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Visceral Glomerular Epithelial Cells Can Proliferate *in Vivo* and Synthesize Platelet-Derived Growth Factor B-Chain

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In glomerular diseases associated with antibody- and complement-mediated injury to endothelial and mesangial cells, cell proliferation is an important early response that precedes matrix accumulation and glomerulosclerosis. In contrast, in diseases in which the visceral glomerular epithelial cell (vGEC) is the principal target of injury, cell proliferation is not a recognized consequence, although vGECs respond* in vitro *to a variety of growth factors and inflammatory mediators. To explore the possibility that low levels of vGEC proliferation may occur and participate in such diseases, serial studies were done in the passive Heymann nephritis model of membranous nephropathy, in which the vGEC is the primary target of antibody- and C5b-9-mediated injury. The results showed mitotic figures and positive staining for the proliferating cell nuclear antigen in cells whose location defined them as vGECs. The proliferating cell nuclear antigen-positive cells at the edge of the capillary wall were confirmed to be vGECs by double-immunostaining with antibodies to SPARC/osteonectin, which preferentially label vGECs in passive Heymann nephritis. Proliferation of vGECs* in vivo *was preceded by increased glomerular expression of platelet-derived growth factor (PDGF) B-chain protein and messenger RNA, both of which localized to vGECs. PDGF B-chain protein and messenger RNA were also detected in cultured vGECs. PDGF receptor β-subunit protein or messenger RNA could not be demonstrated in vGECs* in vivo *or* in vitro*, and no growth response of cultured vGECs to PDGF was noted. These results suggest that proliferation of vGECs does occur in a model of glomerular injury induced by antibody and C5b-9 on vGECs. VGEC proliferation and production of PDGF may be involved in the restoration of the capillary wall but could also contribute to local capillary wall injury and proliferation of other cells in Bowman's capsule, interstitium, and tubules. (Am J Pathol 1993, 142:637–650)

Recent studies demonstrate that acute antibody- and complement-mediated injury to the mesangial cell *in vivo* results in a phase of mesangial cell proliferation that is followed by an increase in messenger RNA (mRNA) and protein for a variety of extracellular matrix components.[1,2] Mesangial cell proliferation has also been recognized as an early event in the course of chronic glomerular diseases associated with the development of mesangial matrix expansion and glomerulosclerosis.[3–6] Similary, endothelial cell proliferation is seen when inflammatory injury occurs on the inner aspect of the capillary wall.[7–9] In experimental membranous nephropathy in rats (Heymann nephritis) glomerular injury occurs as a consequence of antibody- and complement (C5b-9)-mediated injury to the visceral glomerular epithelial cell (vGEC) and also leads to accumulation of excess extracellular matrix with thickening of the glomerular basement membrane (reviewed in references 10 and 11). However, in contrast to mesangial and endothelial cells, turnover of normal vGECs is very low.[12] Moreover, diseases involving the vGECs, such as both human and experimental membranous nephropathy, minimal-change nephrotic syndrome, and aminonucleoside nephrosis, are generally regarded as nonproliferative

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disorders. In fact, vGEC proliferation is not a recognized feature of any clinical or experimental glomerular disease.

To test the hypothesis that some vGEC proliferation may occur and participate in vGEC immune injury just as mesangial and endothelial cell proliferation occurs in response to injury to these cells, we conducted serial studies in the passive Heymann nephritis (PHN) model of membranous nephropathy, using the expression of the proliferating cell nuclear antigen (PCNA) as a marker of cell proliferation *in vivo*.[13] Proliferating vGECs in PHN were identified by immunostaining for SPARC/osteonectin, which has been shown to be a marker of injured vGECs in this model.[14]

We also studied the production and gene expression of platelet-derived growth factor (PDGF) and of the PDGF receptor (β-subunit) in vGECs during the course of PHN and in cultured vGECs. PDGF is a potent mitogen for cells of mesenchymal origin such as mesangial cells and fibroblasts and is also synthesized by these cells, resulting in paracrine and autocrine signaling pathways (reviewed in references 15 and 16). In experimental mesangioproliferative glomerulonephritis we and others have obtained evidence that mesangial cell injury *in vivo* is associated with increased production of PDGF and that PDGF plays a central role in the regulation of the pathological mesangial cell proliferation.[17–19]

Our results provide the first evidence for vGEC proliferation in response to immune glomerular injury and demonstrate an increase in abundance of PDGF mRNA and protein in vGECs during PHN. However, no evidence for either a response of normal vGECs to PDGF *in vitro* or PDGF receptor expression in vGECs *in vivo* or *in vitro* was obtained. These findings suggest that vGEC proliferation and growth factor production may be important features of several vGEC diseases that are generally considered noninflammatory.

Materials and Methods

Experimental Design

In Vivo *Studies*

Forty-two male Sprague-Dawley rats (Tyler Laboratories, Bellevue, WA) weighing 190 to 210 g at the start of the experiment were studied. PHN was induced in 24 of the rats by intravenous injection of sheep antibody to Fx1A, prepared as described in detail elsewhere.[20] Controls included six normal Sprague-Dawley rats, six rats injected with anti-Fx1A but depleted of complement with cobra venom factor (CVF) before disease induction (see below), and two rats each injected intravenously with either 1 μg or 10 μg of lipopolysaccharide (*Escherichia coli* L3129; Sigma Chemical Co., St. Louis, MO) or 1 ml of normal sheep serum (Triple J Farms, Redmond, WA). Twenty-four–hour urinary protein excretion was determined in the normal controls, in six randomly selected PHN rats each at days 3, 5, 10, and 15, and in the PHN/CVF rats at day 5. After the urine collection the rats were sacrificed and renal biopsies were obtained. Rats injected with lipopolysaccharide or normal sheep serum were sacrificed at day 5 after the injection and renal biopsies were also obtained. Biopsies were studied to quantitate and localize proliferating glomerular cells, monocyte/macrophages, neutrophils, lymphocytes, cells expressing PDGF B-chain protein and mRNA, and cells expressing PDGF receptor. After the renal biopsies, glomeruli were isolated using a differential sieving procedure as described.[1] Three separate glomerular preparations were obtained from the kidneys of two rats each at days 0, 3, 5, 5 (CVF), 10, and 15 after injection of anti-Fx1A. The isolated glomeruli were used to extract total RNA for Northern analysis. Northern blots were probed using complementary DNAs (cDNAs) specific for histone H2b.1-H3.2, PDGF B-chain, PDGF receptor β-subunit, and 28 S rRNA.

