Intrinsic renal cells are the major source of interleukin-1β synthesis in normal and diseased rat kidney


Department of Nephrology, Monash Medical Centre, Clayton, Victoria, Australia

Abstract

Background. A number of studies have demonstrated a pathological role for interleukin-1 (IL-1) in experimental models of glomerulonephritis, but the cellular pattern of renal IL-1 production remains poorly characterized. The aim of this study, therefore, was to identify the cell types expressing IL-1 in normal and diseased rat kidney.

Methods. Renal IL-1β expression was examined in normal rats and during a 21-day time course of rat accelerated anti-GBM glomerulonephritis by northern blotting, in situ hybridization and double immunohistochemistry.

Results. Interleukin-1β mRNA expression was readily detectable in normal rat kidney by northern blot analysis and in situ hybridization. Immunohistochemistry staining demonstrated constitutive IL-1β expression by glomerular endothelial cells and cortical tubular epithelial cells. There was a marked increase in whole kidney IL-1β mRNA in rat anti-GBM glomerulonephritis. Glomerular IL-1β immunostaining was upregulated, being expressed by podocytes, mesangial cells and infiltrating macrophages, and was particularly prominent within glomerular crescents. Double staining with the ED1 antibody showed IL-1β expression in up to 13% of glomerular macrophages, whereas 48% of macrophages within crescents stained for IL-1β. However, the most marked increase in IL-1β expression was seen in cortical tubular epithelial cells, particularly in areas of tubular damage. In situ hybridization confirmed that tubular IL-1β staining was due to local cytokine synthesis rather than protein absorption.

Conclusions. This study has identified constitutive IL-1β expression by glomerular endothelium and tubular epithelial cells in normal rat kidney. In addition, the marked upregulation of IL-1β expression by intrinsic glomerular cells and tubules in rat anti-GBM disease suggests an important role for these cells in IL-1 dependent crescent formation and tubulointerstitial injury.

Key words: crescent formation; endothelium; glomerulonephritis; interleukin-1β; macrophages; tubules

Introduction

Interleukin-1 (IL-1) is a cytokine with a wide range of proinflammatory actions, including: endothelial cell activation, induction of chemokines, leukocyte adhesion molecules, cytokines, and inducible nitric oxide synthase [1]. There are two distinct molecular forms of IL-1, termed IL-1α and IL-1β, which share a similar range of activities and bind to the same receptors [1,2]. Activated macrophages are a major source IL-1, although a number of other cell types have also been shown to synthesize IL-1 [1].

The prominent macrophage infiltration seen in most forms of glomerulonephritis suggests that IL-1 may play a role in the disease process [3]. Glomerular production of bioactive IL-1 was demonstrated in a study of human rapidly progressive glomerulonephritis [4], while increased renal IL-1β mRNA expression has been described in the development of murine lupus nephritis [5]. A pathological role for IL-1 in experimental glomerulonephritis has been identified by a number of approaches (reviewed in 6) Administration of IL-1 has been shown to exacerbate proteinuria and decrease creatinine clearance in rats with anti-GBM glomerulonephritis and rabbit immune-complex nephritis [7,8]. Blocking the action of IL-1 by administration of anti-IL-1 antibodies or the IL-1 receptor antagonist (IL-1ra) has been shown to inhibit the induction of acute renal injury in some, but not all studies, of rat anti-GBM glomerulonephritis [9–12]. However, in longer term experiments, IL-1ra treatment was shown to not only inhibit the development of rat crescentic anti-GBM glomerulonephritis, but it was also able to suppress the progression of established crescentic disease [13,14].

Given the importance of IL-1 in the mediation of
renal injury, relatively few studies have examined the cellular pattern of IL-1 expression in normal or diseased kidney. It has generally been assumed that infiltrating macrophages are the major source of IL-1 expression in glomerulonephritis, while the potential contribution of resident kidney cells for IL-1 production has been largely overlooked. Therefore, we examined the cellular pattern of IL-1β expression during the evolution of rat crescentic anti-GBM glomerulonephritis using a combined approach of northern blotting, in situ hybridisation and double immunohistochemistry staining.

