Induction of macrophage inflammatory protein 2 gene expression by interleukin 1 β in rat lung

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Abstract

Background – Recruitment of inflammatory cells in the lungs may contribute to tissue injury as a result of mediators released from these cells. Interleukin 1 β (IL-1 β) is a potent inducer of neutrophil accumulation, a process that may require local protein biosynthesis. Macrophage inflammatory protein 2 (MIP-2) is a ~6 kD heparin binding protein and is a member of the C-X-C superfamily that causes significant neutrophil chemotaxis and activation in vitro. A study was performed to determine whether IL-1 β could induce the expression of MIP-2 in the lungs of Brown-Norway rats.

Methods – rhIL-1 β (500 U) or 0.9% NaCl was injected intratracheally and bronchoalveolar lavage (BAL) cells and lung tissues were evaluated for MIP-2 mRNA expression after RNA extraction by Northern blot analysis. MIP-2 probe was prepared from cDNA obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) of BAL cells obtained from a rat treated with lipopolysaccharide.

Results – There was no detectable MIP-2 mRNA in the lungs of control rats but a marked enhancement of the expression at four hours with no expression at 12 hours and a slight expression at 24 hours. IL-1 β induced a significant influx of neutrophils into BAL fluid with a transient increase in macrophages. In situ hybridisation of lungs using MIP-2 cDNA probe labelled with digoxigenin showed expression of MIP-2 mRNA in airway mononuclear cells and airway epithelium at four hours after IL-1 β ; at 24 hours the signal had nearly gone.

Conclusion – IL-1 β induces the expression of MIP-2 mRNA in rat lung. MIP-2 may be one chemokine that could contribute to IL-1 β induced neutrophil influx.

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of well characterised factors such as interleukin 1 β (IL-1 β), tumour necrosis factor (TNF), and leukotriene B₄ have been implicated in this response. IL-1 β produced by macrophages may promote local pulmonary inflammation by inducing chemotaxis and activation of both neutrophils and macrophages.^{1,2} When administered to the lungs of various species, IL-1 β induces an influx of neutrophils to the lungs.^{3,4} There is evidence to suggest that IL-1 β induced neutrophil accumulation is an indirect mechanism, unlike the chemoattractant leukotriene B₄.⁵

Chemokines are a large group of chemotactic and proinflammatory cytokines. They represent a family of polypeptides with four conserved cysteine residues which can be subdivided into two large groups depending on whether or not there is an intervening amino acid between the two cysteines yielding the C-X-C (or the α) and C-C (or the β) families. Chemokines of the C-X-C type include IL-8 and are predominantly chemotactic in vitro for neutrophils. In vitro studies have shown that endothelial cells exposed to IL-1 β release chemotactic factors for the neutrophil and that this activity is accounted for by the C-X-C chemokine, IL-8, which is closely related to macrophage inflammatory protein 2 (MIP-2).⁶ Other C-X-C chemokines such as MIP-2, PF-4 (platelet factor 4), and GRO/MGSA (melanoma growth stimulating factor) may also be involved as neutrophil chemoattractants.⁷

MIP-2, together with MIP-1, have been identified as secretory products of mouse macrophages.⁸ It has been characterised as a 6 kD heparin binding protein and is a member of the C-X-C group or IL-8 family of the chemokine superfamily.⁹ MIP-2 is chemotactic for neutrophils in vitro, and induces a localised neutrophilic inflammatory response when administered to mice and rabbits.^{10,11} MIP-2, together with MIP-1, has recently been shown to be increased in the lung tissue of rats exposed to mineral dust.¹² We have investigated whether MIP-2 expression is increased in IL-1 β -induced pulmonary inflammation and have determined the localisation of MIP-2 mRNA expression in the lungs.