In Vitro *Studies*

In vitro studies were performed to investigate whether cultured vGECs are able to produce PDGF B-chain mRNA and protein and whether these cells express PDGF receptors. Expression of PDGF B-chain and PDGF receptor β-subunit mRNA was analyzed in total cellular RNA obtained from nonstimulated vGECs or vGECs subjected to sublytic anti-Fx1A and complement exposure or stimulation with interleukin-1β (IL-1β). The expression of PDGF B-chain and PDGF receptor β-subunit protein in resting or IL-1β-stimulated cultured vGECs was analyzed by immunofluorescent staining as well as by enzyme-linked immunoassay in vGEC extracts (PDGF B-chain). Finally, to analyze whether vGECs express functioning receptors for PDGF the growth responsiveness of cultured vGECs to various concentrations of recombinant human PDGF-BB (a kind gift of C. Hart, Zymogenetics, Seattle, WA) was determined by measuring the rate of cellular incorporation of [^{3}H]thymidine (see below).

Complement Depletion

Rats were depleted of complement with CVF (*Naja naja kaounthia*; Diamedix Corporation, Miami, FL).[21]

Serum C3 levels were measured by radial immunodiffusion[21] and were maintained at <10% of baseline values throughout the study period.

Renal Morphology

Tissue for light microscopy and immunoperoxidase staining was fixed in methyl Carnoy's solution[1] and embedded in paraffin. Three-micron sections were stained with the periodic acid-Schiff reagent and counterstained with hematoxylin.

Electron Microscopy

Tissue for electron microscopy was fixed, processed, and examined as described previously.[22] Two specimens each, containing six to eight glomeruli, obtained from rats with PHN at day 3, 5, 10, and 15 were analyzed.

Immunohistochemistry

Four-micron sections of methyl Carnoy's solution-fixed biopsy tissue were processed by an indirect immunoperoxidase technique as described previously.[1] Primary antibodies included 19A2 (Coulter, Hialeah, FL), a murine monoclonal IgM antibody against human PCNA, which is expressed by actively proliferating cells[13]; ED1 (Bioproducts for Science, Indianapolis, IN), a murine monoclonal IgG to a cytoplasmic antigen present in rat monocytes, macrophages, and dendritic cells; RP-3 (kind gift of F. Sendo, Yamagata, Japan), a murine monoclonal IgG antibody to rat neutrophils[23]; OX-22 (Accurate Chemical Corporation, Westbury, NY), a murine monoclonal antibody to the high molecular weight form of the rat common leukocyte antigen expressed on B lymphocytes and most T lymphocytes; ascites 2, a murine monoclonal antibody to a synthetic peptide from domain I of murine SPARC (SPARC peptide 1.1, amino acids 5 to 23)[24] (kind gift of A. Gown, Seattle, WA); PGF-007 (a kind gift of Mochida Pharmaceutical, Tokyo, Japan), a murine monoclonal antibody to a 25-amino acid peptide located near the carboxyl terminus of the human PDGF B-chain[25]; and a rabbit polyclonal antibody to the β-subunit of the PDGF receptor as described elsewhere[17] (kind gift of R. Seifert, Seattle, WA). For all biopsies, negative controls consisted of substitution of the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody or normal rabbit IgG.

For each biopsy >20 consecutive cross-sections of cortical glomeruli containing >20 discrete capillary segments each were evaluated by one of the authors, who was unaware of the origin of the slides. Mean values per biopsy were calculated for the number of proliferating ($PCNA^+$) cells and for the various leukocyte populations. Glomerular staining for PDGF B-chain was quantitatively assessed by counting the cells located in the periphery of the capillary loop that exhibited positive cytoplasmic staining, expressed as positive cells/glomerular cross-section.

Immunohistochemical Double-Staining

Double-immunostaining for the identification of the type of proliferating cells was performed as reported previously,[22] by first staining the sections for proliferating cells with 19A2, an IgM monoclonal antibody to PCNA, using an indirect immunogold procedure. Sections were then incubated with an IgG1 monoclonal antibody against SPARC/osteonectin.[24] We have previously shown by immunohistology and immuno-electron microscopy that SPARC in the normal glomerulus is preferentially expressed by vGECs.[14] Using the same techniques as well as immunoblotting of glomerular protein extracts and Northern analysis, we have also demonstrated that during PHN a marked and selective increase of the glomerular SPARC expression in vGECs occurs.[14] In this model immunostaining for SPARC, therefore, appears to be a suitable way to localize vGECs in glomerular cross-sections. Although SPARC expression has also been detected in the mesangium in association with mesangial cell proliferation (unpublished observations), no detectable staining for SPARC is present in the mesangium in PHN. In the present study cells were identified as proliferating $SPARC^+$ cells if they showed positive nuclear staining for PCNA and if the nucleus was completely surrounded by cytoplasm positive for SPARC. Proliferating cells in which the $PCNA^+$ nucleus did not border on a cytoplasm positive for SPARC were classified as $SPARC^-$. Proliferating ($PCNA^+$) cells that could not be clearly identified as $SPARC^+$ or $SPARC^-$ were considered nonclassifiable. Negative controls included omission of either of the primary antibodies, in which case no double-staining was noted.

In Situ *Hybridization for PDGF B-Chain mRNA*

In situ hybridization was performed on 4-μm sections of biopsy tissue fixed in buffered 10% formalin, utilizing a digoxigenin-labeled antisense complementary RNA probe for the murine PDGF B-chain (transcribed from a genomic DNA clone kindly provided

by C. Stiles, Boston, MA), as described.[26] Detection of the complementary RNA probe was performed with an alkaline phosphatase-coupled antidigoxigenin antibody (Genius nonradioactive nucleic acid detection kit; Boehringer-Mannheim, Mannheim, Germany) with subsequent color development. Controls consisted of hybridization with a sense probe to matched serial sections or deletion of the probe, antibody, or color solution.[26]

Rat vGEC Culture

Rat vGECs were established in culture from isolated glomeruli as described previously.[27] Morphological and immunofluorescent characterization of the cells demonstrated a polygonal shape with cobblestone appearance upon confluency and positive immunostaining with anti-Fx1A but no staining with anti-factor VIII or anti-Thy-1.1, which stain endothelial and mesangial cells, respectively.[27] Furthermore, in contrast to a recent report[28] on the characterization of cells obtained from glomerular outgrowths, the cultured vGECs used for this study also stained positively with a monoclonal antibody to podocalyxin (generously provided by M. Farquhar, San Diego, CA).[27] Finally, puromycin aminonucleoside exhibited toxic effects on the cells.[27]

vGECs were maintained in 100-mm tissue culture dishes (Corning Glassworks, Corning, NY) coated with bovine type I dermal collagen (Collaborative Research Inc., Bedford, MA). The cell culture medium consisted of K1 medium with 2% Nu-Serum (Collaborative Research Corp., Bedford, MA).[29] Cells were passaged by scraping and replating.

