Overexpression of extracellular matrix proteins in renal tubulointerstitial cells by platelet-activating-factor stimulation

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Abstract

**Background.** One common feature of renal diseases is the development of interstitial fibrosis, but the mechanism of this process remains undefined. We hypothesized that platelet-activating factor (PAF), a classical acute inflammatory mediator involved in the pathogenesis of renal damage, acts on renal tubulointerstitial cells, contributing to the development of fibrosis. For this reason we evaluated the effect of PAF on matrix regulation and cell-growth-related events in tubulointerstitial cells.

**Methods.** *In vitro* studies were conducted with two tubulointerstitial cell lines: renal tubulop epithelial cells (NRK 52E) and interstitial fibroblasts (NRK 49F). The effect of PAF on extracellular matrix gene expression was determined by Northern blot. Fibronectin synthesis was quantified by metabolic labelling and immunoprecipitation. Cell growth changes were evaluated by fluorescence-activated cell-sorting analysis (cell cycle and size) and total protein content by $[^3]H$leucine incorporation.

**Results.** In renal tubulop epithelial cells and interstitial fibroblasts, PAF increased fibronectin mRNA expression. PAF-effect on the expression of collagen genes differed depending on the cell type studied. In tubulop epithelial cells there was an increase in type I and IV collagen mRNA levels, while only type I collagen was increased in fibroblasts. The overexpression of matrix proteins induced by PAF was completely blocked by preincubation of cells with the PAF receptor antagonist, BN52021. The PAF-induced upregulation of fibronectin expression was correlated with the increase in fibronectin synthesis. These effects were not associated with an increase in hyperplasia (characterized by changes in cell cycle) either in tubulop epithelial cells or in interstitial fibroblasts. Moreover, PAF did not induce tubular hypertrophy (changes in protein content and cell size).

**Conclusions.** Our data suggest that PAF could be a mediator involved in extracellular matrix accumulation and, therefore, participate in the formation of renal interstitial fibrosis.

Key words: platelet-activating factor; tubulointerstitial fibrosis; matrix production

Introduction

Renal interstitial fibrosis develops in all progressive renal diseases. The release of growth factors, cytokines, and lipid mediators by infiltrating and resident renal cells during renal damage is considered the main mechanism responsible for interstitial fibrosis [1]. Platelet-activating factor (PAF) is a biologically active phospholipid mediator involved in acute inflammatory and immune responses [2,3], but its role in chronic pathology remains undefined. Several studies suggest that PAF participates in the pathogenesis of renal damage [4–6]. It has been demonstrated that PAF infusion induces proteinuria [7]. In experimental models of nephrosis in rats an increase in glomerular PAF production preceded maximal proteinuria [8–10] and treatment with PAF receptor antagonists prevented or reduced proteinuria [11]. Infiltrating and resident renal cells can synthesize PAF in response to several stimuli, including cytokines, vasoactive compounds and PAF [12–14]. Moreover, PAF is rapidly generated after tissue injury, initiating several processes, such as chemotaxis of monocytes and synthesis of cytokines [4–6], that could contribute to the development of interstitial fibrosis. Recently we have demonstrated that in cultured glomerular mesangial cells, PAF increases matrix production through the synthesis of endogenous TGF-β [15], suggesting a role for PAF in the accumulation of mesangial matrix that occurs during glomerulosclerosis. Also, in cultured glomerular epithelial cells we have observed that PAF inhibits proteoglycan production [16]. This effect could be an explanation for the mechanism of PAF-induced proteinuria through the reduction of negative charges in the glomerular basement membrane. However, a direct effect of PAF on matrix production in renal tubulointerstitial cells has not been evaluated.
In the present study, we have examined whether PAF acts on renal tubulointerstitial cells (tubuleepithelial cells and interstitial fibroblasts), regulating cell growth and extracellular matrix production, two key features of progressive renal diseases.

**Methods**

**Materials**

All culture reagents were purchased from Gibco BRL (Paisley, UK). Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) was obtained from Calbiochem (San Diego, CA, USA) and stored in a 1 mM ethanol. PAF receptor antagonist, BN52021 (a gift from Dr Pierre Braquet, Institut Henri Beaufour, Les Plessis-Robinson, France) was dissolved in dimethyl sulphoxide and added to the medium to reach a final concentration of less than 0.01%. Polyclonal antifibronectin antibody was obtained in rabbits by immunization against human fibronectin [17]. [35S]Methionine (1000 Ci/mmol) and [32P]dCTP (3000 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK). All other chemicals were from Sigma (Sigma Chemical, St Louis, MO).