**Materials and methods**

**Anti-GBM disease**

Passive accelerated anti-GBM glomerulonephritis was induced in inbred male Sprague–Dawley rats (150–200 g) as previously described [13]. Briefly, rats were immunized s.c. with 5 mg of normal rabbit IgG in Freund’s complete adjuvant and then injected i.v. with rabbit anti-rat GBM serum 5 days later (termed day 0). Groups of five animals were killed on days 1, 7, 14 or 21. In addition, a group of five normal animals was examined (also referred to as Day 0).

**Probes**

A 1.26-kb fragment of rat IL-1β cDNA was amplified by reverse transcription PCR, restriction mapped, and then cloned using the pMOSBlue T-vector kit (Amersham). Anti-sense digoxigenin (DIG)-labelled cRNA probes were prepared using a T7 RNA polymerase kit (Boehringer Mannheim GmbH, Mannheim, Germany). A 358-bp fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified by reverse transcription PCR and DIG-labelled using the High-Prime random priming kit (Boehringer Mannheim GmbH). For in situ hybridization, anti-sense (5′ TTGTCGTTGCTGCTGTCTGA 3′) and sense (5′ AAATGCCTCGTGCTGTCTGA 3′) oligonucleotides were tailed with dUTP-DIG using terminal deoxynucleotidyl transferase according to the manufacturer’s instructions (Boehringer Mannheim GmbH). Probes were precipitated and incorporation of DIG was determined by dot blot.

**Northern blotting**

Northern blotting was performed as previously described [15]. Briefly, total cellular RNA was prepared by acid–phenol–chloroform extraction and 20–μg samples were denatured with glyoxal and dimethylsulphoxide, size fractionated on 1.2% agarose gels and capillary blotted onto Hybond-N membranes (Amersham International, Buckinghamshire, UK). Membranes were hybridized overnight at 68°C or 42°C with DIG-labelled rat IL-1β cRNA or rat GAPDH cDNA probes respectively, in 5× SSPE/50% formamide/0.2 mg/ml herring sperm DNA/0.5% SDS/5× Denhardt’s solution/1% blocking agent (Boehringer Mannheim GmbH). Following hybridization, membranes were washed finally in 0.1× SSC/0.1% SDS at 68°C or 0.2× SSC/0.1% SDS at 42°C. Bound probes were detected using sheep anti-DIG antibody (Fab) conjugated with alkaline phosphatase which was developed using CPD-star enhanced chemiluminescence (ECL) (Boehringer Mannheim). ECL emissions captured on Kodak XAR film were measured by densitometry using the Cue 2 Image Analyzer programme (Olympus).

**In situ hybridization**

In situ hybridization followed a previously described protocol [15]. Cryostat sections (6 μm) of tissue fixed in 2% paraformaldehyde–lysine–periodate (PLP) were adhered to silanated glass slides, air dried, hydrated in PBS, treated with 0.2 M HCl for 10 min, washed in PBS, incubated with 0.3% Triton X-100 in PBS for 5 min, washed in PBS, digested with 1 μg/ml proteinase-K at 37°C for 30 min, washed in PBS and post-fixed in 4% paraformaldehyde for 5 min and washed again. Sections then were prehybridized for 2 h and then hybridized overnight at 37°C with 1 ng/ml sense or anti-sense DIG-labelled oligonucleotides in 4× SSC. 0.2 mg/ml herring sperm DNA, 0.2 mg/ml yeast RNA, 5× Denhardt’s solution in 50 mM sodium phosphate buffer pH8.0. Sections were washed twice in 2× SSC at room temperature and twice in 0.2× SSC at 37°C. Bound probe was then detected using alkaline phosphatase-conjugated F(ab) fragment of sheep anti-DIG using NBT/X-phosphate to develop a purple/blue colour according to the manufacturer’s instructions (Boehringer Mannheim GmbH). Sections were counterstained with methyl green and mounted in an aqueous medium. Hybridization specificity was demonstrated by the lack of signal with the sense probe and the ability of excess unlabelled anti-sense, but not irrelevant, oligonucleotide to abolish the signal.

**Antibodies**

The following monoclonal antibodies (mAb) were used in this study: MCA 1397, raised against recombinant rat IL-1β (Serotec, Oxford, UK) [16]; ED1, anti-rat CD68 labels most macrophages [17]; RECA-1, labels all rat endothelium (Serotec) [18]; 73.5, mouse anti-human CD45R used as an isotype control. Peroxidase- and alkaline-phosphatase-conjugated goat anti-mouse IgG, mouse peroxidase anti-peroxidase complexes (PAP), and mouse alkaline phosphatase anti-alkaline phosphatase complexes (APAAP) were all purchased from Dakopatts, Glostrup, Denmark.

