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＜新聞報道＞

2011 年 08 月 01 日　「ナノ DDS 技術による革新的低侵襲治療的血管新生療法の橋渡し研究」について、24 年度に医師主導型治験を開始する旨の記事が日本経済新聞に掲載された。

【研究成果の刊行物・別刷】

○をつけた論文の別刷あるいは資料を次のページ以降に添付します。

Nanoparticle-Mediated Delivery of Nuclear Factor κB Decoy Into Lungs Ameliorates Monocrotaline-Induced Pulmonary Arterial Hypertension

Satoshi Kimura, Kensuke Egashira, Ling Chen, Kaku Nakano, Eiko Iwata, Miho Miyagawa, Hiroyuki Tsujimoto, Kaori Hara, Ryuichi Morishita, Katsuo Sueishi, Ryuji Tominaga, Kenji Sunagawa

Abstract—Pulmonary arterial hypertension (PAH) is an intractable disease of the small pulmonary artery that involves multiple inflammatory factors. We hypothesized that a redox-sensitive transcription factor, nuclear factor κB (NF-κB), which regulates important inflammatory cytokines, plays a pivotal role in PAH. We investigated the activity of NF-κB in explanted lungs from patients with PAH and in a rat model of PAH. We also examined a nanotechnology-based therapeutic intervention in the rat model. Immunohistochemistry results indicated that the activity of NF-κB increased in small pulmonary arterial lesions and alveolar macrophages in lungs from patients with PAH compared with lungs from control patients. In a rat model of monocrotaline-induced PAH, single intratracheal instillation of polymeric nanoparticles (NPs) resulted in delivery of NPs into lungs for ≤14 days postinstillation. The NP-mediated NF-κB decoy delivery into lungs prevented monocrotaline-induced NF-κB activation. Blockade of NF-κB by NP-mediated delivery of the NF-κB decoy attenuated inflammation and proliferation and, thus, attenuated the development of PAH and pulmonary arterial remodeling induced by monocrotaline. Treatment with the NF-κB decoy NP 3 weeks after monocrotaline injection improved the survival rate as compared with vehicle administration. In conclusion, these data suggest that NF-κB plays a primary role in the pathogenesis of PAH and, thus, represent a new target for therapeutic intervention in PAH. This nanotechnology platform may be developed as a novel molecular approach for treatment of PAH in the future. (***Hypertension.* 2009;53:877-883.**)

Key Words: pulmonary hypertension ■ lung ■ inflammation ■ leukocytes

Pulmonary arterial hypertension (PAH) is an intractable disease of the small pulmonary arteries that results in a progressive increase in pulmonary vascular resistance, right ventricular failure, and, ultimately, premature death.[1–3] Because its mortality remains high even after the introduction of prostacyclin infusion therapy (which has raised the 5-year survival rate to ≈50%), the development of a more effective and less invasive therapy for PAH is urgently needed.

Recent evidence suggests an important role of monocyte chemoattractant protein (MCP) 1–mediated inflammation in the mechanism of PAH.[4–8] However, the therapeutic benefits of MCP-1 blockade were not optimal for clinical application.[5,6] During the inflammatory process of PAH, several inflammatory factors (eg, MCP-1, interleukin [IL] 1, IL-6, and tumor necrosis factor [TNF] α) are overproduced, leading to a vicious circle.[1–3] A redox-sensitive transcription factor, nuclear factor κB (NF-κB), is known to regulate expression of chemokines such as MCP-1 and multiple inflammatory cytokines such as IL-6 and TNF-α. Blockade of NF-κB by transfection of NF-κB "decoy" oligodeoxynucleotides may attenuate the vascular pathology associated with reduced expression of NF-κB–dependent genes.[9–12] However, no previous study has addressed the specific role of the NF-κB pathway in the pathogenesis of PAH. Therefore, we hypothesized that controlled local delivery of NF-κB decoy into lungs, targeting a battery of multiple important inflammatory cytokines, would be a favorable therapeutic approach for PAH. To this end, we have recently developed bioabsorbable polymeric nanoparticles (NPs) formulated from a poly-(ethylene glycol)-*block*-lactide/glycolide copolymer (PEG-PLGA).[13–15]

The primary aim of this study was to investigate the role of the NF-κB pathway in the pathogenesis of PAH. We first examined the activity of NF-κB in patients with PAH. We then used a rat model of monocrotaline (MCT)-induced PAH to examine whether NP-mediated delivery of the NF-κB decoy can attenuate the development of PAH.

