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Growth factor expression in a murine model of cryoglobulinemia

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Growth factor expression in a murine model of cryoglobulinemia.

Background. Increased expression of growth factors including platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) are thought to play pivotal roles during mesangial expansion and glomerulosclerosis. Thymic stromal lymphopoietin (TSLP) transgenic mice develop mixed cryoglobulinemia and a membranoproliferative glomerulonephritis (MPGN). Here we describe the renal expression of isoforms of PDGF and TGF-β in relation to changes in extracellular matrix (ECM) components and markers of cell proliferation and activation in this model.

Methods. A total of 123 mice, including 61 TSLP transgenic mice and 62 wild-type controls, were sacrificed at defined intervals. PDGF-A chain, -B chain, PDGF α- and β-receptor (β-R) and TGF-β1 mRNA were analyzed by in situ hybridization. Expression of α smooth muscle actin (αSMA), collagen type I, collagen type IV, laminin, and a marker of proliferating cells (PCNA) were assessed by immunohistochemistry. Slides also were studied by combined immunohistochemistry and in situ hybridization with an antibody that recognizes monocytes/macrophage and with riboprobes that detect PDGF B-chain, PDGF β-R or TGF-β1 mRNA.

Results. Increased numbers of proliferating glomerular cells appeared early in the disease course, associated with de novo expression of αSMA. Expression of PDGF B-chain and β-R mRNA was increased in the mesangium and in parietal epithelial cells of TSLP transgenic mice and correlated with the number of PCNA positive cells. Increased TGF-β1 mRNA expression paralleled the deposition of type IV collagen. A significant proportion of Mac-2 positive macrophages expressed TGF-β1 mRNA, while only a small percentage of glomerular macrophages expressed PDGF B-chain mRNA. No PDGF β-R mRNA expression by macrophages was detected.

Conclusion. TSLP transgenic mice develop a membranoproliferative glomerulonephritis in which glomerular cell proliferation and matrix deposition are associated with an increased expression of PDGF B-chain, PDGF β-R and TGF-β1. These findings extend the paradigms covering these growth factors established in the rat Thy 1 model of mesangiolysis and repairs to a murine model of progressive glomerulonephritis closely resembling human MPGN.

Key words: thymic stromal lymphopoietin, glomerulonephritis, cryoglobulinemia, membranoproliferative glomerulonephritis, PDGF, TGF-β.

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Cryoglobulinemia is a systemic disease with a wide spectrum of manifestations [1, 2]. Up to 55% of patients with mixed cryoglobulinemia develop renal involvement [1–4]. The typical renal manifestation of mixed cryoglobulinemia is a membranoproliferative glomerulonephritis (MPGN) characterized by deposits of immune complexes in glomerular capillary walls and mesangial areas, increased glomerular cellularity, mesangial matrix accumulation, and thickening and splitting of peripheral capillaries. Additionally, intraluminal thrombi composed of cryoprecipitable immune complexes, monocyte/macrophage infiltration, and an organized substructure of the immune deposits in the form of microfibrils and/or microtubules are typical morphological features that can be present in cryoglobulinemic glomerulonephritis [5–7]. Extraglomerular microscopic vasculitis also can be present in same cases. The pathogenesis of this important form of glomerular injury is still incompletely understood.

Recent studies have demonstrated that platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) may be of major importance for the development of mesangial expansion and deposition of extracellular matrix (ECM) in settings of glomerular injury [8, 9]. PDGF, which can be released from activated platelets, monocytes/macrophages, and mesangial cells, is an important mediator of mesangial cell proliferation, and is involved in ECM expansion [10–15]. TGF-β promotes glomerular injury through the accumulation of ECM, leading to glomerulosclerosis. Increased synthesis of normal glomerular ECM components (type IV collagen, laminin and proteoglycans), abnormal ECM components (type I and III collagens), as well as reduction of ECM degradation has been ascribed to TGF-β [16–19].

Thymic stromal lymphopoietin (TSLP) is a cytokine that was isolated from conditioned medium of a thymic

stromal cell line, and supports B cell growth and differentiation [20]. We have recently reported that transgenic mice, overexpressing TSLP, develop mixed cryoglobulins and MPGN [21]. Renal involvement in TSLP transgenic mice is characterized by monocyte/macrophage infiltration, marked ECM expansion, increased glomerular cellularity, and prominent accumulation of subendothelial and mesangial electron-dense deposits closely resembling the morphological features of human MPGN [21]. This new model of a membranoproliferative type of glomerular injury gave us the opportunity to describe the sequence of cellular events involving the growth factors mentioned above. We further assessed the expression of alpha-smooth muscle actin (αSMA; a marker of activated mesangial cells), and accumulations of several components of the ECM, and correlated these with the expression patterns of PDGF and TGF-β1. We also evaluated monocyte/macrophage expression of the growth factors PDGF-B chain and TGF-β1, as well as PDGF β-receptor (PDGF-β-R) by combined immunohistochemistry and in situ hybridization.

METHODS

Breeding

Breeding, and genotyping of the TSLP transgenic mouse strain has previously been described in detail [21]. The study was performed after backcrossing to a C57BL6 background for more than eight generations. Male TSLP transgenic mice were than mated with wild type C57BL6 females. The procedures used were approved by the local Animal Care Committee of the University of Washington.

Experimental design

A total of 123 mice including 61 TSLP transgenic mice and 62 wild-type controls were used. Male mice were sacrificed at monthly intervals up to seven months of age ($N = 75$), female mice were sacrificed at 1, 1.5, 2, 2.5 months of age ($N = 48$).

These mice have been evaluated by immunohistochemistry for αSMA, collagen type I, collagen type IV, and laminin. Expression of PDGF-B chain, PDGF β-R, TGF-β1 mRNA expression were evaluated by in situ hybridization. Additionally, a preliminary series of 27 TSLP transgenic and 16 wild-type controls were evaluated for PDGF-A chain and PDGF α-R, PDGF-B chain, PDGF-β-R, TGF-β1 mRNA by in situ hybridization.

Renal morphology

Renal tissue was fixed in part in 10% neutral-buffered formalin and in methyl Carnoy's solution as described previously [22]. Fixed tissues were processed, embedded in paraffin according to standard protocols and sectioned at 4 μm. Slides were stained with periodic acid methenamine silver (silver), with periodic acid-Schiff's (PAS), and hematoxylin and eosin (H&E) using standard histologic procedures.

Immunohistochemistry

Four micrometer sections of formalin- and methyl Carnoy's-fixed, paraffin embedded tissue were processed by an indirect immunoperoxidase technique as previously described [21, 23]. Sections were deparaffinized in xylene, rehydrated in graded ethanol and then incubated in 3% hydrogen peroxide for five minutes to block endogenous peroxidase. Endogenous biotin was blocked using the Avidin-Biotin blocking Kit (Vector Laboratories, Burlingame, CA, USA). Antigen retrieval was performed by heat treatment. Sections were sequentially incubated with 10% normal serum, the primary antibody diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin for one hour, followed by the biotinylated secondary antibody (Vector Laboratories), and the avidin-biotin-horseradish peroxidase (HRP) complex (Vector). The immunoreaction was visualized by 3,3′-diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) with nickel chloride enhancement, resulting in a black color product. After methyl green counterstaining, the slides were dehydrated, and coverslipped in Histomount (National Diagnostics, Atlanta, GA, USA).

Antibodies utilized to detect specific proteins or cell types are listed in Table 1. The specificity of each antibody clone has been established as referenced. For all samples, negative controls for the immunohistochemistry included substituting for the primary antibody an irrelevant IgG from the same species or phosphate-buffered saline (PBS).

All slides were scored by an observer who was blinded to the origin of the histologic specimen. Semiquantitative scoring of glomerular expression of αSMA, type I and IV collagen, and laminin was performed using five grades as follows: 0 = absent staining; 1 = mesangial staining involving less than 25% of the area examined; 2 = segmental mesangial staining involving 25 to 50% of mesangial areas present; 3 = mesangial staining involving 50 to 75% of the areas examined; and 4 = diffuse mesangial staining involving more than 75% of areas examined as previously described and illustrated [22]. The number of PCNA positive cells in the glomerular tufts was counted at a magnification of ×400. Both the number of the intraglomerular positive cells and the number of glomeruli were counted and the data were presented as positively stained cells per glomerular cross section (gcs). Cells outside the glomerular tufts were not included. At least 20 consecutive cross sections of glomeruli were examined using each of the above antibodies. The morphometric analysis of glomerular cell numbers has been described previously [21].

Molecular probes

The molecular probes used in this study have previously been described and used for in situ hybridization [30]. These included probes for:

Table 1. Antibodies used to detect specific antigens in biopsy tissue

Antigen [references]	Clone	Primary antibody	Secondary antibody	Dilution of primary antibody
PCNA (Ab-1) [24]	PC10	Mouse IgG monoclonal antibody anti-human PCNA (Oncogene Research Products, Cambridge, MA, USA)	Biotin-conjugated monoclonal rat anti-mouse IgG2a (Caltag Laboratories)	1:100
α-smooth muscle actin [25]	1A4	Mouse monoclonal IgG2a anti-human α-smooth muscle actin (DAKO, Carpinteria, CA, USA)	Biotin-conjugated monoclonal rat anti-mouse IgG2a (Caltag Laboratories)	1:25
Type I collagen [26]		Goat polyclonal antibody anti-human collagen I (Southern Biotechnology, Birmingham, AL, USA)	Biotinylated rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA, USA)	1:1000
Type IV collagen [27]		Goat polyclonal antibody anti-human collagen IV (Southern Biotechnology)	Biotinylated rabbit anti-goat IgG (Vector Laboratories)	1:600
Laminin [28]		Rabbit polyclonal antibody anti-rat laminin (mouse cross react) (Chemicon, Temecula, CA, USA)	Biotinylated goat anti-rabbit IgG (Vector Laboratories)	1:800
Mac-2 [29]	M3/38	Rat IgG monoclonal antibody anti-mouse Mac-2 (CEDARLANE, Hornby, Ontario, Canada)	Biotinylated goat anti-mouse IgG, mouse adsorbed (Vector Laboratories)	1:5000

All antigens were immunostained using methyl Carnoy's fixed tissue, except PCNA and Mac-2 on formalin fixed tissue.

