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CLINICAL INVESTIGATION

IL-1 receptor antagonist inhibits monocyte chemotactic peptide 1 generation by human mesangial cells

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IL-1 receptor antagonist inhibits monocyte chemotactic peptide 1 generation by human mesangial cells. The elicitation of neutrophils and monocytes from the circulation into the inflamed glomerulus is a key process in the pathogenesis of proliferative glomerulonephritis. The aim of this study was to determine the factors which regulate the expression and synthesis of the monocyte specific chemotaxin, monocyte chemotactic peptide 1 (MCP-1). Mesangial cells in culture did not constitutively express MCP-1, but could be induced to express both MCP-1 mRNA and antigenic MCP-1 by either stimulation with IL-1α or TNFα, which are also stimuli for interleukin 8 (IL-8/NAP-1) expression and release. Pre-treatment of mesangial cells with the IL-1 receptor antagonist (IL-1ra) induced dose-dependent inhibition of both the expression of MCP-1 and IL-8 mRNA as well as the release of both chemotactic peptides in response to IL-1α, while the receptor antagonist had no significant effect on TNFα induced MCP-1 and IL-8 generation. This study demonstrates that the IL-1 receptor antagonist was four times more effective at inhibiting the IL-1 induced expression and release of IL-8 compared to that of MCP-1. These results suggest that mesangial cell-derived MCP-1 may play an important role in the recruitment of monocytes in glomerular inflammation and that an IL-1 receptor antagonist may have therapeutic potential for the treatment of glomerulonephritis.

The accumulation of polymorphonuclear leukocytes and monocytes within the inflamed glomerulus, accompanied by proliferation of resident mesangial cells and expansion of the mesangial matrix are characteristic of immunologically-mediated glomerulonephritis [1, 2]. The migration of inflammatory cells into an extravascular site require a series of coordinated signals, which include the generation of a chemotactic gradient by the cells of the extravascular compartment. The nature of the stimulus and the subsequent spectrum of chemotatic factors produced, determine the specific leukocyte population elicited to the inflammatory site [3]. This hypothesis is supported by the recent characterization of a large family of structurally related peptides which for the most part are potent chemotactic stimuli with considerable target cell specificity [3, 4]. To date the best characterized member of this family is interleukin-8, which is a potent neutrophil chemotaxin [4].

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Mesangial cells on exposure to the pro-inflammatory cytokines IL-1α and TNFα express and release IL-8, as well as the mesangioproliferative cytokine IL-6 [5–8]. These findings support the idea that the mesangial cell has an active role in the propagation of inflammatory response within the glomerulus and can no longer be considered as having a bystander role in these events.

Another member of this family of chemotactic peptides is the monocyte specific chemotaxin known as monocyte chemotactic peptide 1 [3, 9]. We now report that human mesangial cells can express MCP-1 mRNA and release the MCP-1 peptide in response to the same stimuli that induce the formation of IL-8 in mesangial cells, namely IL-1α and TNFα. Since the most potent stimulus for the induction and release of both IL-8 and MCP-1 was IL-1 we have also examined the characteristics of the IL-1 receptors on mesangial cells which are coupled to the generation of these chemotactic cytokines. The nature of the receptors were identified pharmacologically by utilizing the novel recombinant 17 kDa polypeptide IL-1 receptor antagonist, which appears to have a higher affinity for type I, as compared to the type II IL-1 receptor on human cells [10, 11].

Methods

Materials

Human recombinant IL-1α (specific activity 2.1×10^7 U/ml) and human recombinant TNFα (specific activity of 6×10^7 U/ml), were gifts from Hoffman La Roche and Ernst-Boehringer, respectively. The human recombinant IL-1ra was supplied by Synergen Inc. All culture reagents (endotoxin free) and plastics were from Gibco and Nunc, respectively.