Methods

ANIMAL TREATMENT AND RETRIEVAL OF CELLS
Inbred male pathogen-free Brown-Norway rats (250-350 g) were anaesthetised with an intra-

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Inflammatory cells such as neutrophils and monocytes are recruited to the airway and lung in response to various stimuli, and a number

peritoneal injection of 2 mg/kg midazolam and an intramuscular injection of 0.4 mg/kg Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone). The soft tissues of the neck were bluntly dissected in order to expose the trachea, following which 0.9% NaCl (100 μ l) or human recombinant IL-1 β (500 U) were injected. After 4, 12, and 24 hours rats were administered an overdose of pentobarbitone (100 mg/kg intravenously), and bronchoalveolar lavage (BAL) was performed with 2 ml aliquot of saline for 10 times through a polyethylene tube introduced through the tracheotomy. Lavage fluid was centrifuged (500 g for 10 minutes at 4°C) and the pellet was resuspended in 0.5 ml Hank's balanced salt solution. Using Kimura stain (dilution 1:10), total cell counts were made in a Neubauer chamber (American Optical Corp, Southbridge, Massachusetts, USA) using light microscopy. Differential cell counts were performed after cytospin preparations and staining with May-Grunwald stain. BAL cells were identified as macrophages, neutrophils, eosinophils, lymphocytes, basophils, and epithelial cells by standard morphology. Five hundred cells were counted and the percentage and absolute numbers of each cell type were calculated. After BAL, lung tissue was removed and stored at -70°C.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) AND SEQUENCING

For the preparation of the MIP-2 probe a Brown-Norway rat was administered lipopolysaccharide (*Escherichia coli*, Sigma; 100 μ g intratracheally). Total cellular RNA from rat BAL cells recovered four hours later was isolated according to the method of Chomczynski and Sacchi.¹³ Total cellular RNA from BAL cells recovered from lipopolysaccharide stimulated rats was reversely transcribed in a 20 μ l reaction volume with 1 $\times$ PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl, 5 mM MgCl₂), 2 mM dNTP, 1 U RNasin (Promega, Madison), 17-base oligo-dT as primer, and 7.5 U reverse transcriptase at 42°C for 60 minutes. PCR was performed on 1 μ l of reverse transcriptase product at a final concentration of 1 $\times$ PCR buffer, 250 μ M dNTP, 0.5 μ M each of sense and antisense primers, and 2.5 U Taq polymerase (Promega, Southampton, UK) in a total volume of 40 μ l using a thermal cycler (Techne, Cambridge, UK). The primers were designed from the published sequences for murine MIP-2 as follows¹⁴: sense-5'GGCA-CAATCGGTACGATCCAG and antisense-5'ACCCTGCCAAGGGTTGACTTC. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: sense-5'TCCCT-CAAGATTGTCAGCAA and antisense-5'AGATCCACAACGGATACATT.¹⁵ The PCR reagents were overlaid with mineral oil and amplification was carried out through 24–26 cycles of denaturation at 95°C for one minute, annealing at 60°C for one minute and extending at 72°C for two minutes. The PCR products were electrophoresed in 2% agarose gels to visualise the MIP-2 and GAPDH bands. The sizes of the PCR products generated were

287 bp for MIP-2 and 309 bp for GAPDH. The MIP-2 PCR product was analysed by sequencing. The PCR product was excised and purified using GeneClean II (Stratagene, Luton, UK). Cycle sequencing was performed on 100 ng PCR product and 1 pmol of each primer. Cycling conditions were 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C using exo pfu- (Stratagene, Cambridge, UK). The purified product was used as cDNA probe for Northern blot analysis. Sequence analysis of MIP-2 cDNA was homologous to rat MIP-2 but not to other chemokines.

NORTHERN BLOT ANALYSIS

The 287 bp MIP-2 PCR product purified from agarose and a 1272 bp PstI fragment from rat GAPDH cDNA were labelled by random priming using [α -³²P]dCTP (3000 Ci/mmol, Amersham, UK). Total cellular RNA from rat lung or BAL cells was subjected to electrophoresis on a 1% agarose/formaldehyde gel and blotted onto Hybond-N membranes (Amersham, UK). The prehybridisation and hybridisation were carried out at 42°C in buffer containing 5 $\times$ Denhardt's solution, 5 $\times$ standard saline citrate (SSC), 50 mM Na₂HPO₄, 0.1% sodium dodecyl sulphate (SDS), 250 μ l/ml sonicated denatured salmon sperm DNA, and 50% formamide. Each blot was washed to a stringency of 0.1 $\times$ SSC/0.1% SDS for 20 minutes at 55°C exposed at -80°C for seven days to Kodak X-OMAT S film. Autoradiographic bands were quantified by densitometric scanning (PDI, New York, USA).