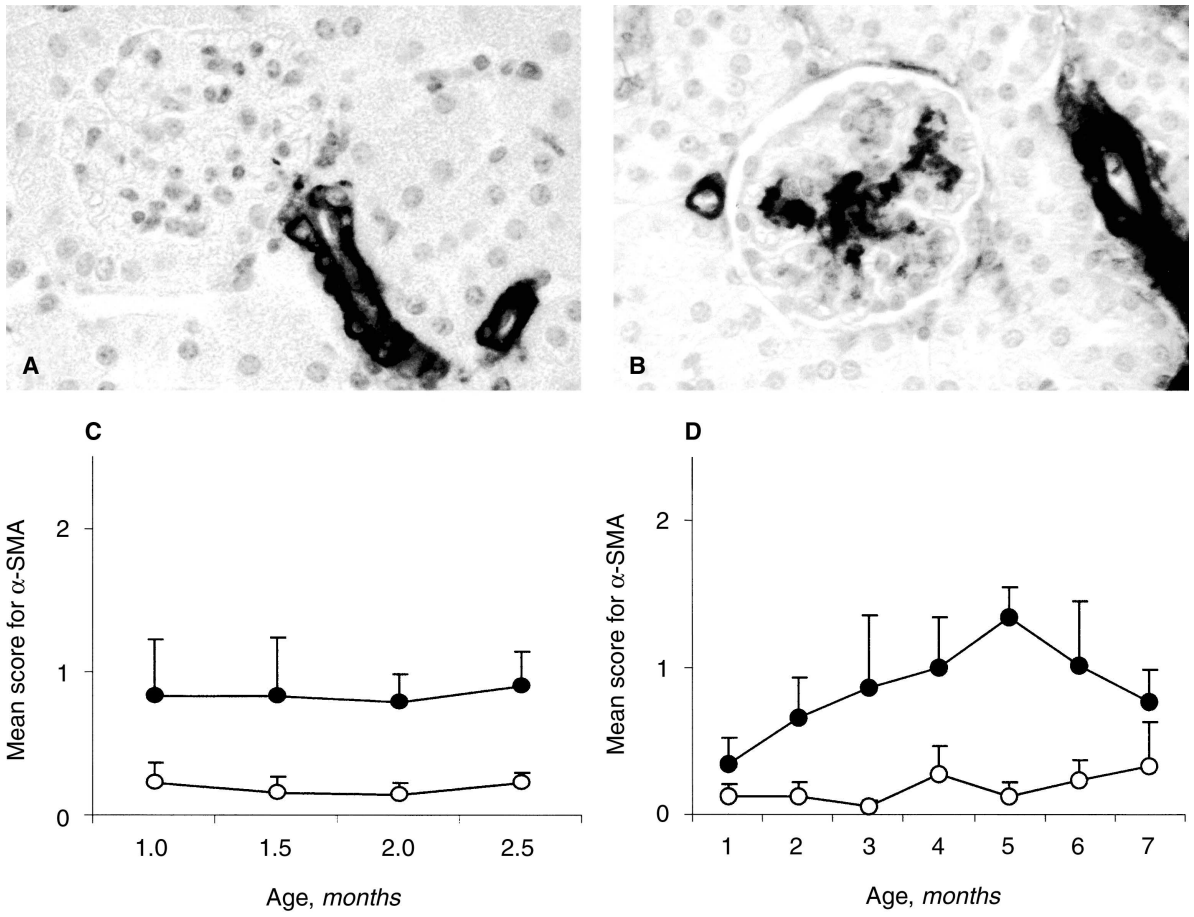

Fig. 1. α-Smooth muscle actin (αSMA) expression in thymic stromal lymphoprotein (TSLP) transgenic mice and wild-type controls. Immunohistochemistry for αSMA in a wild type female (*A*) and a TSLP transgenic female at the age of 2.5 months (*B*; original magnification ×400). Glomerular staining is absent in the wild-type control, whereas the TSLP transgenic mouse shows strong expression of αSMA in a mesangial pattern. Both wild-type and transgenic mice show constitutive expression of αSMA in vascular smooth muscle cells of arteries and arterioles. Time course of αSMA scores in female (*C*) and male mice (*D*). Symbols are: (○) control; (●) transgenic mice.

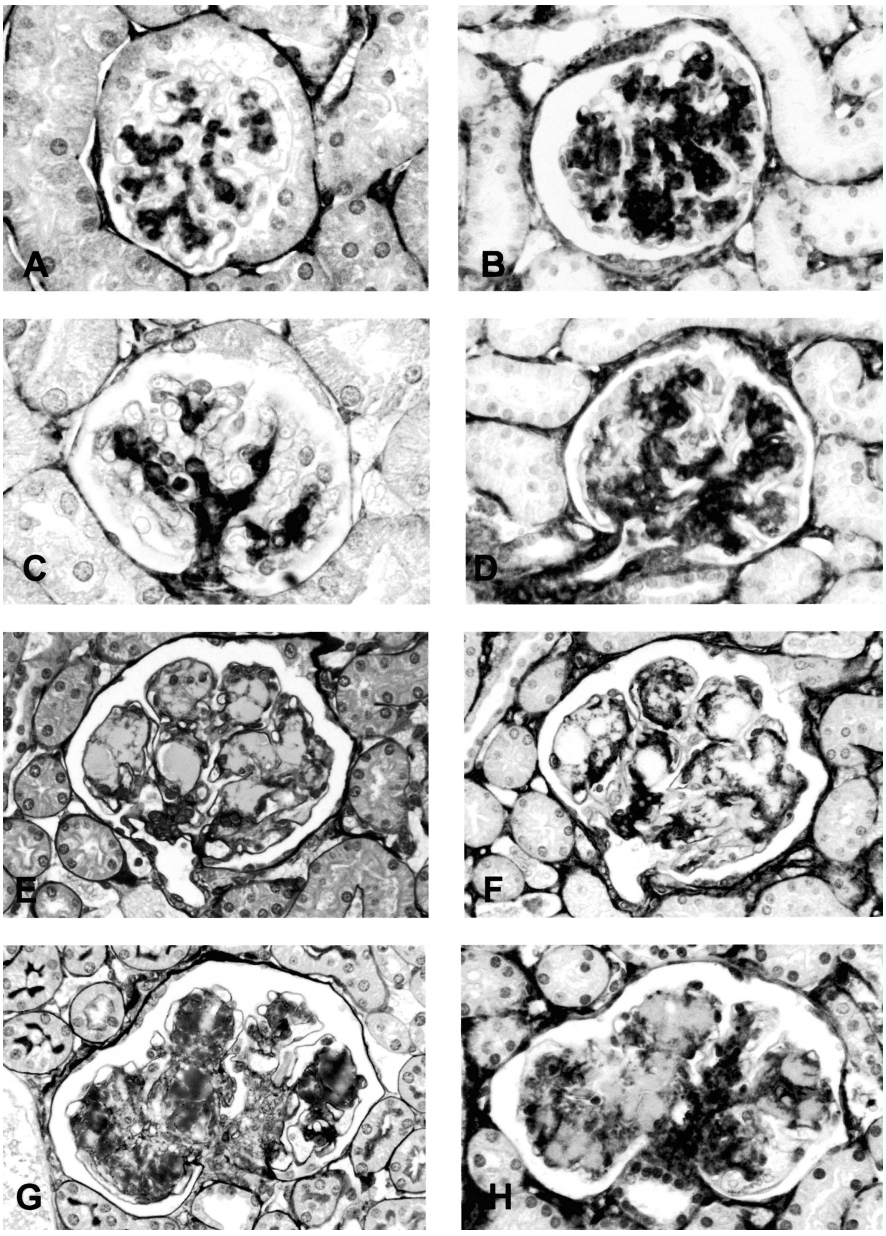

Fig. 2. Immunohistochemical staining for extracellular membrane (ECM). (*A–D*) Immunohistochemistry for type IV collagen (A, B) and laminin (C, D) in a wild-type control (A, C) and a TSLP transgenic female at the age of 2.5 months (B, D; all original magnification ×400). TSLP transgenic mice demonstrated an increase in collagen IV and laminin in a mesangial pattern as compared to wild type controls. (*E–H*) Immunohistochemistry for type IV collagen (F), and laminin (H) and silver stains (E, G) performed on serial sections of a TSLP male at the age of 5 months (all orig. ×400). The widening of the mesangial matrix in this case was mainly due to massive immune deposits, which are silver negative and do not stain for type IV collagen and laminin. Note the positive staining in the remaining mesangial matrix.

PDGF-A chain: A 536 nucleotides (nt) SmaI fragment of the mouse PDGF A-chain cDNA (GenBank Acc. #M29464: nt 1 to 906) was cloned into the SmaI site of pGEM-3Zf+ (Promega Biotec, Madison, WI, USA). The full-length probe is 592 nt.