**Immunohistochemistry**

Single and double immunohistochemistry staining was performed as previously described [14,19]. Cryostat sections (6 μm) of PLP-fixed tissues were treated with microwave oven heating for 10 min in 0.01 M sodium citrate pH 6.0 at 800 watts to enhance antigen retrieval, washed in phosphate-buffered saline (PBS), preincubated with 10% normal goat serum (NGS), 10% fetal calf serum (FCS) in PBS for 20 min, drained, incubated with anti-IL-1β mAb in 1% BSA in PBS overnight at 4°C, washed (×3) in PBS and endogenous peroxidase inactivated in 0.3% H2O2 in methanol. Sections were incubated with peroxidase-conjugated goat anti-mouse IgG, mouse peroxidase anti-peroxidase complexes (PAP), and mouse alkaline phosphatase anti-alkaline phosphatase complexes (APAAP) were all purchased from Dakopatts, Glostrup, Denmark.

For two-colour immunohistochemistry, sections were stained with ED1 or RECA-1 mAbs using 3 layer PAP method and developed with diamobenzidine as described above (without the initial microwave step). Next, sections were treated for 10 min in a microwave oven to block antibody cross-reactivity and inactivate endogenous alkaline phosphatase, preincubated as above and then incubated...
Sequentially with anti-IL-1β mAb, alkaline phosphatase-conjugated goat anti-mouse IgG and mouse APAAP, developed with Fast Blue BB Salt (Ajax Chemicals, Melbourne, Australia), and mounted in aqueous medium.

Interleukin-1β was also detected by staining acetone-fixed or ethanol-fixed snap frozen tissue and this was used for double staining with the RECA-1 mAb. Negative controls employed an irrelevant isotype-matched mAb (73.5, anti-human CD45R). Some sections were lightly counterstained with periodic acid Schiff reagent minus haematoxylin.

Quantitation of kidney IL-1β protein expression

Glomerular staining with the anti-rat IL-1β mAb was scored in 25 glomerular cross-section per animal as follows: (0.5) <25% glomerular cells labelled; [1] 25–50% glomerular cells labelled; [2] 50–75% glomerular cells labelled; and [3] >75% glomerular cells labelled. Tubular epithelial cell IL-1β staining was scored in 300–400 tubules per animal in consecutive high-power fields (×400) moving from the outer to the inner and back to the outer cortex. A positive tubule was defined as a tubular cross-section containing two or more labelled cells. In addition, the total number of ED1+ cells and the number of ED1+ IL-1β+ cells were counted in 25 glomerular cross-sections per animal in double stained sections. All scoring was performed on coded slides.

Statistical analysis

Results of Northern blot densitometry and quantitation of antibody staining were analysed using a two-sided student’s t test, except for semiquantitative scoring of glomerular IL-1β immunostaining which was analysed by a non-parametric Mann–Whitney U test.

Results

Interleukin-1 expression in normal rat kidney

Northern blotting demonstrated the presence of a single 1.7-kb species of IL-1β mRNA within total cellular RNA extracted from whole rat kidney (Figure 1). In situ hybridization of tissue sections found constitutive IL-1β mRNA expression by glomerular cells, some cortical tubular epithelial cells and occasional interstitial cells (Figure 2a). Immunohistochemistry demonstrated the presence of IL-1β protein within normal kidney. Glomerular IL-1β expression had a distinctive endothelial pattern (Figure 2b), which was confirmed by double staining with the endothelial-specific RECA-1 mAb (Figure 2c). In the tubulointerstitium, weak IL-1β staining was evident in some proximal and distal tubules and the thick ascending loop of Henle, accounting for approximately 40% of all cortical tubules. Interestingly, tubular IL-1β staining was very weak on acetone-fixed snap frozen tissue (Figure 2b), but was enhanced by microwave treatment of PLP-fixed tissue sections on which tubular IL-1β expression was scored. In addition, some blood vessel endothelium showed IL-1β staining.

