The expression of two alternative transcription forms of platelet-derived growth factor-A chain in the normal human kidney and in glomerulonephritis

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Abstract

Purpose: **Up to now, a role of platelet-derived growth factor (PDGF)-AA in glomerulonephritis (GN) remains unclear. PDGF-A chain may be produced in two forms, as a result of the alternative splicing.**

Material and methods: **We examined the expression of this growth factor in the renal tissue of 57 patients with GN and seven normal kidneys (NK). The gene expression of PDGF-A was examined by reverse transcriptase-polymerase chain reaction. Sets of primers allowing distinction between the two forms of transcripts were used. Specificity of the PCR products was confirmed by restriction enzyme analysis and sequencing. The expression of PDGF-AA/AB was also evaluated by immunohistochemistry.**

Results: **Compared to NK, the expression of PDGF-A gene was higher in the renal tissue with GN. This expression was higher in non-proliferative GN (NPGN) than in proliferative forms of GN (PGN) (1.24±0.34 vs. 0.86±0.14). In NK, both forms of transcripts (N=4) or only the short one (N=3) were found. In 45.5% of patients with NPGN, only the short form could be detected. In contrast, in 68.6% of patients with PGN both or only the longer form of transcripts were found. In NK, a faint staining for PDGF-AA/AB was observed within glomerular capillaries, whereas a statistically significant increase in this protein expression was particularly stated in NPGN.**

Conclusions: **These results suggest that the production of the longer PDGF-A chain variant is associated with glomerular cells' proliferation. However, the higher expression of PDGF-AA/AB protein in NPGN could indicate an essential role of this growth factor in the maintaining the glomerular architecture.**

Key words: platelet-derived growth factor-A chain, alternative splicing, glomerulonephritis, endothelial cells, maintaining the glomerular architecture.

Introduction

Platelet-derived growth factor-A (PDGF-A) is believed to be a mediator of smooth muscle cell hyperplasia in hypertension and atherosclerosis [1-4]. Increased expression of this growth factor has also been shown in arteries of rejected renal grafts [5-6]. Recently, PDGF-A involvement in the development of interstitial fibrosis in the renal tissue with diabetic nephropathy has been suggested [7]. Though, the results regarding the expression and a role of PDGF-A in the evolution of glomerulonephritis (GN) remain unclear up to now. In previous studies, we demonstrated the expression of PDGF-A mRNA in IgA nephropathy (IgAN) by the reverse transcriptase-polymerase chain reaction (RT-PCR) [8]. Later, Terada et al. [9], using a similar method, have confirmed the expression of mRNA for this growth factor in various forms of GN. In this study, the expression of PDGF-A gene in IgAN did not differ from that found in the non-proliferative forms of GN (NPGN). However, the results concerning the expression of PDGF-A protein in different morphological forms of GN are conflicting so far [10-11]. In the study of Taniguchi et al. [10], performed on the renal tissue with features of IgAN, the expression of PDGF-AA has been localized to the mesangial areas. In IgAN, mesangial staining for this growth factor has also been observed by Stein-Oakley et al. [11]. Since only a faint glomerular staining for PDGF-AA has been found in focal-segmental glomerulosclerosis (FSGS) in the latter study, it has been suggested that PDGF-AA might

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Table 1. **Histological and clinical data of patients at the time of renal biopsy**

Morphology	No of patients	Sex (F/M)	Age (y) Mean/Range	Serum Creatinine μmol/L (Mean ± SEM)	Proteinuria (g/24 h)
Non-proliferative GN	22	13/9	36 (11-71)	91.9 ± 76.9	5.0 ± 1.1
IMGN	14	7/7	45 (11-71)	117.5 ± 55.7	5.5 ± 1.4
FSGS	8	6/2	18 (13-22)	64.5 ± 11.5	3.2 ± 0.3
Proliferative GN	35	19/16	33 (9-64)	83.2 ± 7.1	3.9 ± 1.0
MesPGN	32	18/14	32 (9-64)	78.6 ± 11.5	4.3 ± 1.1
MPGN	3	1/2	31 (16-60)	97.3 ± 9.7	1.2 ± 0.2

NOTE: Values expressed as mean (range); mean ± SEM, or number of patients.
Abbreviations: IMGN, idiopathic membranous GN; FSGS, focal-segmental glomerulosclerosis; MesPGN, mesangial proliferative GN, MPGN, membranoproliferative GN

be involved in the proliferative response of mesangial cells in IgAN [11]. However, Alpers et al. [12] have earlier shown that podocytes of the mature human kidney express the PDGF-AA protein. Recently, Yang et al. have not only confirmed this finding in the normal human kidney, but also demonstrated a significant increase in the podocyte expression of PDGF-AA in the Denys-Drash syndrome [13].