**Cell cultures**

Two cell lines obtained from the American Type Culture Collection (Rockville, MD, USA): NRK52E (ATCC CRL 1571) and NRK49F (ATCC CRL 1570) which are epithelial and fibroblastic clones respectively, isolated from the normal rat kidney line NRK [18]. NRK52E cells are flat and polyhedral with distinct nuclei and nucleoli, characteristic of epithelial-like cells. NRK-49F cells are fusiform in shape, with fibroblastic morphology.

Cells were grown in RPMI 1640 medium buffered with 25 mM HEPES at pH 7.4 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine in the presence of 10% FCS and cultured at 37°C in 5% CO2 atmosphere.

**[3H]leucine incorporation**

Cells were plated in 24-well plates, incubated in a serum-free medium for 48 h, and then stimulated for 24 h with PAF. For the last 6 h, cells were pulsed with 1 µCi [3H]leucine. The cells were washed three times with ice-cold PBS and then precipitated twice with 10% trichloroacetic acid (TCA), redissolved in 0.5 M NaOH with 0.1% Triton X-100, and counted in 1 ml of scintillation liquid [19]. Results are expressed as percentage increase vs control (unstimulated cells; considered as 100).

**Cell-cycle analysis by fluorescence-activated cell sorting (FACS)**

For determination of cell size and DNA content, quiescent subconfluent cells in 75-cm² Petri dishes were incubated for 24 h with PAF (10⁻⁸ M) in serum-free RPMI. Ten percent FCS was used as a positive control. At the end of the incubation period, cells were washed three times with PBS and trypsin. After centrifugation, the cellular pellet was treated with 100 µg/ml ribonuclease A and DNA was stained with propidium iodide 100 µg/ml in 0.005% Nonidet P-40. The stained specimen was kept in the dark at 4°C before flow cytometry. Samples were analysed with a EPICS-C flow cytometer (Coulter, Hialeah, FL, USA). The cell cycle was measured using the PARA-I program [20].

**RNA isolation and Northern blot analysis**

For RNA studies, quiescent cells grown in 75-cm² Petri dishes were incubated with PAF (10⁻⁸ to 10⁻¹⁰ M) in serum-free RPMI. After the incubation period, total RNA was extracted by the Chomczynski and Sacchi method [21] and quantitated by absorbance at 260 nm. Equal amounts of RNA (10–30 µg) were denatured and electrophoresed in a 1% agarose-formaldehyde gel and transferred to nylon membranes (Genescreen, New England Nuclear, Boston, MA, USA). RNA was fixed to the nylon membrane by baking for 90 min at 80°C.

The cDNA probes used were (z1)I (HF677) and (z1)IV (pCVIV-1PE16) collagens (ATTC). Probes were prepared as described previously [22] and radiolabelled by nick translation (Boehringer Mannheim, Germany) with [32P]dCTP. The cDNA probe of rat fibronectin (SR270), used as a positive control, was radiolabeled by a random priming method (Boehringer Mannheim) with [32P]dCTP.

The membranes were prehybridized for 4 h at 42°C in a hybridization solution (50% formamide, 1% SDS, 5× SSC, 1× Denhardt’s, 0.1 mg/ml denatured salmon sperm DNA and 50 mM sodium phosphate buffer pH 6.5) and hybridization was carried out at 42°C for 16–18 h in fresh hybridization solution with 20% dextran sulphate and [32P]dCTP-denatured probe. The membranes were washed using 2× SSC, 0.1% SDS for 30 min at room temperature and then twice with 0.2× SSC, 0.1% SDS at 55°C for 15 min. Autoradiographies were scanned by densitometry (Image Quant densitometer, Molecular Dynamics, Sunnyvale, CA, USA). Ethidium bromide staining was used as RNA quality control. Autoradiographic signals obtained with the 28S cDNA probe served as control for equal loading of the gel. All values were calculated as the ratio of mRNA vs 28S, and the value for cells in control conditions was considered as unity. Other lanes on the same gel were expressed as n-fold increase over this value.