vGEC Synthesis of PDGF

vGEC expression of PDGF B-chain mRNA was examined in resting vGECs (passages 12 to 21) and in cells sensitized with sublytic concentrations of anti-Fx1A or normal sheep IgG followed by exposure to sublytic complement concentrations, as described previously.[30] In brief, vGECs were allowed to reach subconfluency in K1 medium. After washing, the cells were incubated with anti-Fx1A or normal sheep IgG for 15 minutes on ice to allow binding of antibody to the cells without patching and capping of the antigen-antibody complexes.[30] The concentration of antibody was selected on the basis of pilot studies that demonstrated that it provided the maximal amount of antibody IgG binding to vGECs in culture without inducing cytotoxicity, as assessed by lactate dehydrogenase release.[30] After duplicate washing, 2% pooled, normal, fresh frozen human serum or heat-inactivated serum (30 minutes at 56 C) was added as a complement source. This serum concentration was selected as the highest concentration to which cells sensitized with maximal sublytic antibody concentrations could be exposed without any cytotoxicity, as assessed by lactate dehydrogenase release.[30] Cells were then incubated at 37 C for 90 minutes, washed twice, and incubated for an additional 72 hours in K1 medium. Additional cultures included vGECs stimulated for 72 hours with 10^{-8} mol/l recombinant human IL-1β (a kind gift of S. Dower, Immunex Corp., Seattle, WA) and nonstimulated vGECs. After the stimulations total RNA was extracted from the cell layer (see below).

In additional experiments rat vGECs were seeded into eight-well chamber slides (Nunc, Naperville, IL) and allowed to reach subconfluency in K1 medium. Nonstimulated cells or cells stimulated for 24 hours with IL-1β (10^{-8} mol/l) were then fixed for 10 minutes at −20 C in acetone and air dried. For immunohistochemical analysis, the slides were first stained with the PGF-007 or PDGF receptor antibody or irrelevant monoclonal or polyclonal IgG. This was followed by a biotinylated anti-mouse IgG or anti-rabbit IgG antibody (Vector, Burlingame, CA) and finally streptavidin-fluorescein isothiocyanate (Amersham, Arlington Heights, IL).

To analyze further whether cultured vGECs contain PDGF B-chain, confluent cultures were stimulated for 24 hours with 10^{-8} mol/l recombinant human IL-1β and were then lysed by the addition of 1 mol/l acetic acid and 1 mol/l sodium chloride. After homogenization of the cells the supernatants were concentrated 10-fold using Centricon 10 devices (Amicon, Danvers, MA). After repeated washing of the concentrated supernatants with 10 mmol/l phosphate-buffered saline, pH 7.4, and reconcentration, the PDGF content was determined by using an enzyme-linked immunoassay, as described previously.[31]

Response of vGECs to PDGF

To determine whether vGECs proliferate in response to PDGF, vGECs were seeded into 24-well tissue culture dishes (Corning) coated with a collagen matrix (see above) and were incubated in K1 medium with 2% Nu-Serum. After the cells reached subconfluency, the medium in half of the wells was replaced with serum-free K1 medium. Five hours later the cells were stimulated with various concentrations of human recombinant PDGF-BB (a kind gift of C. Hart, Zymogenetics). Each well also received 2.5 μCi of [*methyl*-^{3}H]thymidine (DuPont Company,

Wilmington, DE). After 20 hours of stimulation the radioactivity incorporated into the cell layer was determined as described previously.[32]

Preparation of RNA and Northern Analysis

Total RNA was extracted from cultured vGECs or isolated glomeruli with RNAzol B, following the manufacturer's instructions (TelTest, Friendswood, TX), and was further purified by LiCl precipitation as described.[5] For Northern analysis the RNA was thawed, denatured, electrophoresed (15–20 μg/lane) through a formaldehyde/agarose gel, and transferred to a nylon filter (Hybond N; Amersham) as described.[22] The genomic DNA and cDNA probes used for Northern analysis were as follows. 1) For histone H2b.1-H3.2, a 1.7-kilobase (kb) mouse genomic fragment (pH312) containing histone H2b.1-H3.2 genes subcloned into pUC7 was used to detect the 0.5-kb histone H2b.1-H3.2 transcripts[33] (kindly provided by D. Bowen-Pope, Seattle, WA). 2) For PDGF B-chain, plasmid p3–4a, a clone containing almost the full length of rat PDGF B-chain cDNA, was used to detect the 3.4-kb rat PDGF B-chain transcript (kindly provided by C. M. Giachelli, Seattle, WA). 3) For the PDGF receptor β-subunit, a 5.1-kb *Eco*RI fragment of mouse PDGF receptor β-subunit cDNA[34] was used to detect the 5.7-kb rat PDGF receptor β-subunit transcript (the probe was a kind gift of D. Bowen-Pope, Seattle WA). 4) For 28 S ribosomal RNA, a bovine 280-base pair cDNA probe was used to detect 28 S ribosomal RNA[35] (kindly provided by L. Iruela-Arispe and H. Sage, Seattle WA). All probes were labeled with deoxycytidine [α-^{32}P]triphosphate (3000 Ci/mmol; New England Nuclear, Boston MA) by random primer extension. Total RNA isolated from glomeruli of rats with anti-Thy-1.1 nephritis at day 3[17] served as positive controls.

Membranes were prehybridized and hybridized as described[22] and autoradiograms were obtained and read by linear densitometry.[22] All Northern analyses were repeated two or three times with glomerular RNA preparations obtained from different sets of animals. Some membranes were rehybridized with additional probes (up to a maximum of four times).[22]

Statistical Analysis

All values are expressed as mean ± standard deviation. Statistical significance (defined as $P < 0.05$) was evaluated using the Student's *t*-test or one-way analysis of variance with modified *t*-tests performed using the Bonferroni correction.[36]

Results

Basic Characteristics of the PHN Model

After the injection of anti-Fx1A antibody, vGEC and endothelial swelling were noted by light microscopy at days 3 and 5, followed by vGEC vacuolization as well as glomerular capillary loop dilation at day 10 and to a lesser degree at day 15. Ultrastructural examination of glomeruli at day 10 showed numerous discrete subepithelial electron-dense deposits in peripheral capillary walls, with little evidence of alterations of the underlying basement membranes. VGEC foot processes overlying immune deposits were generally effaced; foot processes overlying uninvolved segments of the capillary wall were frequently preserved. Glomerular endothelium and mesangium were morphologically normal. Low grade but significant proteinuria developed at day 3 in PHN (Table 1). At all subsequent time points marked proteinuria was present (Table 1). The histological changes as well as the proteinuria were prevented in rats depleted of complement before disease induction (Table 1).

