Molecular Expression of the \( \alpha \)-Chemokine Rabbit GRO in Escherichia coli and Characterization of Its Production by Lung Cells in Vitro and in Vivo

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GRO proteins are \( \alpha \)-chemokine cytokines that attract neutrophils and stimulate the growth of a variety of cells. Previously, we observed that rabbit alveolar macrophages transcribe the genes for at least two GRO homologues. In order to study the role of GRO cytokines in lung inflammation, we cloned the predominant rabbit GRO cDNA (RabGRO) from alveolar macrophages, expressed bioactive recombinant protein (rRabGRO) in Escherichia coli, and developed a sensitive and specific enzyme-linked immunosorbent assay for RabGRO protein. We found that rabbit AM express and secrete GRO in vitro in response to both exogenous (e.g. lipopolysaccharide, heat-killed Staphylococcus aureus, and crystalline silica) and endogenous inflammatory stimuli (e.g. tumor necrosis factor-\( \alpha \)) as determined by both radioimmunoprecipitation and enzyme-linked immunosorbent assay. Biologically significant amounts of GRO are present in vivo in the bronchoalveolar lavage fluid of rabbits with E. coli pneumonia; by in situ hybridization, GRO mRNA is detectable in infiltrating pulmonary leukocytes and bronchial epithelial cells. These results indicate that GRO chemokines are likely to be important mediators of the inflammatory response that accompanies acute infectious processes in the lungs.

Human GRO-\( \alpha \), -\( \beta \), and -\( \gamma \) are members of the \( \alpha \)-chemokine family of proteins, which also includes interleukin-8 (IL-8)\(^*\) (1). Like most \( \alpha \)-chemokines, GRO proteins are potent neutrophil chemoattractants and activators (2). In addition to their effects on neutrophils, these cytokines have many other biological activities, including regulatory effects on melanoma cell growth (3), fibroblast collagen production (4), monocyte activation and adhesion to endothelial cells (5, 6), myelopoiesis (7), and angiogenesis (8, 9).

It is likely that GRO proteins are relevant in lung inflammation, as human alveolar macrophages (AM) express GRO mRNA and protein in response to stimulation with lipopolysaccharide (LPS) (10), toxic shock syndrome toxin-1 (11), and tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) (12). Although these in vitro studies suggest that GRO chemokines are mediators of the lung response to both exogenous and endogenous inflammatory stimuli, demonstration of their biological relevance in vivo is lacking.

Rabbits, like humans and in contrast to rodents (1), produce a spectrum of \( \alpha \)-chemokines that includes IL-8 (13) and GRO (6, 14, 15). We reported previously the molecular cloning of two distinct rabbit GRO cDNA homologues from LPS-stimulated AM (RabGRO and rabbit permeability factor-2 or RPF2) (15). Of the two transcripts, RabGRO was the predominant AM-derived GRO homologue. We now report the expression in Escherichia coli of bioactive recombinant RabGRO protein (rRabGRO) and the development of a sensitive and specific RabGRO immunonassay. Using this assay, we have found that rabbit AM secrete native GRO protein in vitro in response to stimulation with a spectrum of biologically relevant agonists. We also report that biologically significant quantities of GRO protein are present in the bronchoalveolar lavage fluid (BALF) of rabbits with acute bacterial pneumonia and identify the sites of GRO mRNA expression in the lungs. These data suggest that GRO chemokines make important contributions to acute inflammatory responses in the lungs.

