DNA MICROARRAY ANALYSIS OF PULMONARY FIBROSIS THREE MONTHS AFTER EXPOSURE TO PARAQUAT IN RATS

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ABSTRACT — Although paraquat (PQ) is known to induce pulmonary fibrosis, how it does so is not entirely clear. To elucidate the mechanisms involved, the profile of gene expression in the lung at three months after exposure to PQ (7 mg/kg, s.c., daily for eight administrations) was investigated in rats using a DNA microarray. Changes in gene expression that were considered to reflect damage to the lung, a change in the balance of electrolytes and fluid, and alveolar remodeling were observed. The products of these genes were: CSF-1 receptor, which is a receptor of inflammatory cytokines that activates monocyte/macrophages; TGF-beta type II receptor, which is a receptor of TGF-betas involved in wound healing and fibrosis; a subunit of Na+/K+-ATPase, an amiloride-sensitive cation channel, and a subunit of the potassium channel, all of which regulate the alveolar fluid balance and play a role in clearing lung edema; the adenosine A2a receptor, which has a protective function in the lung and interacts with dopamine D1 and D2 receptors to regulate the function of amiloride-sensitive cation channels; coflin, which is involved in the depolymerization and cleavage of actin filaments; LIM motif-containing protein kinase 1, which negatively regulates the activity of coflin; SHPS-1, which regulates the integrin-mediated reorganization of the cytoskeleton; and sodium channel beta 2, which is involved in cell adhesion and migration. These results indicate that PQ-induced pulmonary fibrosis does not merely terminate as cicatrices three months after the discontinuation of PQ treatment, but that dynamic functional change continues in the lung.

KEY WORDS: Paraquat, Pulmonary fibrosis, DNA microarray, Gene expression profiling

INTRODUCTION

Paraquat (1,1-dimethyl-4,4'-bipyridium chloride, PQ), a para-substituted quaternary bipyridyl cation, is a herbicide that was introduced commercially in 1962. Although very effective as a herbicide, it is a serious hazard to humans and animals. PQ induces pulmonary fibrosis in humans, monkeys, dogs and rats (Murray and Gibson, 1972; Akahori and Oehme, 1983; Hampson and Pond, 1988), but not in rabbits or guinea pigs (Butler and Kleinerman, 1971; Dubaybo and Thet, 1986). The mechanisms of PQ-induced pulmonary fibrosis have not been fully elucidated.

Previously (SATOMI et al., 2004), we examined the profile of gene expression in the lung at the subacute phase of PQ's toxicity (i.e. two days after the last administration of PQ at a dosage of 7 mg/kg, once per day for 8 days) in rats to investigate the genes involved in the priming of PQ-induced pulmonary fibrosis because it has been well documented that this fibrosis develops after the acute phase of PQ's toxicity (Toner et al., 1970). Using a DNA microarray, we found 49 genes that were expressed differently in the PQ-treated groups compared to the vehicle-treated control group as potential keys to the elucidation of the mechanism of pulmonary fibrosis induced by PQ. However, to inves-
tigate the relationship between the genes and PQ-induced pulmonary fibrosis thoroughly, another study in the fibrotic phase was needed.

Lung collagen contents in PQ-treated rats increased from the subacute phase of PQ toxicity and markedly increased collagen levels were observed in the lungs three months after discontinuation of PQ administration (Akahori and Oehme, 1983). Comparison of the gene expression profile three months after discontinuation of PQ administration with that during the subacute phase is expected to provide further information about the genes involved in PQ-induced pulmonary fibrosis.

In the present study, we examined the profile of gene expression at three months after PQ treatment as the fibrotic phase in rats, using DNA microarrays to identify the genes involved in PQ-induced pulmonary fibrosis.

MATERIALS AND METHODS

Chemicals
Parquat dichloride was obtained from Sigma-Aldrich Fine Chemicals (USA). It was diluted with saline to a final concentration of 2 mg/mL, and used for administration at 7 mg/kg.