Glomerular Cell Proliferation Starts at Day 5 after Induction of PHN

Cell proliferation was assessed by immunostaining for PCNA, which is expressed from the late G_1-phase

Table I. *Proliferating Glomerular Cells (PCNA⁺), Glomerular Monocytes/Macrophages (ED1⁺), and Glomerular Cells Immunostaining for PDGF B-Chain in Normal Rats, in Rats with PHN at Day 3, 5, 10, and 15 after Disease Induction, and in Rats with PHN at Day 5 that Had Been Complement Depleted (CVF) before Disease Induction**

Day	Proteinuria (mg/24 hours)	Proliferating cells (PCNA⁺)/glomerular cross-section	Monocytes/macrophages/ glomerular cross-section	PDGF B-chain-positive cells/glomerular cross-section
0	5 ± 2	0.66 ± 0.12	2.14 ± 0.46	0.48 ± 0.43
3	14 ± 9†	1.05 ± 0.30	3.20 ± 0.59	7.38 ± 1.32†
5	163 ± 68†	2.14 ± 0.84†	2.67 ± 0.89	2.69 ± 1.65
10	324 ± 117†	2.13 ± 1.26†	5.37 ± 1.51†	0.69 ± 0.38
15	208 ± 58†	1.08 ± 0.41	2.85 ± 1.39	1.37 ± 0.80
5/CVF	8 ± 4	0.73 ± 0.21	2.76 ± 0.57	0.47 ± 0.25

* Data are mean ± standard deviation ($n = 6$ each).
† $P < 0.05$ versus control (day 0).

of the cell cycle to the early G_2-phase.[13] A significant increase in PCNA$^+$ cells could be demonstrated in PHN at days 5 and 10 after induction of the disease (Table 1). The PCNA$^+$ nuclei were often located at the outer side of peripheral capillary loops and were larger and rounder than the PCNA$^+$ nuclei of mesangial cells noted in previous studies.[1] Complement depletion before disease induction prevented the increase in PCNA staining at day 5 (Table 1). No increased glomerular PCNA staining was noted in rats at day 5 after the injection of either lipopolysaccharide or normal sheep serum (data not shown).

Glomerular Cell Proliferation in PHN Is not Related to Leukocyte Infiltration

To examine whether the increase in glomerular PCNA staining was related to an infiltration of proliferating leukocytes, the sections were also stained for neutrophils (RP-3), lymphocytes (OX-22), and monocytes/macrophages (ED-1). No significant increases of either neutrophils or lymphocytes above the normal range (0.10 ± 0.02 RP-3$^+$ cells/glomerular cross-section and 0.62 ± 0.11 OX-22$^+$ cells/glomerular cross-section) were observed at the various time points of PHN. Glomerular monocyte/macrophages showed a transient, low-grade increase above the normal range during the autologous phase of PHN (day 10), ie, after the onset of increased glomerular cell proliferation and proteinuria (Table 1).

Increased Glomerular Cell Proliferation Reflects Predominantly vGEC Proliferation in Early PHN

Periodic acid-Schiff staining of the renal biopsies obtained at the various time points revealed rare mitotic figures in vGEC locations at day 5 and to a lesser degree at day 10 (Figure 1). In contrast, no mitoses in vGEC locations were discovered in normal rats or at any other time point of PHN.

To investigate further whether vGEC proliferation was responsible for the increase in glomerular PCNA staining during PHN, double-immunostaining was performed. Double-immunostaining for PCNA and podocalyxin, a frequently used vGEC marker *in vitro*,[27,28] did not prove useful for determining the type of proliferating cell because some immunostaining for podocalyxin *in vivo* is present along the glomerular basement membrane and in endothelial cells.[14,37] Therefore, the sections were double-immunostained with PCNA and SPARC. As described elsewhere,[14] SPARC is constitutively expressed in

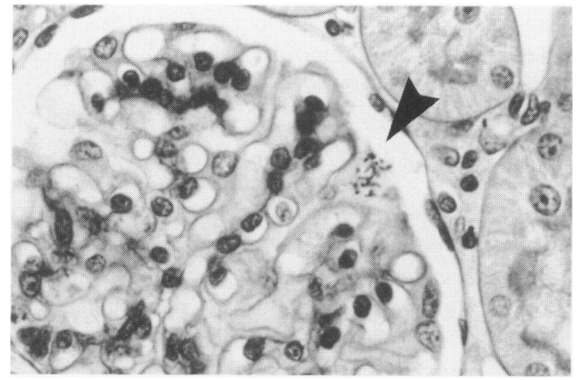

Figure 1. *Periodic acid-Schiff stain of a renal biopsy obtained from a rat at day 5 after induction of PHN. A mitosis (arrowhead) can be seen in a cell on the outer surface of the capillary wall. × 1000.*

vGEC cytoplasm, and only very weak staining is present within the remainder of the glomerular tuft. During PHN, SPARC expression is selectively upregulated in vGECs at all of the time points examined in the present study.[14] As shown in Figure 2a, in normal glomeruli no double-immunostaining of cells for PCNA and SPARC was apparent. In contrast, at day 5 multiple cells that stained positively for both PCNA and SPARC were noted along the periphery and within the glomerular tuft (Figure 2b). Quantification of double-immunostaining cells (Figure 3) showed that no changes from the normal pattern occurred in PHN at day 3. At day 5, nearly 50% of all proliferating (PCNA$^+$) glomerular cells double-labeled with the SPARC antibody, whereas the number of PCNA$^+$/SPARC$^-$ cells did not increase significantly (Figure 3). These changes were prevented in complement-depleted rats examined at day 5 (Figure 3). At day 10, both the number of PCNA$^+$/SPARC$^+$ glomerular cells and the number of PCNA$^+$/SPARC$^-$ cells had increased above the normal range (Figure 3). At day 15 only very few PCNA$^+$/SPARC$^+$ glomerular cells persisted, whereas PCNA$^+$/SPARC$^-$ cells still remained elevated above the normal range (Figure 3).