IN SITU HYBRIDISATION

In situ hybridisation was carried out on lungs obtained from Brown-Norway rats treated with either 0.9% NaCl or IL-1 β at four and 24 hours. Lungs were removed and inflated with OCT and fixed in 4% paraformaldehyde, followed by storage at -70°C until used. Cryostat sections (10 μ m) were cut onto sterile RNase-free glass slides. Sections were treated with 0.3% Triton X-100 and digested with Proteinase K (1 μ g/ml for 30 minutes at 37°C). Sections were acetylated using 0.25% acetic anhydride for 10 minutes and then dehydrated using sequential washes in increasing concentrations of absolute ethanol. The MIP-2 cDNA probe obtained by RT-PCR was labelled with digoxigenin (DIG) using a random priming kit (Boehringer Mannheim, Lewes, Sussex, UK). The denatured probe (1 μ g) was labelled with DIG-dUTP using Klenow enzyme at 37°C for 60 minutes. Tissue sections were dried before hybridising overnight in 75 μ l hybridisation solution (5 $\times$ SSC, 50% formamide, 5% blocking reagent, 0.1% N-laurylsarcosine, 0.02% SDS) at 42°C. Following hybridisation the sections were washed in 4 $\times$ SSC at room temperature for five minutes before increasing the stringency of washes up to 2 $\times$ SSC/50% formamide at 40°C for 4 $\times$ 15 minutes. Specifically bound labelled probe was then detected. Slides were washed for one minute in solution A (150 mM NaCl, 100 mM Tris-HCl, pH 7.5), 30 minutes in solution B (solution A

Figure 1 Effect of IL-1 β on the number of (A) macrophages and (B) neutrophils recovered by bronchoalveolar lavage in Brown-Norway rats. Rats were treated with saline ($\square$) or IL-1 β ($\blacksquare$) intratracheally and lavaged at four ($n=3$), 12 ($n=2$), or 24 ($n=2$) hours afterwards. Saline treated rats showed predominantly similar population profiles in terms of macrophage and neutrophil cell counts. There was a significant increase in macrophages at 12 hours, with an increase in neutrophils at all time points. * $p<0.05$ compared with control.

plus 5% FCS, 0.3% Triton X-100), and finally overnight in solution C (solution A plus 1% FCS, 0.3% Triton X-100) containing 1:500 dilution of alkaline phosphatase-linked anti-digoxigenin polyclonal antibody. The presence of alkaline phosphatase-linked antibody conjugate was detected using nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP). Briefly, sections were washed in solution A (2×15 minutes) followed by solution D (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM MgCl₂) containing 2.4 mg/ml levamisole. Colour development was then started by incubating the sections in solution D containing 350 μ g/ml NBT and 40 μ g BCIP for six hours. The reaction was stopped by washing in 1 $\times$ TE solution (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) before the slides were dehydrated in ethanol, washed in xylene, and stained with haematoxylin. Controls were performed using RNase (20 μ g/ml; Promega) and DNase (50 μ g/ml; Promega) pretreated sections and sections with no probe added.

Mean (SE) total and differential cell counts in BAL fluid ($\times 10^6$ cells) in animals treated with saline (S) or IL-1 β (I) at 4, 12, and 24 hours after treatment

Group (n)	Total cells	Macrophages	Neutrophils	Lymphocytes	Eosinophils
S4 (3)	1.70 (1.95)	1.65 (0.24)	0.01 (0.002)	0.03 (0.01)	0.00 (0.00)
I4 (3)	18.40 (6.17)*	2.73 (0.89)	15.30 (6.57)*	0.26 (0.06)	0.00 (0.00)
S12 (3)	2.73 (0.24)	2.12 (0.42)	0.13 (0.07)	0.01 (0.00)	0.00 (0.00)
I12 (3)	12.03 (0.52)*	5.73 (0.60)*	5.86 (0.72)*	0.09 (0.08)	0.08 (0.08)
S24 (2)	1.90 (0.21)	1.78 (0.327)	0.007 (0.001)	0.06 (0.01)	0.00 (0.00)
I24 (2)	19.5 (5.4)*	1.98 (0.51)	17.01 (3.81)*	0.37 (0.17)	0.02 (0.02)

* $p<0.05$ versus corresponding control.