Methods

Histopathologic and Immunohistochemical Examination of Human Lungs

Human lung tissue was obtained from autopsy specimens from 4 patients whose deaths were attributed to idiopathic PAH and 2

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patients whose deaths were attributed to nonlung disease (Figure S1, available in the online data supplement at http://hyper.ahajournals.org). Additional details are provided in the online data supplement.

Preparation of NPs

The NF-κB decoy oligodeoxynucleotides labeled with or without fluorescein-isothiocyanate (FITC) were prepared as described previously.[10,11] The decoy is directed against the NF-κB binding site in the promoter region that corresponds with NF-κB–responsive genes and works to inhibit binding of this transcription factor to the promoter region.[10,11] PEG-PLGA NPs encapsulated with FITC, NF-κB decoy, or FITC-labeled NF-κB decoy were prepared using an emulsion solvent diffusion method.[13,14] The average diameter of PEG-PLGA NPs was 44 nm. To measure FITC release kinetics, FITC-NP was immersed in Tris-EDTA buffer, and the released FITC was measured. Additional details are provided in the online data supplement.

In Vivo Experiments With a Rat Model of MCT-Induced PAH

Rats were SC injected with 60 mg/kg of MCT, which induces severe PAH within 3 weeks.[5,16,17] In the prevention protocol, animals were assigned to either an untreated control group or a group that received a single intratracheal instillation of NF-κB decoy alone (50 μg), FITC-NP (1000 μg of PEG-PLGA), or NF-κB decoy NPs (50 μg of NF-κB decoy per 1000 μg of PEG-PLGA) immediately after MCT (n=6 each). For intratracheal instillation, a volume of 0.1 mL of phosphate buffer suspension of NP or NF-κB decoy was injected gently into the trachea of animals accompanied by an equal volume of air. The biodistribution of FITC in the lung was also examined 3, 7, and 14 days after intratracheal instillation of FITC only, FITC-NPs, or FITC-labeled NF-κB decoy NPs in rats injected with MCT. In the treatment protocol, rats were divided into 2 groups (rats treated with a single intratracheal instillation of phosphate buffer and rats treated with NF-κB decoy NPs; n=33 each) 21 days after MCT injection, when severe PAH had been established.

Hemodynamic Measurements

Three weeks after MCT administration, the animals were anesthetized with sodium pentobarbital, and then polyethylene catheters were inserted into the right ventricle (RV) through the jugular vein and the carotid artery for hemodynamic measurements. RV systolic pressure and systemic blood pressure were measured with a polygraph system (AP-601G, Nihon Kohden).[5]

Assessment of Right Heart Hypertrophy and Pulmonary Arterial Remodeling

After systemic arterial and RV pressure had been recorded, the animals were euthanized, and the lungs and heart were isolated. The RV wall was dissected from the left ventricle (LV) and ventricular septum (S). The wet weight of the RV and LV+S was determined, and RV hypertrophy was expressed as follows: RV/(LV+S).[5]

The lungs were perfused with a solution of 10% phosphate buffered formalin (pH 7.4). At the same time, 10% phosphate buffered formalin (pH 7.4) was administered into the lungs via the tracheal tube at a pressure of 20 cm H_2O. These specimens were processed for light microscopy by routine paraffin embedding. The degree of remodeling (muscularization) of the small peripheral pulmonary arteries was assessed by double immunohistochemical staining of the 3-μm sections with an anti–α-smooth muscle actin antibody (dilution 1:500, clone 1A4, Dako) and anti–platelet endothelial cell adhesion molecule 1 (M-20) antibody (dilution 1:100, Santa Cruz Biotechnology) modified from a protocol described elsewhere.[18]

To assess the type of remodeling in the muscular pulmonary arteries, microscopic images were analyzed. In each rat, 30 to 40 intra-acinar arteries were categorized as muscular (ie, with a complete medial coat of muscle), partially muscular (ie, with only a crescent of muscle), or nonmuscular (ie, with no apparent muscle). The arteries were counted and averaged within a range of diameters from 25 to 50 μm.