PDGF-B chain: An 806 nt EcoRI fragment of the mouse cDNA that contains a 752 nt fragment of mouse B-chain was a gift from C. Stiles (Dana-Farber Cancer Institute, Boston, MA, USA). It was inserted into the EcoRI site of pGEM-3Zf+ (Promega Biotec). The full-length probe encodes for the whole PDGF B-chain molecule.

PDGF α-R: A 1636 nt EcoRI fragment of the mouse cDNA (GenBank Acc. #M57683: nt 201 to 1837) was cloned into the EcoRI site of pGEM-3Zf+ (Promega Biotec). The full-length probe is 1692 nt.

PDGF β-R: A 2075 bp PstI fragment of the mouse cDNA (GenBank Acc. #X04367: nt 1667 to 3742) was inserted into the PstI site of pGEM-3Zf+ (Promega Biotec). The full-length probe is 2131 nt.

TGF-β1: (gift from H.L. Moses, Dept. Cell Biology, Vanderbilt University, Nashville, TN, USA) [31]. A 974 nt fragment of the mouse cDNA was inserted into the SmaI site of pGem7Zf+ (Promega Biotec).

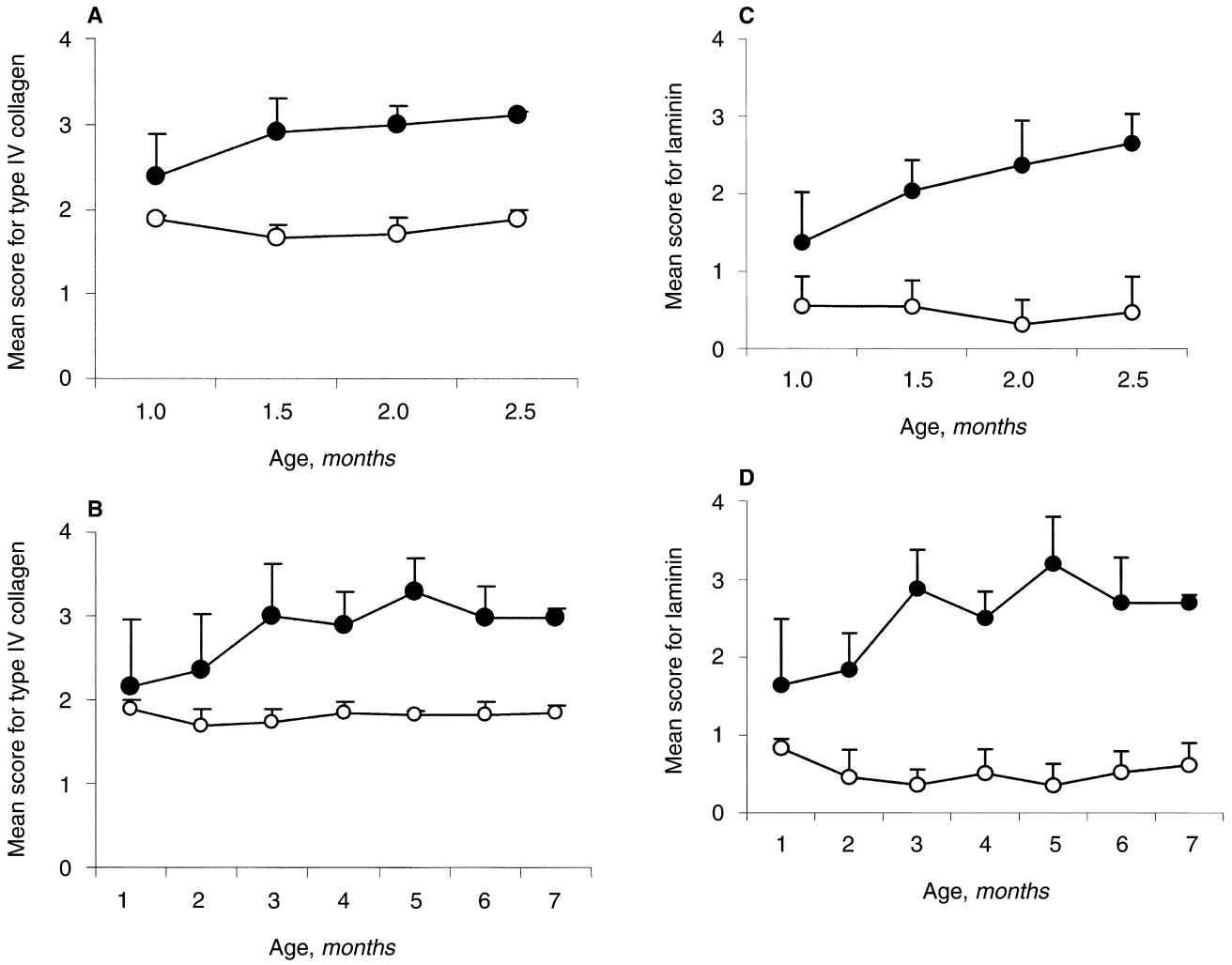

Fig. 3. Immunohistochemical scores for ECM and PCNA. Time course of mean scores for collagen type IV (*A*, *B*), laminin (*C*, *D*) and mean number of PCNA positive cells per glomerular cross section (gcs, *E*, *F*) in female (A, C, E) and male mice (B, D, F). Symbols are: (○) control mice; (●) transgenic mice.

After linearization ^{35}S antisense and sense (negative control) riboprobes were produced, using reagents from Promega, and ^{35}S UTP, from NEN (Boston, MA, USA) [32].

In situ hybridization

The in situ hybridization protocols have recently been described in detail [33, 34]. Negative controls included hybridization performed on replicate tissue sections using the sense riboprobe.

The intensity of intraglomerular expression for each transcription was graded semiquantitatively by an observer blinded to origin of the histologic specimen as follows: 0 = absent, 1 = mild (a few positive cells), 2 = moderate (several positive cells in a segmental distribution within the glomerulus), and 3 = severe (many positive cells in a diffuse distribution).

Combined immunohistochemistry and in situ hybridization

To identify monocyte/macrophage expression of specific growth factors and receptors (PDGF B-chain, PDGF-β-R, or TGF-β1), combined immunohistochemistry and in situ hybridization were performed as previously described [33]. Slides were first hybridized with antisense and sense riboprobes for detection of TGF-β1, PDGF-B chain or PDGF-β-R mRNA. After stringency washes, slides were immunostained for macrophages (Mac-2) using the DAB detection system without nickel chloride enhancement to produce a brown reaction product. Following immunostaining, slides were washed, dehydrated and dipped in NTB-2 emulsion.

Statistical analysis

All values are expressed as the mean ± standard deviation (SD). Using the SPSS® program, Version 10.0 for Windows (SPSS Inc., Chicago, IL, USA), the non-parametric Mann-Whitney U-test was used to compare the means between groups. The number of macrophages expressing PDGF-B chain or TGF-β1 mRNA was compared for each of the study time points by one-way analy-

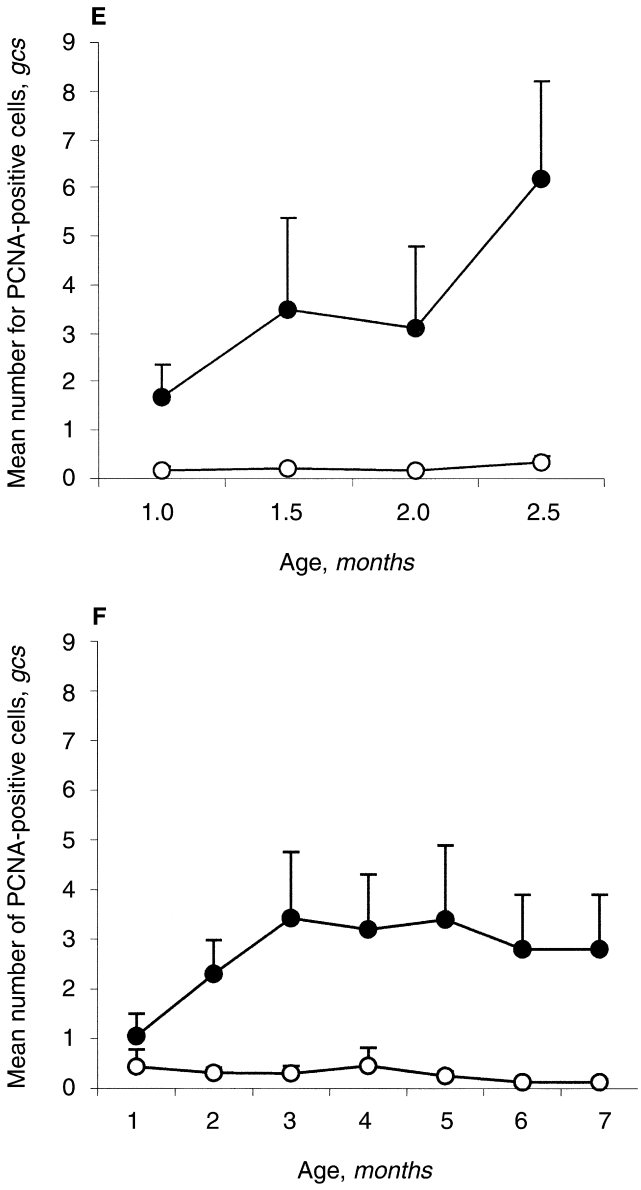

Fig. 3. (Continued)

sis of variance (ANOVA) using Tukey's post-hoc test. Additionally, the non-parametric Spearman rank correlation coefficients was used to determine the association between PDGF mRNA score and the number of glomerular proliferating cells among each group.