DATA ANALYSIS

Data are presented as mean (SE). Statistical analysis of results was performed by the Mann-Whitney U test for stepward comparison. p values of <0.05 were considered to be significant.

Results

IL-1 β -INDUCED NEUTROPHIL INFILTRATION IN BAL FLUID

Animals treated with IL-1 β demonstrated a significant increase in total cell numbers, characterised by significant neutrophilia in BAL fluid with an increase in macrophages at 12 hours (fig 1). There was no significant difference in the number of eosinophils and lymphocytes among the groups studied. The values of total and differential cell counts are summarised in the table.

Figure 2 (A) Northern blot analysis of MIP-2 mRNA from lungs of Brown-Norway rats following intratracheal administration of IL-1 β to three rats at four, 12, and 24 hours. Control rats (C) did not receive IL-1 β . The Northern blots for each of the three rats in each group were probed with MIP-2 and GAPDH cDNA. The estimated size for MIP-2 mRNA transcripts is 1.4 kb. The top line indicates the time points (4, 12, and 24 hours) for each of the lanes. (B) Mean densitometric data normalised to GAPDH mRNA signal with a maximum expression at four hours, no expression at 12 hours, and minimal degree of expression at 24 hours.

Figure 3 (A) Northern blot analysis of MIP-2 mRNA from bronchoalveolar lavage cells obtained from Brown-Norway rats treated intratracheally with IL-1 β . The groups of rats are those as described in fig 1. (A) Representative autoradiographs of Northern blots probed with MIP-2 and GAPDH cDNA at four, 12, and 24 hours. mRNA was extracted from 2×10^6 bronchoalveolar lavage cells. (B) Mean densitometric data normalised to the GAPDH mRNA. There was a maximal expression of MIP-2 mRNA at four hours with lesser expression at 12 and 24 hours. The MIP-2 mRNA transcript was estimated to be 1.4 kb.

IL-1 β -INDUCED MIP-2 mRNA EXPRESSION IN LUNG AND BAL CELLS

MIP-2 mRNA signal was barely detectable in RNA obtained from saline-treated rat tissue either at baseline or any of the other time points studied. MIP-2 mRNA signal was detected from IL-1 β -treated rat lung tissue at four hours with disappearance of the signal at 12 hours, but a barely visible signal at 24 hours (fig 2). Similarly, there was a significant appearance of MIP-2 mRNA at four hours in BAL cells ($p < 0.05$) with some expression at 12 and 24 hours (fig 3).

IN SITU HYBRIDISATION FOR MIP-2 IN LUNG

MIP-2 mRNA was localised by in situ hybridisation in intrapulmonary airways of rats treated with IL-1 β after four hours. Expression was prominent in the airway epithelium and mononuclear cells (fig 4B). Control tissues from rats treated with 0.9% NaCl showed no expression (fig 4A). In addition, sections from rats exposed to IL-1 β obtained after four hours showed no expression when the sections were pretreated with RNase (fig 4C). However, DNase treatment had no effect on IL-1 β -induced expression of MIP-2 mRNA.

At 24 hours after IL-1 β administration there were fewer mononuclear cells in the airway sub-mucosa expressing MIP-2 mRNA and little expression in airway epithelium (fig 4D-F).

Discussion

We have found that mRNA for MIP-2 is rapidly and markedly induced in rat lung and in cells obtained by BAL following in vivo stimulation by IL-1 β . This expression occurred mostly at four hours with little or no expression at 12 and 24 hours. Mononuclear cells within the airway wall expressed MIP-2 mRNA with some contribution by epithelial cells. The increase in neutrophil influx at four hours coincided with the maximal expression of MIP-2 mRNA. Thus, our study supports the potential for MIP-2 to contribute, at least in part, to the neutrophil influx and activation observed after IL-1 β administration. In addition we observed a transient increase in macrophage numbers in BAL fluid following IL-1 β , an effect that could be secondary to the release of other chemokines such as MIP-1.