Histopathologic and Immunohistochemical Analysis

The degrees of monocyte infiltration were evaluated by immunostaining with the ED-1 (analogue of human CD68) antibody against monocytes. For quantification, a blind observer counted the number of ED-1–positive cells in 10 fields.[4] Monocytes were also subjected to immunostaining with antibodies against FITC, an epitope (α-p65) on the p65 subunit of NF-κB, or nonimmune mouse IgG. The α-p65 monoclonal antibody recognizes an epitope on the p65 subunit that is masked by bound inhibitor of κB (I-κB).[9] Therefore, this antibody exclusively detects activated NF-κB.[12]

Electrophoretic Mobility-Shift Assays

Nuclear extracts were prepared from the whole-lung homogenates using a nuclear extract kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Thermo Science) according to the manufacturer's instructions. The protein was measured using a BCA Protein Assay kit (Thermo Science). For NF-κB activation, a nonradioactive electrophoresis mobility-shift assay kit (AY1030, Panomics) was used according to the manufacturer's instructions. Five μg of nuclear protein were incubated for 30 minutes at room temperature with a biotinylated oligonucleotide containing the NF-κB binding site, and then the samples were separated on a nondenaturing polyacrylamide gel and blotted onto a positively charged nylon membrane. After blotting, the oligos on the membrane were fixed using a UV cross-linker oven. Then, the membrane was incubated with streptavidin-horseradish-peroxidase solution at room temperature for 15 minutes and with detection reagents for 5 minutes. Nuclear proteins that were bound to the NF-κB binding site were detected by chemiluminescence with the use of the LAS-1000 detection system (Fujifilm).

Real-Time Quantitative RT-PCR

Real-time PCR amplification was performed with the rat cDNA with the use of the ABI PRI8:21 PM 7000 Sequence Detection System (Applied Biosystems), as described previously.[12] TaqMan primer/probes for MCP-1, TNF-α, IL-1, IL-6, intercellular adhesion molecule 1, and GAPDH, which served as the endogenous reference, were purchased from Applied Biosystems (Assay-on-Demand gene expression products Rn00580555, Rn99999017, Rn00580432, Rn00561420, and Rn00564227 and TaqMan Rodent GAPDH Control Reagents, respectively).

Intracellular Delivery of NPs Incorporated With an FITC-Labeled NF-κB Decoy to Human Monocytes and Pulmonary Arterial Smooth Muscle Cells

The human monocyte cell line THP-1 was obtained from the German Collection of Micro-organisms and Cell Cultures and was used between passages 4 and 8. Cells were cultured in RPMI 1640 with 10% FBS in a humidified atmosphere of 5% CO_2 in air. The cell density was adjusted to 10^6 cells per milliliter in 1 mL of serum-free medium in 35-mm-diameter dishes. The cells were serum deprived 24 hours before the experiment. The growth medium was replaced with FITC-conjugated NF-κB decoy encapsulated PEG-PLGA NP suspension medium (0.5 mg/mL) and then further incubated for 1 hour. At the end of the experiment, the cells were washed 3 times with PBS to eliminate excess NPs that were not incorporated into the cells. Then, the cells were fixed with 10% cold methanol, and nuclei were counterstained with propidium iodide. Cellular uptake of FITC-conjugated NF-κB decoy-encapsulated PEG-PLGA NPs was evaluated by fluorescence microscopy.

Human pulmonary artery smooth muscle cells (PASMCs) were obtained from Cambrex Bio Science, Inc, and cultured as described previously. Cells were used between passages 4 and 8. Human PASMCs were seeded on chambered cover glasses and incubated at 37°C/5% CO_2 until the cells were subconfluent. The following treatments were performed in the same manner.