Mesangial cell preparation and culture conditions

Portions of macroscopically normal renal cortex were obtained from human kidneys immediately after surgical nephrectomy for renal carcinoma. Human MC were obtained from glomeruli isolated by differential sieving and collagenase treatment as described previously [5]. Cell cultures were maintained in Waymouth's MB752/1 supplemented with 15% heat-inactivated fetal calf serum, penicillin-steptomycin (10 U/ml and 10 μg/ml) and fungizone [0.5 μg/ml (complete MC-medium)]: all cells in these studies were used between passages 2 and 6. Mesangial cell identity was confirmed by using a series of cell

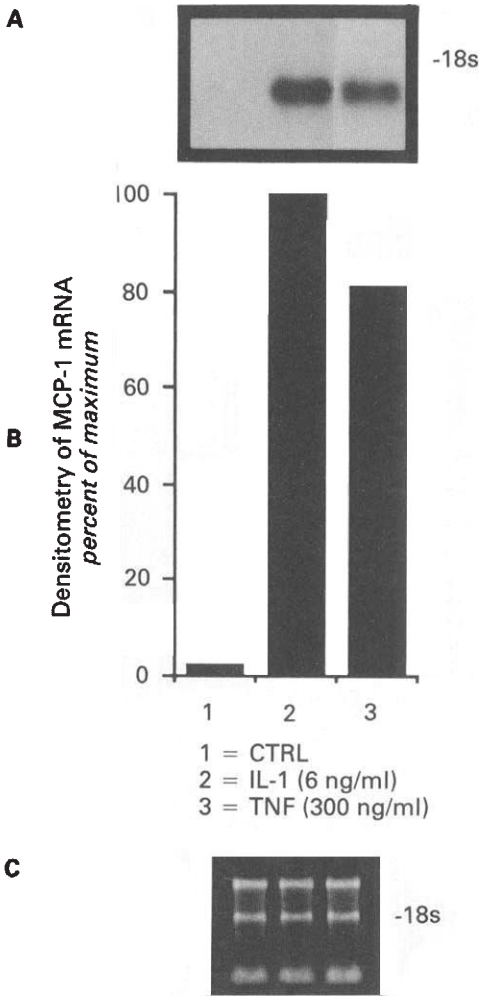

Fig. 1. *Expression of mesangial cell-derived MCP-1 mRNA in response to IL-1α and TNFα.* **A.** Represents Northern blot analysis of MCP-1 mRNA by mesangial cells 6 hours postexposure to either medium alone (1), IL-1α (2) or TNFα (3). Laser densitometry of each respective Northern blot panel **B**, (AU · mm = is the actual absorbance of the density of the autoradiograph for each of the bands of mRNA determined by the laser per mm), and **C**, 28S and 18S ribosomal RNA demonstrating equal loading of RNA. These results are representative of three experiments.

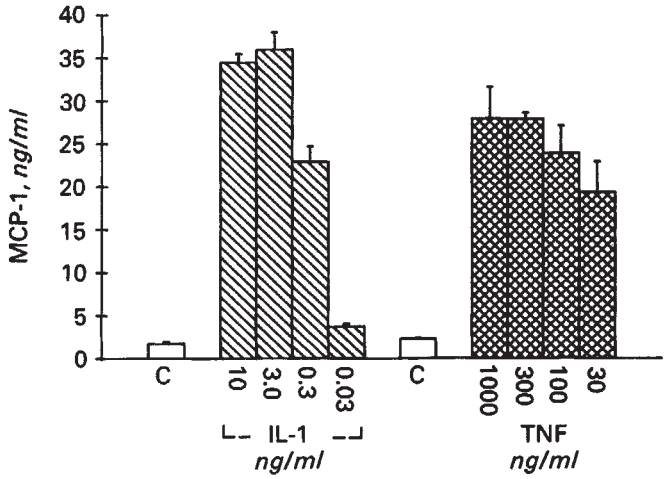

Fig. 2. *Dose-dependent induction of MCP-1 generation by IL-1α and TNFα-stimulated mesangial cells.* Antigenic MCP-1 levels were determined by ELISA following 18 hours stimulation with appropriate dose of IL-1α (0.03 to 10 ng/ml), TNFα (30 to 1000 ng/ml) or medium control. Data shown are from a representative experiment, similar results were obtained in three other experiments (all experiments were performed in duplicate). Results are the mean ± SEM of quadruplet samples.

markers according to the method described previously [5]. In addition, MC displayed the characteristic hillock structures in culture as described by Sterzel et al [12]. Electron microscopy of cell cultures revealed the presence of prominent bundles of peripheral microfilaments with focal attachment bodies (dense bodies) along their course, a feature which is associated with mesangial and smooth muscle cells and is absent in fibroblasts [13].

