/dl)	Proteinuria (g/L)	Serum Albumin (g/dl)
1	M	34	B (Africa)	Minimal change disease (1968), end-stage FSGS (1973)	1973	Early posttransplantation relapse of heavy proteinuria	Nephrotic syndrome	32 d	3.0	26	3.2
					1975	Stable	Nephrotic syndrome	50 d	2.0	2–6	3.1
					1975	Persistent proteinuria	Persistent proteinuria	13 mo	2	2	3.8
					1975	Stable	Systematic RB at 2 yr	2 yr	1.7	0.4	3.9
					1977	Stable	Systematic RB at 4 yr	4 yr	1.7	0.5	3.6
2	M	31	B (French Caribbean)	End-stage FSGS (1977)	1980	Profuse proteinuria from day 22 and development to ESRD	Transplant nephrectomy	137 d	HD	16	2
3	M	23	W (France)	FSGS (1984)	1984	Nephrotic syndrome, renal insufficiency	Relapse of nephrotic syndrome	6 mo	3.3	16	2.2
					1986	End-stage renal failure	Transplant nephrectomy	18 mo	9		
		30			1992	Second transplantation (1992)	Anuria	22 d	Anuric		
					1992	Anuria	Persistent anuria	42 d	Anuric		
4	M	35	W (France)	FSGS (1988)	1992	Anuria	Anuria	19 d	Anuric	8	3.2
					1992	HD until day 70	Profuse proteinuria	68 d	HD	12	1.9
					1993	ESRD	Transplant nephrectomy	11 mo	HD	19	1.6
5	F	50	W (France)	End-stage FSGS, severe vascular lesions (1992)	1992	Poor renal function	Poor renal function	20 d	2	0.2	ND
					1996	Return to HD after 4 yr	Increasing renal insufficiency	4 yr	3.9	3	3.2
					1997	End-stage renal failure	Transplant nephrectomy	4.5 yr	HD		
6	M	41	W (France)	FSGS in 1/5 glomeruli (1975)	1998	Surgical complication	Transplant nephrectomy	27 d	Heavy proteinuria for 3 d followed by anuria		

[a] FSGS, focal segmental glomerulosclerosis; B, black; W, white; Tx, transplantation; ESRD, end-stage renal disease; HD, hemodialysis; RB, renal biopsy.

MLC48A8, a gift of Pierre Ronco, M.D., INSERM U 489, Hôpital Tenon, Paris, France), an antisynaptopodin mAb, clone G1D4 (Progen Biotechnik, Heidelberg, Germany), an anti– glomerular epithelial protein-1 (anti–GLEPP-1) mAb (Biogenex, San Ramon, CA), an anti-C3b receptor mAb (complement receptor-1 [CR-1], clone J3D3) (13,14) (a gift of Michel Kazatchkine, M.D., INSERM U 430, Hôpital Broussais, Paris, France), an anti–Wilm's tumor protein-1 (WT-1) polyclonal antibody (pAb; C19, Santa Cruz Biotechnology, Santa Cruz, CA), and an anti-vimentin mAb (Dakopatts, Trappes, France).

Cytokeratin (CK) polypeptides were labeled by AE1/AE3 (Dakopatts), C2562 (Sigma Aldrich Chimie, St. Quentin Fallavier, France), CK22 (Biomeda, Foster City, CA), and AEL-KS2 (Argen-Biosoft, Varhilles, France) mAb. Also used were mAb with different specificities for macrophage-associated epitopes: an anti-CD68 mAb, clone PGM1 (Dakopatts); an anti-macrophage HAM56 mAb (Dakopatts); and an anti-86KD protein mAb, clone 25F9 (Valbiotech, Paris, France). Anti–HLA-DR (CR3/43; Dakopatts) and anti-CD16 (GRM1) mAb (Harlan Sera-Lab, Loughborough, UK) were used as activation markers of macrophagic cells (15).

Immunostaining was performed by the ABC alkaline phosphatase (Vector Laboratories, Burlingame, CA) or peroxidase procedure as follows: (*1*) digestion by pronase (anti–CR-1, PGM1, HAM 56, AE1/AE3, C 2562, CK22, and AEL-KS2 anti-CK mAb) or microwave processing for 5 min in ethylenediaminetetraacetate buffer (pH 8), 0.1 M (WT-1, synaptopodin, and GLEPP-1), (*2*) blocking with normal horse or goat serum diluted 1:20 for 20 min, (*3*) 30-min incubation with the primary anti-human mAb or pAb, (*4*) after washing in TRIS buffer (pH 7.4), 30-min incubation with biotinylated secondary antibody, a horse anti-mouse Ab to detect the mAb or a goat anti-rabbit Ab to detect the pAb, and (*5*) after washing, 30-min incubation with a preformed avidin-biotinylated alkaline phosphatase or streptavidin peroxidase complex. The indicator system was developed with Vector Red, aminoethylcarbazol, or diaminobenzidine substrates. Slides were counterstained with hematoxylin.

Negative procedures were (*1*) replacement of the primary mAb with antibody-diluting buffer; (*2*) replacement of the primary mAb by anticytomegalovirus mAb, clone E13 (Clonatec Biosoft, Paris, France) or replacement of the primary pAb by an anti-von Willebrand factor pAb (Dakopatts); and (*3*) replacement of the primary mAb by normal mouse serum or replacement of the primary pAb by normal rabbit serum.

Study of Cell Apoptosis and Proliferation. The *in situ* DNA nick end labeling (TUNEL) technique (16) was used to detect apoptosis. Paraffin-embedded sections were processed in a microwave oven (750 W) for 5 min, three times in citrate buffer (2 × SSC, pH 6.2). A TUNEL kit (ApoTag, Oncor, Gaithersburg, MD) was used according to the standard procedure recommended by the manufacturer. The procedures were revealed using peroxidase and aminoethylcarbazol substrate.

Cell proliferation was assessed by proliferating cell nuclear antigen (PCNA) immunohistochemistry (17). It was carried out using the PC 10 mAb (Dakopatts), diluted 1:200. Antigen retrieval was done in a microwave oven for 5 min in ethylenediaminetetraacetate buffer 0.1 M (pH 8). The intensity of microwave oven treatment necessary for specific labeling without background or nonspecific labeling was obtained in positive control tissues, fixed and processed in the same conditions, *i.e.*, intestinal epithelium for PCNA and reactive lymph nodes for TUNEL.

Cell Counts. Quantitative assessment of the glomerular lesions and of cell labeling was performed at ×16 magnification by scanning the whole renal tissue sections. The total number of glomeruli showing each type of lesion (cellular, sclerotic, ischemic, and obsolescent) was counted. Regarding cell labeling, (TUNEL, PCNA, and CD68), the total number of positive cells was counted in Bowman's space and along the external aspect of the tuft. Cell density was obtained by dividing the total cell number by the total number of glomeruli. The total number of positive free cells that drifted in the tubular lumens was counted. To compare samples of variable sizes, this number was divided by the total number of glomeruli.

Confocal Laser Microscopy. Confocal laser microscopy was used to specify epitope colocalization on three transplanted kidneys removed surgically. For this purpose, in each case, three sets of slides were tagged with one antibody, or with another antibody, or with both antibodies. Digitized images captured with one or the other antibody made a clear distinction between the two epitopes in each pair.

For double-labeled IF, deparaffinized sections were first incubated with a primary mAb (AE1/AE3 anti-CK, or anti-CD68 PGM1, or anti-podocalyxin) and then with the biotinylated anti-mouse IgG as described above. Subsequently, the sections were incubated with streptavidin-cyanin-2 (Amersham, Les Ulis, France) at a dilution of 1:400 for 30 min. After rinsing in phosphate-buffered saline (PBS), the sections were incubated with the complementary primary antibody (AE1/AE3 anti-CK, or anti-CD68 PGM1, or anti-podocalyxin mAb) so that the three following couples were obtained: anti-podocalyxin + AE1/AE3 anti-CK, anti-podocalyxin + anti-CD68, and AE1/AE3, anti CK + anti-CD68. After the sections were rinsed in PBS buffer, they were incubated with an anti-mouse IgG antibody tagged with cyanin-3 (Amersham), at a dilution of 1:400 for 30 min. The sections were rinsed in PBS buffer and mounted in Immu-Mount (Dakopatts). They were examined using a confocal microscope Leica TCS SP (Leica Microsystems, Heidelberg, Germany) powered with an argon-krypton laser beam, with excitation at 488 nm for cyanin-2 (detection, 500 to 550 nm) and at 568 nm for cyanin-3 (detection, 580 to 680 nm).

