Preliminary Communication

Over-expression of platelet-derived growth factor in human diabetic nephropathy

Robyn G. Langham1, Darren J. Kelly1, Julie Maguire2, John P. Dowling3, Richard E. Gilbert1 and Napier M. Thomson2

1University of Melbourne St Vincent’s Hospital Department of Medicine, Fitzroy, 2Department of Anatomical Pathology and 3Monash University Department of Medicine, Alfred Hospital, Prahran, Australia

Abstract

Background. The pathogenetic mechanisms responsible for progressive renal impairment of diabetic nephropathy are still poorly understood, despite its growing incidence. Increasing evidence suggests that growth factors may contribute to the initiation and progressive fibrosis of diabetic nephropathy. In this study, the gene expression and protein distribution of platelet-derived growth factor-A and -B (PDGF-A and PDGF-B) in human diabetic nephropathy were examined.

Methods. PDGF-A and PDGF-B mRNA levels in surplus renal biopsy tissue from seven patients with overt diabetic nephropathy and six nephrectomy samples were examined using quantitative reverse transcription–polymerase chain reaction (RT–PCR). In addition, each sample was also examined immunohistochemically to quantify and localize peptide expression of each PDGF isoform.

Results. Gene expression of PDGF-A and PDGF-B mRNA were increased 22- and 6-fold, respectively, in biopsies from patients with diabetic nephropathy compared with control tissue. Immunostaining also demonstrated increased peptide expression of both PDGF-A and PDGF-B in diabetic nephropathy, with each isoform showing a specific pattern of tissue distribution.

Conclusions. The findings of increased gene and protein expression of PDGF in renal biopsies from patients with diabetic nephropathy imply a potential role for this prosclerotic growth factor in the development of the progressive fibrosis that characterizes human diabetic kidney disease.

Keywords: diabetic nephropathy; platelet-derived growth factor; RT–PCR

Introduction

Pro-sclerotic growth factors have been consistently implicated in the pathogenesis of fibrotic organ injury including that of the kidney. While transforming growth factor-β (TGF-β) has been the focus of much attention in diabetic renal disease, other growth factors, such as platelet-derived growth factor (PDGF), have been shown to be up-regulated in the experimental setting [1].

PDGF is a 28–32-kDa basic glycoprotein comprising of two peptide chains designated A and B, that exist in disulphide linkage either as heterodimer or homodimers [2,3]. PDGF induces a variety of cellular responses that may be relevant to the pathology of renal disease, including matrix production, chemotaxis and cell proliferation [4]. More recently, two other PDGF isoforms have been identified (PDGF-C and PDGF-D); however, their pathophysiogical impact in kidney disease states is unclear [5,6]. Many of the pathogenetic factors implicated in diabetic complications induce PDGF, including not only glucose but also advanced glycation end-products [7] and angiotensin II [8]. However, the expression of PDGF in human diabetic nephropathy has not been explored. We therefore sought to examine both gene expression and tissue peptide distribution of PDGF-A and PDGF-B in human diabetic renal disease.

Subjects and methods

Study subjects

Reverse transcription–polymerase chain reaction (RT–PCR) studies were performed on surplus renal biopsy tissue taken for diagnostic purposes from seven patients with Type 2 diabetic nephropathy. Biopsies were immediately snap-frozen in liquid nitrogen and stored at −80°C until analysed. Control tissue was obtained from six patients who underwent nephrectomy for the treatment of tumour. Paraffin-embedded
formalin-fixed tissue was used for immunohistochemical studies. The studies were approved by the Human Ethics Review Committee of the Alfred Group of Hospitals, Prahran, Australia.

**RNA extraction**

Total RNA was extracted from snap-frozen renal biopsies using the RNeasy kit (Qiagen, Georgetown, MD) modified from the guanidium cyanate extraction method [9] with silicon membrane spin columns to allow greater yield from very small tissue samples [10]. The amount of RNA recovered was quantified and its quality verified by UV absorbance spectrophotometric examination at 260 and 280 nm.

**Competitive RT–PCR**

One microlitre of RNA was reverse transcribed to cDNA along with a known amount of a synthetic internal standard RNA, pAW109 [11]. The pAW109, or competitor, is a linear array of sequences of upstream primers of multiple target genes (including PDFG-A and PDGF-B) followed by the complementary sequences to their downstream primers in the same order. In each instance the target gene contains a 50 bp insertion, yielding a product of different size from the respective wild type gene of interest. Each sample was reverse-transcribed five times with varying concentrations of competitor.

For the RT–PCR reaction, GeneAmp RNA PCR kit (Perkin Elmer, Boston, MA) was used. Reverse transcription was performed with the cloned Murine Leukaemia virus (MuLV) reverse transcriptase at 2.5 U/μl. A 20 μl mix of MgCl₂ (5 mM), 1 × PCR buffer, dNTP (1 mM), RNase inhibitor (1 U/μl), MuLV, Oligo dT (2.5 mM) and 1 μl of both RNA and competitor was overlaid with 20 μl mineral oil and underwent annealing for 10 min. The mixture was incubated at 42 °C for 15 min to allow reverse transcription, followed by denaturation of enzyme at 99 °C for 5 min and then cooling to 4 °C.