Because PCNA immunostaining becomes positive during the late G_1-phase of the cell cycle[13] and because cells may become arrested or leave the cell cycle in the G_1-phase, we have also analyzed total glomerular RNA for the expression of histone H2b.1-H3.2 mRNA, which is primarily expressed during the subsequent S-phase of the cell cycle.[38,39] Glomerular histone H2b.1-H3.2 mRNA expression showed a minor increase at day 3 after the induction of PHN (1.2 ± 0.1-fold over normal controls; $n = 3$), which was further increased at day 5 (2.3 ± 0.6-fold) and was maximal at day 10 (3.5 ± 1.3-fold). At day 15 glomerular histone H2b.1-H3.2 mRNA expression

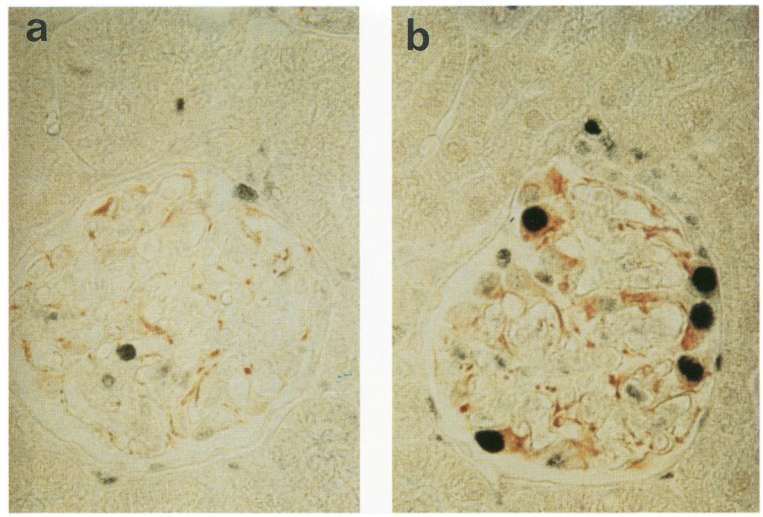

Figure 2. *Double-immunostaining of renal biopsies, obtained from a normal rat* (a) *and a rat at day 5 after induction of PHN* (b), *for proliferating cells (PCNA⁺) and SPARC/osteonectin, a marker of injured vGECs.[14] Proliferating cells are identified by their black nucleus, whereas SPARC⁺ cells are identified by their red cytoplasm. It is evident that in the normal rat* (a) *the proliferating glomerular cells do not double-label for SPARC. In contrast, at day 5 after the induction of PHN* (b) *several cells at the edge of the capillary loop show positive staining for both PCNA and SPARC (arrowheads), indicating that they are vGECs. ×400.*

was no longer different from controls (1.1 ± 0.1-fold). Complement depletion reduced the changes observed at day 5 (1.2 ± 0.1-fold).

The present study does not allow us to answer the question of whether vGEC proliferation serves merely to replace cells lost after C5b-9-mediated injury or whether an absolute increase of vGEC numbers occurs during PHN. In preliminary analyses we have noted no significant changes in the total glomerular cell counts during early PHN. However, if there were small increases in podocyte numbers it is unlikely that they would be detected, given the variability of glomerular cross-sectional cell counts. Direct counting of vGECs by quantitation of only SPARC⁺ cells was not possible, because SPARC is a cytoplasmic antigen expressed by the highly interwoven podocytes; this does not allow delineation and counting of individual cells by light microscopy. Furthermore, as mentioned above, SPARC expression in the glomerulus is not exclusively confined to vGECs.

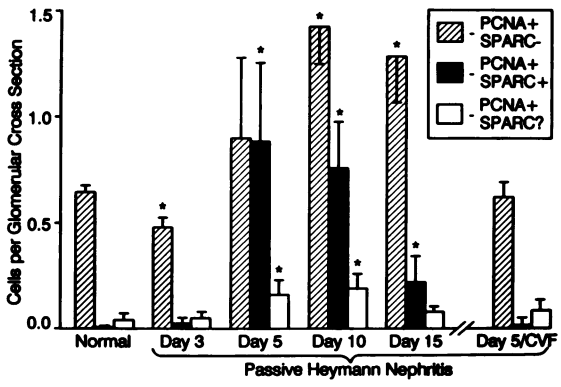

Figure 3. *Quantitative analysis, based on the double-immunostaining for PCNA and SPARC (see Materials and Methods and Figure 2), of the type of proliferating glomerular cells in normal rats, in rats with PHN, and in rats with PHN that were depleted of complement (CVF) before the induction of disease. *, P < 0.05 versus normal.*

PDGF B-Chain Gene and Protein Expression in vGECs Increases in PHN

In normal glomeruli, only rare cells along the periphery and within the glomerular capillary loops exhibited positive cytoplasmic staining for PDGF B-chain (Figure 4a and Table 1). The number of cells in the periphery of the capillary loop that stained positively for PDGF B-chain increased significantly above normal at day 3 of PHN (Figure 4b and Table 1). At day 5 of PHN this number of positive cells was still elevated above normal, although the difference did not reach statistical significance (Table 1). At all other time points no difference was noted between diseased and control rats (Table 1).

Northern analysis of total glomerular RNA obtained from rats with PHN demonstrated a marked increase of PDGF B-chain mRNA expression at day 3 after disease induction; this was not observed at any of the other time points examined (Figure 5). The glomerular localization of PDGF B-chain mRNA was analyzed by *in situ* hybridization. As shown in Figure 6a, in renal biopsies obtained from control rats staining in most glomeruli was absent. Rarely, staining localized to the cytoplasm of glomerular cells in variable locations. At PHN day 3 the expression of PDGF B-chain mRNA increased in the majority of glomeruli (Figure 6, b and c). Probe accumulations at this time point were frequently detected in cells in the periphery of the capillary loops (Figure 6, b and c) as well as in parietal epithelial cells (Figure 6c). At