Animals
The animals utilized in this experiment were 28 specific pathogen-free male rats (Crl: Wistar), 6 weeks of age, obtained from Charles River Japan. Prior to the experiment, these rats were acclimated (24 ± 2°C, 55 ± 15% humidity) in racks under positive pressure, in a clean-air room using HEPA filters for 1 week. During the experiment, an illumination cycle 12L: 12D (i.e. illumination for 12 hr) was used, and CRF-1 pellets (Oriental Yeast Co., Ltd., Japan) and water were freely available. All experimental procedures involving animals and related protocols were approved by the Committee on Animal Care of the TEIJIN Institute for Biomedical Research.

Administration of PQ and grouping of rats
Based upon the report by Akahori and Oehme (1983), PQ was administered s.c. to 24 rats at a dosage of 7 mg/kg, once per day for 8 days. Four control rats were given saline s.c. in volumes equivalent to the dose of PQ for 8 days. Two days after the final administration, i.e. Day 9, PQ-treated rats were divided into two experimental groups. Group 1 consisted of rats that demonstrated a loss of body weight. Group 2 consisted of rats that did not exhibit a loss of body weight (no toxic signs were evident after eight PQ administrations). The saline-treated control group (Group 3) was also maintained for the same experimental period.

Six rats of Group 1, four rats of Group 2, and two rats of Group 3 were used on Day 9 in our previous study (Satomi et al., 2004). The surviving five rats in Group 1 and one rat in Group 2 and two rats of Group 3 were examined three months after exposure to PQ to determine gene expression and collagen content in this study (Table 1).

Lung samples
Before dissection, these animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). After exsanguination was completed, the lung samples were quickly removed and weighed. The left lung was cut in half. The upper half and lower half of the left lung were used to examine collagen content and for gene expression profiling respectively. These specimens were quickly frozen in liquid nitrogen and maintained at −80°C until the amount of collagen was measured or RNA was extracted.

Lung collagen content
Hydroxyproline content was measured as an index of the amount of collagen, which reflects the degree of pulmonary fibrosis. The specimens were minced and then homogenized for 2 min at 4°C in sufficient deionized water to yield a 10% mixture (weight/volume). The hydroxyproline content of homogenates was assayed using the methods of Jamall et al. (1981).

The specimens obtained on Day 9 were also examined in this study.

Isolation of total RNA
Total RNA specimens were extracted from each lung sample using Isogen (Nippon Gene Co., Ltd., Japan) following the instructions provided. The extracted total RNA specimens were purified with an

<table>
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<tr>
<th>Table 1. Assignment of animals to experimental groups.</th>
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<tr>
<td>Group</td>
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<tr>
<td>1*</td>
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<td>2**</td>
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<td>3***</td>
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*Rats with subacute toxicity that lost body weight.
**A rat that did not exhibit body weight loss.
***Control rats.
Microarray analysis of rat lung at 3 months after PQ treatment.

RNeasy Mini Kit (Qiagen Inc., USA). The concentrations of total RNA were determined using a Shimadzu spectrophotometer; model UV 2200 (Japan).

Hybridization to DNA microarray and DNA microarray scanning

The Atlas Glass Rat 1.0 Microarrays (Clontech Laboratories, Inc., USA) on which 1,090 genes were spotted were used to examine differentially expressed genes after PQ treatment. The reverse transcription, fluorescent labeling and hybridization were essentially performed as recommended by Clontech Laboratories, Inc., with a protocol designed for individual sampling. Five micrograms from each total RNA sample was reverse-transcribed into a cDNA probe with the Atlas PowerScript Fluorescent Labeling Kit (Clontech Laboratories, Inc.). The Cyanine 3 (Cy3) labeling reaction was performed in a 50% dimethylsulfoxide solution containing 0.05 mg/μL of Cy3 monofunctional dye (Amersham Biosciences, USA). Thereafter, the Cy3-labeled cDNA probe was purified using a QIAquick PCR Purification Kit (Qiagen Inc.). The individual cDNA probe population was then separately hybridized with an identical DNA microarray. The hybridization was carried out in 2 ml of hybridization buffer containing cDNA probes at 50°C for 18 hr. The glass slides were washed with GlassHyb Wash Solution (Clontech Laboratories, Inc.) at room temperature for 10 min. Next, these slides were washed with a 10% GlassHyb Wash Solution of 1xSSC at room temperature for 10 min, twice and then with 0.1xSSC at room temperature for 10 min. After a rinse with deionized water and removal of the water by centrifugation, the glass slides were scanned using a GenePix4000A (Axon Instruments, Inc., USA) containing a 532 nm laser for the Cy3 measurement. Scans were set to a pixel resolution of 10 μm, a laser power of 100% and a photomultiplier tube voltage of 700 V for the laser.