In the subsequent amplification by GeneAmp PCR process with AmpliTaq Gold DNA polymerase at 0.075 U/μl, a 25 μl mix of MgCl₂ (1.5 mM), 1 × PCR Buffer, primers, each at 0.1 pmol/μl and 2.5 μl of RT mix was overlaid with oil and amplified on a Peltier Thermal Cycler, PTC-200. The amplification profile was: PDGF-A: 40 cycles at 64 °C anneal/extend, PDGF-B: 45 cycles at 72 °C and 64 °C anneal/extend, step-down. Each reaction went to plateau phase.

The PCR protocol was validated to ensure reproducibility for each primer pair; coefficient of variation measured at 3% for PDGF-A and 7.2% for PDGF-B.

**Primers**

Anti-sense primers were synthesized by Perkin Elmer (Boston, MA), according to published sequences [11]. Sense primers were synthesized by Bresatec (Adelaide, Australia) and were fluorescein labelled (Fl).

Primers sequences were as follows. PDGF-A: sense Fl- TTGGCCACCTTGACGCTGCG; antisense GCCCATTC GGAGGAAGAG. PDGF-B: sense Fl- TTCTCACCTGGA CAGGTCG; antisense GAAGAGCCTGGGTTCCCTG.

**Immunohistochemistry**

PDGF-A and PDGF-B peptide distribution was assessed immunohistochemically, as described previously [12] using rabbit anti-human antibodies specific for the PDGF-AA and PDGF-BB peptides (Genzyme, Allston, MA). Tissue sections (4 mm) thick were prepared from paraffin-embedded tissue of biopsies matching samples stored for PCR analysis. Immunostaining was then performed using a modified three-layer immunoperoxidase technique.

Appropriate negative (diluent and non-immune rabbit serum) and positive controls (α-smooth muscle actin) were included in each immunoperoxidase staining run, with no staining observed with the negative controls.

**PCR analysis**

PCR products were analysed using fluorometric scanning after electrophoretic separation on 2% agarose gel (Figure 1). Each PCR reaction yielded product for the native messenger RNA or target molecule (mRNA) and the internal control molecule (aw109). Results were analysed using linear regression of the graph Log(mRNA/aw109) vs Log(aw109) in each instance. The point of equivalence (ratio = 1) was then calculated and from this the copy number of mRNA molecules in the original RNA sample (Figure 2). The final copy number of target mRNA was expressed as molecules (copy number) per nanogram total RNA.

**Fig. 1.** Fluorometric scan of RT–PCR products following agarose gel electrophoresis.

**Fig. 2.** Linear regression analysis of RT–PCR products. Circles, log(mRNA/aw109)copy no.; triangles, predicted values.
**Image analysis**

The magnitude of immunostaining was quantified using computer-assisted image analysis as described previously [13]. In brief, for each tissue section, images from three non-overlapping, randomly selected fields were examined by light microscopy (Olympus BX-50, Olympus Optical, Tokyo, Japan) and digitized using a high-resolution camera (Fujix HC-2000, Fujifilm, Tokyo, Japan). All images were obtained using a 20× objective lens. Digitized images were then captured on a Power Macintosh G3 computer (Apple Computer Inc., Cupertino, CA) equipped with an in-built graphic board and opened using analytical software (Adobe Photoshop 5.0, Adobe Systems, San Jose, CA). An area of brown on an immunoperoxidase-stained section was selected for its colour range and the proportional area of tissue with this range of colour was then quantified such that the magnitude of immunolabelling was expressed as the proportional area of the tissue section, which stained brown.

**Statistical analysis**

Linear regression was used to identify the point of equivalence for each RNA sample ($P<0.05$). Gene expression analysis showed a skew distribution and log transformation was performed. Data were then analysed by ANOVA, significance expressed as $P<0.05$. Data obtained from image analysis of the immunohistochemistry were normally distributed and were analysed by ANOVA.

**Results**

**Clinical data**

The seven biopsies obtained for analysis were from patients with Type 2 diabetes and overt diabetic nephropathy. Analysis of patient records revealed a significant degree of renal impairment with a mean serum creatinine of 0.18±0.05 mmol/l, mean urinary protein of 2.1 g±1.1/l/day. All patients were receiving treatment for hypertension (four with angiotensin-converting enzyme inhibitors, two with calcium antagonists, one with diuretic alone).

**RT–PCR**

Gene expression analysis revealed a 22- and 6-fold upregulation of PDGF-A and PDGF-B mRNA, respectively, in diabetic nephropathy biopsies compared with control tissue (Table 1).