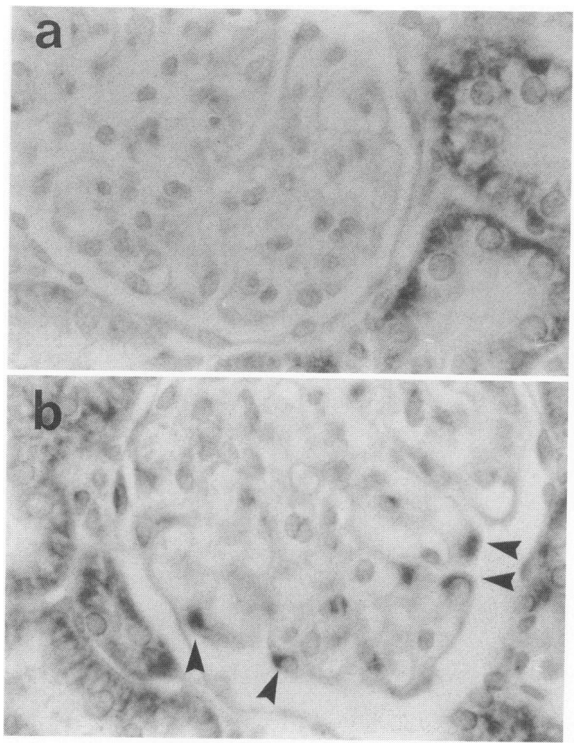

Figure 4. *Immunostaining of renal biopsies, obtained from a normal rat* (a) *and a rat at day 3 after the induction of PHN* (b), *for PDGF B-chain protein. In normal rats no cells at the periphery of the glomerular capillary loop exhibit positive staining for PDGF B-chain. In contrast, at day 3 after the induction of PHN multiple cells at the edge of the glomerular tuft stain positively (arrowheads). Immunoperoxidase stain with methyl green counterstain. × 1000.*

day 5 after disease induction, increased glomerular hybridization for PDGF B-chain mRNA, in a pattern comparable to that observed at day 3, was present in only one of six renal biopsies. At all other time points and in complement-depleted rats no changes from the normal hybridization pattern were noted.

Cultured vGECs Synthesize PDGF B-Chain

As shown in Figure 7, cultured vGECs constitutively expressed a transcript that hybridized with the PDGF B-chain cDNA. A similar PDGF B-chain transcript was expressed by proliferating mesangial cells *in vivo*, which served as a positive control (Figure 7). The expression of PDGF B-chain mRNA in cultured vGECs could be up-regulated by stimulation with recombinant IL-1β (Figure 7). In contrast, exposure of anti-Fx1A–sensitized vGECs to sublytic C5b-9 attack did not increase PDGF B-chain mRNA expression above control values (data not shown).

Figure 8*b* shows that immunostaining of cultured vGECs for PDGF B-chain became positive after 24 hours of IL-1β stimulation. In contrast, in nonstimulated vGECs PDGF B-chain protein was only barely detectable using the immunofluorescent method (Figure 8*a*). Analysis of cellular extracts by enzyme-linked immunoassay of IL-1β-stimulated vGECs confirmed the presence of small amounts of PDGF in these cells (2 to 3 ng of PDGF B-chain/10^6 cells).

There Is No Evidence for Expression of PDGF Receptor β-Subunit by vGECs in Vivo *or* in Vitro

In agreement with previous results,[17] immunostaining for PDGF receptor β-subunit showed positive staining in the mesangium of normal glomeruli, whereas no staining of cells in vGEC locations was apparent. No change of this staining pattern was observed at the various PHN time points examined. Northern analysis for glomerular expression of mRNA for the PDGF receptor β-subunit also failed to reveal significant changes during the course of early PHN (data not shown).

In nonstimulated or stimulated (see above) vGECs *in vitro*, no mRNA for PDGF receptor β-subunit was detectable (data not shown). Furthermore, immunostaining of cultured vGECs for the PDGF receptor β-subunit was negative. The absence of PDGF receptor β-subunit on cultured vGECs was further supported by the lack of a mitogenic effect of PDGF-BB at concentrations of up to 200 ng/ml (data not shown).

Discussion

The present study provides evidence that significant proliferation of vGECs can occur *in vivo* in response

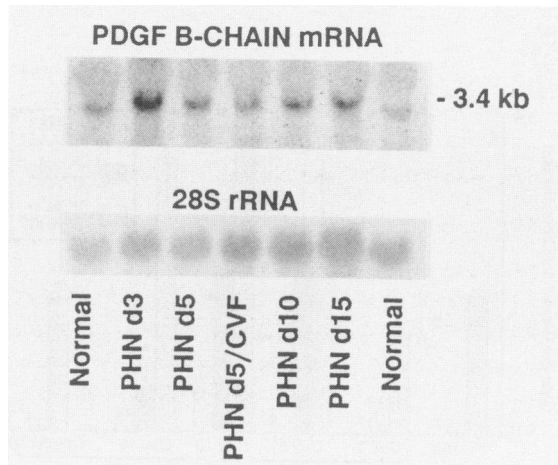

Figure 5. *Expression of PDGF B-chain mRNA in total glomerular RNA obtained from two sets of normal rats, rats with PHN at day 3, 5, 10, and 15 and complement-depleted rats (CVF) with PHN at day 5. PDGF B-chain mRNA expression increases during the course of PHN. The changes are prevented in complement-depleted rats. One representative experiment is shown.*