Northern blot analysis demonstrated a progressive increase in whole kidney IL-1β mRNA expression during the progression of rat anti-GBM glomerulonephritis (Figure 1), with an eightfold increase in the relative ratio of IL-1β to GAPDH mRNA seen on day 14 of disease compared to normal rats (P<0.01). A marked increase in glomerular IL-1β mRNA expression in disease was also shown by in situ hybridization. In particular, IL-1β mRNA was strongly expressed within glomerular crescents (Figure 2d). This increase in IL-1β gene expression was reflected by a substantial
increase in glomerular IL-1β protein as detected by immunohistochemistry staining (Figures 2e, 3a). Glomerular endothelial cells, podocytes, and parietal epithelial cells all showed IL-1β staining. This was most prominent within damaged areas such as focal proliferative lesions and crescents. In addition, some glomeruli displayed a distinct mesangial IL-1β staining pattern (Figure 2e, f).

A significant glomerular macrophage infiltrate was evident on day 1 of anti-GBM disease, which progressed during the disease course (Figure 4a). Few macrophages showed IL-1β expression during the early stages of the disease, but 13% of glomerular ED1+ macrophages were IL-1β-positive by day 21 (Figure 4b). Interestingly, a significantly higher percentage of ED1+ macrophages showed IL-1β expression within crescents compared to those in the glomerular tuft on day 21 of disease (48.3±5.9% vs 13.1±1.1% respectively; P<0.01).

**Tubulointerstitial interleukin-1β expression in anti-GBM disease**

*In situ* hybridization showed a dramatic increase in IL-1β mRNA expression by tubular epithelial cells in anti-GBM disease. In addition, numerous IL-1β mRNA+ interstitial cells were seen (Figure 2d). Antibody staining showed a significant increase in IL-1β expression by tubular epithelial cells in anti-GBM disease, both in terms of the number of IL-1β+ tubules and the...
intensity of tubular staining (Figures 2e, 3b). Tubular IL-1β staining was strongest in damaged tubules. Interstitial cells, including infiltrating ED1+ macrophages, also expressed IL-1β mRNA and protein, but tubular epithelial cells were the major source of IL-1β in anti-GBM disease.

**Discussion**

This study has used three independent techniques to demonstrate constitutive IL-1β expression by intrinsic rat kidney cells and to show that intrinsic renal cells are the major source of the marked increase in IL-1β expression seen during the development of rat crescentic anti-GBM glomerulonephritis.

The demonstration of constitutive IL-1β mRNA expression in normal rat kidney using northern blotting and in situ hybridization is consistent with previous northern blotting studies showing IL-1β gene expression in normal mouse, rat and human kidney [5,20,21]. However, other studies have failed to detect IL-1β mRNA in normal human kidney by in situ hybridization [22,23], and Northern blot analysis of glomerular IL-1β expression failed to detect IL-1β mRNA in normal glomeruli isolated from Sprague–Dawley rats [12,24]. Presumably these differences are due to the sensitivity of the techniques employed. For example, using a random-primed 120-bp rat IL-1β cDNA probe we failed to detect IL-1β mRNA in whole rat kidney by Northern blotting (D. J. Nikolic-Paterson, unpublished results); however, this was easily achieved using a 1.26-kb IL-1β cRNA probe.

Constitutive glomerular IL-1β production was restricted to the endothelium. While glomerular endothelial IL-1α expression has been described following administration of endotoxin to rats [25], constitutive glomerular endothelial IL-1β staining has not been detected in studies of normal human kidney [22,26]. The reason for this difference is not clear. It could be a species difference, since this is the first study to localize IL-1β protein in rat kidney. Alternatively, it may reflect a unique aspect of the epitope of the IL-1β molecule recognized by the anti-rat IL-1β monoclonal antibody employed in the current study.

There was a dramatic increase in glomerular IL-1β expression during rat anti-GBM glomerulonephritis as shown by in situ hybridization and immunohistochemistry. Although double immunostaining demonstrated IL-1β expression by infiltrating macrophages, it was the intrinsic cells (podocytes, mesangial cells and endothelium) which were the major source of glomerular IL-1β production during disease. Studies of human glomerulonephritis have demonstrated significant glomerular IL-1β mRNA and protein expression, particularly within cellular crescents, although this expression has mainly been attributed to infiltrating macrophages rather than intrinsic glomerular cells [22,23,26,27]. The more prominent IL-1β expression seen by intrinsic glomerular cells in anti-GBM disease compared to that of studies of human glomerulonephritis is probably due to two factors. First, the rat model of anti-GBM glomerulonephritis is a more severe and rapidly progressive disease compared to most cases of human glomerulonephritis examined. Indeed, the most appropriate comparison would be with patients with anti-GBM antibodies and crescentic glomerulonephritis, such as in Goodpasture’s disease.

