Anti-monocyte chemoattractant protein-1 gene therapy attenuates pulmonary hypertension in rats

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Ikeda, Yasuhiro, Yoshikazu Yonemitsu, Chu Kataoka, Shiro Kitamoto, Terutoshi Yamaoka, Ken-Ichi Nishida, Akira Takeshita, Kensuke Egashira, and Katsuo Sueishi. Anti-monocyte chemoattractant protein-1 gene therapy attenuates pulmonary hypertension in rats. Am J Physiol Heart Circ Physiol 283: H2021–H2028, 2002.—Monocyte/macrophage chemoattractant protein-1 (MCP-1), a potent chemoattractant chemokine and an activator for mononuclear cells, may play a role in the initiation and/or progression of pulmonary hypertension (PH). To determine whether blockade of a systemic MCP-1 signal pathway in vivo may prevent PH, we intramuscularly transduced a naked plasmid encoding a 7-NH2 terminus-deleted dominant negative inhibitor of the MCP-1 (7ND MCP-1) gene in monocrotaline-induced PH. We also simultaneously gave a duplicate transfection at 2-wk intervals or skeletal muscle-directed in vivo electroporation (EP) to evaluate whether a longer or higher expression might be more effective. The intramuscular reporter gene expression was enhanced 10 times over that by EP than by simple injection, and a significant 7ND MCP-1 protein in plasma was detected only in the EP group. 7ND MCP-1 gene transfer significantly inhibited the progression of MCT-induced PH as evaluated by right ventricular systolic pressure, right ventricular hypertrophy, medial hypertrophy of pulmonary arterioles, and mononuclear cell infiltration into the lung. Differential effects of longer or higher transgene expression were not apparent. Although the in vivo kinetics of 7ND MCP-1 gene therapy should be studied further, these encouraging results suggest that an anti-inflammatory strategy via blockade of the MCP-1 signal pathway may be an alternative approach to treat subjects with PH.

MCP-1; monocrotaline; electroporation

PULMONARY HYPERTENSION (PH) is an intractable disease with sustained elevations of pulmonary arterial pressure (mean pressure >25 mmHg at rest) affecting about one to two cases of primary PH (PPH) in a million persons of the general population (1, 15, 17). Because the prognosis of PH is poor, many efforts have been extensively conducted in clinic. Recent promising clinical studies using epoprostenol (prostacyclin) have demonstrated a significant improvement in survival time for patients with PH (3, 8, 9); however, it seems still distant from curative treatment. Furthermore, although cardiopulmonary transplantation has been effective for PH, related complications including bronchiolitis obliterans occur and retransplantation is needed (19). More effective and less invasive therapy based on the pathophysiology of PH should thus be developed.

Current limitations to treat PPH, however, include less knowledge of the exact mechanisms in the initiation and progression of the disease. For instance, although the pathological changes in PH include intimal thickening and/or medial and intimal hypertrophy of arteries, luminal thrombosis, and plexogenic pulmonary arteriopathy (PPA) (4–6, 15), no precise evidence is now available as to whether these untoward events lead to the disease or may be the result of the disease. No relevant animal model of PPH is available.

Although monocrotaline (MCT)-induced PH has been well accepted as an experimental model of PH (4–6), for instance, only medial hypertrophy of pulmonary arteries is noted, not other important findings, such as PPA lesions, suggesting some questions regarding the relevancy of this model.

Some common histological findings between humans and animals, on the other hand, have also been suggested. An exclusive perivascular inflammatory cell infiltrate, including monocyte/macrophages, was found in the PPA of PH patients (11, 22, 23) as well as in a rat model of MCT-induced PH (20, 21). Evidence shows that transient elevation of plasma levels of monocyte chemoattractant protein-1 (MCP-1) are associated in the early phase; therefore, this may play a role not only in the inflammatory response of MCT-induced PH (10) but also of human PPH. Although the role of MCP-1-
mediated inflammatory reaction in human PPH is still controversial, it is likely to be significant to clarify the role of inflammatory processes in the initiation and/or progression of the disease.