The gene encoding PDGF-A chain is located on the 7p22 position of chromosome 7, and spans 7 exons [14-15]. PDGF-A chain primary transcripts may be spliced into the two different mRNAs, omitting or including the 69 base pairs' (bp) long sequence of exon 6 [15-16]. The sequence of exon 6 has been shown to be responsible for coding the retention motif [17-18]. Transcription of the long form of PDGF-A chain has been suggested to contribute to smooth muscle cells' hyperplasia in atherosclerotic lesions [20]. This is thought to be a consequence of an increased binding of PDGF-AA dimers containing the sequence encoded by the exon 6 to the cell surface- and matrix-associated glycosaminoglycans [19].

The aim of our study was to examine the gene and protein expression of PDGF-A in the normal kidney and in different morphological forms of GN. Patients with proliferative (PGN) and non-proliferative forms of GN (NPGN) were included into the study. Our study revealed the existence of two differentially spliced forms of PDGF-A chain in the human renal tissue. Moreover, the long form of PDGF-A transcript was more often detected in PGN than in NPGN. In addition, glomerular endothelial cells were revealed to express the PDGF-AA/AB protein in the normal kidney. An essential increase in the expression of this protein was observed in GN, particularly in the absence and/or in the association with mild glomerular cells' proliferation.

Material and methods

Patients

Fifty-seven patients with biopsy-proven GN were included into the study: 32 with mesangial proliferative GN (MesPGN), 3 with membranoproliferative GN (MPGN), 14 with idiopathic membranous GN (IMGN) and 8 with FSGS. The diagnosis was based on morphological and immunopathological examinations of renal biopsy specimens. Histological and clinical data of patients are summarized in *Tab. 1*.

Renal biopsies

Renal biopsy material remaining after the immunopathological diagnosis was used in this study. Informed consent was obtained from all the patients. Tissue preparations for immunohistochemistry and RNA isolation were performed as described elsewhere [8]. The RT-PCR analysis of PDGF-A chain gene expression was performed on fragments from the biopsy tissue of all the patients. All these fragments were also checked for the existence of two alternatively spliced PDGF-A chain transcripts. The amounts of tissue remaining after the diagnosis were sufficient for immunohistochemical evaluation of PDGF-AA/AB protein in 17 patients: 11 with MesPGN, 1 with MPGN, 3 with IMGN, and in 2 with FSGS. Normal-appearing kidney tissue from patients undergoing tumor nephrectomy (N = 7) served as controls for both methods used.

RNA isolation and RT-PCR

Total RNA was extracted from renal biopsy specimens and subsequently reverse-transcribed into cDNA as previously described [21]. Sets of primers with lengths of the amplified PCR products and annealing temperatures for particular pairs of primers are listed in *Tab. 2*. For semiquantitative analysis of PDGF-A gene expression, primers' pair 1 was selected, which amplified the sequence spanning exons 1 through 4. The gliceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The sequences of 5' and 3' primers for GAPDH were GAGTCAACGGATTTGGTCGT and GTTGTCATGGATGACCTTGG [22], respectively. For the analysis of both short and long forms of PDGF-A chain transcripts, primers' pairs 2 and 3 were selected, which amplified the sequence spanning exons 3 through 7 (see *Tab. 2*). The reaction mixture was subjected to 40 cycles of PCR amplification. The amplification profile consisted of an initial denaturation step at 96°C for 5 min, followed by denaturation at 94° for 1 minute, annealing at the appropriate temperature (*Tab. 2*), and extension at 72°C for 1 minute and 30 seconds. The PCR products were electrophoretically separated on 2% agarose gel (Serva Electrophoresis, Heidelberg, Germany) and visualized by ethidium bromide staining.