Second, the biopsy of human glomerulonephritis often occurs after a peak, or flare, of disease and as such may miss the peak of IL-1β expression.

In situ hybridization and antibody staining can identify local IL-1β synthesis, but it cannot distinguish whether the cytokine is being stored or is in the process of secretion. Therefore, the relative contribution of intrinsic cells and infiltrating macrophages to IL-1β secretion within the injured glomerulus is difficult to assess. A link between glomerular macrophage infiltration, renal injury, and increased glomerular secretion of bioactive IL-1 has been established in experimental glomerulonephritis [5,28–30]. Not only can infiltrating glomerular macrophages secrete IL-1 directly, but they may also contribute indirectly to glomerular IL-1 production through stimulating the synthesis and release of IL-1 by intrinsic glomerular cells.

The strong IL-1β expression by tubular epithelial cells in rat anti-GBM disease was an unexpected finding. This was due to local IL-1β synthesis rather than absorption, as shown by in situ hybridization. Tubular IL-1β staining has been described previously in human glomerulonephritis [22,23], and this is consistent with a emerging trend that tubular epithelial cells are capable of synthesizing a wide range of cytokines, including tumour necrosis factor-α (TNF-α). Tubular IL-1β expression was most prominent in areas of damage, suggesting that it may play a role in lesion formation.

The cellular pattern of IL-1β expression identified in this study of rat anti-GBM disease has a number of implications for the pathogenic mechanisms of this cytokine. First, secretion of stored IL-1β by endothelium may mediate the very rapid upregulation of monocyte chemotactic protein-1 (MCP-1) and ICAM-1 (CD54) expression seen following deposition of anti-GBM antibody [31,32]. Second, the presence of strong IL-1β expression within cellular crescent provides a mechanism for stimulation of tissue factor-like procoagulant activity by epithelial cells and the marked upregulation of MCP-1, ICAM-1, VCAM-1 (CD106) and basic fibroblast growth factor expression by crescent cells and parietal epithelial cells [33]. This also explains the impressive ability of IL-1ra treatment not only to prevent glomerular crescent formation in this disease, but also to reverse crescent formation in established disease [13,14]. Third, many in vitro studies have demonstrated that IL-1 stimulates tubular epithelial cell production of inflammatory mediators such as TNF-α, MCP-1, IL-8, ICAM-1, VCAM-1, and inducible nitric oxide synthase [34]. Therefore the strong upregulation of tubular IL-1 expression, particularly in areas of tubular damage, may be an important trigger for the production of these inflammatory mediators in rat anti-GBM glomerulonephritis. This line of argument is supported by the ability of IL-1ra...
treatment to virtually abrogate interstitial leukocytic infiltration and tubulointerstitial damage even in the presence of moderate proteinuria [13,14]. However, it should be noted that while leukocytic infiltration was evident in areas with strong tubular IL-1β expression, this expression per se is not sufficient to induce leukocyte accumulation since there is constitutive IL-1β expression in normal kidney and in intact areas of the diseased kidney without infiltration. One further consequence of increased tubular IL-1β expression may be modulation of renal handling of fluid and electrolytes, as suggested by in vitro studies on proximal tubular cells [35].

In conclusion, this study has demonstrated that intrinsic renal cells constitutively express IL-1β and that they are the major source of the increased IL-1β production seen during rat anti-GBM glomerulonephritis. Given the ability of IL-1ra treatment to inhibit the induction and progression of rat anti-GBM disease, IL-1 production by intrinsic renal cells is likely to play an important role in crescent formation and tubulointerstitial injury in experimental glomerulonephritis.

Acknowledgements. This work was supported in part by NH&MRC grant No. 930827 and the Baxter Extramural Grant Program.

References

21. Tovey MG, Content J, Gresser I et al. Genes for IFN-beta-2 (IL-6), tumor necrosis factor, and IL-1 are expressed at high levels in the organs of normal individuals. J Immunol 1988; 141: 3106–3110

Received for publication: 14.10.96
Accepted in revised form: 11.2.97