MCP-1, a member of the C-C chemokine family, has a potent chemotactic molecule for monocytes (12, 16, 18) and activates the receptor CCR2 as a dimer (12). Our recent study has demonstrated that a 7-NH$_2$ terminus-deleted mutant gene, namely 7ND MCP-1, is a potent dominant negative inhibitor for MCP-1 (26, 27). We reported that the intramuscular gene delivery of 7ND MCP-1 prevented vascular remodeling in rats with a chronic blockade of nitric oxide production (7), as well as atherosclerotic plaque formation of apolipoprotein E-deficient mice in vivo (13), which suggests that this approach might be effective in treating subjects with PH.

In the present study, we determined whether the intramuscular injection of a naked plasmid DNA encoding 7ND MCP-1 might prevent the disease progression of MCT-induced PH in a rat model. To evaluate the kinetics of this strategy, we examined three different intramuscular gene therapy protocols: 1) simple naked DNA injection; 2) duplicate injection to prolong transgene expression; and 3) simple injection with electronic pulses, which is well known as an effective technique to markedly increase intramuscular transgene expression (2).

**METHODS**

**Plasmid DNA**

Plasmid pCMV-luciferase was prepared as described previously (25). Human 7ND MCP-1 cDNA with a FLAG epitope was constructed as previously described (7). These plasmids, purified by equilibrium centrifugation in CsCl-ethidium bromide gradients were suspended in Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA; pH 8.0) at a concentration of 1.0 mg/ml.

**Animals and Experimental Protocol**

Adult male Sprague-Dawley rats (6–8 wk old, 250–350 g) were separated into six groups as follows: 1) buffer-injected control (C group, n = 8); 2) MCT injection without gene transfer (MCT group, n = 34); 3) MCT + gene transfer of luciferase once (Lucif group, n = 9); 4) MCT + gene transfer of 7ND MCP-1 once (7ND group, n = 34); 5) MCT + 7ND MCP-1 twice at 2-wk intervals (7ND×2 group, n = 21); and 6) MCT + in vivo electroporation once (EP group, n = 27). The experimental protocol is summarized in Fig. 1. Seventy-two thigh muscles were used for direct comparison study of pCMV-luciferase gene transfer, and luciferase activity was measured as previously described (25). The following animal experiments were reviewed by the Committee of Ethics on Animal Experiments in the Faculty of Medicine, Kyushu University. The National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, Revised 1985) was also followed.

Intraperitoneal injections of pentobarbital were given during the following procedures. The rats were given a single subcutaneous injection of MCT (60 mg/kg, Wako; Osaka, Japan). Rats were maintained under the humanized conditions throughout. Right ventricular (RV) hypertrophy and RV systolic pressure was measured as previously described (25). RV systolic pressure was measured using a clinically available electric pressure meter (NEC, Tokyo, Japan) via direct puncture.

**Intramuscular Gene Transfer**

Direct injection. Three days before gene transfer, 500 μl of 0.25% bupivacaine were injected into both thigh muscles, and a total 500 μl solution of plasmid DNA (total 500 μg) was subsequently injected at the same sites (24).

Electroporation. Electronic pulses were delivered using a standard square-wave electroporator (CUY21, BEX; Tokyo, Japan) at 100 V, 50-ms pulse length and six pulses, soon after plasmid DNA injection (total 500 μg) (2). The steel electrode, a pair of 2.5 × 0.5-cm parallel plates, was brought into contact with the muscle in parallel orientation with respect to the muscle fibers.

**Western Blot Analysis**

Cytoplasmic fraction was subjected to Western blot analysis using a primary monoclonal mouse anti-FLAG antibody (dilution 1:200, Stratagene; La Jolla, CA) and visualized by fluorescence using the HNPP-Fast Red TR System (Takara; Tokyo, Japan).

**Enzyme-Linked Immunosorbent Assay**

Commercially available ELISA systems (Bio Source; Camarillo, CA) were used to detect human MCP-1 and rat MCP-1 according to the manufacturer’s instructions. Cross-reactivity of each species was determined as ~3% (data not shown).