Morphology: Definitions and Cell Markers

Glomerular Lesions. The lesions observed by light microscopy fell into four subsets defined as follows.

Cellular Variant. This variant was segmental and occasionally global in the tuft. Some podocytes were swollen and vacuolated and contained protein reabsorption droplets. Others appeared as large round cells that contained a voluminous nucleus, occasionally multinucleated. Pseudocrescents adjacent to the tuft and not to Bowman's capsule were composed of large cells that were not surrounded by extracellular matrix. The capillaries underlying the podocyte lesions were often collapsed.

Synechiae between the tuft and Bowman's capsule were rare but, when present, helped distinguish this variant from the typical appearance of "collapsing glomerulopathy." Detached large round cells could be found free in Bowman's space and in the tubular lumens. In noncollapsed loops, foam cells, hyaline deposits, and cell debris occasionally were found. Part or all of the tuft was covered by an alignment of cobblestone-like cells.

Scar Variant. Sclerosis appeared as a localized scar of the tuft surrounded by cuboid cobblestone epithelial cells that differed from the swollen or large round cells observed in cell FSGS. The localized scars within the glomerular tuft were not always associated with hyalinosis and/or with adhesions to Bowman's capsule. Apart from synechiae, scars were covered by an alignment of cobblestones that adhered to the underlying glomerular basement membrane (GBM) and to that of Bowman's capsule. The space formed between two adhesions was lined by a layer of continuous cells with no demarcation between parietal and visceral epithelium, yielding the appearance of a pseudotubule. Once again, large round cells could be found free in Bowman's space.

Ischemic Glomeruli. Glomerular ischemia was characterized by retraction toward the hilum of a paucicellular tuft, with widening of Bowman's space and thickening and splitting of Bowman's capsule. Epithelial cells that covered the tuft were rare and did not show hypertrophy, vacuolization, or cobblestone alignment. Detached cells were not present in Bowman's space.

Glomerular Obsolescence. The diagnosis of end-stage FSGS reasonably still could be made on globally sclerotic glomeruli given the presence of hyaline and lipid deposits within the tuft and of some pseudotubules that still partitioned Bowman's space.

Immunohistochemical Markers. *Podocytes.* Podocytes were identified (Table 2) by six epitopes: podocalyxin (18), synaptopodin (19), GLEPP-1 (20,21), WT-1 (22,23), CR-1 (13,14), and vimentin (18,24,25).

CK Markers. CK polypeptides were labeled by AE1/AE3 C2562 (used as a marker of parietal epithelial cells [PEC]) (26), CK22, and AEL-KS2 mAb.

Macrophages. Macrophage-associated epitopes studied were an anti-CD68 mAb, clone PGM1; an anti-macrophage HAM56 mAb for the detection of monocytes and macrophages; and an anti-86KD protein mAb, clone 25F9 that recognizes mature macrophages but not monocytes (27). Anti-HLA DP-DQ-DR (CR3/43) and anti-CD16 (GRM1) mAb were used as activation markers of macrophagic cells (15).

Results

Clinical

In all six patients (Table 1), the primary renal disease was corticosteroid-resistant nephrotic FSGS. In patient 1, a first renal biopsy was interpreted as MCD. The subsequent evolution was development to end-stage renal disease. Renal biopsy disclosed a majority of obsolescent glomeruli with some exhibiting remaining changes of advanced FSGS. This patient followed a benign course after transplantation. Despite early relapse of nephrotic syndrome, renal function remained compatible with medical treatment and at the time when renal biopsies were carried out between 13 d and 4 yr after transplantation, renal function was stable with creatinine levels of 1.7 mg/dl. In the five other patients, pretransplantation biopsies showed FSGS. Patients 2 and 5 followed a rapid course to end-stage renal disease. In patients 2, 3, 4, and 5, relapse of FSGS led to transplantectomy at 4.6 mo, 18 mo, 11 mo, and 4.5 yr, respectively. In patient 6, diuresis resumed after transplantation, with abundant proteinuria. Transplantectomy on day 27 was necessitated by a surgical complication. Patient 2 underwent two transplantations with relapse of FSGS on each allograft.

Light Microscopy

The changing distribution of the different types of glomerular lesions over the course of successive biopsies and nephrectomies is shown in Table 2. A majority of specimens showed a mixture of the cellular variant of FSGS, with segmental or global distribution in the glomerular tufts, the scar-variant of

Table 2. Distribution of FSGS lesions on posttransplantation renal histology in six patients with relapse after transplantation

Patient No.	Interval Since Tx	Total No. of Glomeruli	Cell FSGS	Scar FSGS	Ischemic Glomeruli	Obsolescent Glomeruli	Normal Glomeruli
1							
posttransplant RB	5 min	5	0	0	0	0	5
posttransplant RB	32 d	7	0	0	0	0	7
posttransplant RB	50 d	14	2	0	0	0	12
posttransplant RB	13 mo	17	2	3	0	1	11
posttransplant RB	2 yr	9	0	2	0	0	7
posttransplant RB	4 yr	28	0	2	1	2	23
2							
transplant nephrectomy	137 d	46	43	3	0	0	0
3							
first transplant RB	6 mo	15	5	0	0	0	10
transplant nephrectomy	18 mo	123	6	34	48	32	3
second transplant RB	22 d	19	0	4	2	4	9
second transplant RB	42 d	23	0	2	2	4	15
4							
posttransplant RB	19 d	25	0	0	3	3	19
posttransplant RB	68 d	33	6	0	0	1	26
transplant nephrectomy	11 mo	166	4	51	59	31	21
5							
posttransplant RB	20 d	16	0	0	2	0	14
posttransplant RB	4 yr	16	4	3	2	3	4
transplant nephrectomy	4.5 yr	141	28	4	23	29	57
6							
transplant nephrectomy	27 d	279	44	15	7	16	197

FSGS, along with ischemic, obsolescent, and normal glomeruli. The interpretation of the lesions was not biased by histologic changes suggesting rejection. Of note, silver methenamine staining did not show gaps in the glomerular or tubular basement membranes or in Bowman's capsule. In the seven controls, the renal tissue was normal.

Immunohistochemistry

Controls. Podocyte markers used were expressed by fetal podocytes from the stage of S-shaped or capillary loop stage onward, in normal adult kidneys and in kidneys with MCD (Table 3). In the fetus, PEC also expressed podocalyxin and GLEPP-1. In adult control kidneys, occasional PEC in some

Table 3. Antibodies used for identifying the glomerular cell epitopes in normal control and fetal kidneys[a]

Antibody	Specificity	Expression in Normal Adult Kidney	Expression in Fetal Kidney
Podocyte markers			
MLC4818	Podocalyxin	Podocytes, endothelial cells	Podocytes and PEC as of S-shaped stage, endothelial cells
G1D4	Synaptopodin	Podocytes	Podocytes as of capillary loop stage
anti-GLEPP-1	GLEPP-1	Podocytes, some PEC	Podocytes and PEC as of S-shaped stage
C19 (pAb)	WT-1	Podocytes	Restricted to podocytes at capillary loop stage
J3D3	CR-1	Podocytes	ND
V9	Vimentin	Podocytes, endothelial cells (faintly), PEC (faintly), myocytes	ND
Cytokeratin markers			
AE1/AE3	CK 1-6, 10, 15, 19	None on podocytes, none or very rare on PEC, strongly on distal tubule, collecting ducts, thin limbs of Henle's loop	None on podocytes, none on PEC, + on distal tubules
C 2562	CK 1, 4-6, 8, 10, 13, 18, 19	None on podocytes, numerous PEC, proximal tubule, thin limbs of Henle's loop, distal tubule, collecting duct	None on podocytes, very rarely on PEC, proximal tubule, thin limbs of Henle's loop, distal tubule
CK 22	CK 1-8, 10, 11, 13, 15-19	None on podocytes, numerous PEC, proximal tubule, thin limbs of Henle's loop, distal tubule, collecting duct	None on podocytes, very rarely on PEC, proximal tubule, thin limbs of Henle's loop, distal tubule
AEL-KS 2	CK 1-19	None on podocytes, numerous PEC, proximal tubule, thin limbs of Henle's loop, distal tubule, collecting duct	None on podocytes, + on sparse PEC, proximal tubule, thin limbs of Henle's loop, distal tubule
Macrophagic markers			
PGM1	CD68 (monocyte/macrophage)	Rare circulating and interstitial cells	Few, interstitial
HAM56	Monocyte/macrophage	Rare circulating and interstitial cells	Few, interstitial
25F9	86KD protein, monocyte/macrophage	None	Few, interstitial
CR3/43	HLA-DP DQ DR	Rare circulating and interstitial cells, glomerular and peritubular endothelial cells	None
GRM 1	CD16, activated macrophages, NK	None	None

[a] PEC, parietal epithelial cells; pAb, polyclonal antibody; CK, cytokeratin(s); ND, not determined.