Table 2. **Sets of primers and PCR conditions used in the study**

No	Forward 5'-3'	Reverse 3'-5'	Size (bp)	Anneal. temp. (°C)
1	ATGAGGACCTTGGCTTGC Exon 1 (839-856) [23]	GGGACAGCTTCCTCGATGCT Exon 3/4 (1097-1117) [9]	278	56
2	AGCATCGAGGAAGCTGTCCC Exon 3/4 (1097-1017) [9]	TTCTCCCGAGTGTTCTCGGAC Exon 7 (1607-1628) [24]	531 462	62
3	CTCCCGCGTCCACCACCGCAGCGTC Exon 4 (1265-1278) [25]	TTCTCCCGAGTGTTCTCGGAC Exon 7 (1607-1628) [24]	363 294	56

Location for primers is based on the published NCBI sequence (accession no NM_002607)

For PCR products obtained with primers' pair 1 and for the short form of transcript, original cDNA for the short variant of PDGF-A chain in the plasmid puC13 (kindly supplied by Dr. Ch. Betsholtz, University of Uppsala, Sweden) [14] linearized with SmaI was used as a positive control. Negative controls consisted of buffer alone and/or non-reverse-transcribed sample RNA.

Analysis of PCR products

To confirm the specificity of PCR products obtained with the pair 2 of primers, restriction enzyme analysis was performed. The PCR products were purified using the Clean-Up kit for DNA purification (A&A Biotechnology, Gdynia, Poland), according to the manufacturer's instructions. The kit is based on the of DNA ability to absorb to silica-coated surfaces in the presence of chaotropic salts. After purification, the PCR products were resuspended in 25 μL of diethyl pyrocarbonate (DEPC) treated water (Fermentas, Vilnius, Lithuania) and subjected to digestion by the MboI restriction endonuclease (Fermentas). Briefly, 25 μl of purified PCR product was added into the reaction mixture containing MboI specific restriction buffer, 2 U of MboI and the DEPC treated water to the final volume of 30 μL. Overnight incubation at 37°C was performed. After digestion, products were separated on 8% non-denaturing polyacrylamide gel (Serva) and silver stained according to the method of Budowle et al. [26].

For the PCR products obtained with the third set of primers, cycle sequencing of the long and short forms of transcripts (in both directions) was performed by the means of fmol® DNA Cycle Sequencing System (Promega Biotech). Briefly, 5-10 fmol of the purified PCR product was dissolved in the mixture containing 5× sequencing buffer (250 mM Tris-HCl, pH 9.0 at 25°C; 10 mM $MgCl_2$), 1.5 pmol of CY5 (indodicarbocyanine 5-1-O- (2--cyanoethyl)-(N, N-diisopropyl)-phosphoramidite)-end-labeled primer, and 5U of Sequencing Grade *Taq* DNA Polymerase, and DEPC water to the final volume of 16 μL. Then, the mixture was separated into 4 tubes, 4 μL to each, containing 2 μL of the deoxyribonucleoside triphosphates (dNTP) supplemented with a limiting amount of a different dideoxyribonucleoside triphosphate (ddNTP). The amplification profile consisted of an initial denaturation at 95°C for 2 minutes, followed by denaturation at 95°C for 30 seconds, annealing at 42°C for 30 seconds and extension at 70°C for 1 minute. Products were then separated on the Repro Gel™ Long Read (Amersham Biosciences, Uppsala, Sweden) in 0.5 × TBE buffer (890 mM Tris-borate, 890 mM boric acid, 20 mM EDTA) at 55°C for 750 minutes using an automated sequencer Alf® Express II (Amersham Biosciences). Data obtained by sequencing were analyzed by the means of Alf Win™ Sequence Analyser 2.10 software (Amersham Biosciences) and compared to that published in the NCBI sequence database.

Immunohistochemistry

The immunostaining procedure was performed on acetone-fixed cryostat sections using the alkaline phosphatase – anti-alkaline phosphatase method as previously described [8,21]. A rabbit anti-human polyclonal antibody against PDGF-AA (Chemicon International, Temecula, USA) was applied in this study. An antibody to CD31 (Dako A/S, Glostrup, Denmark) was used as a marker of endothelial cells. Control experiments were conducted by omitting the incubation with the primary antibody, as well as with substitution of the primary antibody with non-immune murine serum.

Statistical analysis

Results of RT-PCR and immunohistochemical evaluation of PDGF-A expression were analyzed in particular groups of patients according to their morphological classification. Images of ethidium bromide-stained bands for PDGF-A and GAPDH cDNAs were photographed using a system with camera supported with a darkroom (Vilbert Loumart, Torcy, France). The intensity of the bands was densitometrically measured with the BioGENE computer software (Vilbert Loumart). All PDGF-A signals were normalized to mRNA levels of GAPDH and expressed as a ratio.