**Immunohistochemistry**

Immunohistochemistry for monocyte/macrophage labeling was done using a primary monoclonal mouse anti-rat ED1 IgG antibody (Serotec; Raleigh, NC) diluted 1:500, and signals were then developed using an avidin-biotinylated peroxidase complex method. For quantification, two blinded observers counted the number of ED1-positive cells on the same nine high-power fields (×400) in the lung of each animal.
Fig. 2. Evaluations of transgene expression via gene transfer techniques. A: intramuscular gene expression efficiency via injection of pCMV-luciferase plasmid DNA with or without electronic pulse in vivo. Muscles were subjected to luciferase assay at 3, 10, 17, and 24 days after gene transfer. Electric pulse-mediated intramuscular gene transfer showed an ∼15-fold greater gene expression compared with the direct injection once on day 3. RLU, relative light units. *P < 0.01 vs. direct injection once; **P < 0.05 vs. direct injection once. B: Western blot analysis of 7ND MCP-1 protein expression using a FLAG-specific antibody in vitro. A dose-dependent increase of 7ND MCP-1 protein with FLAG sequence was observed (lanes 2 and 3) in cytoplasmic fractions of THP-1 cells subjected to lipid-mediated gene transfer. Cytoplasmic protein of THP-1 cells, which strongly express endogenous MCP-1 under LPS stimulation, showed negative results (lane 1), thereby indicating a transfected cDNA-specific gene product in lanes 2 and 3.

Statistical Analyses

All values are expressed as means ± SD. Data were analyzed using one-way ANOVA, and, where appropriate, Student’s t-test with Scheffe’s adjustment for multiple comparisons was used.

The survival rate, determined by Kaplan-Meier’s method, was calculated on day 28. The statistical significance of the survival experiments was determined using the log rank test.

RESULTS

Transgene Expression Via Intramuscular Injection With or Without Electric Pulses

We first determined intramuscular gene transfer efficiency of direct injection or in vivo electroporation using pCMV-luciferase plasmid DNA. These muscles were subjected to luciferase assay at 3, 10, 17, and 24 days after the gene transfer. Electric pulse-mediated intramuscular gene transfer showed an ∼15-fold greater gene expression compared with the direct injection of plasmid DNA once on day 3 [Fig. 2A; direct injection: 3.8 × 10⁵ relative light units (RLU)/mg protein (n = 10) and electroporation: 5.8 × 10⁶ RLU/mg protein (n = 8), respectively, P < 0.001]. Transgene expressions using both techniques were gradually decreased and showed almost equal levels with an apparent significant expression on day 24. Gene transfer of luciferase done twice showed an increase in intramuscular gene expression on day 17 that was approximately fivefold greater than the direct injection once [once: 1.6 × 10⁴ RLU/mg protein (n = 10) and twice: 7.5 × 10⁴ RLU/mg protein (n = 8), respectively, P < 0.05] on day 17; however, even at that time, the level was still lower than that seen with electroporation.

To determine whether our construct of the 7ND MCP-1 gene could be translated in mammalian cells efficiently, in vitro lipid-mediated 7ND MCP-1 gene transfer was done. We used an anti-FLAG antibody to exclude 7ND MCP-1 from endogenous expression of wild MCP-1 protein. Western blot analysis using the cytoplasmic protein of THP-1 cells, which strongly expressed endogenous MCP-1 under LPS stimulation, showed no expression of MCP-1 with the FLAG (Fig. 2B), whereas a dose-dependent increase in 7ND MCP-1 protein was observed in Western blot analysis using

Table 1. Expression and time course of human-derived 7ND MCP-1 protein

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma, pg/ml</th>
<th>Muscle, ng/g protein (Day 5)</th>
</tr>
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<tr>
<td></td>
<td>Day 3</td>
<td>Day 10</td>
</tr>
<tr>
<td>Lucif</td>
<td>&lt;20(5)</td>
<td>&lt;20(6)</td>
</tr>
<tr>
<td>7ND</td>
<td>&lt;20(8)</td>
<td>&lt;20(6)</td>
</tr>
<tr>
<td>7ND × 2</td>
<td>&lt;20(6)</td>
<td>&lt;20(6)</td>
</tr>
<tr>
<td>EP</td>
<td>482.73 ± 366.21(7)*</td>
<td>64.16 ± 72.56(8)*</td>
</tr>
</tbody>
</table>

Values are means ± SD. Numbers in parentheses are the numbers of animals. 7ND MCP-1; 7-NH₂ terminus-deleted mutant gene (7ND) of monocYTE/macrophage chemotactrant protein-1 (MCP-1). The following groups are shown: monocrotaline (MCT) + gene transfer of luciferase (Lucif); MCT + gene transfer of 7ND MCP-1 once (7ND); MCT + gene transfer of 7ND MCP-1 twice at 2-wk intervals (7ND × 2); and MCT + in vivo electroporation (EP). *P < 0.01 vs. the 7ND group.