RESULTS

Morphology

Glomerular pathologic alterations of TSLP transgenic mice during the time course of the study, which covers first 2.5 months of age in female and the first seven months of age in males, were described in a previous study [21]. Renal lesions were characterized by mesangial expansion due to matrix and immune complex deposition, thickened and split capillary walls with prominent immune deposits and cellular interposition and macrophage influx, which are typical features of cryoglobulinemic MPGN. All TSLP transgenic mice demonstrated a widening of the mesangial area after the age of one month, which was more prominent in female than in males at early time points. Massive deposition of immune complexes were present also in the capillary walls of both genders of transgenic mice, which sometimes led to complete capillary occlusion with progression of the disease. In the most severe cases, the mesangium was expanded and most of the capillary lumina were occluded. In contrast, globally sclerotic glomeruli were not found in either gender of transgenic mice even at late time points. The progression of renal lesion and the disease course were more pronounced and more accelerated in female than in male mice.

Glomerular expression of α-smooth muscle actin is increased in TSLP transgenic mice

α-Smooth muscle actin is expressed by smooth muscle cells of the arterial wall in normal kidneys, as well as by mesangial cells during renal development, and by activated mesangial cells during glomerular injury [22]. All renal specimens from TSLP transgenic mice and controls demonstrated intense staining for αSMA by smooth muscle cells in arterial walls in the interstitium. In most controls αSMA staining was restricted to these sites (Fig. 1A). However, a few glomeruli in control animals demonstrated mesangial αSMA expression, usually in a segmental distribution within the glomerular tuft. In contrast, TSLP transgenic mice of both genders exhibited increased αSMA expression in a mesangial pattern beginning at the age of one month. Female TSLP transgenic mice demonstrated a constant increase of αSMA protein expression through the course of the disease (0.9 ± 0.2 in TSLP transgenic mice vs. 0.2 ± 0.1 in controls at month 2.5, $P < 0.01$, Fig. 1C). αSMA immunostaining in male transgenic mice was only slightly elevated at one month of age, but rose progressively and peaked at month 5 (1.3 ± 0.2 in TSLP transgenic mice vs. 0.1 ± 0.1 in controls, $P < 0.05$), and then gradually decreased (Fig. 1D). At the age of one month female mice demonstrated higher scores than male mice. No apparent αSMA expression was observed in interstitial cells of TSLP transgenic mice as well as of control mice, and tubular epithelium was negative in the well preserved tubulointerstitium.

Deposition of extracellular matrix is associated with increased type IV collagen and laminin protein expression in TSLP transgenic mice

The area and percentage of glomerular ECM was assessed by morphometric analysis on silver methenamine

stained sections [21]. An increased deposition of extracellular matrix was demonstrated early in the disease course and reached a plateau in both genders [21]. To assess the sequential contribution of individual ECM components, immunohistochemical staining was performed for type I collagen, type IV collagen and laminin. Control mice of both genders demonstrated immunostaining of type IV collagen in the glomerular and tubular basement membranes. Glomerular staining for type IV collagen in control mice was focal in distribution, which had no apparent change during the ages studied (Figs. 2A and 3 A, B). A similar staining pattern for laminin was observed in control kidneys (Figs. 2C and 3 C, D). In contrast, TSLP transgenic mice demonstrated increased staining for type IV collagen and laminin predominantly in the mesangium (Fig. 2 B, D, F, H). In the most severe cases, the mesangium was widened due to both matrix accumulation and massive deposition of immune deposits, at times associated with complete capillary occlusion [21]. Although the expanded mesangial area in transgenic mice was due to increased matrix deposition, this did not advance to global sclerosis, as such glomeruli were absent throughout the time course of the disease that was studied [21]. Areas occupied by immune deposits, as determined by correlative electron microscopy, did not stain with the silver methenamine reagent, and these areas also were unreactive with antibodies to detect both collagen type IV and laminin accumulation (Fig. 2 F, H). In female TSLP transgenic mice, mesangial immunostaining for type IV collagen and laminin rose gradually from one month of age up to month 2.5 (type IV collagen, 3.1 ± 0.1 in TSLP transgenic mice vs. 1.9 ± 0.1 in controls, $P < 0.01$; Fig. 3A; laminin, 2.7 ± 0.4 in TSLP transgenic mice vs. 0.5 ± 0.4 in controls, $P < 0.01$; Fig. 3C). In male TSLP transgenic mice, laminin and type IV collagen scores reached a plateau at month 3 (type IV collagen, 3.0 ± 0.1 in TSLP transgenic mice vs. 1.9 ± 0.1 in controls, $P < 0.01$; Fig. 3B; laminin, 2.7 ± 0.1 in TSLP transgenic mice vs. 0.6 ± 0.3 in controls, $P < 0.05$; Fig. 3D). Glomerular staining for collagen I was absent in both controls and TSLP transgenic mice (data not shown).

An increased number of proliferating glomerular cells characterizes the increased glomerular cellularity in TSLP transgenic mice

A significant increase in PCNA positive cells was demonstrated beginning at month 1 in both genders of TSLP transgenic mice, and was more pronounced in females at this time point (Fig. 4). In male TSLP transgenic mice, glomerular cell proliferation reached a plateau at three months of age (3.4 ± 1.3 PCNA positive cells/gcs vs. 0.3 ± 0.2 in controls, $P < 0.05$), while female transgenic mice had a gradual elevation up to month 2.5 (6.2 ± 2 PCNA positive cells/gcs vs. 0.3 ± 0.1 in controls, $P <$ 0.05). PCNA positive nuclei were present in the mesangium, at the periphery of capillary loops, and occasionally in parietal epithelial cells.

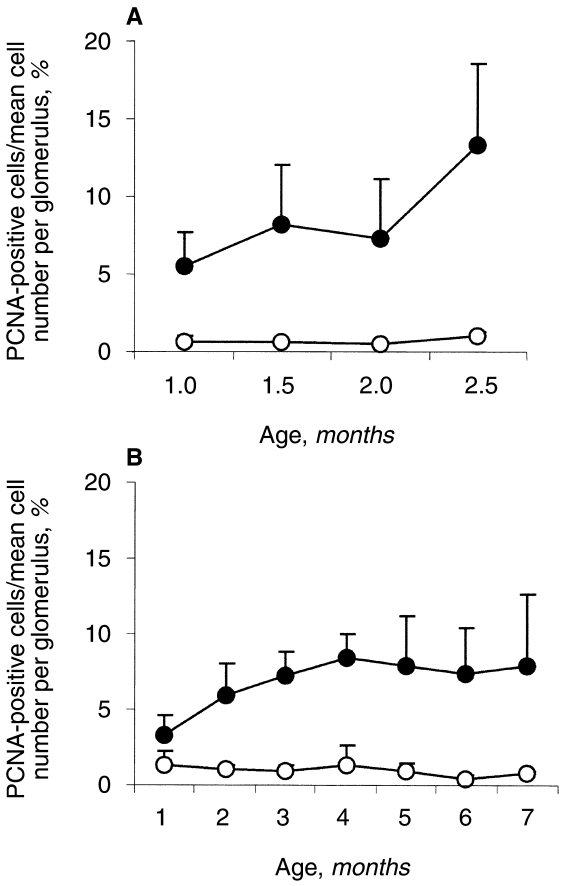

Fig. 4. Percentage of PCNA positive cells to the total glomerular cells in female mice (*A*) and male mice (*B*). The proportion of PCNA positive cells to the cell number increased with the time course of the disease in both genders of TSLP transgenic mice (●), but not in control mice (○).

Although an increase in PCNA positive cells per glomerulus was demonstrated in TSLP transgenic mice, the total cell number per glomerulus was increased significantly in TSLP transgenic mice [21]. Therefore, we assessed the percentage of PCNA-positive cells with respect to the total glomerular cell number. The proportion of PCNA-positive cells compared to the total glomerular cells in female transgenic mice increased progressively to month 2.5 (5.5 ± 2.1% in month 1, 13.3 ± 5.2% in month 2.5). Male transgenic mice had a gradual increase in percentage of proliferative cells up to month 7 (3.3 ± 1.3% in month 1, 7.8 ± 4.3% in month 7). In contrast, the proportion of PCNA-positive cells per glomerulus did not change in control groups.

PDGF-B chain and PDGF-β-R mRNA is increased in TSLP transgenic mice and correlates with glomerular cell proliferation

Representative in situ hybridization results including sense controls are illustrated in Figure 5 and Figure 6.