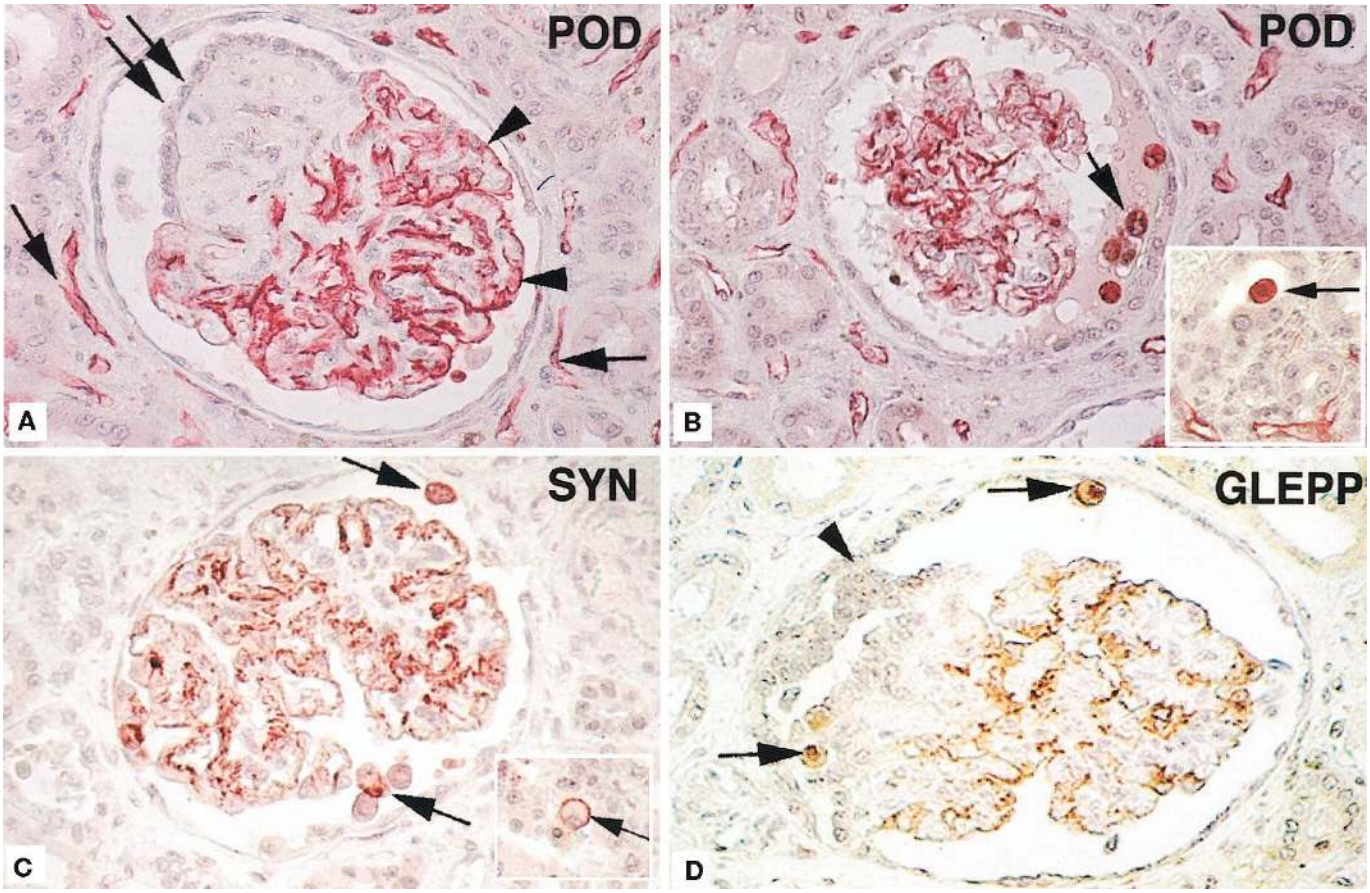

Figure 1. Patient 6: Transplant nephrectomy 27 d after transplantation. (A) Antipodocalyxin (anti-POD) monoclonal antibody (mAb) tags vascular endothelium (arrows) and podocytes in the preserved lobules (arrowheads). In the lobule changed into a segmental scar, the cobblestone-like epithelial cells, presumably podocytes (double arrow), are podocalyxin negative. In this lobule, the capillaries have disappeared and no endothelial cells are labeled by the anti-podocalyxin mAb. (B) Antipodocalyxin mAb. The glomerular tuft is collapsed. Some detached large round cells (arrow) that are free in Bowman's space are still tagged by the antibody. This identifies these cells as podocytes. A similarly tagged cell (arrow) is found free in a tubular lumen (inset). Note that the podocytes attached to the tufts and the interstitial capillary endothelium are tagged by the antipodocalyxin mAb. (C) Antisynaptopodin mAb. The glomerular tuft is fairly preserved. Free large round cells in Bowman's space (arrows) and in a tubular lumen (arrow) (box) are labeled, showing their podocyte origin. Note the normal positivity of podocytes attached to the tuft. (D) Anti–glomerular epithelial protein-1 (anti–GLEPP-1) mAb. Free large round cells in Bowman's space (arrows) are positive for GLEPP-1, which demonstrates their podocyte origin. Hyperplastic cells forming the pseudotubule structure (arrowhead) with a synechia are negative. Note that the normal podocytes surrounding preserved tuft lobules are positive. Magnifications: ×200 in A through D; ×150 in B inset.

glomeruli expressed GLEPP-1, usually at the hilar reflection. The mAb used to characterize macrophagic cells did not tag any titular nephron cells or cells free in the urinary space.

In the fetus, the anti-CK mAb stained neither podocytes nor PEC, with the exception of very rare PEC that were labeled with C2562, CK22, and AEL-KS2. The tubular cells were labeled as in adult controls. In adult controls, the AE1/AE3 mAb labeled no podocytes but did faintly label very rare PEC. The CK22, AELSK2, and C2562 mAb were uniformly negative on podocytes. They tagged a number of PEC in a mostly segmental fashion. AE1/AE3 labeled only the distal tubules. The other mAb strongly tagged collecting ducts, distal tubules, and the thin limbs of Henle's loops. The proximal tubules were also, although less frankly, positive. The anti-CK mAb thus could be arranged in order of progressively broader positivity, both in the fetal and adult kidneys, as shown in Table 3: AE1/AE3, CK22, C2562, AEL-KS2. Note that although PEC stained progressively more extensively with successive anti-CK mAb, normal podocytes were negative for all.

On TUNEL and PCNA preparations, no cell nuclei were labeled in the glomerular cells or in the cells found within the tubular lumens. Regarding TUNEL, in lymph node sections (control tissue), positive nuclei were observed only in reactive germinal centers. Concerning PCNA, in intestinal epithelium (control tissue), positive nuclei were observed in the proliferative part of this tissue, *i.e.*, in the bottom and the lower part of Lieberkühn's crypts.