Experimental protocol

For experiments MC (5×10^5/well) were plated on to gelatin coated 24-macrowell tissue culture plates and 80 cm^2 tissue culture flasks (for Northern blots). Twenty-four hours before stimulation confluent cell cultures were washed and cultured in serum-free medium (without fetal calf serum or growth factors). Growth arrested cultures were pre-treated with the appropriate dose of IL-1ra (10 to 1000 ng/ml) or media control for 15 minutes at 37°C, before the addition of sub-maximal concentration of either IL-1α (6 ng/ml) or TNFα (300 ng/ml), which were selected from previous dose-response studies [5]. Cultures were incubated for a further 18 hours for quantitation of extracellular MCP-1 and IL-8. Expression of mRNA for both peptides was determined by Northern blot analysis six hours post-stimulation with either cytokine.

Enzyme-linked immunoadsorbent assay (ELISA) for IL-8

Extracellular IL-8 activity of culture supernatants was measured using a double-ligand ELISA method [14]. The detection limit of this assay is 50 pg/ml. Anti-human IL-8 mouse monoclonal antibody, human recombinant IL-8 and anti-human IL-8 goat polyclonal antibody conjugated to alkaline phosphatase were supplied by Sandoz, Vienna, Austria. The substrate p-nitrophenyl phosphate (Sigma) was dissolved in 10% diethanolamine buffer pH 9.8 to a final concentration of 1 mg/ml. The reaction was stopped with 50 μl/well of 3 M NaOH when the desired extinction had been reached.

Northern blot analysis

Total cellular RNA from mesangial cells was isolated using a modification of the method of Chirgwin et al and Jonas, Sargent and Davis [15, 16]. RNA from MC was extracted and separated by Northern blot analysis using the method described by Strieter et al [17]. Briefly, mesangial cells were solubilised in a solution consisting of 25 mM Tris, pH 8.0 containing 4.2 M guanidine isothiocyanate, 0.5% Sarkosyl and 0.1 M 2-mercaptoethanol. After homogenization, the above suspension was added to an equal volume of 100 mM Tris pH 8.0 containing 10 mM EDTA and 1% SDS. The RNA was then extracted with chloroform-phenol and chloroform-isoamyl alcohol. The RNA was alcohol-precipitated and the pellet dissolved in 10 mM Tris, 0.1 mM EDTA buffer with 0.1% Sarkosyl. The concentration of RNA was determined by obtaining the absorbance at A_{260} and