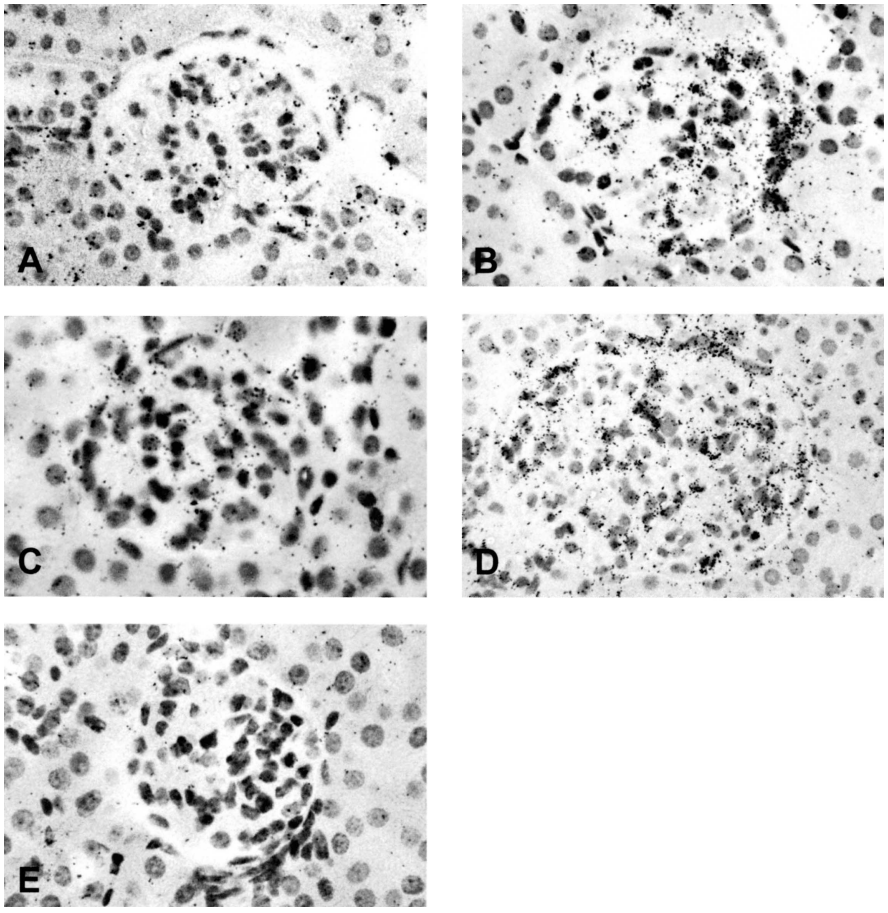

Fig. 5. In situ hybridization for PDGF-B chain mRNA. In situ hybridization using a PDGF-B chain antisense riboprobe (*A–D*) and a sense riboprobe (*E*) performed on tissue sections from wild-type controls (A, female; C, male) and a 2.5-month-old TSLP transgenic female (B) and a TSLP transgenic male at month 6 (D, all orig. ×400). TSLP transgenic mice showed a prominent increase in number of cells and intensity of the signal in the mesangium and in parietal epithelial cells. Minimal background accumulation of silver grains is present in E.

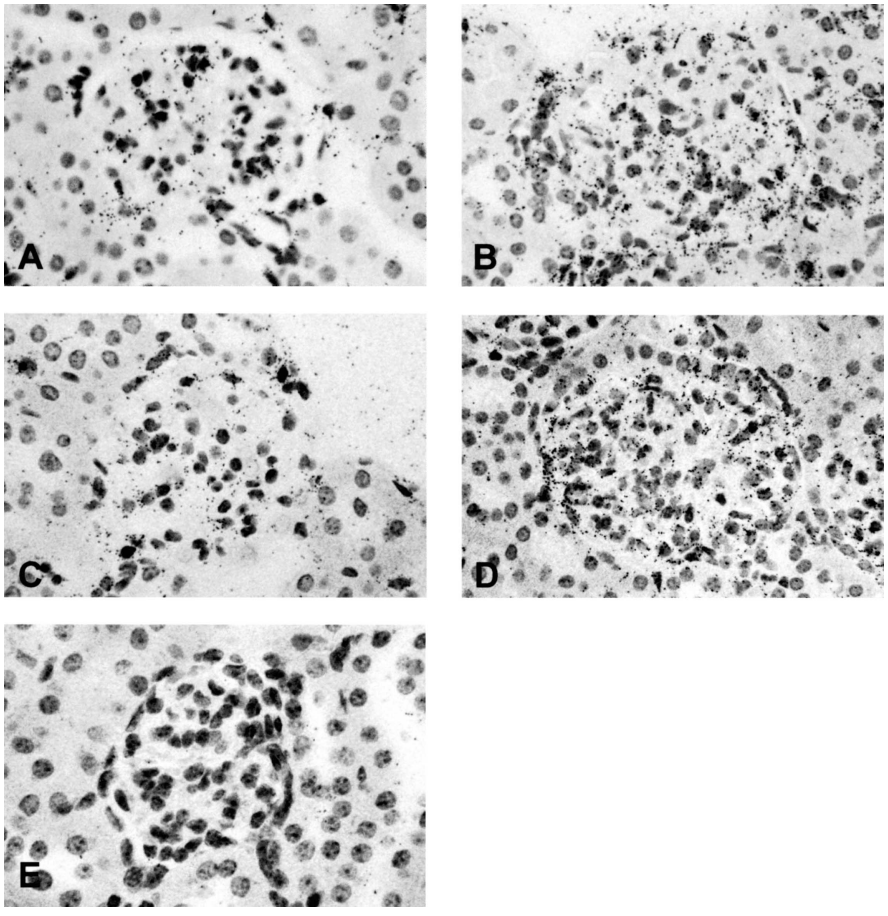

Fig. 6. In situ hybridization for PDGF-β-R mRNA. In situ hybridization using a PDGF-β-R antisense riboprobe (*A–D*) and a sense riboprobe control (*E*) performed on tissue sections from wild-type controls (A, female; C, male) and a 1.5-month-old TSLP transgenic female (B) and a TSLP transgenic male at month 3 (D, all orig. ×400). Up-regulated expression of PDGF-β-R mRNA is demonstrated in the TSLP mice of both genders. Minimal background accumulation of silver grains is present in E.

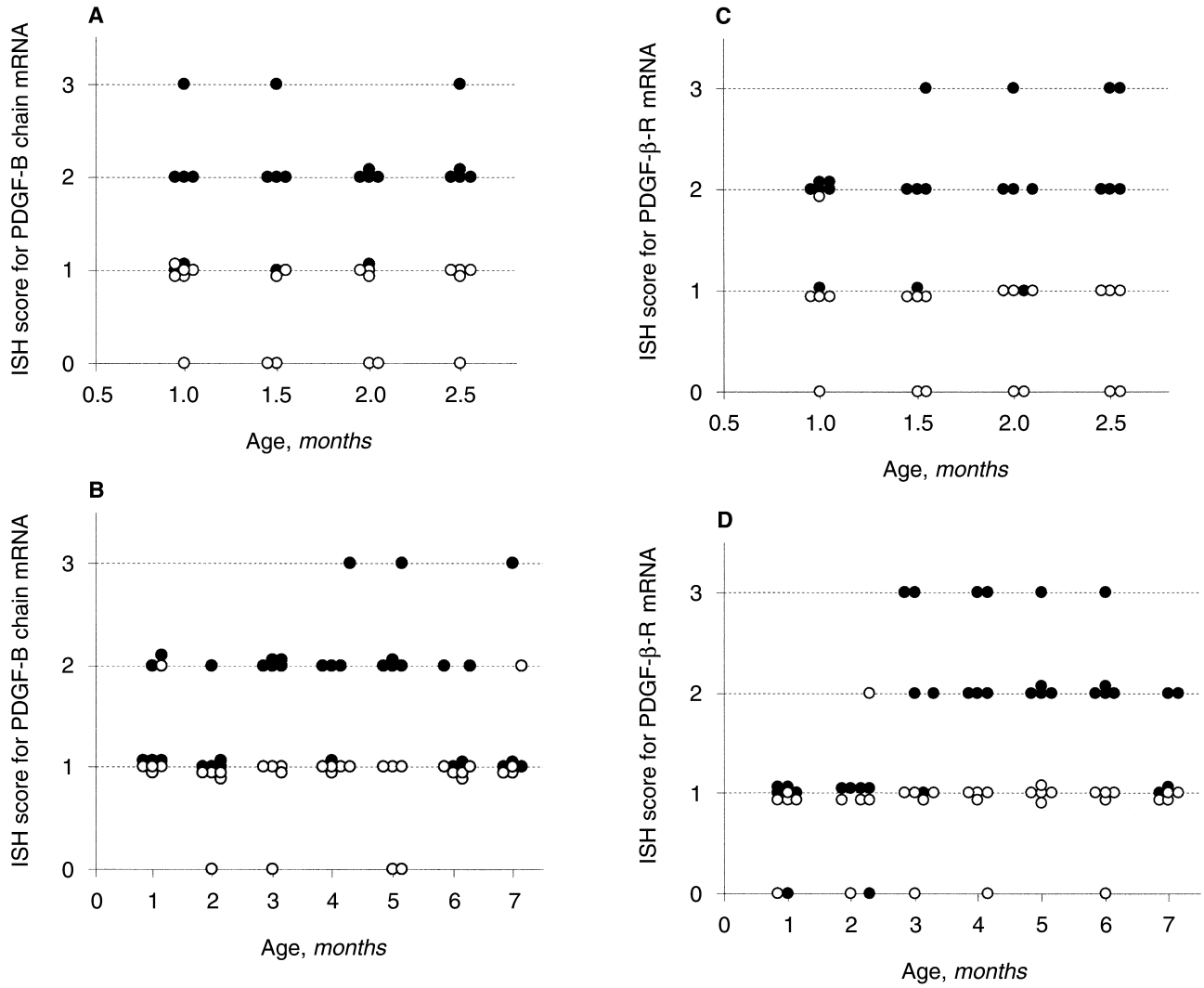

Fig. 7. Semiquantitative PDGF mRNA scores. Semiquantitative analysis of glomerular PDGF-B chain mRNA (*A*, *B*) and PDGF-β-R mRNA (*C*, *D*) in female (A, C) and male mice (B, D) demonstrating higher levels of expression for both PDGF-B chain and its receptor in TSLP transgenic mice. Symbols are: (●) transgenic mice; (○) control mice.