Patients with Relapse of FSGS on Transplanted Kidneys. *Podocyte Markers: Podocalyxin, Synaptopodin, and GLEPP-1.* The results were identical with the three markers. Normal or large

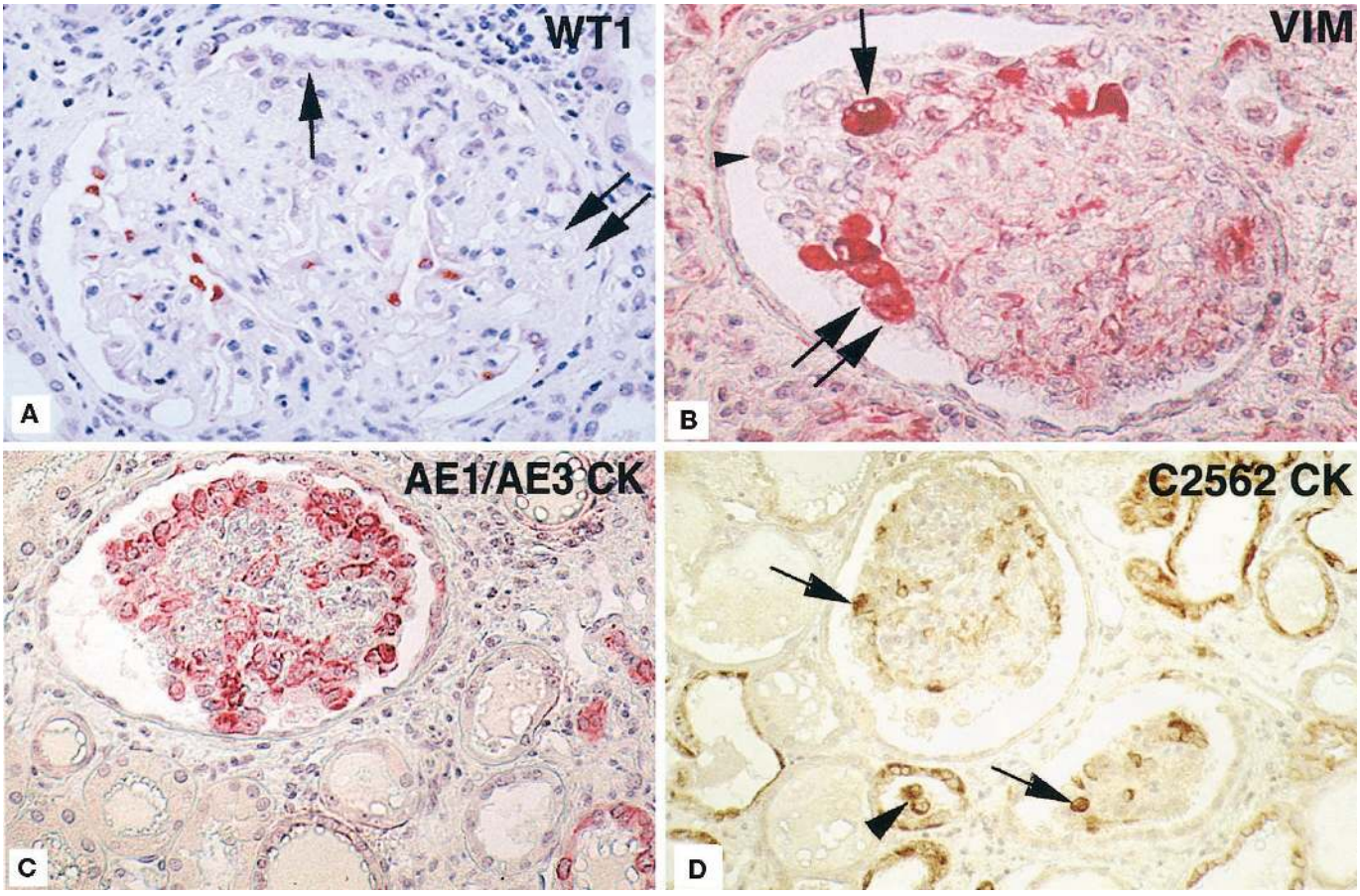

Figure 2. Transplant nephrectomy. (A) Patient 4, 11 mo after transplantation. Wilm's tumor protein-1 (WT-1) tags podocyte nuclei in preserved lobules, whereas cobblestone-like cells (arrow) and synechiae (double arrows) are not labeled. (B) Patient 6, 27 d after transplantation. Antivimentin mAb. This glomerulus is undergoing a severe process of cell focal segmental glomerulosclerosis (FSGS) with marked podocyte hyperplasia (arrowhead). Some voluminous round cells still express vimentin. This is especially true for those still attached to the outer aspect of the tuft (double arrow) but also for some detached cells (arrow) within a pseudocrescentic cluster of vimentin negative cells. (C and D) Patient 2, 137 d after transplantation. (C) AE1/AE3 anticytokeratin (anti-CK) mAb. All podocytes crowding at the periphery of the tuft in this glomerulus have acquired an intense labeling for these CK. Note that some parietal epithelial cells of Bowman's capsule are faintly labeled. Some tubular epithelial cells are also labeled, which is normal in the distal tubule (lower right quadrant). (D) C2562 anti-CK mAb. In two glomeruli, some podocytes (arrows) still adhering to the tuft have acquired positivity for these CK. Note that the parietal epithelial cells (PEC) are negative and that no synechia is observed. The atrophic tubular epithelium is inconstantly labeled. Two positive cells (arrowhead) are free in the tubular lumen. Magnifications: ×250 in A and B; ×150 in C and D.

vacuolated podocytes attached to the GBM were labeled. The cobblestones were negative (Figure 1A). In the cellular variants of FSGS, most podocytes were not tagged. Some large round cells that were attached to the tuft or free in Bowman's space (Figure 1, B through D) and in the tubular lumens (Figure 1, B and C) were strongly positive. Very few PEC were also labeled with podocalyxin and GLEPP-1. Podocalyxin was expressed on the glomerular and extraglomerular endothelial cells.

WT-1. WT-1 nuclear tagging was negative on swollen and vacuolated podocytes, on the cobblestone-like cells (Figure 2A), and on the large round cells that were free in Bowman's space and in tubular lumens.

Vimentin. Podocytes that were still attached to the tuft were vimentin positive. Cobblestone-like cells were negative. Some of the hypertrophic-hyperplastic large round cells that formed part of the pseudocrescent that was still in contact with the GBM or detached from the tuft strongly expressed vimentin (Figure 2B). A few parietal epithelial cells were faintly labeled. Some epithelial tubular cells and arterial myocytes were tagged.

CR-1. Swollen vacuolated podocytes that kept contact with the GBM were CR-1 positive. Cobblestone-like cells that lined the glomerular tuft and detached large round cells had lost this labeling (not shown).

CK Labeling. Podocytes and PEC were often tagged by these mAb in FSGS kidneys. All of the anti-CK mAb labeled diffusely (Figure 2C) or sparsely (Figure 2D) cells (presumably podocytes) that clumped at the periphery of the tuft in retracted glomeruli, even where no synechiae were found and in areas where PEC did not express CK. Cobblestones that surrounded the synechiae and formed pseudotubules often were tagged by the anti-CK mAb (Figure 3A). Nevertheless, these cobble-