Lipopolysaccharide-Induced Activation of Human Monocytes

Bacterial lipopolysaccharide (serotype 0111:B4; Sigma) was added at 1 μg/mL to the cells as indicated for each experiment. NF-κB decoy at 5 μg/mL, NF-κB decoy-encapsulated NPs containing 0.1 mg/mL of PEG-PLGA NP and 5 μg/mL of NF-κB decoy, or the vehicle alone was added to the wells simultaneously. Four hours later, the cells were washed 3 times with PBS. NF-κB pathway activity was measured using a TransAM NF-κB p65 ELISA-based assay kit (Active Motif). Nuclear extracts of THP-1 were prepared with the NE-PER kit (Pierce) according to the manufacturer's protocol. All of the procedures were carried out at 4°C. Protein concentration was determined by BCA assay, and 20 μg of protein from each sample were used in the assay. Samples were placed along with 30 μL of binding buffer on a 96-well plate to which oligonucleotides containing an NF-κB consensus binding site had been immobilized. Plates were incubated for 1 hour on a shaker. During this time, the activated NF-κB contained in the sample specifically bound to this nucleotide. The plate was then washed, and the NF-κB complex bound to the oligonucleotides was detected using a primary antibody (100 μL diluted 1:1000 in antibody binding buffer for 1 hour) that is directed against the NF-κB p65 subunit. The plate was then washed again, 100 μL of secondary antibody (diluted 1:1000 in antibody binding buffer) conjugated to horseradish peroxidase was added, and the plate was incubated for 1 hour. The plate was washed again, and 100 μL of developing solution were added. The plate was incubated for 4 minutes away from direct light, 100 μL of stop solution were added, and the plate was read using a plate reader at 450 nm.

Human PASMC Proliferation Assay

Human PASMCs were seeded on 96-well culture plates at 1×10^4 cells per well (n=6 per group) in smooth muscle cells–basal medium with 10% FBS. After 24 hours, the cells were starved for 48 hours in serum-free medium to obtain quiescent nondividing cells. After starvation, 10% FBS was added. Also, a concentration of 1 mg/mL of NF-κB decoy only, NF-κB decoy-encapsulated PEG-PLGA NPs (0.05 mg/mL of PEG-PLGA and 1 mg/mL of decoy), or FITC-encapsulated PEG-PLGA NPs was added to each well. Cells were incubated for another 24 hours after addition of 5′-bromo-2′-deoxyuridine. 5′-Bromo-2′-deoxyuridine incorporation was evaluated by an ELISA kit from Calbiochem.

Statistical Analysis

All of the results are expressed as the mean±SEM. Statistical analysis of differences was performed by ANOVA followed by Bonferroni's multiple comparison test. The survival rates were determined by the Kaplan–Meier method. $P<0.05$ was considered statistically significant.

Results

Activation of NF-κB Expression in Patients With PA6H and in MCT-Induced PAH Rats

Localization of NF-κB activation was examined by immunohistochemical studies in lung tissue from patients using the antibody against α-p65.[9] An intense immunoreactivity of α-p65 was noted primarily in alveolar macrophages and to some extent in small pulmonary arterial lesions (mainly in smooth muscle cells in the medium) from 4 patients with PAH (Figure 1A and Figure S1A). This NF-κB activation was associated with positive staining of MCP-1 and IL-6. In contrast, none at all of α-p65 was detected in 2 control patients whose deaths were not attributed to lung disease (Figure S1B).

In MCT-induced PAH rats, activation NF-κB was noted mainly in alveolar macrophages and weakly in pulmonary artery lesions 7 days after MCT administration (Figure 1B and 1C). An electrophoretic mobility-shift assay was performed to detect the DNA binding activity of NF-κB (Figure S2). The binding activity of the lung increased in rats after MCT injection, which peaked on day 3 and decreased on day 7.