A preliminary series of animals (**Methods** section) was studied by in situ hybridization for expression of PDGF A-chain, PDGF-B chain and their receptors, PDGF-α-R and β-R. Glomerular expression of PDGF A-chain was not detected and PDGF-α-R was present in a very low number of glomeruli, whereas a prominent induction of PDGF-B chain and PDGF-β-R became apparent. Therefore, these two factors were chosen for further study in the current prospective animal series.

Glomeruli of wild-type controls showed either absent or weak mesangial expression of PDGF-B chain (Fig. 5 A, C) and PDGF β-R mRNA (Fig. 6 A, C). In contrast, TSLP transgenic mice exhibited increased expression of PDGF-B chain and PDGF β-R in both the mesangium and the parietal epithelial cells (Fig. 5 B, D). Expression of PDGF-B chain and PDGF β-R mRNA was found in scattered tubulointerstitial cells both in wild-type and TSLP transgenic mice without apparent differences between the two groups. As shown in Figure 7, the glomerular expression patterns of PDGF-B chain and PDGF-β-R mRNA in controls did not significantly vary over time between the two genders. In contrast, the expression of PDGF-B chain or PDGF-β-R mRNA reached a plateau after three months of age in TSLP transgenic males (Fig. 7 B, D), whereas female mice demonstrated high expression for each probe as early as the first month of age and this remained elevated for the duration of time course of the study (Fig. 7 A, C). In TSLP transgenic mice, there was a moderate to strong positive correlation between the number of glomerular proliferating cells and the intensity of glomerular expression for PDGF B-chain (Spearman $r = 0.63$, $P = 0.002$ for females;

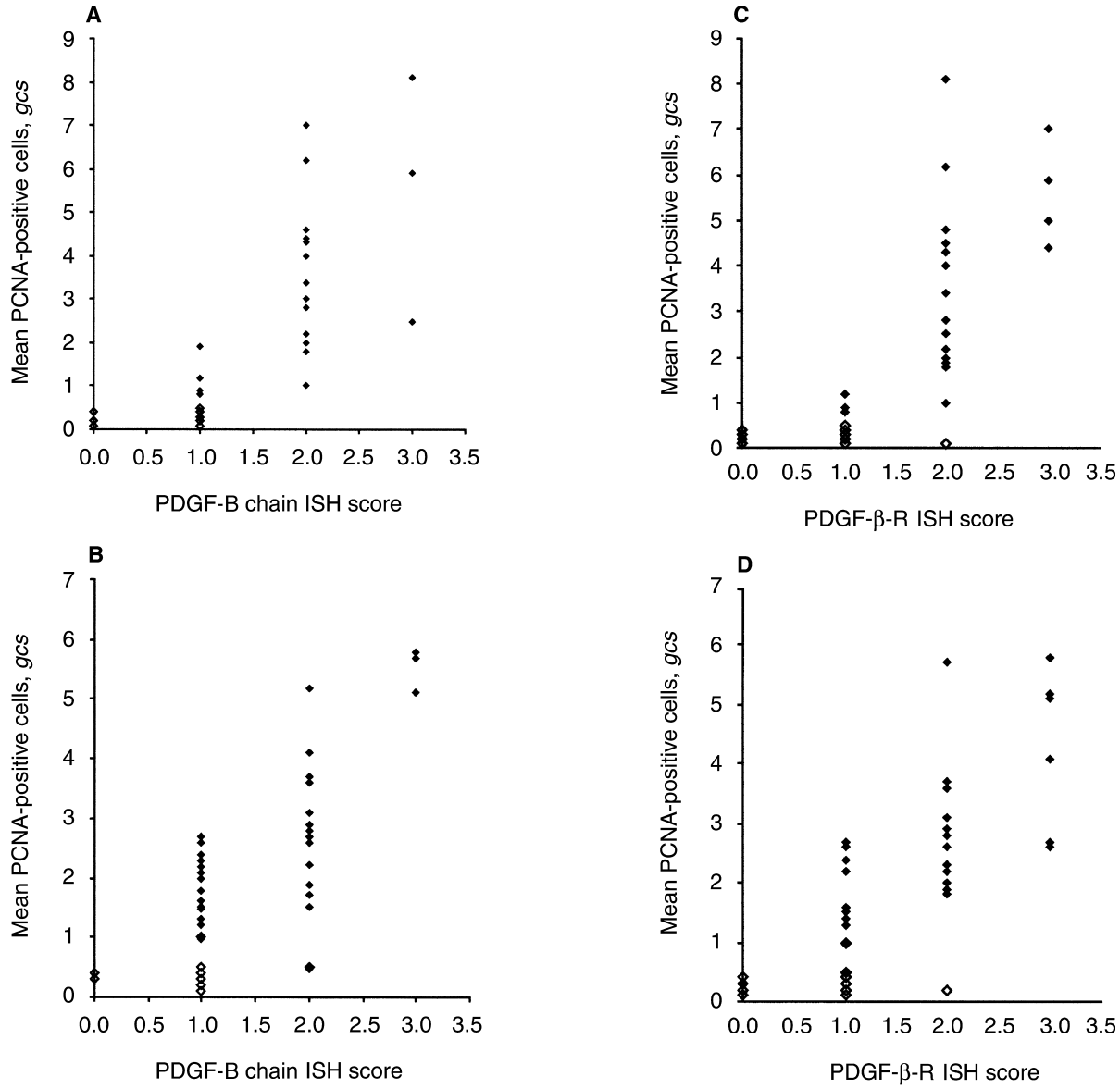

Fig. 8. (*A*) Correlation between PCNA-positive cells per glomerular cross section (gcs) and PDGF-B chain mRNA scores in female mice. (Transgenic mice, $r = 0.63$, $P \leq 0.01$; Control mice, $r = 0.23$, $P = 0.31$). (*B*) Correlation between PCNA-positive cells and PDGF-B chain mRNA scores in male mice (transgenic mice, $r = 0.64$, $P \leq 0.001$; control mice, $r = -0.05$, $P = 0.76$). (*C*) Correlation between PCNA-positive cells and PDGF-β-R mRNA scores in female mice (transgenic mice, $r = 0.70$, $P \leq 0.001$; control mice, $r = -0.18$, $P = 0.42$). (*D*) Correlation between PCNA-positive cells and PDGF-β-R mRNA scores in male mice (transgenic mice, $r = 0.75$, $P \leq 0.001$; control mice, $r = 0.16$, $P = 0.37$). The correlation between cell proliferation as measured by PCNA expression and PDGF synthesis was significant in both genders of TSLP transgenic mice. Symbols are: (◆) transgenic mice; (◇) control mice.

Fig. 8A, Spearman $r = 0.64$, $P < 0.001$ for males; Fig. 8B) or PDGF-β-R mRNA (Spearman $r = 0.70$, $P < 0.001$ for females; Fig. 8C, Spearman $r = 0.75$, $P < 0.001$ for males; Fig. 8D). In contrast, the association between those variables in control groups was very weak (PDGF B-chain, Spearman $r = 0.23$, $P = 0.31$ for females, Fig. 8A, and Spearman $r = -0.05$, $P = 0.76$ for males; Fig. 8B; PDGF-β-R, Spearman $r = -0.18$, $P = 0.42$ for females, Fig. 8C, and Spearman $r = 0.16$, $P = 0.37$ for males; Fig. 8D). Although a few control mice showed elevated levels of PDGF-B chain and PDGF-β-R mRNA expression, they did not manifest a significant glomerular proliferative response (Fig. 8).

TGF-β1 mRNA is increased in TSLP transgenic mice

As TGF-β1 is thought to be of major importance during the deposition of ECM, the expression of TGF-β1 mRNA was studied by in situ hybridization. Weak expression of TGF-β1 mRNA in a mesangial pattern was detected in control mice (Fig. 9 A, C). Female transgenic mice showed increased glomerular expression of TGF-β1 mRNA beginning at the age of 1.5 months (Figs. 9B and