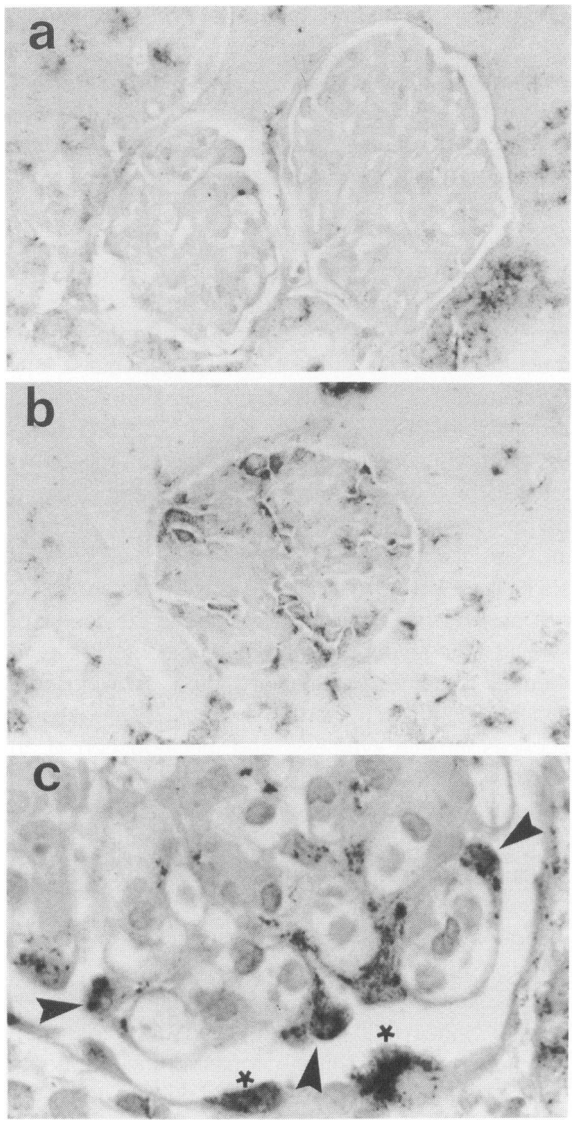

Figure 6. *In situ hybridization for PDGF B-chain mRNA in renal biopsies obtained from a normal rat* (a) *and rats at day 3 after the induction of PHN* (b *and* c). *In the normal rat few grains are observed overlying glomerular cells* (a). *At day 3 after the induction of PHN multiple glomerular cells exhibit positive hybridization signals* (b). *Many of these cells are vGECs, as suggested by their location at the edge of the capillary loop* (c, *arrowheads*). *Some parietal epithelial cells also show cytoplasmic hybridization for PDGF B-chain mRNA* (c,*) a *and* b, ×*200;* c, ×*1000.*

to antibody- and C5b-9-mediated vGEC injury. Thus, during PHN we have observed mitotic figures and nuclear PCNA staining in cells at the periphery of the capillary loop and we have detected a parallel increase in glomerular histone mRNA expression. More importantly, nuclei of proliferating cells localized to the periphery of the capillary loops, and half of the glomerular cells with nuclear PCNA staining also exhibited positive immunostaining for SPARC/osteonectin. Although SPARC/osteonectin can be produced by other glomerular cells, for example mesangial cells, in the normal glomerulus SPARC is expressed mainly by vGECs and is selectively up-regulated in vGECs in PHN, thus serving as a marker of injured vGECs in this model.[14]

Both human and experimental membranous nephropathy have classically been considered to be nonproliferative glomerular lesions, and vGEC proliferation has not been previously reported.[40] This may be because proliferation is low grade and transient, and conventional techniques to detect it are relatively insensitive. In the present study we have analyzed cell proliferation by immunostaining for PCNA, which is expressed from the late G_1-phase of the cell cycle through the S-phase and early G_2-phase.[13] Immunostaining for PCNA is likely to be more sensitive in detecting cell proliferation *in vivo* than autoradiography with [^{3}H]thymidine, which labels only S-phase cells, or the identification of mitotic figures. Autoradiography with [^{3}H]thymidine has previously been the main technique utilized to detect vGEC proliferation in various glomerular diseases. Studies using

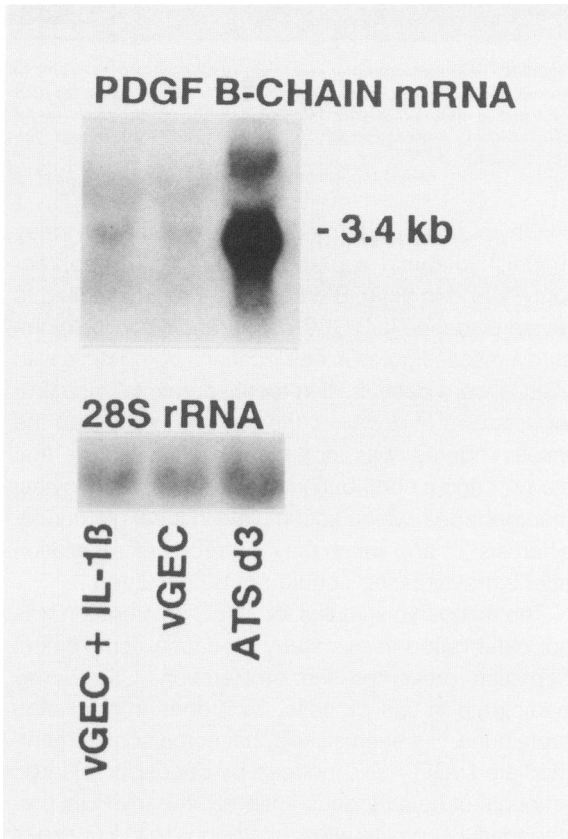

Figure 7. *Expression of PDGF B-chain mRNA in total RNA isolated from cultured vGECs. The cells were either left untreated or stimulated with 10^{-8} mol/l IL-1β for 24 hours. A 3.4-kb transcript of similar size as that observed in total glomerular RNA obtained from rats with anti-Thy-1.1 nephritis (ATS d3)[17] is observed in nonstimulated vGECs. Expression of PDGF B-chain mRNA in vGECs increases after stimulation with IL-1β.*

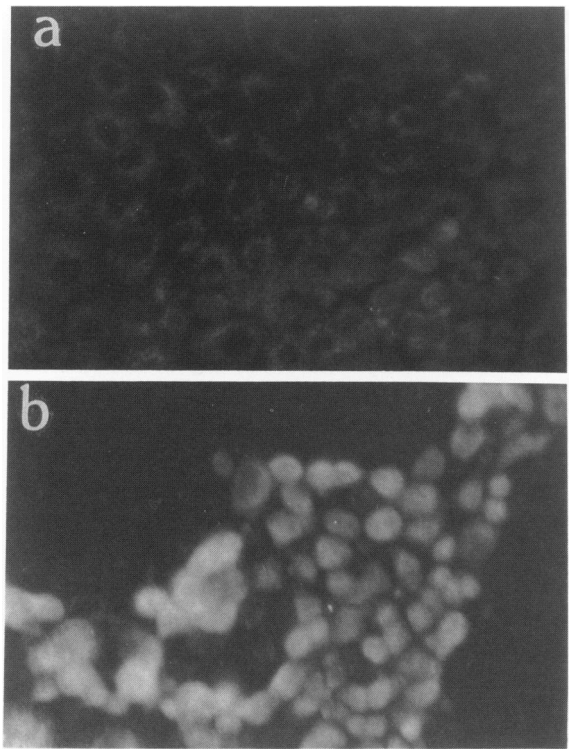

Figure 8. *Immunostaining of cultured vGECs for PDGF B-chain. In resting vGECs only trace staining for PDGF B-chain is present* (a) *After 24 hours of stimulation with 10^{-8} mol/l IL-1β, immunostaining for PDGF B-chain increases in the cytoplasma of vGECs* (b). *Immunofluorescent stain, ×400.*

[^{3}H]thymidine, performed in the remnant kidney model,[41] diabetic nephropathy,[42] after uninephrectomy,[42] and in anti-GBM nephritis,[7] have all failed to reveal evidence for vGEC proliferation *in vivo*. In one study, mitotic figures in cell locations compatible with vGECs were described in focal segmental glomerular sclerosis.[43] However, the cell type exhibiting the mitotic figures was not clearly identified in that study,[43] and a contribution of glomerular monocytes/macrophages, which accumulate in focal glomerulosclerosis[6,44] and which may undergo cell replication after extravasation,[45] could not be excluded.