MIP-2 is a ~6 kD heparin binding protein which was first identified from a macrophage cell line RAW264.7 stimulated by endotoxin.¹⁰ Its sequence was shown to be most closely related to that of gro/KC gene product which is expressed in transformed or platelet-derived growth factor-treated cells. We used mouse-specific primers for MIP-2 by using RT-PCR of lung lavage cells obtained from a rat treated with lipopolysaccharide.¹⁴ The MIP-2 cRNA was shown to be homologous with the published rat MIP-2 sequence.⁹

MIP-2 may contribute to the recruitment and activation of neutrophils to sites of inflammatory reactions in the lungs. Macrophages obtained from rat lung have been previously shown to express MIP-2 mRNA following treatment with lipopolysaccharide,^{9,16} and following TNF- α treatment in vitro.¹² Our data are consistent with the expression of MIP-2 in mononuclear cells but not in neutrophils in lung sections by using in situ hybridisation. However, it does not exclude the potential for neutrophils also to express MIP-2 mRNA. Macrophages are one likely source of increased expression we observed in vivo. In BAL cells obtained after IL-1 β instillation our results do not establish the relative contribution of macrophages versus neutrophils to the observed increase in MIP-2 mRNA expression. Human neutrophils have been shown to express mRNA for the other C-X-C chemokine, IL-8, and to secrete the protein although this occurs at much lower concentrations than monocytes.¹⁷ Human epithelial cells express mRNA for IL-8 and this can be enhanced by IL-1 β and TNF- α but not by lipopolysaccharide.^{18,19} MIP-2 has been shown to be induced by TNF in rat alveolar macrophages and in rat fibroblasts and epithelial cell lines,¹² and our in situ hybridisation data demonstrate that the airway epithelium expresses MIP-2 mRNA following topical administration of IL-1 β . This supports an important potential role for the airway epithelium

Figure 4 In situ hybridisation showing the expression of MIP-2 mRNA following administration of IL-1 β to Brown-Norway rats. Photomicrographs (A)–(F) are representative pictures of intrapulmonary airways as follows: (A) four hours after 0.9% NaCl administration showing no expression; (B) four hours after IL-1 β with expression in epithelium and submucosal mononuclear cells; (C) four hours after IL-1 β with sections pretreated with RNase resulting in loss of expression; (D) 24 hours after 0.9% NaCl administration; (E) 24 hours after IL-1 β showing no expression in epithelium with some signal in submucosal mononuclear cells; (F) 24 hours after IL-1 β with sections pretreated with RNase. The arrows on micrographs (B) and (D) point to examples of positive cells with dark staining indicating expression of MIP-2 mRNA. The staining procedures used are given in the text.

as a source of chemokines following exposure to external stimuli.

MIP-2 is chemotactic for neutrophils in vitro and can elicit a localised neutrophilic inflammation when injected subcutaneously in mice or when given intracisternally in rabbits.^{10,11} It is therefore plausible that the release of MIP-2 may be responsible for the chemotactic activity of IL-1 β for neutrophils. This activity of IL-1 β has previously been shown to be dependent on the production of new protein.⁵ Expression of IL-8 mRNA in rat cells stimulated with lipopolysaccharide has previously been looked for and not found.⁹ It is possible that other PF-4 family cytokines such as MIP-2 may substitute for IL-8 during inflammation in the rat.

The elicitation of MIP-2 mRNA by another cytokine, IL-1 β , which is a pro-inflammatory cytokine that can be released in many lung inflammatory conditions,^{20–22} is an illustration of the potential cytokine interactions in the cytokine network. Other examples relating to the expression of chemokines include that of MIP-1 α from alveolar macrophages and monocytes induced by IL-1 β ,²³ and of IL-8 from epithelial cells induced by TNF- α .²⁴ This illustrates the complexity of the cytokine network, and these interactions may represent ways in which the various components of the

inflammatory process such as neutrophil recruitment may be amplified.

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