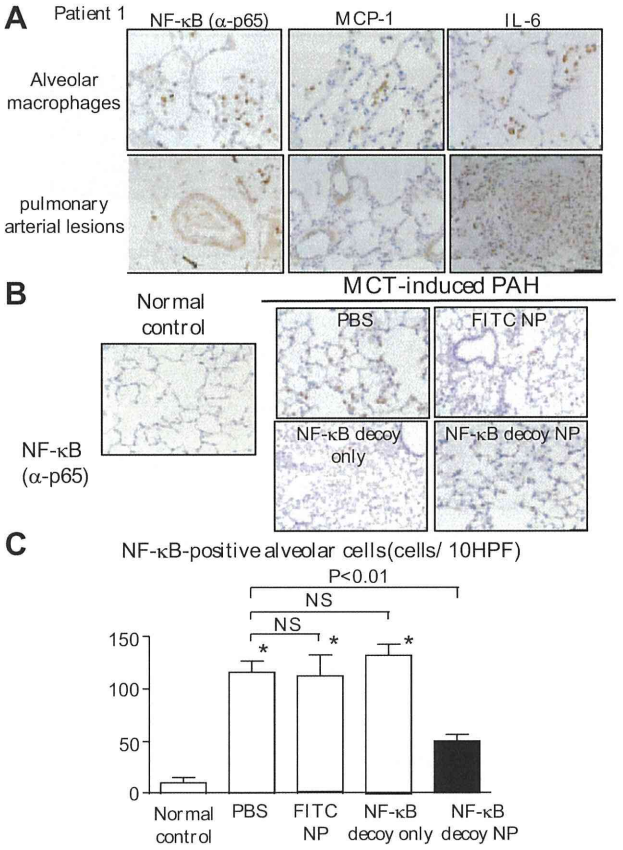

Figure 1. NF-κB activation in patients with PAH and rats with MCT-induced PAH and the effect of intratracheal instillation of NF-κB decoy NPs on NF-κB activation in rats. A, Micrographs of cross sections of the lung from patient 1 stained immunohistochemically with NF-κB (α-p65), MCP-1, and IL-6. Pictures stained with nonimmune IgG control are shown in the inset. Scale bar: 50 μm. B, Micrographs of cross sections of the lung stained immunohistochemically with NF-κB (α-p65) from normal rats and PAH rats 7 days after MCT injection. Scale bar: 50 μm. C, Effects of NF-κB decoy NPs on infiltration of NF-κB (α-p65)–positive cells 7 days after MCT injection. Data are mean±SEM (n=4 each). *$P<0.01$ vs PBS vs normal control.

Effects of Intratracheal Treatment With NF-κB Decoy NP on NF-κB Activation

Single intratracheal instillation of NF-κB decoy NPs, but not FITC NPs or NF-κB decoy only, resulted in marked attenuation of the increased NF-κB (α-p65) activity 7 days after MCT injection (Figure 1B and 1C). Treatment with NF-κB decoy NP markedly attenuated the DNA binding activity of NF-κB after MCT injection (Figure S2).

Because NF-κB was activated in alveolar monocytes and small pulmonary arterial smooth muscle cells in animals and humans with PAH, the effects of NF-κB decoy NPs on NF-κB activity were examined in the human monocyte cell line (THP-1) and in PASMCs in vitro (Figure S3). When those cultured cells were incubated with FITC-labeled NF-κB

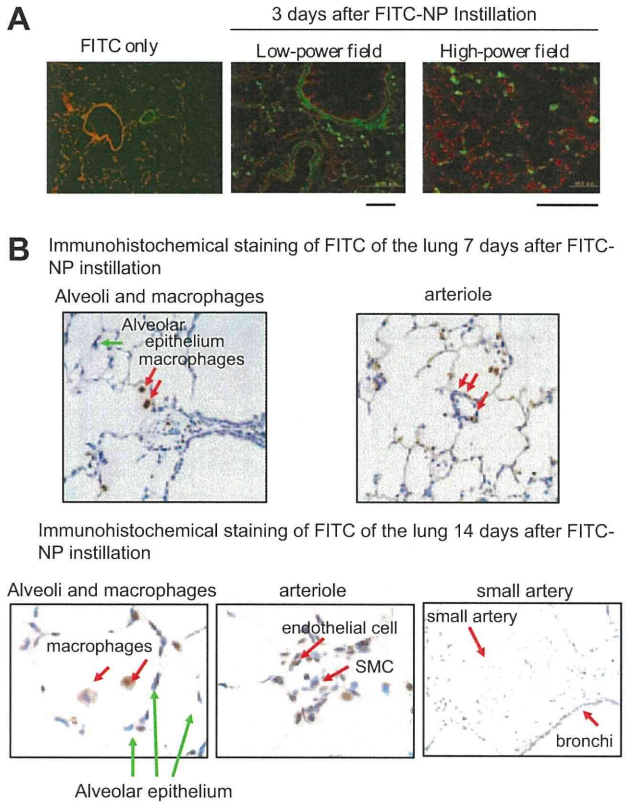

Figure 2. Localization of FITC after FITC-labeled NF-κB decoy NPs postinstillation in the rat lung. A, Fluorescent micrographs of cross sections from lung instillated with FITC only and FITC-labeled NF-κB decoy NPs on day 3 postinstillation. Nuclei were counterstained with propidium iodide (red). Scale bars: 100 μm. B, Micrographs of cross sections stained immunohistochemically against FITC from lung instillated intratracheally with FITC-NPs on days 7 and 14 postinstillation. Scale bars: 100 μm.

decoy NPs for 60 minutes, they were exclusively positive for intracellular localization of FITC. Treatment with NF-κB decoy NPs, but not but with FITC-NPs only or NF-κB decoy only, prevented NF-κB activation in THP-1 cells and attenuated proliferation of human PASMCs.