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cytoplasmic proteins of THP-1 with lipid-mediated gene transfer of 7ND plasmid (Fig. 2B, lanes 2 and 3).

We reported that the intramuscular injection of 7ND plasmid efficiently prevented recombinant MCP-1-mediated intradermal infiltration of monocytes, thus indicating that the 7ND transgene products functioned well (7). However, little information is available regarding the required transgene expression level of 7ND MCP-1 compared with endogenous wild (rat) MCP-1 expression [untreated control plasma: 83.3 ± 19.9 pg/ml (n = 2)]. At various time points after gene transfer, we assessed the protein expression level of 7ND MCP-1 in plasma (days 3, 10, and 17) and muscles (day 5) in rats given an intramuscular gene transfer using human MCP-1-specific ELISA systems (Table 1). In plasma samples of all experimental groups, however, human sequence-specific 7ND MCP-1 protein was significantly detected only in the EP group on days 3 and 10 (P < 0.01). More than 18-fold higher 7ND MCP-1 protein was also obtained in the muscular tis-

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**Fig. 3.** A: typical macroscopic view of a ventricular cross section of the rat heart. An enlarged RV and shifted septum (S) to the left ventricle (LV) in the heart of the MCT-injected rat (middle) are apparent. Note that these are not obvious in the heart treated with 7ND (right), similar to findings in the untreated control heart (left). B–D: measurements of RV systolic pressure (RVSP; B), the wet weight RV-to-LV + S ratio (RV/LV + S; C), and the percent wall thickness of pulmonary arterioles (D). Data were analyzed by one-way ANOVA. *P < 0.05 vs. the MCT group; **P < 0.01 vs. the MCT group; ***P < 0.001 vs. the MCT group. E and F: scattered plot analyses to determine the relationships between RVSP vs. the percent wall thickness of pulmonary arterioles (E) or RV/LV + S vs. the percent wall thickness of pulmonary arterioles (F). Both graphs indicate significant relationships among these parameters.
sue in case of electroporation than that with naked DNA injection ($P < 0.01$). These findings suggested that in vivo electroporation enhanced not only transgene expression in skeletal muscle but also plasma levels of 7ND MCP-1 protein.

Therapeutic Effects of 7ND MCP-1 Gene Transfer

We then assessed the therapeutic effects of 7ND MCP-1 gene transfer by assessing RV systolic pressure (RVSP) via direct needle puncture connected to a clinically available pressure meter. The hearts were harvested, and the wet weight was recorded [RV-to-left ventricle + septum (LV + S) ratio]. The lung was histopathologically and immunohistochemically examined for macrophage infiltration.

7ND MCP-1 gene transfer prevents pulmonary arterial pressure, RV hypertrophy, and vascular remodeling of pulmonary arterioles. On day 21, a marked elevation of RVSP was observed in the MCT group [day 21: 47.8 ± 11.4 mmHg ($n = 9$) and day 28: 82.5 ± 10.6 mmHg ($n = 7$)]. At that time, the elevation of RVSP was significantly prevented in the EP group [38.4 ± 2.3 ($n = 6$), $P < 0.05$ on day 21 but not in other groups on day 21 [7ND: 42.2 ± 4.6 mmHg ($n = 8$), $P = 0.154$]. On day 28, elevation of RVSP in the MCT group [80.64 ± 11.07 mmHg ($n = 8$)] was significantly prevented in the 7ND×2 and EP groups.