**Determination of fibronectin synthesis**

Fibronectin synthesis was measured by metabolic labelling with [35S]methionine and immunoprecipitation with anti-fibronectin antibodies [24]. Quiescent cells were incubated in methionine-free culture medium RPMI with 20 µCi/m of [35S]methionine and PAF (10⁻⁸ to 10⁻¹⁰ M). At the end of the incubation period, supernatant was collected and immunoprecipitated in buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5% Triton X-100, 2 mM PMSF, 2 mM EDTA and 5 mM NEM, pH 7.4) with an excess of antifibronectin antibody (50 µg) for 16–18 h at 4°C. After this incubation, immune complexes were recovered by addition of protein A-Sepharose beads (Pharmacia, Uppsala, Sweden), and incubated for 1 h at room temperature. Beads were washed in extraction buffer. Fibronectin was released by heating at 100°C for 5 min in electrophoresis sample buffer and analysed under denaturing conditions in 7.5% SDS–polyacrylamide gels. The gels were treated with fluorographic reagent (Amplify, Amersham) and autoradiography was performed by standard methods. As a negative control for immunoprecipitation, isotopic normal rabbit IgG (30 µg/ml) was used and recombinant human...
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TGF-β1 (50 pM) was employed as a positive control. As control of cell proliferation, aliquots (20 μl) of cell lysates were taken for determination of DNA content in order to normalize fibronectin production to cell number. Results are expressed as % increase vs control (unstimulated cells; considered as 100).

**Statistical analysis**

Results are expressed as the mean ± SEM. Significance was established using a t test and differences were considered significant if the P value was less than 0.05.

**Results**

**Effect of PAF on extracellular matrix protein mRNA levels in renal tubulointerstitial cells**

Tubuloepithelial cells (NRK 52E) and renal interstitial fibroblasts (NRK 49F) were growth arrested by serum depletion for 48 h. Cells were incubated in serum-free medium with different doses of PAF (10^-6 to 10^-10 M) for increasing times until 24 h. In some experiments TGF-β1 (50 pM) was employed as positive control. RNA was then extracted and gene expression of fibronectin and collagens were determined by Northern blot.

In both cell types, PAF increased fibronectin mRNA levels, with a maximal response found with 10^-8 M PAF after 18 h of incubation (1.7- and 1.9-fold vs control, in tubuloepithelial cells and interstitial fibroblasts respectively, n = 3, P < 0.05), as shown in Figures 1 and 2. The positive stimulatory effect on FN gene expression after 18 h was as potent as 50 pM TGF-β1 (not shown).

In tubuloepithelial cells, PAF augmented the expression of type I and type IV collagen with a maximum increase at 24 h of incubation with 10^-8 M PAF (1.5- and 1.9-fold respectively, n = 3, P < 0.05) (Figures 1A and 1C). In renal interstitial fibroblasts, PAF also increased type I collagen mRNA expression which was maximum at 24 h of incubation (2.3-fold, n = 3, P < 0.05), but no effect was seen on type IV collagen gene expression (Figures 1B and 1C).

**Effect of PAF receptor antagonist on PAF-induced matrix protein gene expression**

PAF displays its effects through binding to specific receptors in target cells [2,3]. Preincubation of cells with the PAF receptor antagonist BN52021 (10^-5 M), a terpene derived from Ginkgo biloba [25], abolished PAF-induced extracellular matrix gene expression (Figure 3). BN52021 alone did not have any significant effect on matrix gene expression. These results indicate that the observed effects of PAF are mediated by its interaction with specific cell membrane receptors.

**Effect of PAF on fibronectin synthesis**

To investigate whether the stimulation of gene expression was accompanied by an increase in protein synthesis, aliquots (20 μl) of cell lysates were taken for determination of DNA content in order to normalize fibronectin production to cell number. Results are expressed as % increase vs control (unstimulated cells; considered as 100).

**Fig. 1A–C.** Time-course of the effect of PAF on extracellular matrix mRNA expression in (A) tubuloepithelial cells and (B) renal interstitial fibroblasts. Cells were incubated for 0–24 h with 10^-8 M PAF. The membranes were hybridized with cDNA probes of fibronectin (FN), type IV collagen (COL IV), type I collagen (COL I) and 28S. Results are expressed as n-fold increase over control (time 0), normalized to the respective 28S bands of a total of three experiments. Figure 1C shows a representative Northern blot. Molecular size markers are shown on the left.
Platelet-activating factor and tubulointerstitial fibrosis

Fig. 3. Effect of PAF receptor antagonist on extracellular matrix mRNA expression in (A) tubuloepithelial cells and (B) renal interstitial fibroblasts. Cells were preincubated with 10 μM BN52021 for 1 h before the addition of 10⁻⁸ M PAF. After 18 h of incubation RNA was isolated and extracellular matrix gene expression was determined by Northern blot as described in the Methods section. Data are expressed as n-fold increase over control (unstimulated cells) normalized to the respective 28S bands of a total of three experiments.