MATERIALS AND METHODS

Expression of rRabGRO Protein—The coding sequence for mature RabGRO protein (219 base pairs, 72 amino acids) (15) was amplified from LPS-stimulated rabbit AM mRNA by reverse transcriptase-polimerase chain reaction techniques using a 5'-primer with a BamH I restriction enzyme site (5'-CGGGATCCGGGCCTCAACCAGG-3') and a 3'-primer with a Bgl II restriction enzyme site (5'-GAAGATCTTCCCTCCCTTCCCCAGG-3'). The primer sequences written in boldface correspond to base pairs coding for RabGRO. The RabGRO polymerase chain reaction product was confirmed by sequencing and directionally cloned into the expression vector pRSET (Invitrogen Co., San Diego, CA). The pRSET vector encodes a fusion protein that contains an N-terminal fusion peptide sequence with polyhistidine residues for Ni\(^2+\) affinity column purification of the fusion protein and an enterokinase cleavage site for removal of the fusion peptide sequence to generate the mature recombinant protein. The transformants were cultured in the presence of isopropyl-\( \beta \)-D-thiogalactopyranoside and M13/T7 bacteriophage to provide the T7 RNA polymerase required for transcription of the rRabGRO fusion protein (rRabGRO-fp) mRNA. After induction for 10 h, the bacterial pellet was resuspended in 20 \( \mu \)l PBS, pH 7.8, with lysozyme (100 \( \mu \)g/ml, Sigma) and then incubated on ice for 15 min with 10 \( \mu \)l EDTA and 1% Triton X-100, sonicated, flash-frozen in liquid nitrogen, and thawed at 37°C three times in succession. The supernatant was dialyzed overnight against PBS, pH 7.8 (M, 1000 limit membrane, Spectrum Medical Industries, Inc., Los Angeles, CA). Following dialy-
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sis, the supernatant was mixed with Ni²⁺-activated resin and incubated at room temperature for 1 h. The resin was then washed with PBS at pH 7.8, 6.0, and 5.0 and then packed into a column. The rRabGRO-fp was eluted from the column in 1.0 M NaCl. The fractions containing the rRabGRO-fp were pooled and dialyzed against 100 mM NaCl over night. The rRabGRO-fp was incubated overnight with enterokinase (Biozyme, South Wales, United Kingdom) at a concentration of 1 unit/µg fusion protein in 10 µl Tris-HCl, pH 8.0 and 10 µl CaCl₂ to cleave the fusion peptide sequence. The reaction mixture was then loaded onto a 4.0 cm (Dynamax-300A, Rainin Co., Emeryville, CA) and reverse-phase high performance liquid chromatography (HPLC) was used at a 5-95% gradient. The resulting HPLC peaks were analyzed by SDS-PAGE, and the N-terminal amino acid sequences were determined by Edman degradation with a 475 Å pulse liquid protein sequencer (Applied Biosystems, Foster City, CA).

Animals—Specific pathogen free female New Zealand White rabbits weighing approximately 3.0-3.5 kg (Western Oregon Rabbit Co., Phillomath, OR) and 6-8-week-old female BALB/c mice (Simonsen Laboratories, Gilroy, CA) were housed in the Seattle Veterans Affairs Medical Center vivarium prior to all experiments. A female goat was purchased locally and housed at the University of Washington vivarium.

Cell Purification—Rabbit AM were recovered by whole lung lavage as described previously (16). Rabbit and human neutrophils were recovered from heparinized venous blood using Ficol-Hypaque density gradient centrifugation (ICN Biomedicals, Aurora, OH). The cells were washed twice in PBS and resuspended at the indicated concentrations for chemotaxis or immunoprecipitation studies.

Chemotaxis Assays—Rabbit and human neutrophil chemotaxis to rRabGRO was measured by the modified Boyden method using 48-well microchemotaxis chambers and nitrocellulose membranes with 3.0-µm pores for neutrophils as described (17). The samples were diluted with PBS, and 25 µl of each sample were added to the bottom well. Controls included 10% zymosan-activated human or rabbit serum (ZAS, positive control) or PBS alone (negative control). Neutrophils were added to the top wells, and the chambers were incubated for 2 h at 37°C in 5% CO₂ and 95% air. After incubation, the chambers were fixed and stained with crystal violet. Rabbit chemotaxis was measured as the total number of leukocytes migrating through the filters in 10 consecutive high power microscopic fields. Results were expressed as percent maximal chemotaxis, in which the average number of leukocytes migrating toward PBS for each chamber was subtracted from each measurement (including ZAS) and then each measurement was divided by the adjusted value for ZAS. The value for ZAS was set as 100% maximal chemotaxis. Results are reported as mean ± S.E.

Endobronchial Instillation of rRabGRO Protein—To test the bioactivity of rRabGRO in the lung, 1.0 µg of LPS-free protein in 1.0 ml sterile, pyrogen-free 0.9% NaCl (approximately 10⁻¹ M solution) was instilled into the right lung of an anesthetized rabbit through an intratracheal catheter directed toward the right mainstem bronchus. For comparison, another rabbit was treated with 0.9% NaCl alone. The catheters were removed immediately following the instillations, and the animals were closely monitored to ensure adequate recovery from the procedure. The rabbits were euthanized 4 or 24 h later, and selective right lung lavage was performed. Bronchoalveolar lavage fluid cell counts and differentials were calculated and GRO protein levels were measured by ELISA.