The mitogenic stimulus for vGEC proliferation was not established in this study. Because complement depletion prevented the proliferation, C5b-9 may participate in this process, as it does in mediating proteinuria.[10] It seems likely that some complement-mediated vGEC lysis induced by deposition of large amounts of heterologous antibody in early PHN preceded vGEC proliferation, analogous to the phase of mesangiolysis that precedes mesangial cell proliferation induced by anti-Thy-1.1 antibody.[1] However, even sublytic C5b-9 attack on cultured vGECs has been shown to induce or increase intracellular calcium, inositol bisphosphate, inositol trisphosphate, and diacylglycerol and to activate phospholipase C and increase prostaglandin and collagen production.[46–49] Although a mitogenic effect of C5b-9 on vGECs has not been directly demonstrated, these data suggest that C5b-9 may induce vGEC proliferation as well as activation. Alternatively, C5b-9 might induce release of other factors by vGECs, or possibly endothelial cells,[50] that are mitogenic for these cells. In the anti-Thy-1.1 model of mesangioproliferative glomerulonephritis up-regulation of PDGF appears to contribute to an autocrine mechanism that maintains mesangial cell proliferation.[17–19] Although our studies show an up-regulation of PDGF expression by vGECs after C5b-9-mediated injury in PHN, PDGF was not mitogenic for vGECs in culture and no PDGF receptors could be demonstrated in vGECs *in vivo* or *in vitro*, a finding consistent with studies by others.[51,52] Glomerular staining for epidermal growth factor, a potent vGEC mitogen *in vitro*,[51] was negative during the course of PHN (J. Floege, unpublished observations). We have recently obtained preliminary data showing that IL-1 is a potent activator of several vGEC functions, such as laminin synthesis and the induction of an NF-κB-like DNA-binding protein (C. Richardson, unpublished observations). Furthermore, IL-1 is synthesized by vGECs (S. Bursten, personal communication) and in mesangial cells is released in response to sublytic C5b-9 attack.[53] Other known mitogens for vGECs that might participate in the proliferative response observed in PHN include insulin,[51] leukotrienes C_4 and D_4,[54] and thrombin.[55] The possibility that vGEC proliferation occurs in response to mitogens derived from circulating cells appears unlikely, in view of the fact that the only evidence for nonglomerular cell participation in our studies was a transient and minimal increase in monocytes/macrophages, which did not occur until well into the autologous phase of the disease and followed rather than preceded the vGEC proliferation observed. However, others, on the basis of depletion studies, have suggested a functional role for infiltrating macrophages in this model.[56]

The pathophysiological significance of the vGEC proliferation demonstrated in this study is also unclear. Several possibilities merit consideration. VGEC proliferation in PHN could occur to replace cells killed by C5b-9-mediated cytotoxicity[57] or cells lost by detachment from the glomerular basement membrane.[58] This thesis is consistent with preliminary observations showing that vGEC proliferation is not limited to PHN but also occurs in concanavalin A/anti-concanavalin A nephritis, nephrotoxic nephritis, and aminonucleoside nephrosis (J. Floege,

unpublished observations) and that vGEC proliferation in all instances followed rather than preceded injury to the capillary wall. Alternatively, proliferation may occur to expand the cell layer necessary to cover a glomerulus undergoing hypertrophy and to prevent the development of intercellular defects in permeability leading to increased transcapillary macromolecule flux, subendothelial hyaline accumulation, and eventually capillary occlusion and glomerulosclerosis.[41,59] Finally, vGECs activated to proliferate may also release increased quantities of hemodynamically active compounds such as prostaglandins[60] or potential inflammatory mediators such as protease[27,61] or oxidants[62] that could contribute to capillary wall damage. The focal nature of the permeability defects demonstrated by ultrastructural tracer studies in active Heymann nephritis[63,64] is consistent with the focal distribution of the proliferating vGECs in this study.

The second major observation of the present study was that vGECs, analogous to various other cells of mesenchymal origin, are capable of synthesizing PDGF B-chain. Previous studies have emphasized the importance of the mesangial cell as a source of PDGF in glomerular disease.[17–19,26] In contrast, in PHN a selective induction of PDGF B-chain mRNA and protein was documented in vGECs and parietal epithelial cells. Similar to vGEC proliferation, the expression of PDGF B-chain mRNA and protein during PHN appeared to be complement dependent. The absence of an increase of PDGF B-chain mRNA in cultured vGECs stimulated with anti-Fx1A and complement suggests that C5b-9 may require other cofactors to affect the PDGF B-chain synthesis.

Despite the lack of an autocrine vGEC response to intrinsic PDGF, the augmented production of this cytokine in disease could have a number of pathophysiological consequences that are independent of vGEC proliferation. Thus, PDGF released from activated vGECs could exert mitogenic effects on neighboring glomerular cells such as mesangial cells,[15] or it could deliver a mitogenic signal to cells within or surrounding the "downstream" part of the nephron. Although proximal tubular cells in primary culture have been found to be nonresponsive to PDGF,[65] it has recently been shown that fibroblasts derived from the renal interstitium proliferate in response to PDGF[66] and may contribute to the development of interstitial fibrosis, which characterizes all progressive forms of renal disease.[67] In fact, studies in human membranous nephropathy,[68] as well as in a variety of other glomerular diseases,[67] have shown that the only predictor of a progressive course is an increase in the tubulointerstitial area, whereas no glomerular morphological predictors were identified.

In conclusion, the present study shows that vGECs *in vivo* can exhibit two previously unrecognized reaction patterns to injury, namely proliferation and increased PDGF B-chain production. Both of these events could play important roles in glomerular diseases with primary vGEC involvement, such as membranous nephropathy. Furthermore, as outlined above, both proliferation of vGECs and PDGF release by these cells might contribute to the progression of chronic glomerular diseases. Further experiments will be necessary to elucidate the factors that initiate the proliferation of vGECs *in vivo* and to study the consequences of interference with either vGEC proliferation or vGEC-derived PDGF synthesis.

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