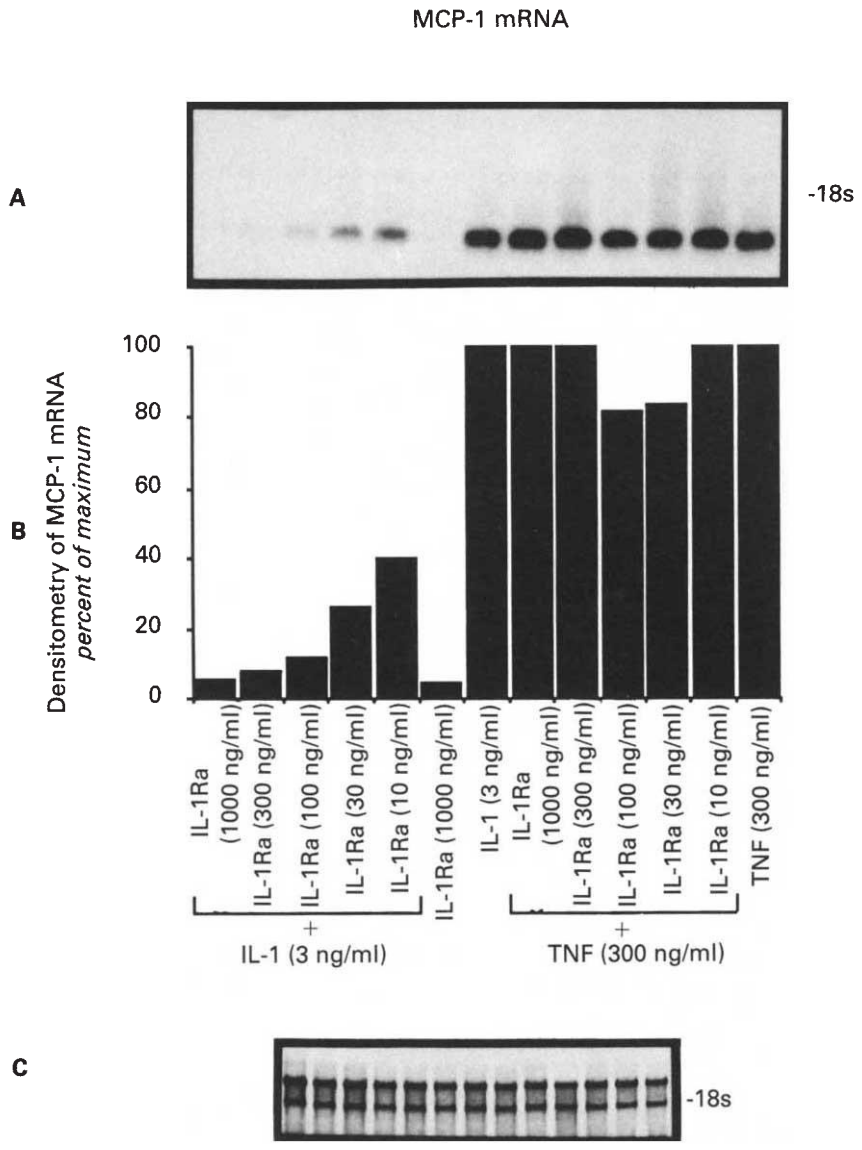

Fig. 3. *Effect of IL-1 receptor antagonist on MCP-1 mRNA expression by mesangial cells in response to IL-1α or TNFα.* Cells were pretreated with the appropriate dose of IL-1ra for 15 minutes, before the addition of either IL-1α or TNFα. Total RNA was extracted five hours post-challenge. **A.** Northern blot of the MCP-1 mRNA expression, laser densitometry of each respective Northern blot panel **B**, and 28S and 18S ribosomal RNA panel **C**. These results are representative of three experiments.

A_{280} nm, so that 10 μg of RNA was loaded into each well of the agarose gel. RNA was separated by Northern blot analysis using formaldehyde, 1% agarose gels, and transblotted to nitrocellulose. The blots were baked under vacuum, prehybridized, and then hybridized with a ^{32}P 5′-end labeled oligonucleotide probe. The 30 mer oligonucleotide probes were complementary to either nucleotides 262-291 or nucleotides 256-285 of published cDNA sequence for IL-8 and MCP-1, respectively [18, 19]. The sequence of the IL-8 probe was 5′-GTT-GGC-GCA-GTG-TGG-TCC-ACT-CTC-AAT-CAC-3′ [18], while the sequence for the MCP-1 probe was 5′-TTG-GGT-TTG-CTT-GTC-CAG-GTG-GTC-CAT-GGA-3′ [19]. Blots were quantitated by laser densitometry [17]. Equivalent amounts of total RNA load per gel lane were assessed by monitoring 18S and 28S RNA.

Enzyme-linked immunoadsorbent assay for MCP-1

Antigenic MCP-1 was measured using a double-ligand method, as previously described [20]. In brief, 96-well ELISA plates (Nunc-Immuno Maxisorb plate, Gibco Ltd) were coated with 50 μl/well of rabbit anti-MCP-1 (3.2 μg/ml) in coating buffer (borate-buffered saline, 50 mM H_3BO_3, 120 mM NaCl, pH 8.6) and incubated overnight at 4°C. The plates were washed three times with 200 μl/well of wash buffer [phosphate-buffered saline (PBS) + 0.5% vol/vol Tween 20]. Nonspecific binding sites were blocked with 2% bovine serum albumin in PBS and incubated for one hour at 37°C. Plates were washed three times with wash buffer, followed by the addition of 50 μl/well in duplicate of either MCP-1 standards (1-1000 pg/ml diluted in wash buffer supplemented with 2% FCS), or culture supernatants (neat, 1:5, and 1:10), incubated for one hour at 37°C. Plates were washed three times with wash buffer, and 50 μl/well of biotinylated rabbit anti-MCP-1 (6 μg/ml in PBS + 2% FCS) was added for 45 minutes at 37°C. Plates were washed three times, followed by the addition of 100 μl/well of 1:5000 avidin-horseradish peroxidase (Dako Ltd) diluted in wash buffer + 2% FCS, and incubated for 30 minutes at 37°C. Following three washes, a 100 μl/well of the chromogen substrate (0.67 mg/ml