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Fig. 4. Immunohistochemical findings indicating monocyte/macrophage infiltration in the lung using ED1 antibody (A) and quantification of a positive cell number (B). A: typical immunohistochemical findings of ED1-positive cells on day 28. The ED1-positive cells (brown-labeled cytoplasm, arrows), indicating macrophages and monocytes, infiltrated into the alveolar spaces (original magnification ×400). B: ED1-positive cells were counted at the same nine high-power fields (×400) in the lung of each animal, and the total cell number was expressed. Data were analyzed using one-way ANOVA. *$P < 0.05$ vs. the MCT group; **$P < 0.01$ vs. the MCT group.
The degree of RV hypertrophy was determined by assessing the wet weight RV-to-LV + S ratio (Fig. 3, A and C). On day 21, no significant difference was observed among groups tested [C: 0.39 ± 0.06 (n = 8); 7ND: 0.38 ± 0.06 (n = 7), P = 0.801; EP: 0.34 ± 0.05 (n = 6), P = 0.073]. On day 28, the RV-to-LV + S ratios of the all 7ND-treated groups were significantly decreased [7ND: 0.49 ± 0.06 (n = 19), P < 0.05; 7ND×2: 0.47 ± 0.07 (n = 12), P < 0.01; EP: 0.48 ± 0.07 (n = 14), P < 0.01] compared with findings in the MCT group [0.54 ± 0.05 (n = 19); Fig. 3C].

On day 28, medial hypertrophy of pulmonary arterioles was significantly reduced in all 7ND-treated groups [7ND: 19.2 ± 3.5% (n = 8), P < 0.01; 7ND×2: 20.2 ± 5.5% (n = 8), P < 0.05; EP: 15.4 ± 3.4% (n = 8), P < 0.001] compared with findings in the MCT group [27.3 ± 4.5% (n = 8); Fig. 3D]. To confirm the physiological significance and relationships among these parameters, we made scattered plot analyses. As shown in Fig. 3, E and F, medial hypertrophy of pulmonary arterioles significantly correlated to the RVSP and RV-to-LV + S ratio (P < 0.01, respectively).

7ND MCP-1 gene transfer prevents monocyte/macrophage recruitment in the lung. To determine the effects of 7ND MCP-1 gene transfer regarding mononuclear cell recruitment in the lung, immunohistochemical analyses for rat ED1-positive cells, indicating macrophages and monocytes, were made. On day 21, an increased number of ED1-positive cells was present in the lung of the MCT group (452.4 ± 95.0 cells/9 fields). At that time, ED1-positive cell infiltration in the EP group (337.5 ± 64.0 cells/9 fields, n = 6) was significantly prevented, compared with findings in the MCT group (P < 0.05). On day 28, MCT-mediated mononuclear cell infiltration (527.4 ± 124.5 cells/9 fields, n = 19) was also significantly prevented in all treatment groups [7ND: 430.8 ± 112.2 cells/9 fields (n = 19), P < 0.01; 7ND×2: 386.4 ± 122.4 cells/9 fields (n = 14), P < 0.01; EP: 446.9 ± 58.6 cells/9 fields (n = 14), P < 0.05; Fig. 4, A and B].

7ND MCP-1 improves the mortality rate of MCT-induced PH rats. As shown in Fig. 5, 10 of 25 rats in the MCT group died by day 21 (n = 1) and day 28 (n = 9) spontaneously [overall survival rate on day 28: 15/25 = 60.00%, and 5 rats in the Lucif group were also dead by day 21 (n = 1) and day 28 (n = 3); overall survival rate on day 28: 5/9 = 55.56%]. Meanwhile, in the three 7ND-treated groups, eight rats died spontaneously by day 28 [7ND: n = 4; 7ND×2: n = 2; EP: n = 2]. Survival analyses for each group showed that 7ND-treated groups had a significantly improved survival rate (overall survival rate on day 28: 7ND: 22/26 = 84.62%, P < 0.05 vs. the MCT group; 7ND×2 19/21 = 90.48%, P < 0.05 vs. the MCT or Lucif group; EP, 19/21 = 90.48%, P < 0.05 vs. the MCT or Lucif group; Fig. 5).

DISCUSSION

Monocyte/macrophage recruitment to inflammatory foci releases various cytokines, growth factors, and chemokines, including MCP-1, resulting in the initiation and/or progression of various diseases via the autocrine/paracrine loop. Recent studies have suggested that recruitment of monocyte/macrophage plays a role not only for MCT-induced PH in rats (10, 20, 21) but also for various human diseases, including primary PH (23). Therefore, we tested a skeletal muscle-directed gene transfer approach, which was shown to be effective in preventing systemic MCP-1 activity (7), to determine whether it would be applicable in a rat model of MCT-induced PH.