Mean glomerular expression scores were calculated for the examined protein, based on the staining intensity (I) and the percentage of glomerular stained area (A) by applied antibody according to the formula: mean glomerular expression score $= I(1) \times A(1)/100\% + I(N) \times A(N)/100\%/N$, where N was the number of glomeruli in the renal specimen. Staining intensity was graded from 0 to 3 points according to the following scale: zero – no immunoreactivity, 1-weak immunoreactivity, 2-moderate intense immunoreactivity, and 3-dense marked immunoreactivity.

All values in the text and figures are presented as mean ± SEM. Non-paired Mann-Whitney test was used to test differences between particular groups of patients. The level of significance was set at $P < 0.05$.

Figure 1. Examples of RT-PCR evaluation of PDGF-A expression (band at 278 bp) in comparison to GAPDH expression (band at 482 bp) in kidney samples: Lanes 1, 2 and 3, normal kidneys, lanes 4 and 5, MesPGN, lanes 6 and 7, IMGN, lane 8, negative control (buffer)

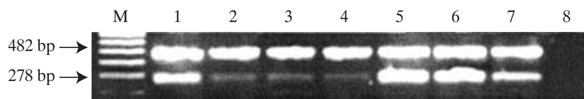


Figure 3. Examples of two differentially spliced variants of PDGF-A chain transcripts in controls and diseased kidneys: lane 1, original PDGF-A chain cDNA without the sequence of exon 6 (positive control); lanes 2 and 4, normal kidneys; lanes 5 and 7, MPGN; lane 6, IMGN, lanes 8, 9, and 12, MesPGN; lanes 10 and 11, FSGS; lane 13, negative control (non-reverse-transcribed RNA)

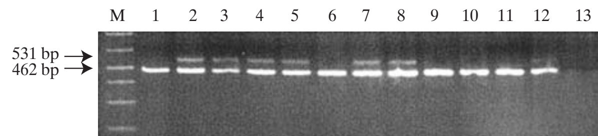


Figure 2. Results of RT-PCR evaluation of PDGF-A chain expression in normal kidneys (NK), proliferative glomerulonephritis (P), and non-proliferative glomerulonephritis (NP)

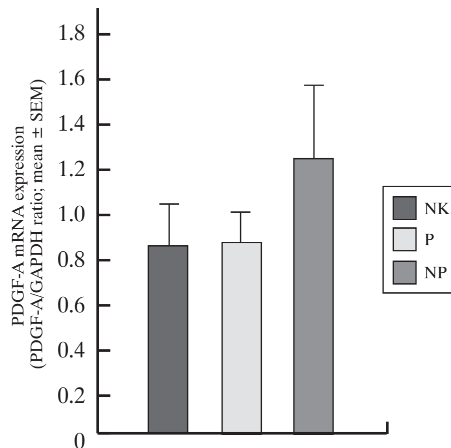
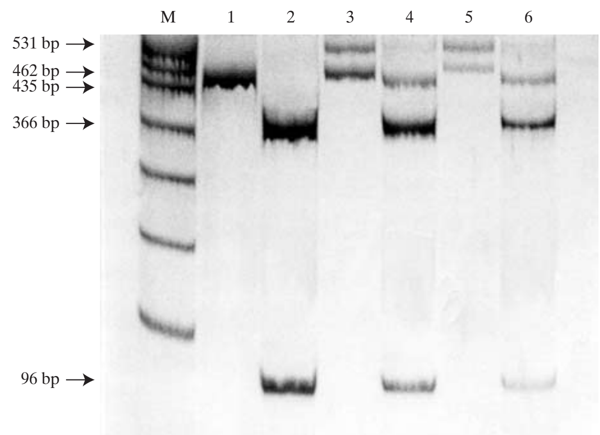


Figure 4. Results of the restriction analysis with MboI of both variants of PDGF-A chain transcripts. The products were separated electrophoretically on 8% polyacrylamide gel and silver stained. Lane 1, short form of PDGF-A chain transcript before digestion (band at 462 bp); lane 2, short form of PDGF-A transcript after digestion with MboI (bands at 366 and 96 bp); lanes 3 and 5, both forms of PDGF-A transcript before digestion (bands at 531 and 462 bp); lanes 4 and 6, both forms of PDGF-A transcript after digestion with MboI (bands at 435, 366 and 96 bp)