Normalization and analysis of DNA microarray data

The images produced by the Axon scanner were acquired and analyzed using the ArrayGauge version 1.3 (Fuji Photo Film Co., Ltd.) software package. The data were further analyzed using Microsoft Excel. Each DNA microarray of the PQ-treated groups was compared to the saline-treated control group. The signal intensity was calculated as the total pixel value minus a global background and reported for each spot in the array. The signal intensities were then normalized to the mean for all of the spots in the array (global normalization). Gene expression was considered to be positive if the fluorescence intensity was higher than the maximum value of fluorescence intensity of the negative control (lambda phage DNA spots) on the array. This enabled a list of expressed genes on each array to be compiled for analysis. A complete list of the genes spotted on this array can be accessed at the website http://www.clontech.com/clontech/atlas/genelists/.

The criterion that we chose for identifying the differentially expressed genes was that their expression must be altered twofold or more between the PQ-treated groups and the saline-treated control group. Also, the differentially expressed genes must be recognized as two or more sets from the PQ-treated groups. A two-fold difference was established as a standard based upon the results of a preliminary experiment in which about 95% of the genes compared exhibited a change in expression of less than two-fold when this type of DNA microarray analysis was performed using identical total RNA as samples (data not shown).

RESULTS

Changes in body weight

The changes in body weights after the administration of PQ are shown in Fig. 1. Group 1 was composed of the five rats that demonstrated a loss of body weight and Group 2 had one rat that did not experience a weight loss. All of these animals were sacrificed three months after the last administration.

Clinical signs and appearances of the lungs following autopsy

In Group 1, three of the five rats exhibited clinical signs, i.e. reddish tearing, nasal hemorrhage, crouching, hypoactivity, piloerection and tachypnea during the period of body weight loss, and pulmonary fibrosis was observed on autopsy. The rat in Group 2 did not show clinical signs or pulmonary fibrosis (Photo 1).

Changes in lung wet weight

The changes in lung weight after administration of PQ are shown in Fig. 2. The data for Day 9 are from the lung specimens reported in our previous study (Satomi et al., 2004) and re-analyzed in this study. At Day 9, lung weight in Group 1 increased remarkably due to pulmonary edema (322.2% vs. Group 3). At three months after PQ-treatment, the lungs remained heavier in Group 1 than Group 3 but the difference was reduced (146.1% vs. Group 3). On the other hand, lung weight in Group 2 was almost equal to that in Group 3 both at Day 9 and at three months after PQ-treatment.
Changes in lung collagen content

The changes in lung collagen content after administration of PQ are shown in Fig. 3. Lung collagen content on Day 9 is data for specimens from a previous study (Satomi et al., 2004). At Day 9, the collagen content of the lung was increased in both Group 1 and Group 2 (191.9% and 157.7% vs. Group 3, respectively). At three months after PQ-treatment, in Group 1, the quantity of collagen in the lung was greater than in Group 3, but in Group 2, it was the same as in Group 3 (172.3% and 97.1% vs. Group 3, respectively).

Analysis of the gene expression profiles

A comparison of expression levels of the genes of the animals from Group 1 or Group 2 with the control animals treated with vehicle indicated that 335 genes from Group 1 and 143 genes from Group 2 were differentially expressed, and 142 genes were observed in both Group 1 and Group 2. Consequently, the number of differentially expressed genes specific to Group 1 was 193 and the number specific to Group 2 was only one: this last gene encoded ATP synthase, a H⁺ transporting, mitochondrial F1 complex, beta polypeptide (Fig. 4).

A functional classification of the differentially expressed genes in Group 1 is shown in Fig. 5. The data for Day 9 are the reanalysis results of our previous study (Satomi et al., 2004). Three months after treatment, the functional classification was diverse. However, on Day 9, functionally unclassified genes were most frequently altered, followed by the genes of apoptosis-associated proteins, oncogenes and tumor suppressor genes as well as the genes of stress response proteins.