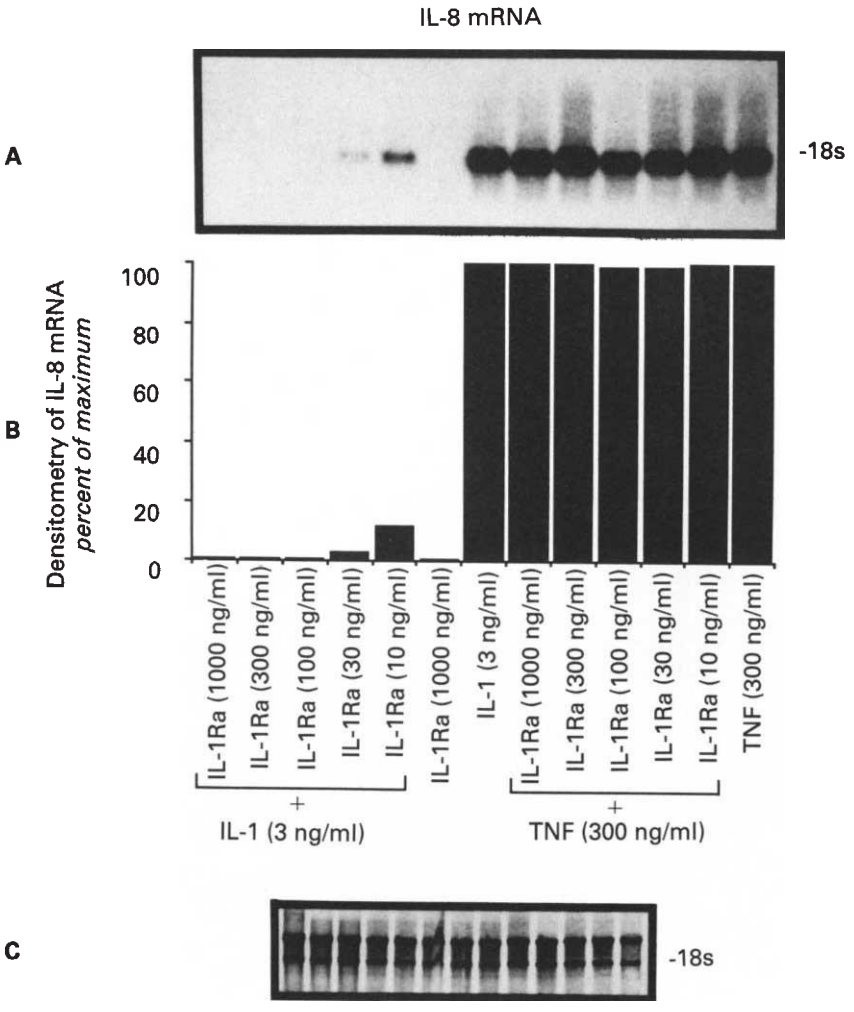

Fig. 4. *Effect of IL-1 receptor antagonist on IL-8 mRNA expression by mesangial cells in response to IL-1α or TNFα.* Cells were pre-treated with the appropriate dose of IL-1ra for 15 minutes, before the addition of either IL-1α or TNFα. Total RNA was extracted five hours post-challenge. **A.** Represents the Northern blot of the IL-8 mRNA expression, laser densitometry of each respective Northern blot panel **B,** and 28S and 18S ribosomal RNA panel **C.** These results are representative of three experiments.

orthophenylenediamine dichloride in 25 mM citrate/phosphate pH 5.0, and 0.0002% hydrogen peroxide) was added and the plates were incubated at room temperature until color development was noted, at which point the reaction was terminated with 50 μl/well of 3 M H_2SO_4. The absorbance was read at 490 nm in an ELISA plate reader.