Results

Evaluation of PDGF-A gene expression and analysis of transcript variants

By RT-PCR, the PDGF-A chain gene expression was stated in both the normal and diseased kidneys (*Fig. 1*). Fragments of the same length were amplified from the original PDGF-A chain cDNA and reverse transcribed cDNAs from biopsy tissue. In addition, cycle sequencing analysis confirmed that the sequence of the obtained PCR product was identical to that deposited in the NCBI database (not shown). Compared to normal kidneys, higher levels of PDGF-A mRNA were observed in GN (0.84±0.19 versus 1.0±0.16) (not shown). The expression of PDGF-A mRNA in NPGN exceeded that in PGN, although the difference between these groups did not reach statistical significance (*Fig. 2*).

Using the primers' pair 2, PCR products of 462 and 531 bp were observed both in the normal kidney (NK) and in the renal tissue with GN (*Fig. 3*). With respect to the shorter form of detected transcripts, fragment of the same length was amplified from the original PDGF-A chain cDNA. Restriction enzyme analysis of these products with MboI resulted in bands of the expected length, 366 and 96 bp for the short form of PDGF-A chain transcript, and 435, 366 and 96 bp when two forms of transcripts were detected (*Fig. 4*).

Both forms of PCR products obtained with the third set of primers were subjected to the sequencing analysis. They disclosed the 100% similarity with the sequences deposited in the NCBI database (*Fig. 5* and *Fig. 6*).

In four out of seven NK examined, both the long and short forms of PDGF-A transcripts were detected, whereas in the remaining three cases only the short form could be observed. Out of 57 patients, both forms of transcripts were found in 31. In 23 of them, only the short form of PDGF-A chain mRNA was present, whereas the long form singly was detected in 3 patients. Interestingly, the expression of PDGF-A mRNA was significantly higher in the group with the only short variant of transcripts detected (*Fig. 7*). Among these patients were all individuals with FSGS, 4 with IMGN and 11 with MesPGN. The short form of PDGF-A chain mRNA was less frequently detected in PGN. In 68.6% of them, both or only the longer form of transcripts were found.

Figure 5. **Sequencing analysis of the long splice variant of PDGF-A chain. An arrow indicates the boundary between the exons 5 and 6**

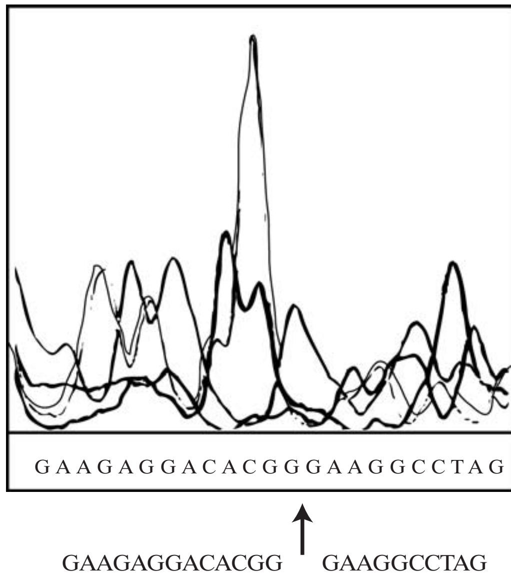


Figure 6. **Sequencing analysis of the short splice variant of PDGF-A. An arrow indicates the boundary between the exons 5 and 7**