Apparently altered genes, whose expression changed two fold or more between Group 1 and Group 3, are listed in Table 2. Twenty-two genes were up-regulated and 24 genes were down-regulated.
Microarray analysis of rat lung at 3 months after PQ treatment.

Photo 1. Photographs of lung specimens from Group 1, Group 2 and Group 3 rats. Pulmonary fibrosis was observed in Group 1 following autopsy.

I-1 through I-5: Group 1 (rats that lost weight due to PQ treatment).
II: Group 2 (a rat that did not exhibit body weight loss).
III-1 and III-2: Group 3 (rats treated with saline as a control).
Fig. 2. Wet weight of the left lung after PQ administration.
Wet weight of the left lung was measured in sacrificed animals at Day 9 and 3 months after PQ-treatment.
Each bar represents the mean ± S.D.,
N equals the number of rats in the group.

Fig. 3. Lung collagen content after PQ administration.
Collagen content is expressed as the amount of hydroxyproline. Lung collagen levels were measured in sacrificed animals at Day 9 and 3 months after PQ-treatment. The amount of collagen in the left lung was then calculated using these measurements.
Each bar represents the mean ± S.D.,
N equals the number of rats in the group.
Microarray analysis of rat lung at 3 months after PQ treatment.

DISCUSSION

Previously, we investigated the profile of gene expression in the lung two days after the last administration of PQ (Day 9) under the same conditions as in the present study (Satomi et al., 2004). In the subacute phase, pulmonary hemorrhage and edema were observed and lung weight and collagen content were remarkably increased in Group 1. In the present study, pulmonary hemorrhage and edema were not observed as shown in Photo 1, but the wet weight of the lung in Group 1 was slightly more than that in Group 3 as shown in Fig. 2. This increase in lung weight would be due to the deposition of collagen, an increase in the amount of extracellular matrix accompanying pulmonary fibrosis, and/or the continuing slight pulmonary edema. The collagen content in Group 1 remained high at three months after PQ treatment. Meanwhile, that in Group 2 was increased at Day 9, but at three months after treatment was the same as in Group 3. This result might indicate that the collagen was eliminated three months after PQ treatment, but in the present study, the number of animals in Group 2 was only one, so the result is inconclusive.

The number of differentially expressed genes in the present study was 335 in Group 1 and 143 in Group 2. Some 142 of the 143 genes detected in Group 2 were common to Group 1 (Fig. 4). On Day 9, again, most of the differentially expressed genes were common to Group 2. Theoretically, the specific altered genes for Group 1 would reflect fibrosis-related genes and those for Group 2 would imply anti-fibrosis-related genes. There were no markedly altered genes in either of the two groups. Therefore, most of the differences in differentially expressed genes between Group 1 and Group 2 would be caused by differences in the severity of PQ's toxicity.

Three months after the treatment the functional classification was diverse. On the other hand, on Day 9, functionally unclassified genes were most frequently altered, followed by the genes of apoptosis-associated proteins, oncogenes and tumor suppressor genes related to cell proliferation, and the genes of stress response proteins (Fig. 5). These results well reflected the cell death, wound healing, and stress responses triggered by PQ at Day 9. On the other hand, the diversity of genes at three months after treatment would reflect the complicated genetic regulation involved in the progression of pulmonary fibrosis.

Consequently, several genes listed in Table 2

![Fig. 4. Graph of the number of differentially expressed genes.](image)

Group 1: 335 genes were differentially expressed in total; 142 of the genes were common to Group 2.

Group 2: 143 genes were differentially expressed in total. Only one gene was differentially expressed in Group 2 specifically.
Fig. 5. Functional classification of differentially expressed genes at three months after PQ treatment (A) and on Day 9 (B). Each value means existence rate (%).
Microarray analysis of rat lung at 3 months after PQ treatment.

Table 2. Apparent gene expression differences three months after PQ treatment in the pulmonary fibrosis group. Genes with a two-fold or greater variation on average between Group 1 and Group 3 are listed.