Results

MCP-1 expression in mesangial cells

We examined the expression of MCP-1 mRNA transcripts in mesangial cells by Northern blot analysis. Figure 1 shows that mesangial cells did not express detectable amounts of MCP-1 mRNA, after six hours in medium alone (Fig. 1A and B). However, after six hours of exposure to either IL-1α (6 ng/ml) or TNFα (300 ng/ml), significant induction in the expression of MCP-1 mRNA was observed with both cytokines (Fig. 1A and B).

MCP-1 peptide synthesis and release by mesangial cells

Figure 2 shows that supernatants derived from MC cultured in the presence of serum-free medium alone did not contain detectable levels (<50 pg/ml) of MCP-1 at 18 hours. While the addition of IL-1α (0.3 to 30 ng/ml) or TNFα (30 to 1000 ng/ml) for 18 hours resulted in a dose-dependent release of MCP-1 peptide from mesangial cells (Fig. 2).

Effect of IL-1 receptor antagonist on cytokine induced MCP-1 and IL-8 mRNA expression of mesangial cells

Having demonstrated that IL-1α was the most potent stimuli for the generation of MCP-1 and IL-8 from mesangial cells, we next wanted to determine the nature of IL-1 receptor coupled to their synthesis. Pre-treatment with the IL-1ra (10 to 1000 ng/ml) for 15 minutes followed by five hours exposure to either IL-1α (3 ng/ml) or TNFα (300 ng/ml) resulted in dose-dependent inhibition of only the IL-1 induced expression of MCP-1 mRNA (Fig. 3A and B), with complete inhibition of MCP-1 mRNA transcripts being achieved with a dose of 100 ng/ml of IL-1ra. In marked contrast, the TNFα induced MCP-1 mRNA expression was unaffected by the IL-1ra in the dose range of 10 to 1000 ng/ml (Fig. 3A and B). The IL-1ra in this concentration range did not exhibit any agonist effects (Fig. 3A and B). Similarly expression of the neutrophil chemotactic peptide IL-8 mRNA transcripts induced by IL-1α was effectively antagonized by IL-1ra. Complete inhibition of IL-8 mRNA expression was

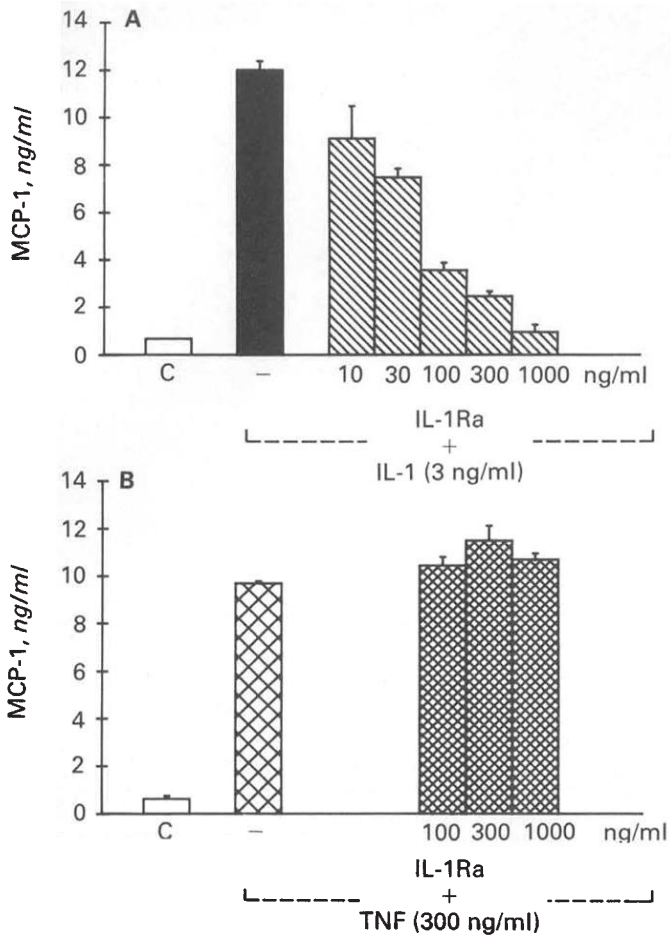

Fig. 5. *Effects of IL-1 receptor antagonist on cytokine-induced release of extracellular MCP-1 by mesangial cells.* Cells were pre-treated with the appropriate dose of IL-1ra for 15 minutes with IL-1ra, before the addition of either IL-1α (**A**) or TNFα (**B**), cultures were incubated for a further 18 hours and supernatants were quantitated for extracellular MCP-1 activity. Data shown are from a representative experiment. Similar results were obtained in four other experiments (all experiments were carried out in duplicate). Results are the mean ± SEM of quadruplet samples.