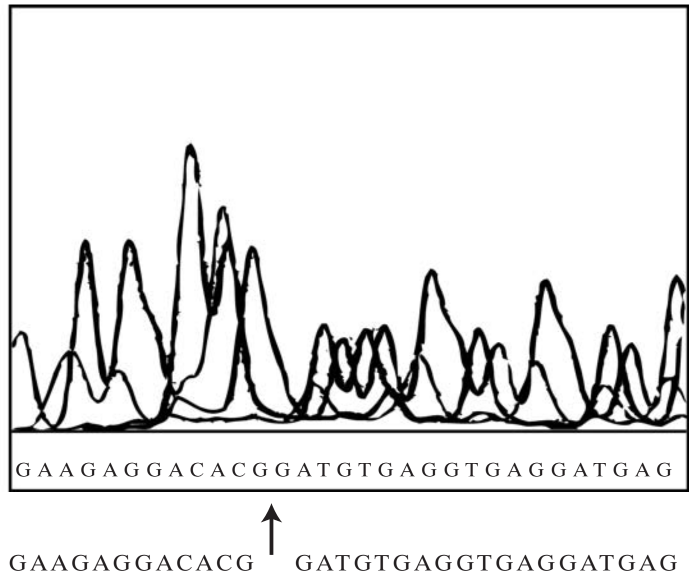
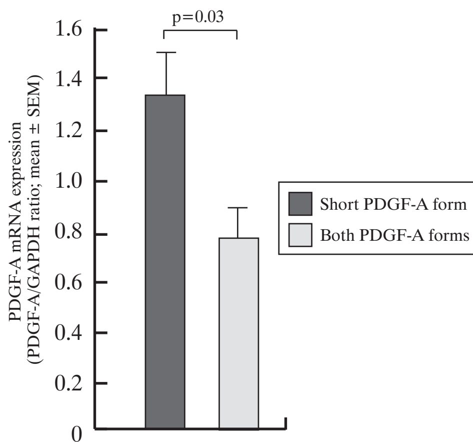


Figure 7. **RT-PCR evaluation of PDGF-A chain expression in patients presenting with only the short splice variant of PDGF-A chain and both splice variants of transcripts**



Evaluation of PDGF-AA/AB protein expression

A faint glomerular immunoreactivity for PDGF-AA/AB was observed in NK (*Fig. 8A*). In the tubulointerstitial compartment, tubular epithelial cells and endothelial cells in the interstitial vessels were positive for PDGF-AA/AB. In GN, the glomerular expression of this protein varied greatly with a staining pattern resembling that for CD31 (not shown). In PGN, the expression of PDGF-AA/AB protein depended on the degree of mesangial cells' proliferative response. Marked immunoreactivity for this growth factor was observed in glomeruli with no or mild mesangial cells' proliferation (*Fig. 8B*), whereas only a faint reaction was noticed in advanced lesions in the course of MPGN (*Fig. 8D*). In NPGN, the glomerular expression of PDGF-AA/AB was increased in early stages of IMGN or FSGS and significantly reduced in the presence of advanced sclerotic alterations (*Fig. 8C*).

Semiquantitative evaluation of the glomerular expression of PDGF-AA/AB in NPGN and PGN, compared to NK, is shown in *Fig. 9*.