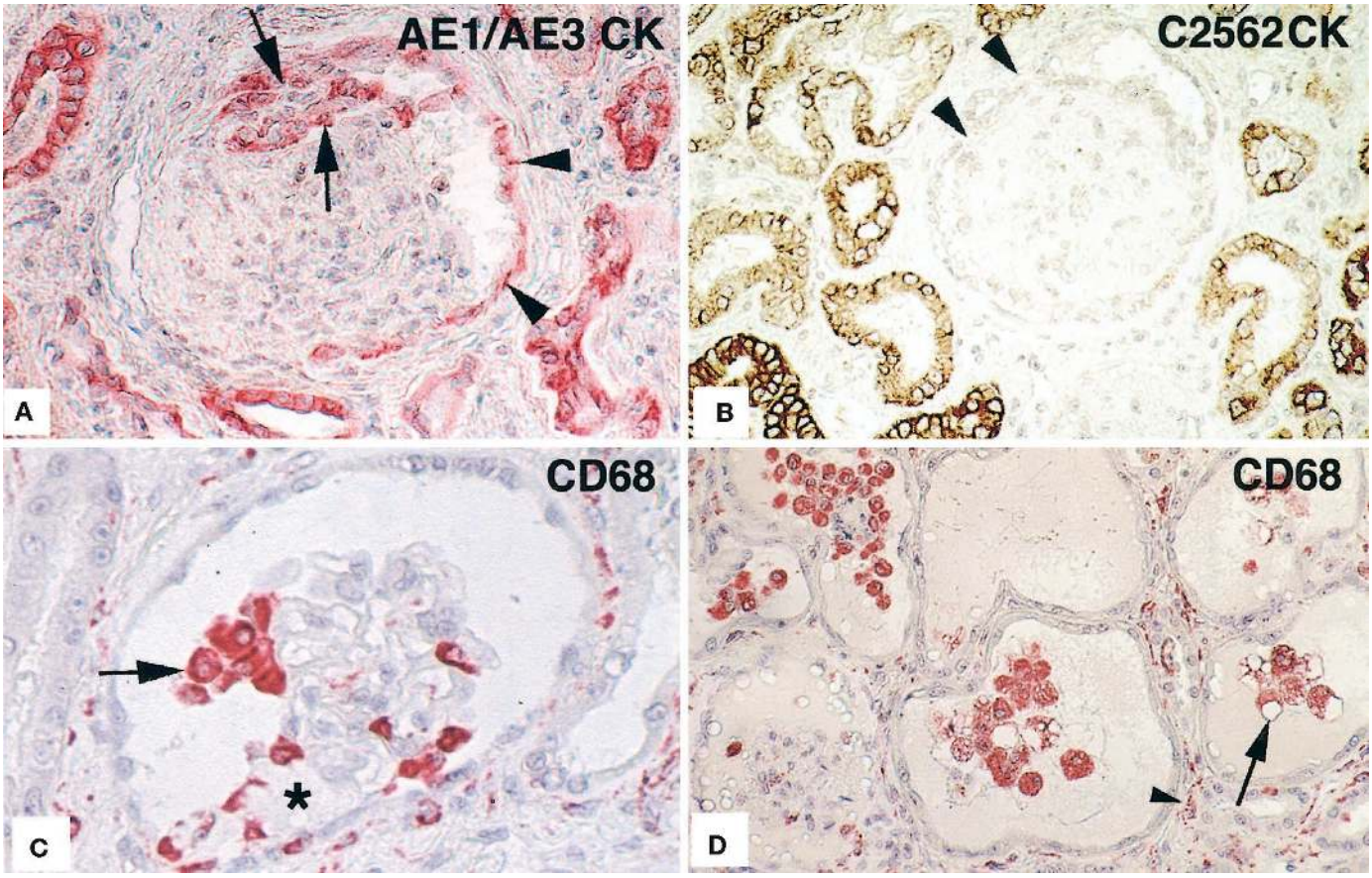

Figure 3. Patient 1: Transplant biopsy at 13 mo. (A) AE1/AE3 anti-CK mAb. The cells lining the pseudotubules between synechiae are AE1/AE3 positive (arrows). This is also true for PEC of Bowman's capsule (arrowheads). The atrophic tubules are positive. (B and C) Patient 6, transplant nephrectomy 27 d after transplantation. (B) C2562 anti-CK mAb. In this scar FSGS lesion, all of the glomerular cells are negative, including the pseudotubule lining between two synechiae. This is in contrast with A. Most of the tubular epithelial cells are strongly labeled, which is normal. (C) PGM1, an anti-CD68 mAb, a marker of the monocyte/macrophage lineage, strongly tags hyperplastic large cells on the external aspect of the tuft (arrow). Positive cells are located around a scar lesion adherent to Bowman's capsule (*). (D) Patient 5, nephrectomy of a failing kidney 4.5 yr after transplantation. PGM1, an anti-CD68 mAb. Numerous large round cells in the tubular lumens are tagged with the PGM1 mAb. Some contain voluminous clear vacuoles (arrow). PGM1-positive cells are present in the interstitium (arrowhead). There is no evidence of macrophage trafficking from the interstitium to within the tubules. Magnifications: ×150 in A, B, and D; ×200 in C.

stones occasionally were not labeled (Figure 3B) by those mAb (C2562, CK22, and AEL-KS2) that usually strongly tag PEC. The large round cells found in Bowman's space and in tubular lumens rarely expressed CK epitopes (Figure 2D).

Macrophagic Epitope Labeling. PGM1 (Figure 3, C and D), HAM56 (Figure 4A), and CR3/43 (Figure 4B) labeled the same cells. Cells that were tagged with these mAb were especially numerous in cell FSGS. These large round cells were aligned at the periphery of the glomerular tuft and free in Bowman's space and in the tubular lumens (Figure 3D). In contrast, cobblestones were not labeled. Spindle-shaped interstitial cells were labeled, without evidence of trafficking through tubular or GBM. Some sparse PEC also expressed the macrophagic CD68 epitope.

The number of glomerular CD68-positive cells varied from case to case, with a density from 0 in paucicellular forms to 4.31 in highly cellular variants. The same was true regarding the tubular lumens, in which the density was 0.12 to 21.52. In the three cases in which early biopsies were available, no positive cells were observed before day 20 posttransplantation. In the six cases studied, CD68-positive cells were detected in the tubular lumens at least in one or several tissue samples during the follow-up period. Regarding glomeruli, CD68-positive cells were observed in five of the six cases.

A marker of macrophage maturation, 25F9, labeled some tubular cells, whereas others were entirely 25F9 negative (Figure 4C). Within some lumens, 25F9 labeled large round cells, some of which were apparently free, whereas others seemed to replace some of the tubular epithelial cells (Figure 4D).

Anti-CD16, another marker of activated macrophages, also tagged a few large round cells within the tubular lumens (Figure 4E). 25F9 and CD16 were invariably negative on interstitial infiltrates.

Confocal Laser Microscopy

Double IF labeling with the AE1/AE3 anti-CK mAb and the anti-human podocalyxin mAb showed that some free cells that drifted in tubular lumens coexpressed CK and podocalyxin