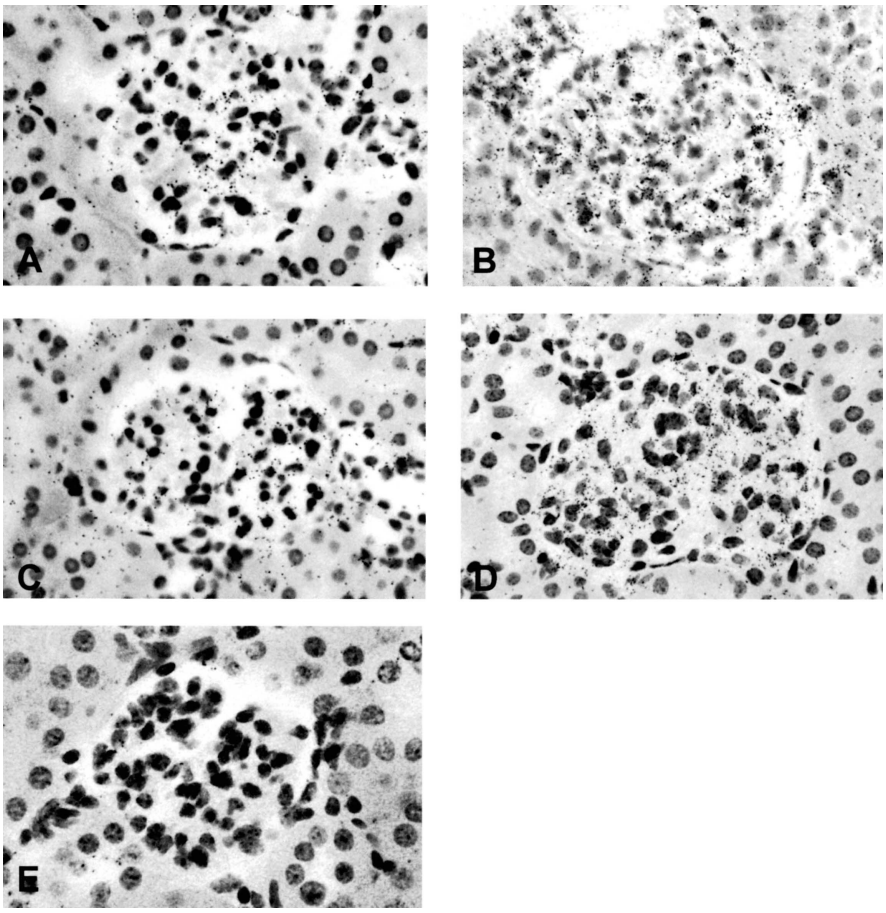

Fig. 9. In situ hybridization for TGF-β1 mRNA. In situ hybridization using a TGF-β1 antisense riboprobe (*A–D*) and a sense riboprobe control (*E*) performed on tissue sections from wild-type controls (A, female; C, male) and a 2.5-month-old TSLP transgenic female (B) and a TSLP transgenic male at the age of 6 months (D, all orig. ×400). Only a weak signal is present in wild-type controls, while there is prominent up-regulated expression of TGF-β1 in TSLP transgenic mice. Minimal background accumulation of silver grains is present in E.

10A). In contrast, TSLP transgenic males demonstrated an increased expression beginning at four months up to seven months of age (Figs. 9D and 10B), but did not show significant changes from the normal expression pattern prior to month 3.

Intraglomerular macrophages express PDGF-B chain and TGF-β1 mRNA but not PDGF-β-R mRNA

To identify glomerular monocytes/macrophages as a potential source of growth factors or the PDGF β receptor, we combined immunohistochemical labeling of Mac-2 expressing macrophages with in situ hybridization for PDGF-B chain, PDGF-β-R, and TGF-β1 mRNA. Renal tissues obtained from control TSLP wild-type mice were not included in this part of the study, because glomerular macrophages in wild-type mice were barely detected in previous experiments [21].

A significant proportion (approximately half) of intraglomerular monocytes/macrophages manifesting Mac-2 antigen expressed TGF-β1 mRNA (Figs. 11C and Fig. 12). In contrast, only a small percentage (less than 10%) of glomerular Mac-2 expressing monocytes/macrophages expressed PDGF B-chain mRNA during any of the time points studied (Figs. 11A and 12). Most of the PDGF-B chain mRNA was expressed by intrinsic renal cells that did not express the macrophage marker (Fig. 11A). Macrophages expressing PDGF-β-R mRNA were not detected in any of the transgenic mice (Fig. 11B). Figure 12 shows the percentage of the glomerular macrophages that express these growth factors at the different time points studied. The proportion of glomerular macrophages expressing PDGF-B chain and TGF-β1 mRNA did not show significant differences when analyzed at different time points.

DISCUSSION

A new transgenic mouse model of cryoglobulinemic MPGN gave us the opportunity to describe the temporal changes in expression of several members of the PDGF family and TGF-β1, in combination with components of the ECM and markers of cell activation/proliferation, in this disease process [21]. The glomerular lesion in TSLP transgenic mice is characterized by widening of the mesangium due to an expanded mesangial matrix and deposition of immune deposits, increased glomerular size with an increase of the absolute glomerular cell number, sub-

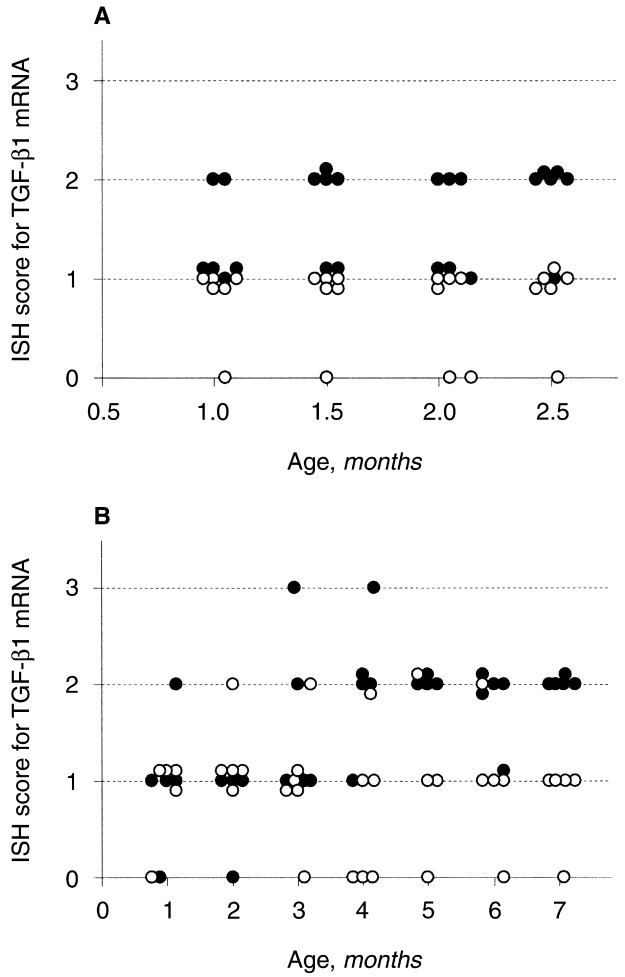

Fig. 10. Semiquantitative analysis of glomerular TGF-β1 mRNA scores in female (*A*) and male (*B*) mice showing higher levels of expression of TGF-β1 in the TSLP transgenic mice. Symbols are: (●) transgenic mice; (○) control mice.

endothelial immune deposits in peripheral capillary walls, and splitting of capillary basement membranes.

We documented that a change in the mesangial cell phenotype characterized by de novo expression of αSMA was found early in the disease course in both genders of TSLP transgenic mice and showed a progressive increase in TSLP transgenic males. This phenotypic change has previously been described in other rodent models of renal injury [8, 25, 35], and in human glomerulonephritis including MPGN [22]. In TSLP transgenic mice this change is likely related to activation of mesangial cells following deposition of immune complexes.

An increase of extracellular mesangial matrix develops early in the development of the disease in TSLP transgenic mice, but does not progress to global glomerulosclerosis, at least within the time frame studied. Here we demonstrated that this matrix accumulation is due in part to deposition of collagen type IV, and laminin, whereas collagen type I was not detectable. These results are consistent with data in human MPGN, which describe increased expression type IV collagen and laminin in the glomerular mesangial areas and the peripheral capillary walls [36, 37].

In contrast to the rare occurrence or absence to the expression of PDGF-A chain and PDGF-α-R, as revealed by our pilot studies, there is prominent induction of PDGF-B chain and the corresponding receptor PDGF-β-R in this disease model. The distribution patterns for this ligand/receptor pair were very similar in mesangial areas, and parietal epithelial cells. PDGF-B chain and β-R correlated significantly with the number of proliferating glomerular cells. Up-regulation of PDGF protein previously has shown to correlate with glomerular mesangial cell proliferation [38–40]. Additionally, the expression of PDGF-B chain and β-R paralleled an increase in extracellular matrix. The importance of PDGF-B chain in the regulation of glomerular extracellular matrix accumulation, as well as in mesangial cell proliferation has been demonstrated by several interventional studies [9, 15, 41]. Experiments with infusion of PDGF-B chain in the anti-Thy1.1 model in rats [41] or transfection of PDGF-B chain cDNA to rats [15] induced selective glomerular mesangial cell proliferation and matrix accumulation redundant [8, 15]. Furthermore, blockade by a neutralizing antibody to PDGF reduced mesangial cell proliferation and largely prevented the increased deposition extracellular matrix in anti-Thy1.1 glomerulonephritis [9]. Here we demonstrate the expression of PDGF-B chain and its receptor in a mouse model with a membranoproliferative pattern of injury and confirm a correlation with glomerular cell proliferation and matrix deposition. Induction of PDGF-B chain was demonstrated earlier in females as compared to males, consistent with more severe lesions that occur in females at earlier time points.

Transforming growth factor-β also has been shown to play an important role during the development of mesangial matrix expansion [16, 19]. Increased expression of TGF-β1 has been reported in several experimental models as well as in human glomerulonephritis [42–48]. One study suggested a potential role for direct stimulation of glomerular cells by immune complexes composed of IgG and IgA, which then trigger the formation of ECM via Fc receptors, utilizing pathways involving TGF-β1 [49]. The actual stimulus for TGF-β1 production in our model or in human disease is not yet known. It is clear that the time course of TGF-β1 mRNA paralleled the expression of extracellular matrix proteins in our model, consistent with established paradigms.

