Down-Regulation of GATA-2 Transcription during Pneumocystis carinii Infection

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Differences in gene expression between Pneumocystis carinii-infected and noninfected rats were examined. Total RNA was isolated from homogenized rat lungs and then subjected to differential display with combinations of oligo(dT) and various arbitrary PCR primers. Approximately 50 differentially expressed bands were observed. Several of these DNA bands were isolated, reamplified, and cloned. The cloned DNA fragments were used as probes to perform Northern hybridization on RNA from P. carinii-infected and noninfected rat lungs. One clone was found to react with a 3-kb mRNA from noninfected but not from P. carinii-infected rat lung, suggesting that the gene represented by this clone was down-regulated during P. carinii infection. The nucleotide sequence of this clone was determined and found to be 97% homologous to the mouse GATA-2 transcription factor. In situ hybridization using RNA probes derived from this clone revealed that alveolar macrophages, resident lung monocytes, and bronchial epithelial cells express the GATA-2 gene in the lung.

Pneumocystis carinii causes pneumonia with a high mortality rate in immunocompromised individuals, such as those with AIDS and organ transplants. Children with severe malnutrition are also susceptible to P. carinii infection. Although P. carinii is an extracellular parasite, it must adhere to the surface of type I pneumocytes to proliferate. However, in an in vitro axenic culture of P. carinii was recently reported (17). Adhesive proteins such as integrins have been shown to enhance binding of P. carinii to type I pneumocytes.

During P. carinii infection, surfactant protein A and integrins are up-regulated (1, 8, 20, 22). In contrast, the production of alveolar macrophage mannose receptor, which is responsible for binding and engulfing P. carinii (6), is reduced (11). The amount of total surfactant phospholipid is also reduced during infection (2, 5, 10, 21, 23, 24, 25), with a decrease in phosphotidylcholine but an increase in sphingomyelin (23, 25).

In order to further understand how host cells respond to P. carinii infection, we performed mRNA differential display to detect genes that are up- or down-regulated during P. carinii infection. Three groups of two rats each were used. The first group was immunosuppressed with dexamethasone (Dex) and then infected with P. carinii (referred to as P. carinii infected hereafter). The second group was immunosuppressed with Dex only (referred to as Dex suppressed hereafter), and the third group was nonimmunosuppressed and noninfected (referred to as normal hereafter). We found that the expression of the GATA-2 transcription factor is severely down-regulated during P. carinii infection. We also determined that ciliated bronchial epithelial cells, alveolar macrophages, and resident lung monocytes are the cell types that normally express the GATA-2 gene in the lung. This the first report of GATA-2 expression in the pulmonary system and in a terminally differentiated immune cell.

Materials and Methods

Animals and infection of animals with P. carinii. Three groups of two rats each were used: normal, Dex suppressed, and P. carinii infected. The Dex-suppressed group served as a control to detect gene expression altered by immunosuppressive treatment with Dex. The normal rats served as the negative control. P. carinii infection in immunosuppressed rats was achieved by transtracheal inoculation of lung homogenate from P. carinii-infected rats. The lung homogenate was shown to contain P. carinii by light microscopy performed on stained smears. Each rat was transtracheally injected with 0.2 ml of lung homogenate containing 10⁆ P. carinii organisms. The rats developed P. carinii pneumonia in 5 to 8 weeks and were then sacrificed.

Isolation of RNA from animals for mRNA differential display. The lungs of P. carinii-infected rats were lavaged with normal saline, and the lavage fluids were assayed for the presence of P. carinii by PCR, using the mitochondrial rRNA gene primers (29). The lavage fluids from both the Dex-suppressed and normal rats were negative in the mitochondrial rRNA gene PCR, indicating that P. carinii infection did not develop in these rats. The lavage fluids from P. carinii-infected rats were positive in the PCR, indicating that the rats were indeed infected with P. carinii. The lungs were perfused from the pulmonary artery with Hanks balanced salt solution to remove blood in order to avoid interference with RNA isolation. Total RNA was isolated from homogenized lung tissue. The isolated RNAs were treated with RNase-free DNase I and then reverse transcribed using various oligo(dT) primers. The cDNAs thus generated were used for mRNA differential display.