Treatment of quiescent cells with PAF for 24 h had no significant effect on TCA-precipitable [³⁵S]leucine incorporation (n=3, P=n.s.) (Figure 5). Ten percent FCS was employed as positive control (n=3, P<0.05). These data indicate that PAF does not modify total cellular protein synthesis in tubuloepithelial cells.

Fig. 2. Dose-response of PAF-stimulation on fibronectin gene expression in (A) tubuloepithelial cells and (B) renal interstitial fibroblasts. Cells were incubated for 18 h in medium alone or in the presence of PAF (10⁻⁶ to 10⁻¹⁰ M). Data are expressed as n-fold increase over control (unstimulated cells) normalized to the respective 28S bands of a total of three experiments.

Effect of PAF on total protein synthesis in epithelial cells

[³H]Leucine incorporation was examined in order to assess the effect of PAF on total protein synthesis.

Discussion

Most recent studies on the progression of renal diseases have shown that the degree of tubulointerstitial damage, rather than the glomerular damage, determine the final outcome of the disease [26]. This indicates that interstitial fibrosis is a common pathway in progressive renal diseases including glomerulonephritis, renal mass ablation, diabetes and hypertension-induced
of type IV collagen, an integral part of the basement membrane [32]. Several studies have shown that cultured renal interstitial cells produce extracellular matrix proteins in response to cytokines, growth factors and vasoactive compounds [33–36]. In this paper, we demonstrate that in two cell lines of renal interstitial cells, PAF, upregulates fibronectin mRNA expression. However, the effect on collagen mRNA levels differed in both cell types, showing an increased expression of interstitial type I collagen in fibroblasts and basement type IV collagen in tubuloepithelial cells. The effect on matrix expression was also observed at the protein level since PAF augmented fibronectin production in tubuloepithelial cells. In both cell types, a maximal increase in fibronectin was also observed at the protein level since PAF augmented fibronectin production in tubuloepithelial cells. In both cell types, a maximal increase in fibronectin production in tubuloepithelial cells. In both cell types, a maximal increase in fibronectin production in tubuloepithelial cells.

Fig. 4. Fibronectin synthesis induced by PAF in tubuloepithelial cells. Effect of PAF receptor antagonist. Cells were metabolically labelled with [35S]methionine, stimulated with PAF alone (10⁻⁶ M to 10⁻¹⁰ M) or in the presence of BNS2021 (10⁻⁸ M PAF), and newly synthesized fibronectin was determined by immunoprecipitation using polyclonal antifibronectin antibody. (A) Representative autoradiography of SDS–PAGE. (B) Densitometric analysis of the fibronectin bands. Results are expressed as percentage increase vs control (unstimulated cells) of densitometric intensity of electrophoretic bands. Mean ± SEM of six experiments. *P<0.05 vs control. **P<0.05 vs PAF-treated cells.
Many factors can stimulate mitogenesis of tubulointerstitial cells and interstitial fibroblasts [28,29,34,35]. We have demonstrated that PAF did not affect the cell cycle either in tubulointerstitial cells or interstitial fibroblasts. Moreover, we have observed that PAF does not modify cell proliferation of cultured mesangial cells [15]. All these data suggest that PAF is not a mitogenic agent, at least under our study conditions. Tubulointerstitial cells are probably the most important cells in renal hypertrophy which is characterized by an increase in cell size and protein content, but not in DNA synthesis [43]. In this paper, we have shown that PAF did not induce tubular hypertrophy, as determined by studies on protein content ([3H]leucine incorporation) and on cell size (FACS).

In summary, our data provide evidence that PAF, a classical mediator of acute inflammation, induces increased matrix production in tubulointerstitial cells and interstitial fibroblasts, two key cells of renal fibrosis. Although further studies are needed, our results suggest that PAF could be an important mediator of renal scarring.

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