<table>
<thead>
<tr>
<th>Up-regulated genes</th>
<th>Mean Ratio</th>
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<tbody>
<tr>
<td>1. colony stimulating factor 1 receptor</td>
<td>9.52</td>
</tr>
<tr>
<td>2. protein tyrosine phosphatase, non-receptor type substrate 1 (SHPS-1)</td>
<td>7.55</td>
</tr>
<tr>
<td>3. glycine transporter 1</td>
<td>6.48</td>
</tr>
<tr>
<td>4. adenylate cyclase 5</td>
<td>4.31</td>
</tr>
<tr>
<td>5. sodium channel beta 2</td>
<td>3.92</td>
</tr>
<tr>
<td>6. transforming growth factor-beta type II receptor</td>
<td>3.72</td>
</tr>
<tr>
<td>7. nuclear receptor subfamily 2, group F, member 6</td>
<td>3.35</td>
</tr>
<tr>
<td>8. LIM-domain containing, protein kinase</td>
<td>3.33</td>
</tr>
<tr>
<td>9. amiloride-sensitive cation channel 2, neuronal</td>
<td>3.00</td>
</tr>
<tr>
<td>10. coflin 1, non-muscle</td>
<td>2.86</td>
</tr>
<tr>
<td>11. adenosine A2a-receptor</td>
<td>2.70</td>
</tr>
<tr>
<td>12. acetylcholine receptor epsilon</td>
<td>2.69</td>
</tr>
<tr>
<td>13. putative G protein-coupled receptor (CNL3)</td>
<td>2.54</td>
</tr>
<tr>
<td>14. acetylcholine receptor beta</td>
<td>2.50</td>
</tr>
<tr>
<td>15. guanine nucleotide binding, protein, alpha inhibiting polypeptide 3</td>
<td>2.46</td>
</tr>
<tr>
<td>16. chemokine-like receptor 1</td>
<td>2.43</td>
</tr>
<tr>
<td>17. neurexin 2</td>
<td>2.33</td>
</tr>
<tr>
<td>18. fucosyltransferase 2</td>
<td>2.24</td>
</tr>
<tr>
<td>19. potassium inwardly-rectifying channel subfamily J, member 5</td>
<td>2.16</td>
</tr>
<tr>
<td>20. glial cell line-derived neurotrophic factor receptor alpha</td>
<td>2.14</td>
</tr>
<tr>
<td>21. G protein-coupled receptor 30</td>
<td>2.11</td>
</tr>
<tr>
<td>22. ADP-ribosylation factor 5</td>
<td>2.08</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Down-regulated genes</th>
<th>Mean Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. cytochrome c oxidase subunit Vb</td>
<td>0.49</td>
</tr>
<tr>
<td>2. inhibin beta-A</td>
<td>0.48</td>
</tr>
<tr>
<td>3. phospholipase C, gamma 1</td>
<td>0.48</td>
</tr>
<tr>
<td>4. rat kidney prostaglandin EP3 receptor</td>
<td>0.48</td>
</tr>
<tr>
<td>5. cytochrome P450 4A3 (CYP4A3); lauric acid omega-hydroxylase; P450-LA-omega 3</td>
<td>0.48</td>
</tr>
<tr>
<td>6. ATPase, Na'K' transporting, alpha 2 polypeptide</td>
<td>0.47</td>
</tr>
<tr>
<td>7. solute carrier family 21 (organic anion transporter), member 5</td>
<td>0.47</td>
</tr>
<tr>
<td>8. interleukin 2</td>
<td>0.45</td>
</tr>
<tr>
<td>9. calcitonin receptor</td>
<td>0.45</td>
</tr>
<tr>
<td>10. ras inhibitor</td>
<td>0.44</td>
</tr>
<tr>
<td>11. lipoprotein lipase</td>
<td>0.43</td>
</tr>
<tr>
<td>12. cytochrome c oxidase subunit VIIa 3</td>
<td>0.43</td>
</tr>
<tr>
<td>13. 3-hydroxy-3-methylglutaryl CoA lyase</td>
<td>0.43</td>
</tr>
<tr>
<td>14. 5-hydroxytryptamine (serotonin) receptor 2 A</td>
<td>0.42</td>
</tr>
<tr>
<td>15. interleukin 13</td>
<td>0.40</td>
</tr>
<tr>
<td>16. adrenergic receptor, alpha 1d</td>
<td>0.40</td>
</tr>
<tr>
<td>17. growth hormone secretagogue receptor</td>
<td>0.35</td>
</tr>
<tr>
<td>18. phospholipase C, gamma 2</td>
<td>0.34</td>
</tr>
<tr>
<td>19. interleukin 15</td>
<td>0.33</td>
</tr>
<tr>
<td>20. adrenergic receptor, alpha 1c</td>
<td>0.31</td>
</tr>
<tr>
<td>21. glucagon receptor</td>
<td>0.31</td>
</tr>
<tr>
<td>22. gonadotropin-releasing hormone receptor</td>
<td>0.25</td>
</tr>
<tr>
<td>23. arginine vasopressin receptor 1B</td>
<td>0.25</td>
</tr>
<tr>
<td>24. cholecystokinin B receptor</td>
<td>0.25</td>
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were considered involved in the lung injury, the change in the balance of electrolytes and fluid, and the alveolar remodeling following exposure to PQ.