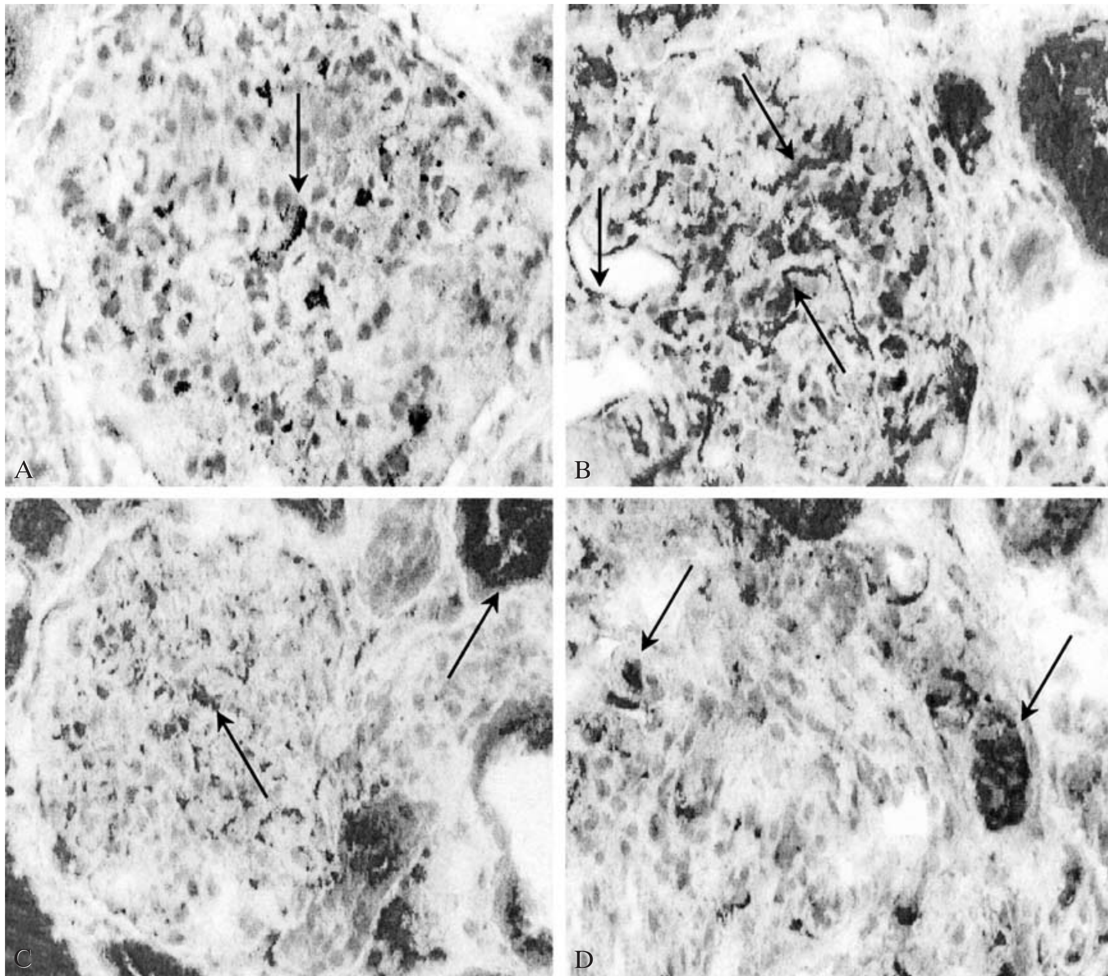
Clinical-pathological correlation

No relationship could be stated between the expression of PDGF-A mRNA or protein and serum creatinine levels or degree of proteinuria in different morphological forms of GN. Intriguingly, a higher expression of PDGF-A mRNA and protein was observed in NPGN. As stated above, the expression of the shorter PDGF-A transcript was more often detected in this group of patients.

Discussion

Our RT-PCR study showed that the gene expression of PDGF-A chain in renal specimens with GN exceeded that in the normal kidney. Previously, only Terada et al. [9] have used a similar method to evaluate the expression of PDGF-A chain mRNA in the renal tissue with GN. Though, the expression of PDGF-A chain mRNA in the normal kidney has not been examined at all in this study. The authors compared the gene expression of PDGF-A chain in IgAN, IMGN, FSGS, and minimal change disease. They stated that the expression of this growth factor was higher in IgAN, but it did not differ significantly from that found in the other types of GN grouped together [9]. In contrast, in our study, a higher expression of PDGF-A chain mRNA was observed in the renal tissue of patients with NPGN than in PGN, although the difference between these groups did not reach statistical significance. One of the reasons for the lack

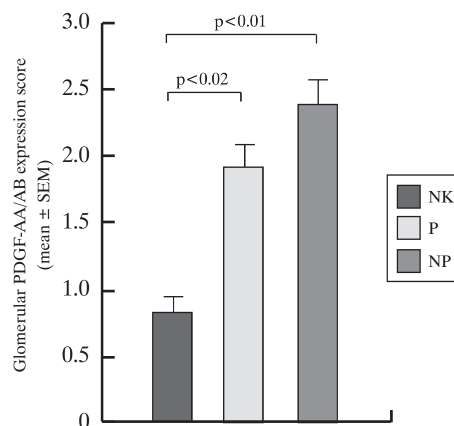
Figure 8. (*A*) Immunoreactivity for PDGF-AA in a normal glomerulus. Some endothelial cells are positive. (*B*) Increased immunoreactivity for PDGF-AA in a glomerulus from a patient with IgA nephropathy and mild mesangial cell proliferation. (*C*) A glomerulus from a patient with an advanced stage of FSGS. The reactivity for PDGF-AA is comparable to that in the normal kidney. (*D*) PDGF-AA immunoreactivity in a glomerulus with features of membranoproliferative GN is even lower than that observed in the normal kidney



of significance might have been the fact that we used the total RNA isolated from the whole remaining biopsy fragment for PDGF-A chain evaluation. However, although Terada et al. performed their study on the RNA isolated only from glomeruli, they amplified the PDGF-A chain sequence spanning exons 3 through 6, and thus they were able to detect only the long transcript [9].