mRNA differential display. The RNAmap Kit B, obtained from GenHunter Co. (Nashville, Tenn.), was used to perform mRNA differential display. Four different sets of oligo(dT) primers (5’T-A,MN-3’) with the following sequences were used for reverse transcription (RT): 5’T-A,MG-3’, 5’T-A,MA-3’, 5’T-MT-3’, and 5’T-MC-3’. These primers are composed of 12 residues followed by a degenerate base M, which includes G, A, and C, and then either G, A, T, or C at the extreme 3’ end. Two hundred nanograms of lung RNA from each animal group was used in each 20-μl RT mixture. The RT mixture contained 25 mM Tris-HCl (pH 8.3), 37.6 mM KCl, 1.5 mM MgCl₂, 50 mM dithiothreitol, 50 μM deoxynucleoside triphosphate, 1 μM T12MN primer, and 100 μM of Moloney murine leukemia virus reverse transcriptase. The reaction was carried out in a thermocycler at 65°C for 5 min, 37°C for 60 min, and 95°C for 5 min. After 10 min at 37°C, the reverse transcriptase was added to each reaction tube. The reverse-transcribed cDNA species were then amplified by PCR with various combinations of a 5’T-A,MN-3’ primer used in RT and one of the five 10-μl random primers (API to -5). The PCR was run in a mixture containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 2 μM deoxynucleoside triphosphate, 200 mM AP primer, 1 μM T12MN primer, 2 μl of RT product, 10 μl of [α-33P]dATP, and 1 U of AmpliTaq (Perkin-Elmer, Foster City, Calif.) at 94°C for 30 s, 42°C for 2 min, and 72°C for 30 s for 40 cycles. The labeled PCR products were electrophoresed on 6% DNA sequencing gels and detected by autoradiography of the gels.

Isolation and reamplification of differentially expressed bands. The DNA in the differentially expressed bands was eluted by boiling the gel slice containing the band for 15 min in 100 μl of water. The eluted DNA was precipitated with...
ethanol, dried, and resuspended in water. To obtain a sufficient amount of DNA for subsequent studies, the eluted DNA of each band was reamplified with the same primer set which produced the isolated band. The reamplified products were electrophoresed on a agarose gels, and the band of the correct size was isolated, purified with a Qiagen II kit (Qiagen, Valencia, Calif.), and then cloned into the TA cloning vector pCRII. PCR was then performed on colonies containing recombinant plasmids using primers which anneal to the vector flanking the cloning site (EcoRI) to determine the sizes of the inserts.

In situ hybridization. Rat lungs were cut into small pieces and then immersed for 24 h in a fixative composed of 4% formaldehyde and 1% glutaraldehyde. The tissues were washed with phosphate-buffered saline (PBS) (pH 7.4) three times for 30 min each and then dehydrated by being passed through a series of increasing concentrations of ethanol. After the removal of ethanol, the tissue was embedded in paraffin. Five-micrometer sections were cut with a microtome (Leitz, Rockleigh, N.J.) and mounted on ProbeOn Plus microscope slides (FisherBiotech, Pittsburgh, Pa.). Each slide contained both immunosuppressed and P. carinii-infected rat lung sections.

GATA-2 riboprobes were used for the in situ hybridization and were produced by in vitro transcription using the TA vector containing the insert (pGATA-2a or pGATA-2s) as the template. Both pGATA-2a and pGATA-2s were linearized with BamHI and transcribed with T7 RNA polymerase to produce antisense (from pGATA-2a) and sense (from pGATA-2s) probes. The BamHI-linearized plasmids were purified by the Qiagen II gel elution method. Riboprobes were labeled with biotin using a biotin RNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). In vitro transcription was performed in a 20-μl reaction mixture containing 1 μg of linearized vector RNA, 2 μl of biotin RNA labeling buffer (10 mM Tris- HCl (pH 7.5), 10 mM CTP, 0.5 mM biotin-UTP, 2 μl of 10X transcription buffer (pH 7.5), and 2 μl (20 U) of T7 RNA polymerase. The reaction mixture was incubated at 37°C for 2 h. Two microliters of RNase-free DNase I (2 U) was then added to the reaction mixture and incubated for 15 min at 37°C to digest the template DNA. Two microliters of 0.2 M EDTA solution (pH 8.0) was then added to stop the reaction. The RNA probes were precipitated by adding 2.5 μl of 4 M LiCl and 75 μl of cold ethanol. After cooling at −70°C for 1 h, the samples were centrifuged at 16,000 × g for 15 min at 4°C. The pellets were washed with 200 μl of cold 75% ethanol, dried, and resuspended in 100 μl of diethyl pyrocarbonate-treated water containing 1 μl of RNase inhibitor (RNasin) (20 U). The concentration of RNA thus produced was determined by spectrophotometry, measuring the absorbance at 260 nm.