As changes in gene expression that accompanied the pulmonary damage, an up-regulation of the expression of the colony stimulating factor 1 (CSF-1) receptor gene and transforming growth factor-beta (TGF-beta) type II receptor gene was observed. CSF-1 is an inflammatory cytokine that activates monocyte/macrophages. The TGF-beta type II receptor is involved in wound healing and fibrosis. The up-regulation of these genes would reflect an activation of their function, thus the inflammation due to PQ continued at the genetic level three months after PQ treatment in the lung.

Na\(^+\) channels in the alveolar epithelium play a critical role in regulating the balance of alveolar fluid and in the clearance of lung edema through the transport of Na\(^+\). The amiloride-sensitive cation channel is a major Na\(^+\) channel, and Na\(^+\)/K\(^+\)-ATPase is important to its activation (Matalon et al., 2002; Dada and Szinajer, 2003; Eaton et al., 2004; Egli et al., 2004; Kemp and Kim, 2004), as are potassium channels (O’Grady and Lee, 2003). The down-regulation of ATPase, a Na\(^+\)/K\(^+\) transporting, alpha 2 polypeptide, and the up-regulation of amiloride-sensitive cation channel 2, a neuronal and potassium inwardly-rectifying channel, subfamily J, member 5 would indicate changes in these functions.

The Adenosine A2a receptor protects against damage in the lung (Hasko et al., 2006) and interacts with dopamine D1 and D2 receptors that regulate amiloride-sensitive cation channels (Fuxe et al., 2005; Salmi et al., 2005; Kudlacek et al., 2003; Helms et al., 2006). The up-regulation of the adenosine A2a receptor gene also would indicate changes in these functions.

As changes related to the pulmonary remodeling following exposure to PQ, the up-regulation of the expression of coflin, which is involved in the depolymerization and cleavage of actin filaments, the LIM motif-containing protein kinase 1, which negatively regulates the activity of coflin (Hotulainen et al., 2005; Prochniewicz et al., 2005; Willis et al., 2005), SHPS-1, which regulates the integrin-mediated reorganization of the cytoskeleton (Inagaki et al., 2000; Maile et al., 2003; Mura-Takebe et al., 2004) and sodium channel beta 2, which is involved in cell adhesion and migration (Kim et al., 2005) were observed.

These results indicate that PQ-induced pulmonary fibrosis does not just terminate as cicatrices three months after the discontinuation of PQ treatment, but dynamic functional change related to PQ-induced pulmonary fibrosis continues in the lung. Besides the genes described above, the differentially expressed genes detected in this study would also involve pulmonary fibrosis. To investigate the relationship between these genes and their function in pulmonary fibrosis is an important theme in the future.

In conclusion, we uncovered 336 genes that were expressed differently in the PQ-treated groups compared to the vehicle-treated control at three months after PQ treatment using a DNA microarray. Of the 336 genes, 335 were differentially expressed in the group with pulmonary fibrosis. A functional classification of these genes indicated diversity. There were injury-related genes, electrolyte and fluid balance-related genes and alveolar remodeling-related genes. In addition to the 49 genes reported in our previous study (Satomi et al., 2004), these genes are valuable for elucidating the mechanism of pulmonary fibrosis induced by PQ. We will next perform studies of the advancing stages of fibrosis, between Day 9 and three months. Such studies will facilitate investigation of the molecular mechanisms underlying the pulmonary fibrosis induced by PQ.

REFERENCES