To our knowledge, our study is the first one, which analyzed the expression of two alternative forms of PDGF-A chain transcripts in the normal kidney and in different morphological forms of GN. Digestion with a restriction enzyme and, more notably, sequencing analysis confirmed the specificity of the obtained PCR products. We observed significantly higher levels of mRNA for PDGF-A chain in cases with the only short form of transcripts detected. Intriguingly, in this group were predominantly patients with NPGN, in particular all the patients with FSGS. In contrast, the long form of PDGF-A chain transcript was more often found in PGN. In the latter respect, also in the study of Terada et al. [9] transcripts for the longer form of PDGF-A chain were more frequently detected in patients

Figure 9. **Evaluation of PDGF-AA glomerular expression in normal kidneys (NK), proliferative glomerulonephritis (P) and non-proliferative glomerulonephritis (NP)**



with IgAN, i.e. the proliferative form of GN, than in those with NPGN. Thus, both studies would suggest that transcription of longer PDGF-A chain form is associated with glomerular cells' proliferation.

In vitro, both forms of PDGF-A chain transcripts have been found in the human smooth muscle arterial cells (VSMC), adult vein endothelial cells (VEC) and monocyte-derived macrophages (M) [19]. In VSMC, the short PDGF-A chain transcript was 8 times more abundant than that of the long form. The long and short PDGF-A chain mRNAs were expressed at similar levels in monocyte-derived macrophages. In contrast, a constant and comparatively high production of the long PDGF-A chain transcript, representing approximately 44% of the total PDGF-A mRNA was observed in VEC [20].

In the latter context, results of our immunohistochemical studies point to the glomerular EC as to the most likely source of PDGF-AA protein in glomeruli. In NK, only trace amounts of this protein could be detected within the glomerular capillaries. An essential increase in the immunoreactivity for PDGF-AA was observed in GN. Furthermore, a significantly higher expression of this growth factor was found in NPGN than in PGN. These results are in disagreement with that obtained by others [10-13]. However, inconsistent are also the results of the above-cited studies. Two different antibodies against PDGF-AA (both polyclonal) were used in these studies. One of them stained mesangial areas in IgAN and gave negative results in NK [10-11], whereas the other stained podocytes both in NK and in the disease condition [12-13]. We used an antibody that in addition to other applications is also recommended for neutralization of the biological activity of human PDGF-AA. Moreover, opposing to all the above studies that were carried out on deparaffinized sections, our study was performed on the acetone-fixed tissue. In the light of these data, both cross-linking effects of formalin on proteins and specificity of the antibodies used to detect the PDGF-AA protein could have impact on the obtained results. Further, one has to realize that an anti-PDGF-AA antibody is able to detect also the PDGF-AB dimer.

Nevertheless, given the specificity of the antibody used, increased expression of PDGF-AA observed in our patients with NPGN appears to be a confusing finding. In vivo, the expression of PDGF-AA has been demonstrated in vascular EC of arteries undergoing acute rejection. The authors suggested that PDGF-AA chain expression might be a marker of EC injury or activation in this condition [5-6]. Recent in vitro results propose a new role for PDGF-AA in vascular remodeling. It has been shown that PDGF-AA and PDGF-BB are released from activated arterial EC. But, PDGF-AA inhibits PDGF-BB-mediated VSMC migration and this is regulated at the receptors level. Namely, PDGF-AA induces the expression of PDGF-α receptor (PDGF-αR) in VSMC [27]. This receptor activates JNK-1 signaling and thus counteracts the activity of PDGF-β receptor (PDGF-βR), i.e. the signaling receptor of PDGF-BB [28].

Increased expression of PDGF-BB and PDGF- R with the mitogenic consequences of this axis has long been implicated in the pathogenesis of GN [8-11,29]. On the other hand, the increased expression of PDGF-αR has been shown in vitro and in vivo on mesangial cells [11,30]. Taking into account the new data on PDGF-AA action, increased expression of this growth factor in GN would indicate its protective role aimed at inhibition of mesangial cell proliferation and maintaining the glomerular architecture.

In conclusion, our study showed the existence of two alternatively spliced variants of PDGF-A chain mRNA in the normal human kidney and in GN. The short form of PDGF-A chain was more frequently detected in NPGN and accompanied by a higher expression of PDGF-AA protein. However, further studies including parallel examination of PDGF-αR and PDGF-BB are necessary to signify the actual role of PDGF-AA in GN.

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