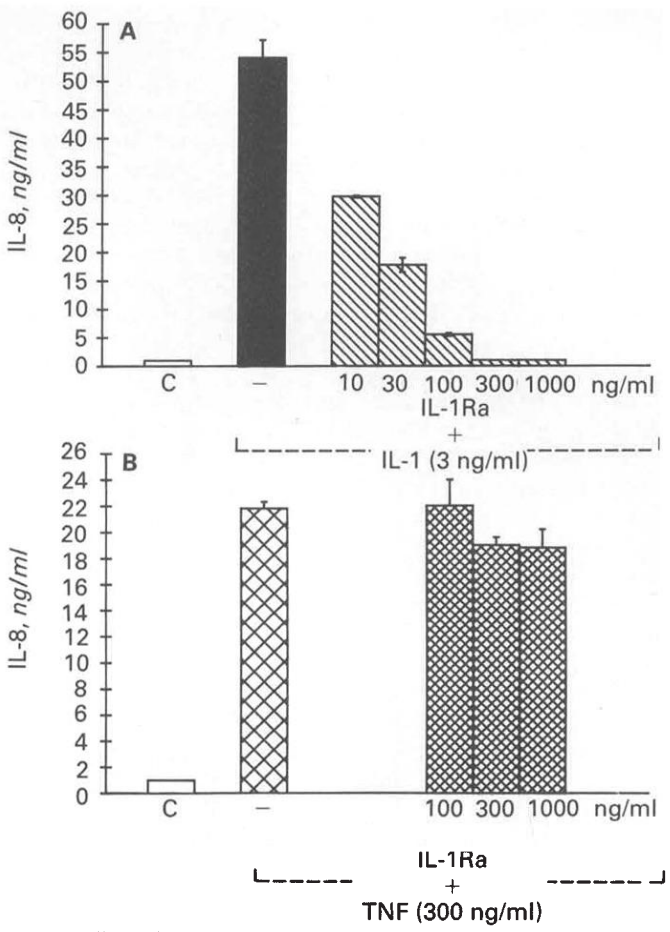

Fig. 6. *Effect of IL-1 receptor antagonist on cytokine-induced release of extracellular IL-8 by mesangial cells.* Cells were treated as described in Figure 5. Supernatants were quantitated for extracellular IL-8 activity. The IL-1α or TNFα induced release of extracellular IL-8 in the presence or absence of the IL-1ra are shown in **A** and **B**, respectively. Data shown are from a representative experiment. Similar results were obtained in four other experiments (all experiments were carried out in duplicate). Results are the mean ± SEM of quadruplet samples.

observed with a dose of 30 ng/ml of IL-1ra (Fig. 4A and B). The TNFα induced IL-8 mRNA expression was unaffected by the IL-1ra at all concentrations used (10 to 1000 ng/ml; Fig. 4A and B).

Effect of IL-1 receptor antagonist on cytokine-induced MCP-1 and IL-8 synthesis and release by mesangial cells

Treatment of mesangial cells with either IL-1α (3 ng/ml) or TNFα (300 ng/ml) for 18 hours induced significant generation of MCP-1 peptide, with a concentration range of 12 ng/ml and 9.7 ng/ml, respectively, compared with unstimulated controls (Fig. 5A and B). Pre-treatment of mesangial cells for 15 minutes with IL-1ra (10 to 1000 ng/ml), resulted in dose-dependent inhibition of IL-1α (3 ng/ml) induced release of MCP-1 (Fig. 5A), whereas, release of this peptide was unchanged in response to TNFα (300 ng/ml; Fig. 5B). Similarly, the IL-1α induced release of IL-8, resulted in dose-dependent inhibition by the IL-1ra, while the receptor antagonist had no significant effect on TNFα induced IL-8 release (Fig. 6A and B). The IL-1ra was a more effective antagonist of IL-1α induced IL-8 release (IC_{50} 12 ng/ml) than MCP-1 release (IC_{50} 54 ng/ml) in mesangial cells. Thus the receptor antagonist was four times more effective at blocking IL-8 than MCP-1 release from mesangial cells.