We found that in all 7ND-treated groups, disease progression was significantly suppressed and survival rates improved, and thus we concluded that this approach is likely to be a strategy to treat diseases related to inflammatory consequences associated with MCP-1 activity. Furthermore, to address the therapeutic effect of this strategy more precisely, we used two additional groups, including duplicate DNA injection to prolong transgene expression and electroporation-mediated muscular gene transfer to enhance transgene expression. No significant differences were observed among these 7ND-treated groups, suggesting that the neutralizing effect of 7ND for endogenous MCP-1 seems not to largely depend on the duration and strength of the transgene expression.

Regarding the level of transgene expression, electroporation showed a constantly higher transgene expression when using the luciferase reporter gene and a higher exogenous gene product level in muscle and plasma than seen in the case of single or duplicate injections (Fig. 2A and Table 1). Regarding the therapeutic outcome, however, no significant differences were found in almost all parameters among these groups when using the same therapeutic gene in vivo (Figs. 3, B and C, and 4B). The exact reason is unclear, but one possible explanation may be that 7ND MCP-1 expression was still too low, even in the EP group, to prevent disease progression completely. To assess this more confidently, we also did another set of experiments to achieve a higher level of 7ND MCP-1 gene transfer to the respiratory tract using a more efficient
viral vector, including the novel recombinant Sendai virus (25).

This notion is supported by findings indicating the requirement of a >30 times greater molar ratio of 7ND MCP-1 for 50% inhibition of MCP-1 activity in vitro (Ref. 26 and our unpublished observations). However, this seems not to be likely because, if these in vitro findings are comparable in in vivo situations, a 15 times higher therapeutic gene expression should be more effective. Another explanation is that the biological effect of 7ND MCP-1 may be independent of plasma concentrations. C-C chemokines, including MCP-1, are locally stored via binding to glycosaminoglycans in vivo (16), suggesting that the lower transgene expression of 7ND might be sufficient to prevent MCP-1 activity. This was supported by our previous data indicating that intramuscular plasmid-mediated 7ND injection could inhibit >80% of recombinant MCP-1 activity in the skin in vivo (7, 13). These kinetic studies of antagonistic effects of 7ND MCP-1 in vivo should be clarified further in the near future.

One more important question is also raised: Does MCP-1 play a role in the initiation and/or progression during MCT-induced PH disease? Although Cowan et al. (5a) reported that elastase inhibitor treatment could reverse established PH, 7ND gene administration could not do so in case of established PH (data not shown). These results suggest that elastase activity may be important during the whole process of MCT-induced PH, whereas MCP-1 might contribute to early but not to late disease initiation or progression in a rat model of MCT-induced PH. This notion seems reasonable because the endogenous MCP-1 level in the plasma showed a peak level 7 days after the MCT injection and declined within 2 wk (10), suggesting that later blockade of the MCP-1 signal pathway is likely to be without effect. However, suppression of early MCP-1 activity may affect the later disease phenotype, because suppression of ED1-positive cell infiltration in the lung was evident in all 7ND treatment groups. Therefore, early inhibition of MCP-1 signaling may prevent the autocrine/paracrine recruitment of monocytes/macrophages in a diseased lung.

In addition, timing of the gene transfer needs to be reconsidered. We injected the 7ND MCP-1 gene at the same time as MCT administration because, as mentioned above, it was seen that the plasma endogenous MCP-1 level showed a peak level 1 wk later. Because transgene expression via the intramuscular route showed a peak 3–5 days later, this gene transfer protocol might not prevent early MCP-1 activity.

In conclusion, intramuscular gene transfer of 7ND MCP-1 cDNA suppressed disease progression of MCT-induced PH and improved the survival rate. These results showed that monocyte/macrophage recruitment and the systemic MCP-1 signal pathway contribute to progression of this disease. Although kinetic studies including the level and duration of transgene expression as well as the timing of gene transfer are needed, this approach can be considered for use to block the systemic MCP-1 signal pathway and to treat subjects with PH. Most importantly, more information regarding that inflammatory process in the initiation and/or progression of human PH subjects should be obtained in the near future.

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