In Situ Hybridization of Lung Tissue from Rabbits with E. coli Pneumonia—Anesthetized rabbits were treated with either a 1.0 ml bolus of 2 x 10¹⁰ colony-stimulating factor E. coli (serotype K1) in sterile, pyrogen-free 0.9% NaCl solution or NaCl alone (n = 8) through an intratracheal catheter directed toward the right mainstem bronchus. The animals were euthanized 4 or 24 h later, and selective right lung lavage was performed. Bronchoalveolar lavage fluid cell counts and differentials were calculated and GRO protein levels were measured by ELISA.

RESULTS

Expression of rRabGRO—The coding sequence for mature RabGRO was amplified by polymerase chain reaction and cloned into the pRSET expression vector. After confirming the sequence for the RabGRO cDNA, the recombinant protein was

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expressed in JM109 E. coli and purified from bacterial lysates by Ni²⁺ affinity chromatography. Analysis by SDS-PAGE confirmed that the purified protein was of the expected size for rRabGRO-fp (approximately 14,000 kDa) (Fig. 1A). The rRabGRO-fp was then treated with enterokinase, and the products were purified by reverse-phase HPLC (Fig. 1B). The HPLC peak labeled “rRabGRO” was found to contain mature rRabGRO of the expected sequence as determined by N-terminal amino acid sequencing.

Bioactivity of rRabGRO—The chemotactic activity of rRabGRO was tested in vitro using rabbit and human neutrophils. For comparison, recombinant human GRO-α (R & D Systems) was also tested. Recombinant RabGRO protein stimulated chemotaxis of rabbit neutrophils at concentrations as low as 3 × 10⁻⁷ M, with maximal activity at approximately 3 × 10⁻⁶ M and a decline in activity at 10⁻⁵ M (Fig. 2A). In contrast, rRabGRO had little chemotactic activity for human neutrophils over the same concentration range. Similarly, human GRO-α showed no significant chemotactic activity for rabbit neutrophils, despite being chemotactically active for human neutrophils (Fig. 2B).

To assess in vivo bioactivity, rRabGRO was instilled bronchially into a rabbit. This produced a brisk influx of neutrophils into the lung (45% neutrophils in the BALF cell count from the treated lungs at 4 h). For comparison, the BALF from the untreated lung in the same rabbit and from the lungs of a rabbit treated with sterile 0.9% NaCl alone contained only 6 and 5% neutrophils, respectively.

Generation and Specificity of Goat Polyclonal Anti-rRabGRO IgG—After isolating total IgG from immune goat serum, the specificity of the anti-rRabGRO IgG was tested by Western analysis using rRabGRO, recombinant rabbit MCP-1, rabbit IL-8 (each produced using similar methods (18)), and recombinant human GRO-α. The anti-rRabGRO IgG reacted strongly with both the fusion protein and the cleaved mature rRabGRO protein (data not shown). Although there was very weak cross-reactivity with human GRO-α, there was no cross-reactivity with either rabbit IL-8 or rabbit MCP-1. The specificity of the goat anti-rRabGRO antibodies for native rabbit GRO protein was further confirmed by RIP (described below).

Expression of GRO Protein by AM—We performed RIP and ELISA measurements of stimulated rabbit AM supernatants to measure the expression of newly synthesized and secreted GRO protein. The immunoprecipitation experiments were conducted using the supernatants of adherent rabbit AM metabolically labeled with [³⁵S]cysteine and stimulated for 19 h with a variety of endogenous and exogenous proinflammatory agents. Immunoprecipitation of these supernatants with the goat anti-rRabGRO IgG resulted in a major band of the expected size (approximately 7 kDa) for each stimulus (Fig. 3). Further analysis of the gels by phosphorimaging allowed quantitation of GRO protein in the supernatants. SiO₂, Al₂O₃, LPS, human TNF-α, and HKSA were each strong inducers of GRO secretion, while ConA and heat-inactivated human TNF-α were very weak stimuli. GRO protein was not detected when RIP was performed with nonimmune goat serum.