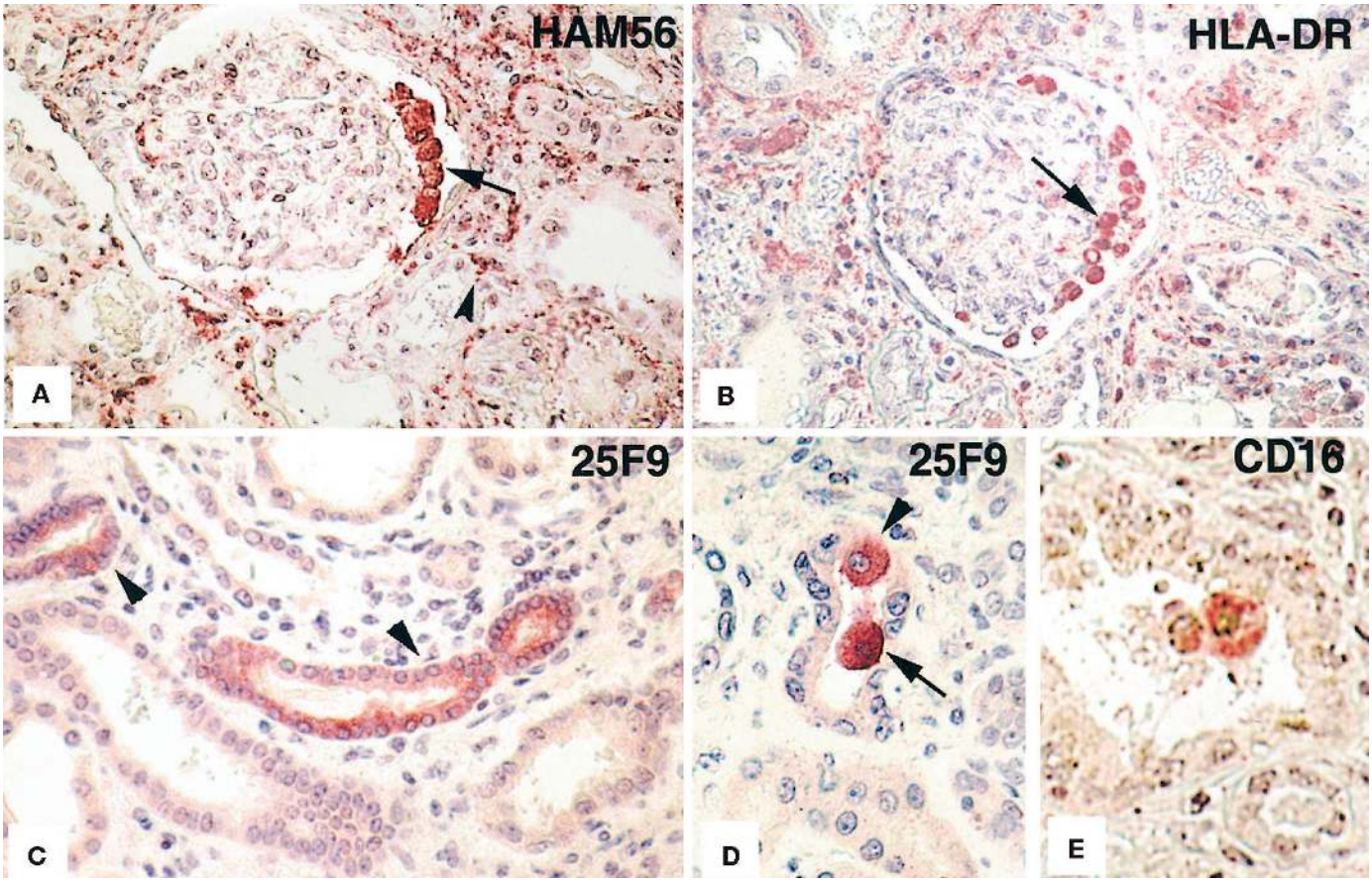

Figure 4. Transplant nephrectomy. (A and B) Patient 2, 137 d after transplantation. (A) HAM56, another marker of the human macrophage, strongly labels large round cells aligned at the periphery of the glomerular tuft (arrow). Note the numerous small spindle-shaped cells in the interstitium (arrowhead). (B) CRD/43, an mAb specific for HLA-DR, labels a row of large round cells at the periphery of the tuft (arrow), indicating that these cells can be considered activated. (C through E) Patient 5, 4.5 yr after transplantation. (C) 25F9 mAb, a marker of macrophage maturation, is detected on all cells of some tubules (arrowheads), whereas other tubules do not exhibit such transdifferentiation. Interstitial inflammatory cells are not tagged by 25F9. (D) 25F9 mAb labels two large round cells in a tubular lumen. One (arrow) is apparently free in the lumen. The other (arrowhead) seems to be inserted between tubular cells or, alternatively, could be a transdifferentiated tubular cell. (E) Anti-CD16 mAb, a marker of activated macrophages, tags large round cells within the tubular lumens. The interstitial inflammatory cells are CD16 negative. Magnifications: ×120 in A and B; ×200 in C and D; ×250 in E.

(Figure 5A). No such coexpression was found on glomerular cells.

Double IF labeling with the antipodocalyxin and the PGM1 (anti-CD68) mAb showed that some cells that were free in the tubular lumens (Figure 5B) and in Bowman's space (Figure 5C) were tagged by PGM1 but also that some of these cells were tagged by both antibodies, indicating co-localization of podocyte and macrophagic epitopes.

Double IF labeling with the AE1/AE3 (anti-CK) and the PGM1 (anti-CD68) mAb showed that some podocytes that were still attached to the tuft strongly expressed AE1/AE3 CK, whereas others, whether attached to the tuft or free in Bowman's space, expressed CD68 (Figure 6A). However, it was also apparent that podocytes that were still attached to the glomerular tuft coexpressed AE1/AE3 CK and CD68 (Figure 6B). A few Bowman's capsule PEC also coexpressed AE1/AE3 CK and CD68. The flow of cells migrating from Bowman's space into the glomerular outlet and progressing along the proximal tubule strongly expressed CD68 (Figure 6C). In other tubular sections, some of the free cells coexpressed AE1/AE3 CK and CD68 (Figure 6D).

Assessment of Apoptosis and Proliferation

Apoptosis (TUNEL). No positive cells were found in the adult control kidneys. In the patients' kidneys, the density of intratubular cell nuclei labeling was variable (density 0 to 0.85). Very rare positive cell nuclei were found in some Bowman's spaces (Figure 7) and on the external aspect of the glomerular tufts (density 0 to 0.20). Overall, apoptosis was inconstantly observed as compared with the number of CD68-positive cells, as shown by assessing the following ratio: Density of TUNEL-positive cells:Density of CD68-positive cells, respectively in the glomeruli and within the tubular lumens, a ratio that yielded 0 to 0.08 and of 0 to 0.15, respectively.

Cell Proliferating Activity (PCNA). In control kidneys, very rare tubular epithelial cell nuclei were positive, and no

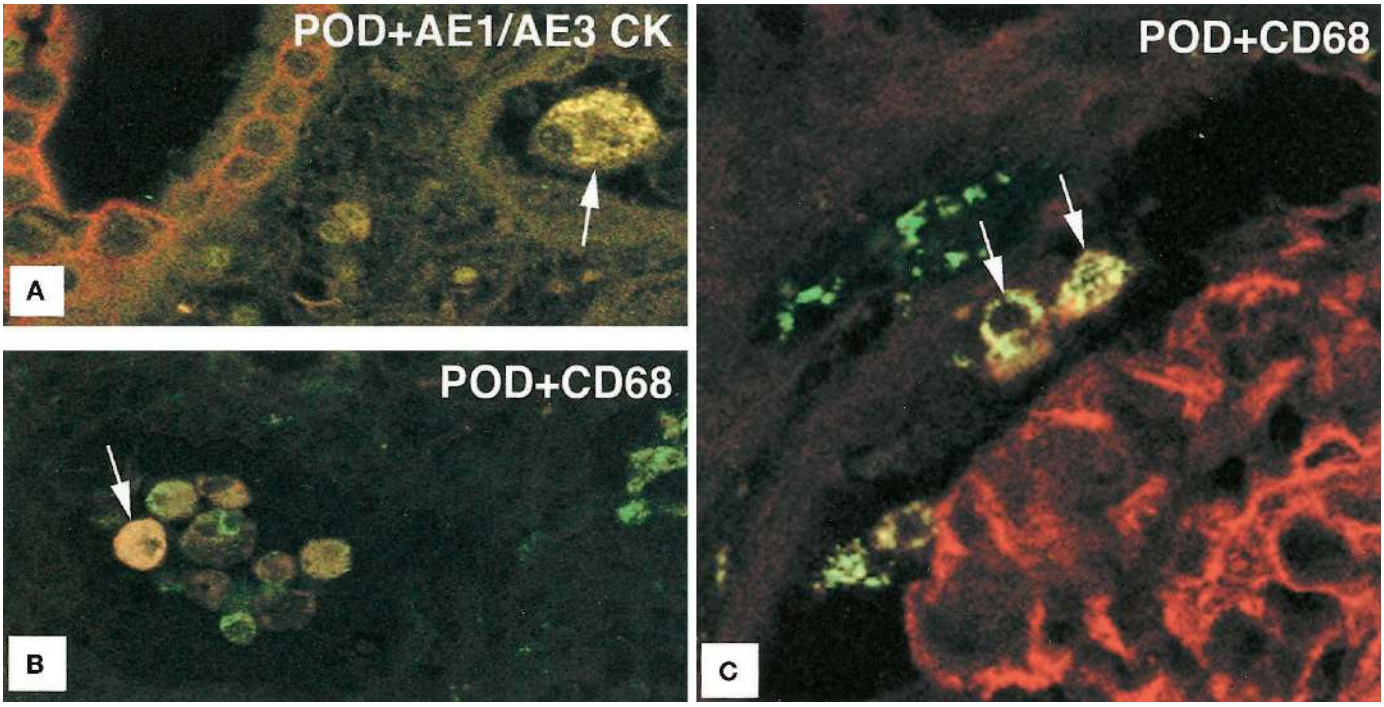

Figure 5. Confocal laser microscopy illustrating double immunofluorescence labeling. Case 2, 137 d after transplantation. (A) Antipodocalyxin (green) and anti-AE1/AE3 CK mAb (red). A distal tubule is labeled red with the AE1/AE3 mAb. A large cell that is free in the tubular lumen (arrow) is tagged by both mAb, this admixture yielding a yellowish color. This demonstrates coexpression on these cells of podocalyxin and AE1/AE3 CK. (B) Anti-podocalyxin mAb (red) and anti-CD68 (PGM1) mAb (green). Large round cells within a dilated tubular lumen exhibit combined labeling with both the antipodocalyxin and the anti-CD68 antibodies. The digitized imaging result of this co-localization is an orange color (arrow). The balance resulting from admixture of the two colors varies from cell to cell: In some, the yellowish hue predominates; in others, the greenish hue predominates. Some cells are purely CD68 positive and appear green. (C) Antipodocalyxin mAb (red) and anti-CD68 (PGM1) mAb (green). Outside Bowman's capsule, a cluster of small interstitial macrophages is labeled by the anti-PGM1 mAb. The glomerular tuft endothelial cells and resident podocytes are normally tagged (red). Some podocytes are free in Bowman's space. Two (arrows) show double positivity, for both podocalyxin and CD68. Magnifications: ×450 in A; ×500 in B and C.

podocytes were tagged. In the patients' kidneys, intratubular cells were labeled (density, 0 to 0.85). Very rare positive cell nuclei (density, 0 to 0.11) were found in some Bowman's spaces and on the external aspect of the glomerular tufts (Figure 8). Overall, proliferating activity was highly variable. The ratio Density of PCNA-positive cells:Density of CD68-positive cells, respectively in the glomeruli and in the tubular lumens, yielded a figure of 0 to 0.53 and of 0 to 0.07, respectively.