The paraffin sections on the ProbeOn Plus microscope slides were deparaffinized, rehydrated, and treated with H2O2 in PBS (pH 7.4) for 15 min at room temperature to inactivate the native peroxidase activity. The sections were digested with 50 μg of proteinase K per ml in PBS at 42°C for 20 min, rinsed with diethyl pyrocarbonate-treated H2O for 2 min, and then treated with 0.25% (vol/vol) acetic anhydride in 0.1 M triethanolamine HCl (pH 8.0) for 10 min at room temperature, followed by washing with neutralizing solution (0.1 M Tris-HCl [pH 7.5], 0.2 M NaCl, 5 mM MgCl2) for 10 min. The sections were then incubated with the hybridization cocktail, which is composed of 1X Denhardt's solution, 50% deionized formamide, 10% dextran sulfate, 4X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate), 50% dithiothreitol, 0.1 μg of mRNA per ml, and 400 ng of labeled riboprobes per ml, in a humidified chamber at 50°C overnight. After hybridization, the sections were washed two times for 15 min each with solution I (2X SSC, 0.1% sodium dodecyl sulfate [SDS], and 0.05% Brij) and then two times for 15 min each with solution II (0.5X SSC, 0.1% SDS, and 0.05% Brij) at room temperature. The final wash was done two times for 5 min each with solution III (0.1X SSC, 0.1% SDS, and 0.05% Brij) at 42°C.

A tyramide signal amplification kit (NEN Science Products, Boston, Mass.) was used to amplify the hybridization signals in tissue sections. The sections were rinsed with solution I and then soaked in 100 μl of TNB blocking buffer (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl, and 0.15% blocking reagent supplied in the kit) for 30 min at room temperature to block the non-specific binding sites of antibodies. After blocking, the sections were incubated for 30 min at room temperature in 100 μl of horseradish peroxidase-conjugated streptavidin (SA-HRP) diluted 1:100 in TNB buffer. The excess SA-HRP was removed by washing three times for 5 min each in TNB buffer (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl, 0.05% Tween 20) at room temperature. The sections were then incubated in 300 μl of biotin-tyramide working solution (1:5 dilution of the biotin-tyramide stock solution with the amplification diluent supplied in the kit) for 10 min at room temperature. After being washed three times for 5 min each in TNB buffer, the sections were again incubated in 100 μl of diluted SA-HRP for 30 min at room temperature. The sections were washed three times for 5 min each in TNN buffer to remove the excess SA-HRP. The hybridization signals were developed by incubating the sections with 1× 3,3′-diaminobenzidine–CoCl2 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 15 min. After the reaction was stopped by rinsing the sections in Tnis-EIDA buffer, the hybridization signal was further enhanced by incubating the sections with 100 μl of 1% osmium tetroxide at room temperature for 5 to 30 s. The sections were washed with water to stop the reaction, counterstained with hematoxylin for 30 s, dehydrated in a graded series of xylene, cleared with xylene, and sealed with Permount under a coverslip. The sections were then examined under a light microscope.

RESULTS

Approximately 50 differentially expressed bands were observed. Some bands were present only in the samples of P. carinii-infected rat lungs; these may represent expressed P. carinii genes or host genes that are turned on or up-regulated by P. carinii infection. There were also bands that were present in samples of Dex-suppressed and normal rats but not in P. carinii-infected rats; these bands may represent host genes that are turned off or down-regulated by P. carinii infection. Seven of the down-regulated bands were isolated, reamplified, and cloned from the differential display gel.