Because adherence alone appeared to be a stimulus for GRO protein production in the RIP studies, we measured GRO levels by ELISA using the supernatants of rabbit AM cultured in polypropylene tubes which were periodically agitated. These results showed significant time- and stimulus-dependent differences in the accumulation of GRO protein in the supernatants of relatively nonadherent AM (Fig. 4). In response to stimulation by HKSA and LPS, significant amounts of extracellular GRO were detected after only 2 h of incubation. For HKSA, GRO increased steadily and was maximal by 20 h of incubation. For LPS, maximal amounts of GRO were detected at 4 h of incubation and the concentration failed to increase further at 20 h. Incubation with SiO₂ caused the secretion of...
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GRO protein levels and percent neutrophils in BALF from rabbits treated with either E. coli or NaCl endobronchially at 4 and 24 h

| Treatment | Time | GRO (ng/ml) | Neutrophils (%) |
|-----------|------|-------------|----------------|
| NaCl      | 4    | <0.022      | 4.5 ± 0.3      |
| E. coli   | 4    | 0.799 ± 0.264 | 81.5 ± 3.7    |
| NaCl      | 24   | 0.060 ± 0.023 | 6.5 ± 1.3    |
| E. coli   | 24   | 0.620 ± 0.195 | 86.3 ± 5.6    |

* n = 4 for each group.
* p < 0.03 compared with NaCl group.
* p < 0.0001 compared with NaCl group.

Alveolar macrophages are important cellular mediators of pulmonary inflammation, in part due to their ability to elaborate a variety of leukocyte chemotactic factors (20). Among these factors, IL-8 is thought by many investigators to be the major neutrophil chemoattractant in the lung (21). However, studies using specific antisera to neutralize IL-8 in the supernatants of activated human AM (22) and BALF from patients with adult respiratory distress syndrome (23) suggest that a significant proportion of AM-derived neutrophil chemotactic activity is due to other non-IL-8 chemoattractants. Possible candidates include the GRO subgroup of α-chemokines, which are closely related to IL-8 and are also potent neutrophil chemotactic activators (2). In this study, our goals were: 1) to express recombinant rabbit GRO protein and develop a specific and sensitive ELISA; 2) to investigate GRO protein expression by rabbit AM in response to stimulation with both endogenous and exogenous inflammatory agents; and 3) to determine whether GRO protein and mRNA can be detected in the lung in vivo during the acute inflammatory response that accompanies bacterial pneumonia.

In order to generate the species-specific reagents necessary to measure rabbit GRO protein, we first produced rRabGRO as a fusion protein in E. coli. We used a prokaryotic expression system for producing the recombinant protein because GRO proteins have been shown not to undergo post-translational sulfation, glycosylation, or phosphorylation (24). RabGRO amino acid sequence alignment with all reported full-length GRO homologues demonstrated identities ranging from 41% for 9E3 (chicken GRO homologue) to 78% for human GRO-β (Table I). Given the high degree of identity with human GRO proteins, it is perhaps surprising that rRabGRO had little chemotactic activity for human neutrophils. However, this confirms previous observations by other investigators that 125I-labeled human GRO-α does not bind to rabbit neutrophils (25) and further underscores the necessity of using species-specific reagents when studying α-chemokines (26). The rRabGRO-fp proved to be a good immunogen for raising specific goat anti-rabbit GRO polyclonal antibodies, as Western

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analysis confirmed that the goat antibodies did not cross-react with either rabbit IL-8 or MCP-1. The antibodies did show a small amount of cross-reactivity for recombinant human GRO-α, which is not surprising given the high degree of homology between these proteins (Table II). As RabGRO and RFP2 are nearly identical at the mature protein level (93% identity), the anti-rRabGRO antibodies are likely to cross-react with RFP2 as well.

We also have shown that rabbit AM express and secrete GRO protein in response to stimulation by LPS, TNF-α, SiO₂, Al₂O₃, and HKSA. Adherence and/or culture conditions alone also appear to stimulate GRO expression. Under nonadherent conditions, LPS, HKSA, and SiO₂ were the most potent inducers of GRO protein secretion, whereas human TNF-α was a weak stimulus. By contrast, human TNF-α was a potent stimulus for GRO protein production in adherent AM. Cell adherence has been shown to induce the expression of both TNF-α and IL-1β in AM (27, 28), and it is possible that increased expression of these cytokines in adherent AM may in turn mediate increased GRO protein expression by these cells. The increase in GRO secretion by rabbit AM in response to the tested stimuli is in agreement with studies performed with AM from other species. In human AM, GRO expression has been described in response to LPS (10, 12), TNF-α (12), and S. aureus (11). In rat AM, the GRO homologue KC (CINC-1) is induced by LPS, and MIP-2 expression increases following stimulation with TNF-α, LPS, and SiO₂ (29). Incubation with rabbit IFN-γ did not cause rabbit GRO expression under nonadherent conditions. It is possible that IFN-γ may actually inhibit GRO protein secretion by AM, as the measured concentrations were less than those observed with 0.9% NaCl alone. Although this finding needs to be confirmed in further studies, it is consistent with a prior report that IFN-γ inhibits KC gene expression in mouse peritoneal macrophages (30).