Discussion

We have demonstrated that cultured human mesangial cells, upon stimulation with either IL-1α or TNFα, release significant quantities of MCP-1 peptide, which is a potent monocyte specific chemotaxin [3]. These results, together with our earlier observations that mesangial cells release the neutrophil chemotaxin, IL-8 [5–8], suggest that mesangial cells have the potential for directing the specific leukocyte population within the glomerulus, a feature which is associated with different forms of immunologically mediated glomerulonephritis [1, 2]. Interestingly, under our experimental conditions, the mesangial cells

displayed a differential synthesis of the two chemotactic peptides, with two- or threefold more IL-8 than MCP-1 being released in response to IL-1. Whether such quantitative differences in chemotactic cytokine generation by mesangial cells accounts for the time dependent elicitation of various leukocytes within the glomeruli in vivo, is at present unknown. However, the mesangial cell clearly has the potential, by differential regulation of IL-8 and MCP-1 generation, to dictate which population of leukocytes are enriched within the glomeruli. At present very little information is available concerning the nature of the signal transduction system involved in the generation of these chemotactic peptides and thus targets for therapeutic intervention are limited.

The participation of infiltrating monocytes in the pathogenesis of human proliferative glomerulonephritis has been recognized for some time [21]. The monocyte has been regarded as a source of procoagulant activity, pro-inflammatory mediators, and matrix degrading enzymes [1, 22]. The accumulation of activated macrophages would provide a positive feedback mechanism via the macrophage derived IL-1 and TNF which would activate the mesangial cells to generate more of the monocyte chemotaxin. Clearly there must be a number of signals which regulate this destructive cycle of events.

To examine this possibility, we have explored the potential of a recombinant form of IL-1 receptor antagonist, which was originally isolated from IgG stimulated macrophages [11]. Our present results indicate that the IL-1 receptor antagonist effectively inhibit, in a dose-dependent manner, IL-1α but not TNFα induced MCP-1 and IL-8 mRNA expression and peptide release. Studies with IL-1ra in other cells demonstrate that approximately 100- to 300-fold excess of IL-1ra is required to produce a 50% inhibition of IL-1 induced response [23, 24]. However, mesangial cells only require a threefold excess of IL-1ra to block IL-1 induced chemotactic cytokine generation. These results indicate that mesangial cells express low numbers of high affinity IL-1 receptors. The data also shows that although the IL-1α induced response for both chemotactic peptides was inhibited by IL-1ra, the antagonist was four times more effective at inhibiting the expression and release of IL-8 than MCP-1.

Recent reports indicate that the IL-1ra blocks the binding of IL-1 to human cells bearing both type I and II receptors, but one requires 10- to 50-fold more antagonist to block the type II receptors [23]. The difference in potency of IL-1ra in blocking the IL-1 type I or II receptors on human cells may be one explanation for our observed difference in blockade of MCP-1 and IL-8 response induced by IL-1α in mesangial cells. The results of this study suggest that the mesangial cell may possess both types of receptors for IL-1, such that the signal for IL-1 induced IL-8 response may be operating via the type I receptor, whereas the signal for IL-1 induced MCP-1 response requires either one (type II receptors only) or both types of receptors. Alternatively, there may be associated accessory binding or signaling proteins on mesangial cells which are different for MCP-1 and IL-8.

Several studies have identified that mesangial cells in response to IL-1, can proliferate as well as synthesize and release other inflammatory mediators [25, 26]. Thus the inhibition of IL-1 action within the inflamed glomerulus is a potential target for the therapeutic control of glomerulonephritis. Glucocorticoids such as dexamethasone are effective inhibitors of IL-1 induced cytokine generation by fibroblasts and macrophages [27, 28], however, dexamethasone does not inhibit IL-1-induced IL-8 generation by mesangial cells [5]. In this study we demonstrate the ability of the recombinant IL-1ra to effectively block IL-1 induced generation of both chemotaxins, MCP-1 and IL-8. Thus the results of this study suggest that the IL-1 receptor antagonist may have therapeutic potential for the treatment of glomerulonephritis.

Acknowledgments

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