To confirm that these clones represent genes that are differentially expressed, a Northern hybridization was performed. The inserts of these clones were isolated, labeled with 32P, and used as probes to react with RNA samples from Dex-suppressed and P. carinii-infected rat lung samples. These two RNA samples (10 μg each) were run side by side on an agarose gel in eight replicates. Each replicate was separately transferred to a Nytran membrane. One of the membranes was reacted with the β-actin gene probe to demonstrate that the same amounts of RNA samples were loaded into each well. The other membranes were each reacted with different probes. The β-actin gene probe gave approximately the same intensity of hybridization signal with both Dex-suppressed and P. carinii-infected RNA samples (Fig. 1), indicating that approximately equal amounts of RNA were loaded. A separate membrane was probed with the β-actin gene probe because it was unknown whether there would be interference from hybridization signals of other probes to mRNA of the same length as β-actin.

All of these probes gave a more intense hybridization signal with the RNA sample from Dex-suppressed rat lung cells than with that from P. carinii-infected rat lung cells (Fig. 1). This result indicates that the genes represented by these probes are down-regulated during P. carinii infection. The gene represented by A8III has the highest level of expression, followed by those represented by the C6l and T6II clones and then by A7I, A7IV, A8I, and A8III. Probe A8II generated two bands that are very close in size (∼1.8 and 1.5 kb). Probes C6l and T6II produced the same hybridization patterns, and the intensities of hybridized bands of P. carinii-infected samples are approximately one-fourth of those of noninfected cells. Probes A8IV and A8III also produced the same hybridization patterns; a weak band was seen in Dex-suppressed RNA samples and no band was seen in P. carinii-infected samples, suggesting that these two genes are expressed at a very low level in Dex-suppressed rats and are severely down-regulated in P. carinii-infected rats.

To identify these genes, the inserts of these clones were sequenced. The sequences thus obtained were compared with all of the sequences in GenBank using the BLAST-n search tool. The sequences of clones C6l and T6II were found to be identical to each other and are 90% homologous to that of the MM-1 gene (GenBank accession no. D989667). The sequences of clones A8IV and A8III were also identical to each other and are 97% homologous to that of the mouse GATA-2 transcription factor gene at the 3' noncoding region (Fig. 2). The sequence of clone A8I was found to be 83% homologous to that of an unidentified gene, KIA0026 (GenBank accession no. D14812). The sequences of clones A8II and A7IV were found to be novel. These two sequences have no significant homology with those of any of the genes in the nucleotide sequence banks.

Among the three known genes identified to be down-regulated during P. carinii infection, the GATA-2 gene is the most well characterized. The function of KIA0026 is unknown.
MM1 was recently identified to be a c-Myc binding protein. GATA-2 is a transcription factor and regulates the development of hematopoietic cells. Therefore, a decision was made to further study the function of the GATA-2 gene in the lung. In situ hybridization using biotin-labeled sense and antisense GATA-2 riboprobes was performed to identify cells expressing the GATA-2 gene in the lung.

No hybridization signal was seen in any sections that reacted with the sense riboprobe, indicating that no nonspecific hybridization occurred in the reaction (Fig. 3). In sections of Dex-suppressed rat lung, cells that showed positive hybridization signals with the antisense GATA-2 probes were located in the epithelium of bronchioles, interstitium, alveolar walls, and alveoli (Fig. 3A). Ciliated bronchial epithelial cells were most prominently stained, indicating high expression of the GATA-2 gene. The cells in the interstitium and alveolar walls that react with the probe had the typical appearance of monocytes, with horseshoe or uniform nuclei and a moderate amount of vacuolated cytoplasm. These resident lung monocytes are commonly referred to as histiocytes. There was no hybridization signal seen in interstitial fibrocytes or endothelial cells in these sections. Cells in the alveoli that reacted with the probe were large and had an amoeboid shape with shaggy cell margins and phagocytic vacuoles in the cytoplasm, characteristic of alveolar macrophages.

In sections of P. carinii-infected lung, the alveolar spaces were filled with an amphophilic foamy amorphous exudate composed of cell debris and P. carinii organisms. The organisms were also seen in the lumen of some small bronchioles. The alveolar septa were wider than those of noninfected rats and contained more inflammatory cells such as neutrophils and lymphocytes. The numbers of bronchial epithelial cells that showed positive hybridization with the antisense GATA-2 probes were much fewer than those seen in noninfected rat lungs (Fig. 3B). The intensity of the brown color in hybridization-positive cells, including ciliated bronchial epithelial cells, monocytes, and alveolar macrophages, was reduced. The numbers of monocytes that reacted with the probes were also reduced.