**Immunohistochemistry**

Minimal immunoreactive PDGF-A and PDGF-B was detected in control tissue (Figure 3A and B). In contrast, tissue sections from patients with diabetic nephropathy showed abundant expression of PDGF-A and PDGF-B in glomeruli and in the tubulointerstitial compartment (Figure 3C–F). In the tubulointerstitium, PDGF-A immunostaining was localized to both glomerular and tubular epithelial cells. In contrast, PDGF-B immunostaining was predominantly extra-cellular, and localized to areas of peritubular, interstitial and periglomerular fibrosis. Image analysis confirmed a significantly greater proportional area showed positive immunolabelling for both PDGF isoforms in the glomerulus and in the tubulointerstitial compartment (Table 2).

**Discussion**

The present study demonstrates a significant increase in the expression of PDGF-A and PDGF-B in human diabetic nephropathy. In addition, translation into PDGF peptide was confirmed by the increased deposition of immunostainable PDGF with each isoform showing a specific tissue pattern of distribution.

Exploration of PDGF in the human kidney has, to date, mostly focused on its role in mesangial cell proliferation with PDGF up-regulation reported in lupus nephritis [14], IgA nephropathy [15] and crescentic glomerulonephritis [16]. However, in addition to its pro-proliferative actions, PDGF also induces extracellular matrix formation in vitro [7] with infusion and transfection studies confirming the pro-sclerotic effects of PDGF in non-diabetic renal disease in the in vivo setting [17,18].

Several studies have suggested that PDGF may be involved in the pathogenesis of diabetic nephropathy. For instance, Nakagawa et al. documented increased expression of PDGF-B and PDGF-receptor $\beta$ in glomeruli in experimental diabetic nephropathy [19]. Furthermore, increased urinary PDGF-B has also been demonstrated in diabetic patients with both macro- and micro-albuminuria [20]. However, although these studies have demonstrated increased PDGF in rodent models and in the urine of patients with diabetic nephropathy, the present study is the first to demonstrate the over-expression and histological distribution of this growth factor in human diabetic kidney disease.

The demonstration of PDGF-B peptide predominantly within areas of extracellular matrix is further confirmation of the importance of the extra-cellular matrix as a potential reservoir of PDGF-B [21]. For instance, Soyombo et al. demonstrated that in endothelial cells, newly synthesized PDGF-B chain is stably associated with the extracellular matrix and is rapidly released by selective proteolytic cleavage [21].

**Table 1. Comparison of PDGF-A and PDGF-B gene expression in diabetic nephropathy and nephrectomy**

<table>
<thead>
<tr>
<th>Condition</th>
<th>PDGF-A</th>
<th>PDGF-B</th>
</tr>
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<tbody>
<tr>
<td>Diabetic nephropathy</td>
<td>7857a</td>
<td>9325b</td>
</tr>
<tr>
<td>(n = 7)</td>
<td>(5328, 11 584)</td>
<td>(4493, 19 352)</td>
</tr>
<tr>
<td>Normal (n = 6)</td>
<td>346</td>
<td>1635</td>
</tr>
<tr>
<td>(243, 492)</td>
<td>(647, 4132)</td>
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Data expressed as copy number per nanogram of RNA, geometric mean (95% confidence interval).

$^aP < 0.001$.

$^bP < 0.05$, diabetic nephropathy vs normal.
In the present study PDGF-A peptide was localized to the cytoplasm of both glomerular and tubular cells. This predominant intracellular localization is similar to the findings of other groups examining PDGF in the kidney [12,22] in which intracellular staining was also noted with the use of two anti-PDGF-A antibodies both different from those used in our study. These findings suggest that, unlike PDGF-B, PDGF-A may not be sequestered in extracellular matrix.

In summary, this study demonstrates a marked increase in both gene and protein expression of PDGF-A and PDGF-B in human diabetic nephropathy, with each isoform showing a specific pattern of tissue distribution. In the context of the prosclerotic effects...
Table 2. Comparison of PDGF-A and PDGF-B immunostaining in diabetic nephropathy and nephrectomy in glomeruli (glom) and tubulo-interstitium (ti)

<table>
<thead>
<tr>
<th></th>
<th>PDGF-A glom ti</th>
<th>PDGF-B glom ti</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic nephropathy</td>
<td>0.84 (±0.11)a</td>
<td>0.72 (±0.26)a</td>
</tr>
<tr>
<td>(n=7)</td>
<td>9.9 (±8.3)b</td>
<td>12.4 (±6.3)b</td>
</tr>
<tr>
<td>Normal (n=6)</td>
<td>0.03 (±0.04)</td>
<td>0.07 (±0.04)</td>
</tr>
<tr>
<td></td>
<td>0.4 (±0.04)</td>
<td>0.5 (±0.14)</td>
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Data expressed as mean percentage area (± SD).

aP < 0.01.
bP < 0.001 diabetic nephropathy vs normal.

of PDGF, these findings suggest a potential role for PDGF in the development of progressive fibrosis of human diabetic nephropathy.

Conflict of interest statement. None declared.

References


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