Discussion

The goal of this study was to demonstrate that cell transdifferentiation accompanies or follows the podocyte insult observed in recurrent FSGS. Despite wide variations among samples and among patients, histologic analysis showed that, basically, the first detectable lesions were cell FSGS and that the glomerular lesions developed to a mixture of cell FSGS with various degrees of tuft contraction and scar FSGS. These findings are in agreement with studies indicating that in primary FSGS as well as in recurrence after transplantation, the initial lesion that leads to FSGS is cellular (2,28,29) and that scar formation follows (8). Glomerular ischemia and glomerular obsolescence, common features of any glomerulopathy that reaches end stage, do not call for particular comment.

Relapse of FSGS Is Characterized by Striking Epithelial Cell Phenotypic Changes Suggesting Transdifferentiation

Loss of Normal Podocyte Epitopes. We and others have shown that the peculiar entity described as "primary collapsing glomerulopathy" (3,4,30) and HIV-associated nephropathy (4) is characterized by podocyte phenotypic dysregulation. In this study, we showed that, similarly, in transplanted kidneys with relapse of classical FSGS, the expression of specific podocyte markers, including podocalyxin, synaptopodin, GLEPP-1, WT-1, CR-1, and vimentin was often but not constantly lost on swollen, large round cells located along the outer aspect of the tuft or free in Bowman's space. In fact, that some of these large cells still adherent to the tuft or free in Bowman's space or drifting within the tubular lumens were indeed podocytes was demonstrated by their strong expression of podocalyxin, synaptopodin, and GLEPP-1. Conversely, expression of podocalyxin, synaptopodin, GLEPP-1, WT-1, CR-1, and vimentin was not found on cobblestones.

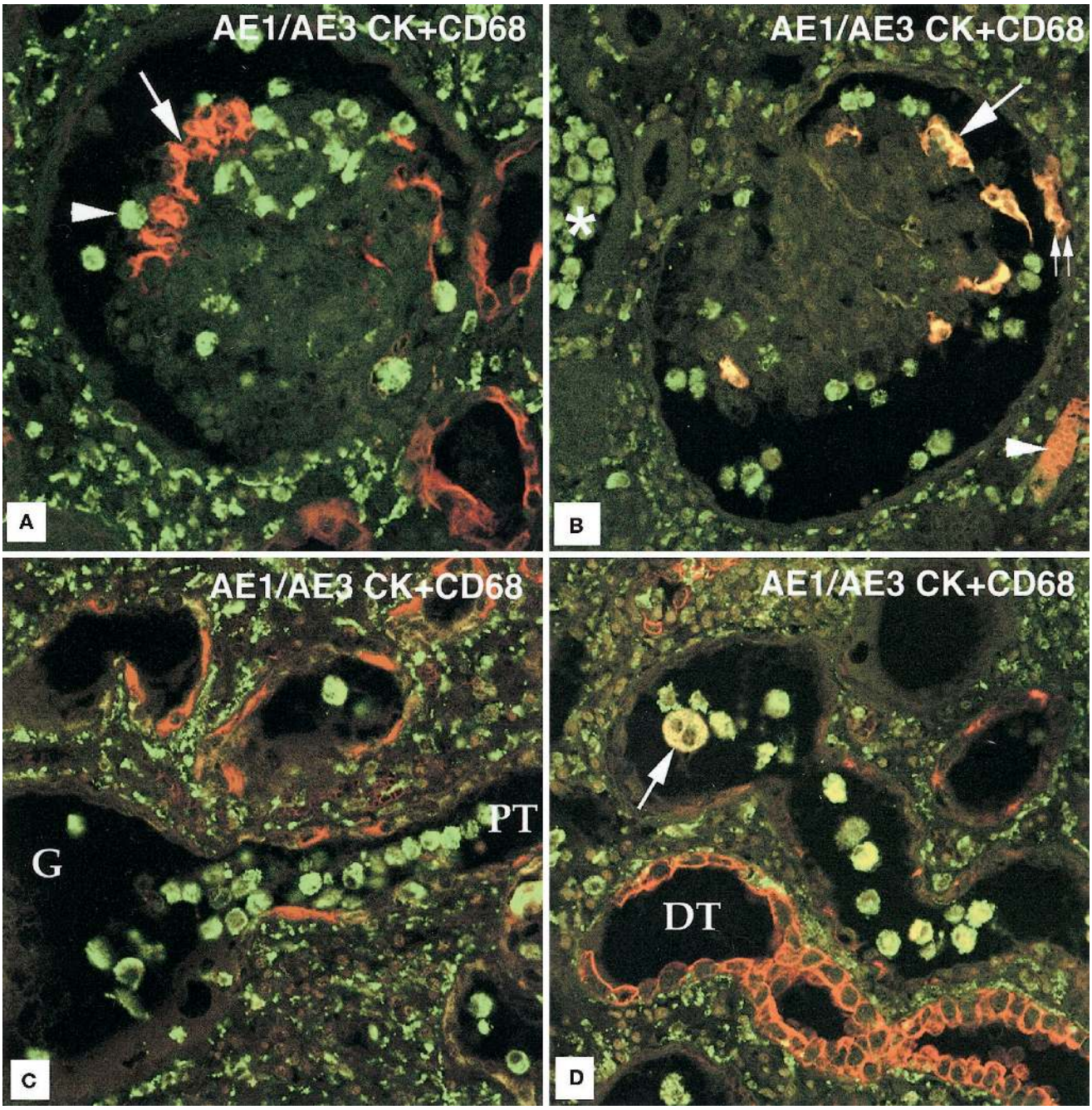

Figure 6. Confocal laser microscopy illustrating double immunofluorescence labeling with anti-CK AE1/AE3 mAb (red) and anti-CD68 (PGM1) mAb (green). (A and B) Patient 5: Transplantectomy at 4.5 yr. (A) Some podocytes still attached to the glomerular tuft strongly express CK AE1/AE3 (arrow), normally absent from podocytes. Other adjacent cells, presumably detached podocytes, appear green (arrowhead), suggesting transdifferentiation into a macrophagic phenotype. Some of these cells are still located in the glomerular tuft area, others drift free in Bowman's space. On this section, no co-localization of both phenotypes is apparent. (B) Podocytes with a normal shape, which are located at the periphery of a glomerular lobule, show co-localization of AE1/AE3 CK and CD68, which yields a bright orange color (arrow). Other round cells, presumably also podocytes, that are free in Bowman's space are green (CD68 positive). Numerous round cells clumping in a tubular lumen (*) are CD68 positive. An atrophic tubule (arrowhead) is tagged by the anti-CK AE1/AE3 mAb. Note at the upper part of the glomerulus that some parietal epithelial cells of Bowman's capsule (double arrow) also coexpress AE1/AE3 CK and CD68. (C and D) Patient 2, 137 d after transplantation. (C) A number of large round cells are leaving the glomerular outlet and are flowing into the proximal tubule. Note the staining (red) of some tubular epithelia with the AE1/AE3 anti-CK mAb. All of the cells in Bowman's space and in the tubular lumen are strongly CD68 positive. There is no clear evidence of co-localization of anti-CK and CD68 epitopes on this particular section. G, glomerulus; PT, proximal tubule. (D) Distal tubular section labeled red with AE1/AE3 anti-CK mAb in the lower part of the image. Numerous large round cells are free in the dilated (presumably proximal) tubular lumen. Using this digitized imaging process, AE1/AE3 CK + CD68 co-localization, a mixture of red and green, appears yellow. The balance between the two colors varies from cell to cell. Co-localization of CK and CE68 is conspicuous on a very large, binucleated cell (arrow) with a bright yellow color. DT, distal tubule. Magnification, ×250.