**DISCUSSION**

In this study, we have investigated differences in gene expression between noninfected and P. carinii-infected rat lungs. The expression of the GATA-2 gene was found to be downregulated during P. carinii infection. This GATA-2 down reg-

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**FIG. 1.** Confirmation of differential expression by Northern blot hybridization. Inserts containing a portion of potential differentially expressed genes were labeled with \(^{32}\)P and used as probes to react with electrophoresed RNA samples from lungs of Dex-suppressed (lanes D) and P. carinii-infected (lanes I) rats.

**FIG. 2.** Sequence comparison between A8IV and GATA-2. The nucleotide sequence of the clone A8IV was compared with all sequences stored in GenBank using the BLAST program. The A8IV sequence (positions 85 to 236) was found to be homologous to the mouse GATA-2 gene at nucleotides 2957 to 3108.
ulation was first detected by mRNA differential display experiments. Identification of the gene was achieved by comparing the nucleotide sequence of the differential displayed product with sequences stored in GenBank (Fig. 2). Down-regulation of the GATA-2 gene was confirmed by Northern blot analysis. The GATA-2 gene in normal rat lung was found to be expressed at a very low level based on the intensity of the RNA band that hybridized with the probe. Its expression in \textit{P. carinii}-infected lung is even lower, as no RNA band was found to react with the probe (Fig. 1). With in situ hybridization, alveolar macrophages, resident lung monocytes, and ciliated bronchial epithelial cells are found to express the GATA-2 gene in the lung (Fig. 3). The expression of the GATA-2 gene in these three types of cells in \textit{P. carinii}-infected lungs is much lower than that in normal rat lung, confirming the results of Northern blot analyses.

GATA-2 is one of the six transcription factors of the GATA family that have been identified. All members of the GATA family contain two copies of zinc finger DNA binding motifs (14). The consensus binding sequence of GATA transcription factors is (A/T)GATA(A/G) (19), which is present in the promoters or enhancers of many genes. GATA-2 plays a crucial role in the development of hematopoietic cells (27). It has been found to be expressed in erythroid and early myeloid cells and regulates the expression of \textalpha-globin and erythropoietin genes (9, 16, 18). It has also been shown to control the expression of the endothelin-1 gene (12), the endothelial nitric oxide synthase gene (30), and the gene encoding the platelet and endothelial cell adhesion molecule-1 (12).

Down-regulation of GATA-2 in alveolar macrophages may have an impact on host defenses against \textit{P. carinii} infection. In vitro, alveolar macrophages have been shown to be activated by the whole organism or the major surface glycoprotein of \textit{P. carinii} to release inflammatory substances such as tumor necrosis factor alpha, prostaglandin \textE2, and leukotriene \textB4 (3, 8, 26). This activation is enhanced by vitronectin or fibronectin, which accumulates in the lung during \textit{P. carinii} infection. Furthermore, alveolar macrophages from normal rat lung are able to bind, phagocytize, and degrade \textit{P. carinii} (6, 13, 15, 28). However, alveolar macrophages appear to have decreased functional abilities during \textit{P. carinii} infection. Using the SCID mouse model, Chen et al. (4) demonstrated that phagocytosis of \textit{P. carinii} is not common. In addition, Hanano et al. (7) showed that activated alveolar macrophages are insufficient to resolve \textit{P. carinii} infection. The mannose receptors of alveolar macrophages are found to be defective in AIDS patients with \textit{P. carinii} pneumonia (11). It is possible that down-regulation of GATA-2 in alveolar macrophages renders them unable to phagocytose \textit{P. carinii}. This hypothesis is consistent with the finding that phagocytosis of \textit{P. carinii} is uncommon in heavily infected lungs.

We also found that GATA-2 is expressed in resident lung monocytes and that this expression is also decreased during \textit{P. carinii} infection. Resident lung monocytes are interstitial...
monocytes in the lung with the potential of becoming alveolar macrophages. Since GATA-2 plays a key role in the development of many types of tissues, down-regulation of the GATA-2 gene in resident lung monocytes may prevent them from becoming alveolar macrophages. Down-regulation of GATA-2 in resident lung monocytes and macrophages may be one of the mechanisms by which *P. carinii* ensures its own survival. Studies are being conducted to test these hypotheses.

REFERENCES


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