The data also show that GRO protein is present in the lungs of rabbits with Gram-negative bacterial pneumonia. All of the rabbits with E. coli pneumonia had elevated concentrations of GRO in BALF, and the amounts correspond to biologically significant levels based on the chemotactic activity of the recombinant GRO protein that we measured in vitro. Moreover, the amount of GRO protein present in the rabbit BALF is roughly equivalent to the levels of IL-8 present in the BALF of patients with the adult respiratory distress syndrome (23). While the in vitro studies suggest that AM are probably a significant source of GRO protein in the lung, many other lung cell types can produce GRO chemokines, and each may be an important contributor to the expression of GRO in the lungs, depending on the in vivo circumstances. In rabbits with Gram-negative bacterial pneumonia, the in situ hybridization studies indicate that airway epithelial cells and tissue inflammatory cells are significant sources of GRO gene expression within 4 h of bacterial entry into the lungs. These results agree with studies by Becker and associates who showed increased GRO mRNA expression in human airway epithelial cells in response to LPS-stimulation (12) and with studies by Xing et al. showing localization of GRO mRNA expression in parenchymal inflammatory cells in LPS-treated rat lung tissue (31). In contrast, Rogivue and colleagues (32) have reported in bovine pneumonia pasteurellosis that GRO protein is detectable in type II alveolar epithelial cells and mesothelial cells, but not in bronchial epithelial cells or pleural fibroblasts. Lung fibroblast cell lines (33) and endothelial cells (6) also have been shown to express GRO homologues in vitro, but we did not find evidence of GRO production by these cell types in vivo in bacterial pneumonia.

Using a cDNA library prepared from LPS-stimulated rabbit AM and Northern analysis, we have shown previously that LPS-stimulated rabbit AM express the genes for two GRO

| RabGRO | RFP2 | ocGRO | GROx | GROy | CINC-1 | CINC-2x | CINC-2β | CINC-3β | ratMIP2 | murMIP2 | KC | CRUGRO | 9E3 | rabL-8 |
|--------|------|-------|------|------|--------|--------|--------|--------|--------|--------|----|--------|----|--------|
| RabGRO | 100  | 93    | 66   | 68   | 78    | 70     | 68     | 56     | 56     | 59     | 59 | 67     | 70 | 41     | 41   |
| RFP2   | 100  | 70    | 70   | 74   | 74    | 70     | 56     | 56     | 59     | 59     | 67 | 68     | 70 | 43     | 39   |
| ocGRO  | 100  | 71    | 74   | 74   | 68    | 56     | 56     | 62     | 62     | 62     | 68 | 68     | 70 | 41     | 41   |
| GROx   | 100  | 88    | 85   | 67   | 59    | 59     | 66     | 64     | 67     | 43     | 38 |        |    |        |      |
| GROy   | 100  | 84    | 71   | 59   | 58    | 59     | 66     | 67     | 70     | 40     | 38 |        |    |        |      |
| CINC-1 | 100  | 61    | 61   | 61   | 66    | 70     | 92     | 86     | 48     | 43     |    |        |    |        |      |
| CINC-2x| 100  | 96    | 75   | 73   | 58    | 66     | 56     | 61     | 41     | 38     |    |        |    |        |      |
| CINC-2β| 100  | 75    | 73   | 58   | 67    | 56     | 66     | 68     | 44     | 41     |    |        |    |        |      |
| ratMIP2| 100  | 84    | 64   | 67   | 64    | 67     | 45     | 45     |        |        |    |        |    |        |      |
| murMIP2| 100  | 67    | 70   | 68   | 68    | 68     | 44     | 41     |        |        |    |        |    |        |      |
| KC     | 100  | 85    | 49   | 46   |    |        |        |        |        |        |    |        |    |        |      |
| CRUGRO | 100  | 45    | 42   |    |    |        |        |        |        |        |    |        |    |        |      |
| 9E3    | 100  | 48    |    |    |    |        |        |        |        |        |    |        |    |        |      |
| rabL-8 | 100  |      |    |    |    |        |        |        |        |        |    |        |    |        |      |
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