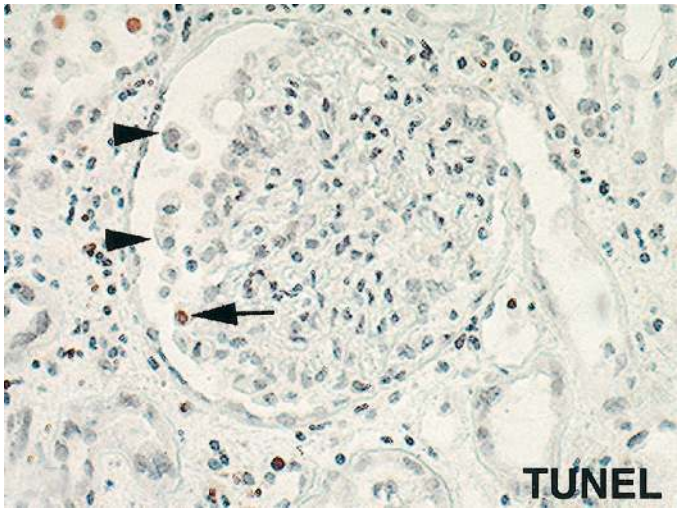

Figure 7. Patient 2: Detection of apoptosis by *in situ* DNA nick end labeling (TUNEL). In this glomerulus showing cell FSGS, numerous round cells that are free in Bowman's space are negative (arrowheads). There is only one positive cell (arrow) at the periphery of the tuft. Note positive intratubular cells in the upper left quadrant. Magnification, ×200.

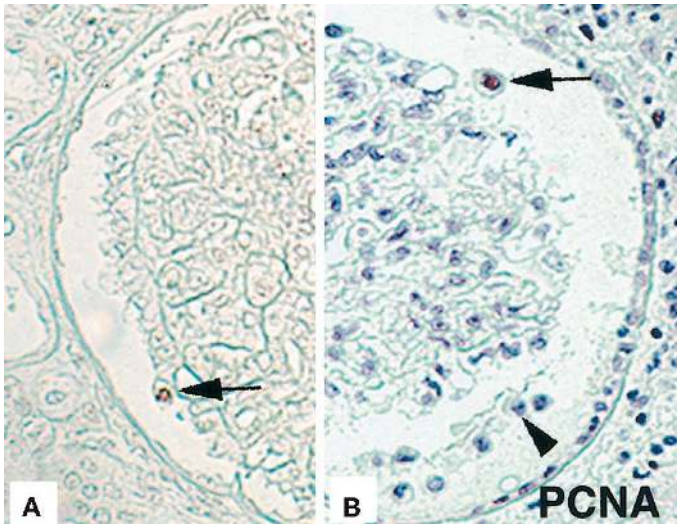

Figure 8. Detection of cell proliferation by immunostaining for proliferating cell nuclear antigen (PCNA). (A) Patient 4: A single proliferating cell is seen (arrow) at the lowermost end of a line of cobblestone-like cells. (B) Patient 2: A PCNA-positive cell is present in Bowman's space (arrow). All other cells that are free in Bowman's space are PCNA negative (arrowhead). Magnification, ×250.

Acquisition of CK Epitopes. Labeling by AE1/AE3 anti-CK mAb of cells that clumped at the periphery of the tuft and on cobblestone-like cells that surrounded the synechiae and formed pseudotubules suggests that in FSGS these cells undergo transdifferentiation. This interpretation is substantiated by the fact that AE1/AE3 mAb did not label (or very faintly labeled) podocytes or PEC in normal adult control kidneys. Labeling of cobblestone-like and pseudotubular epithelial cells by C2562, CK22, and AEL-KS2 might suggest that these cells originate from the PEC of Bowman's capsule, as these antibodies marked most of the PEC in adult control kidneys. This is consistent with the hypothesis that migration of PEC onto areas of the tuft where podocytes have disappeared might be an explanation for cobblestone and pseudotubule formation, as others have suggested (31–33). However, this hypothesis does not adequately explain the presence of CK-positive cells on the glomerular tuft in the absence of synechiae, in particular in collapsed glomeruli and in areas where PEC did not express CK.

Acquisition of Macrophagic Epitopes. A number of large round cells located on the tuft or drifting free in Bowman's space and in tubular lumens expressed both podocyte and macrophagic epitopes, and particularly two epitopes that are recognized by the PGM1 and the HAM56 mAb. That these cells that express epitopes specific for the monocyte/macrophage lineage were endowed with macrophagic attributes was shown by expression of 25F9, which characterizes macrophage maturation, and moreover by expression of HLA-DR and CD16, which characterize cell activation.

We consider it most unlikely that these cells were in fact true bone marrow–derived macrophages that had gained access to the urinary space from the circulation. By silver methamine staining, no gaps could be found in Bowman's capsule or in glomerular or tubular basement membranes. By contrast, small, spindle-shaped macrophages that infiltrated the renal interstitium were not labeled by 25F9 and CD16. Therefore, acquisition of macrophagic epitopes on the large round cells described above suggests that they had undergone transdifferentiation.

The best argument for a process of cell transdifferentiation occurring in the glomerular epithelial cells stems from confocal laser microscopy. With this technique, we found coexpression of (*1*) POD + AE1/AE3, (*2*) POD + CD68, and (*3*) CD68 + AE1/AE3 on cells that drifted in Bowman's space and within the tubular lumens.

The origin of these transdifferentiated cells might be interpreted in several ways. (*1*) Cells that coexpress CD68 or AE1/AE3 CK *and* podocalyxin are transdifferentiated podocytes that have acquired two new epitopes, or, alternatively, (*2*) cells that coexpress CD68 and AE1/AE3 CK originate from PEC or from tubular epithelial cells as well as from podocytes. In fact, some cells of the tubular epithelium occasionally expressed 25F9, an epitope of mature macrophages.

Transdifferentiation of podocytes into macrophagic cells has an experimental counterpart. Orikasa *et al*. (34) cultured podocytes from whole glomeruli in the rat. Between 3 and 11 d of culture, these detached visceral epithelial cells lost both the electron microscopic appearance and some immunohistochemical markers of normal podocytes and acquired morphologic and functional characteristics of macrophages.

Transdifferentiation in the Glomeruli Is Accompanied with Expression of Proliferation and Apoptosis Markers

Our results confirm that in normal human glomeruli, the number of proliferating cells identified by PCNA (35), as well as the number of apoptotic cells identified by TUNEL (36), is nil. By contrast, on allografts undergoing relapse of FSGS, these markers were significantly present. Barisoni *et al.* (4), in

idiopathic collapsing glomerulopathy, similarly found that the cell cycle marker Ki-67 was expressed in regions where the number of epithelial cells on the outer aspect of the tuft was obviously increased. The term *hyperplasia* has been used by several authors regarding cell FSGS (28). This fits with our observations of cell proliferation demonstrated by PCNA and suggests, although without conclusive morphologic evidence, that, contrary to what has been repeatedly shown in the rat (31–33), the human podocyte is likely to undergo cell division. Finally, the demonstration by PCNA and TUNEL that podocytes attached to the tuft as well as cells free in Bowman's space and in some tubular lumens suggests that relapse of primary FSGS is characterized by an increased cell turnover.

In summary, this study showed that relapse of FSGS, which most likely reflects the initial phenomenon that occurs in this variety of idiopathic nephrotic syndrome, is characterized by glomerular visceral epithelial cell dysregulation, or more specific, transdifferentiation. In the cellular variant of FSGS, most podocytes had lost their specific epitopes. This was also true in the so-called scar lesion, where the cobblestone-like epithelial cells did not express podocyte epitopes. Conversely, visceral glomerular epithelial cells acquired CK epitopes that are not expressed in the normal fetal and adult glomeruli. Immunohistochemical and confocal microscopy findings strongly suggested that in primary FSGS, some podocytes, occasionally some parietal epithelial cells, and possibly some tubular epithelial cells undergo a process of transdifferentiation with acquisition of epitopes that are characteristic of activated macrophages.

Note Added in Proof

Since this article was accepted for publication, two important articles that shed new light on the issue of podocyte proliferation in FSGS and in collapsing glomerulopathy (CG) have been published. Barisoni and coauthors (Barisoni L, Mokrzycki M, Sablay L, Nagata M, Yamase H, Mundel P: Podocyte cell cycle regulation and proliferation in collapsing glomerulopathies. Kidney Int 58: 137–143, 2000) showed that loss of cyclin kinase inhibitors p27 and p57 leading to expression of cyclin A may account for the activation of podocyte proliferation in CG. Likewise, Shankland and coauthors (Shankland SJ, Eitner F, Hudkins KL, Goodpaster T, D'Agati V, Alpers CE: Differential expression of cyclin-dependent kinase inhibitors in human glomerular disease: Role in podocyte proliferation and maturation. Kidney Int 58: 674–683, 2000) studied the Cip/Kip family of cyclin-dependent kinase inhibitors p21, p27, and p57 along with the proliferation marker Ki-67. They showed that in proliferating areas of the tuft, both in cell FSGS and in CG, loss of expression of p27 and of p47 and acquired expression of p2 are correlated with expression of Ki-67.

Acknowledgments

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