Our study also established that the intraglomerular monocytes/macrophages may be a source for some of the PDGF-B chain and TGF-β1 activity in the glomerular injury. TSLP transgenic mice develop significant mono-

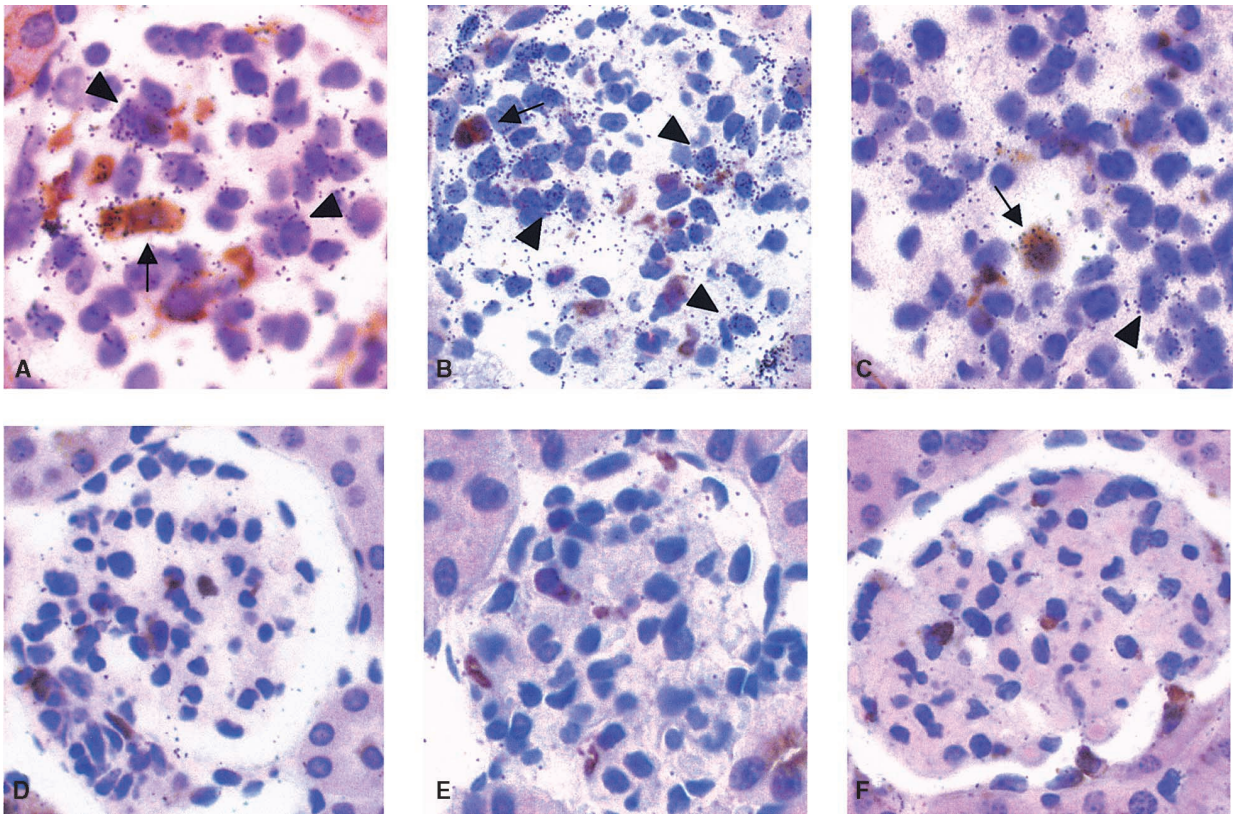

Fig. 11. Combined immunohistochemistry and in situ hybridization. Combined immunohistochemical labeling (brown) of Mac-2 expressing monocytes/macrophages with in situ hybridization (black grains) for PDGF-B chain (*A*), PDGF-β-R (*B*) and TGF-β1 (*C*), and a sense riboprobe control for PDGF-B chain (*D*), PDGF-β-R (*E*), and TGF-β1 (*F*). (A) PDGF-B chain mRNA is present in a Mac-2 expressing macrophage (arrow), but most glomerular cells expressing PDGF-B chain mRNA do not express monocyte/macrophage marker (arrowheads). This glomerulus was obtained from a female transgenic mouse at the age of 2.5 months. (B) Cells with up-regulated PDGF-β-R mRNA were numerous (arrowheads), but these cells could not be identified as monocytes/macrophages (arrow). Immunolabeled monocytes/macrophages in brown revealed no colabeling with the PDGF-β-R mRNA probe. This glomerulus was obtained from a male transgenic mouse at the age of 3 months. (C) TGF-β1 mRNA expressing monocyte/macrophage (arrow) and TGF-β1 mRNA expressing cell which does not express the monocyte/macrophage marker (arrowhead), within a glomerulus obtained from a female transgenic mouse at the age of 2 months. Minimal background accumulation of silver grains is present in D, E and F (original magnification, ×1000).

cyte/macrophage infiltration in glomeruli [21]. Although monocytes/macrophage are known to be one of the major sources of growth factors including PDGF and TGF-β [50, 51], the contribution of infiltrating macrophages compared with that of renal intrinsic cells in the synthesis of growth factors currently has not been established. In our model, a significant proportion of Mac-2 expressing monocytes/macrophages expressed TGF-β1, which suggests increased TGF-β1 activity may be the result of both paracrine secretion and autocrine production in this model. In contrast, the percentage of monocytes/macrophages expressing PDGF B-chain was small in TSLP transgenic mice, and it is it unlikely that they are the major source of increased glomerular PDGF. Previous studies demonstrated that in the rat anti-Thy1.1 model of mesangial proliferative glomerulonephritis, more than 90% of the glomerular cells immunostaining for PDGF B-chain also expressed αSMA, indicating that the majority of cells producing PDGF-B chain were mesangial cells [40].

Based on the above, these are several principal points to be gleaned from this study. First, the findings demonstrating expression and presumptive activities of PDGF-B chain and its receptor and TGF-β1 in this model of MPGN conform to paradigms established in the rat model of anti-Thy1 of mesangiolytic injury and repair. We believe this is important because it demonstrates that these paradigms are generalizable to glomerular and mesangial injuries other than mesangiolysis, and to species other than the rat. The close morphologic similarity between renal injury in the TSLP transgenic mouse and human cryoglobulinemic MPGN means it is likely, though not yet proven, that similar expression of these growth factors occurs at similar stages of injury in the human disease. The predictable injury course in these mice, which occurs notably early in female mice, suggests it would serve as a good model system to test interven-

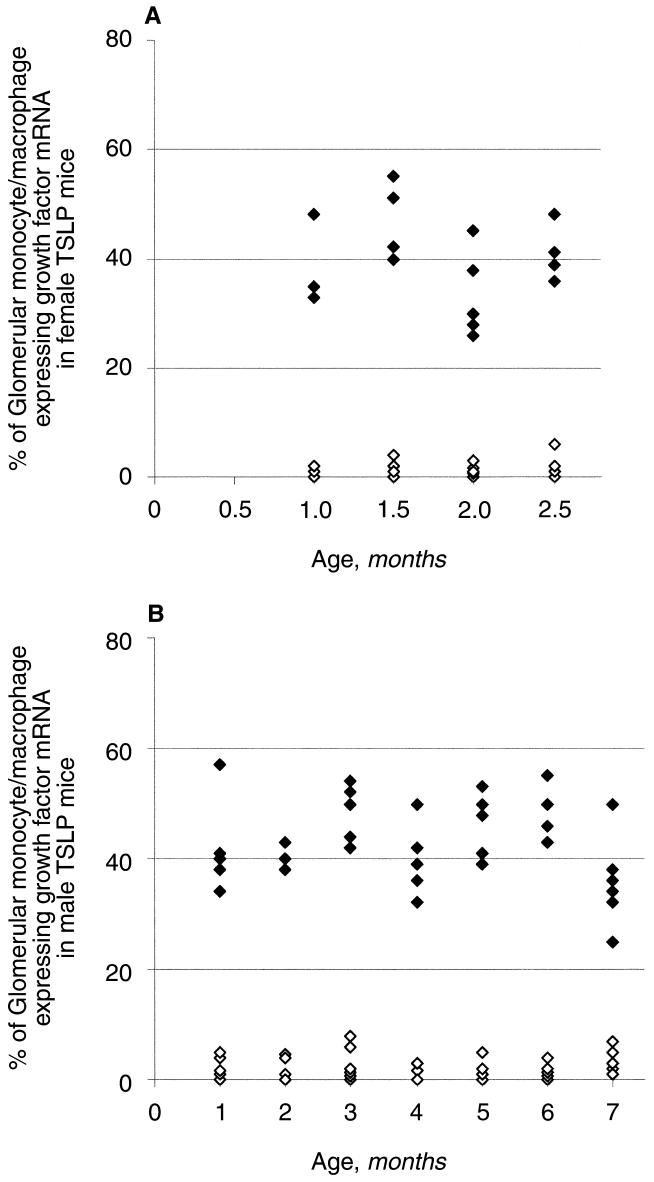

Fig. 12. Extent of intraglomerular monocyte/macrophage and expression of PDGF B-chain (◇) or TGF-β1 (◆) mRNA. Data were obtained from combined immunohistochemistry and in situ hybridization as detailed in the **Methods** section. Data are expressed as percent of the total number of PDGF B-chain positive or TGF-β1–positive macrophages/all macrophages in glomeruli within an entire tissue section of female TSLP mice (*A*) and of male TSLP mice (*B*).

tions aimed at interrupting growth factor pathways in MPGN. These are differences in disease expression in these mice that are gender related, which is not a recognized feature of human cryoglobulinemic MPGN. We hope that further study of this feature may provide useful insights into the pathogenesis and potential treatment of this form of glomerulonephritis. The detailed characterization of a murine model of MPGN should allow it to serve as a baseline system for genetic manipulations aimed at defining the pathophysiology of this disorder.

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