Localization of FITC-Labeled NF-κB Decoy NPs in the Lung of MCT-Induced PAH

Localization of FITC was examined after a single intratracheal instillation of FITC-labeled NF-κB decoy NPs in animals injected with MCT. Histopathologic examination of lung sections showed that strong FITC signals were detected only in FITC-NP–instillated lung 3 days after instillation, whereas no FITC signals were observed in control noninjected lungs or in lungs injected with FITC only (Figure 2A). There were the FITC-positive cells in bronchi and alveoli, alveolar macrophages, and small arteries. Immunofluorescent staining revealed FITC signals localized mainly in small arteries and arterioles, as well as in small bronchi and alveoli, 7 and 14 days after instillation of FITC-NPs (Figure 2B). FITC signals were not detected in remote organs (liver, spleen, kidney, and heart) on days 1, 3, and 7 (data not shown).

Effects of NF-κB Decoy NP on the Development of PAH in the Rat Model of MCT-Induced PAH

As reported previously by us and by other investigators,[5,16,17] the injection of MCT results in severe PAH (increased RV systolic pressure and RV hypertrophy; Figure 3) associated with small pulmonary arterial remodeling (Figure 4) and increased infiltration of ED-1–positive monocytes (Figure 4) 3 weeks after MCT injection. Single intratracheal treatment with NF-κB decoy NPs but not with NF-κB decoy only or FITC-NPs attenuated the development of PAH (Figure 3), small pulmonary arterial remodeling (Figure 4), and inflammation (Figure 4).

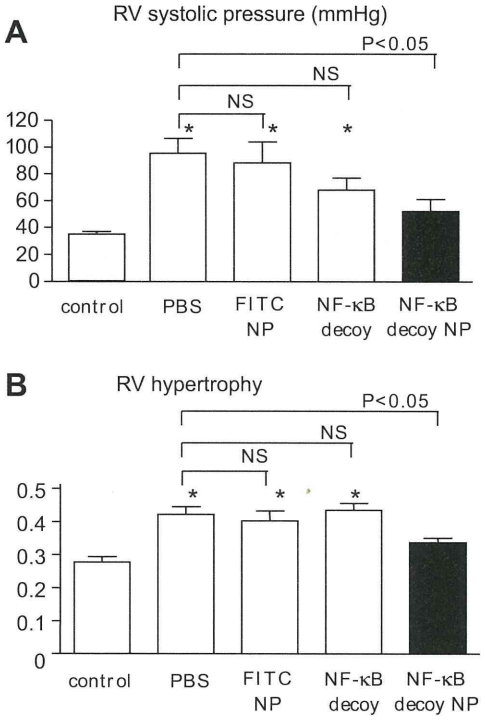

Figure 3. Effects of NF-κB decoy NPs on RV systolic pressure and RV hypertrophy 3 weeks after MCT injection. A, RV systolic pressure 21 days after MCT injection in 4 groups. Data are mean±SEM (n=6 each). *$P<0.05$ vs normal control. B, RV hypertrophy (the ratio of RV/[LV+S]) 21 days after MCT injection in the different treatment groups. Data are mean±SEM (n=6 each). *$P<0.05$ vs normal control.

Effects of NF-κB Decoy NPs on Expression of Proinflammatory Factors

As reported previously,[3,4] MCT-induced PAH was associated with increased gene expression of proinflammatory factors. Treatment with NF-κB decoy NPs significantly reduced the increased gene expression of MCP-1, TNF-α, and IL-1β (Figure 5). NF-κB decoy NPs tended to decrease the expression of IL-6 and intercellular adhesion molecule-1.

In Vitro NP Release Kinetics

An analysis of the in vitro FITC release kinetics from FITC-NP showed an early burst of FITC release such that ≈40% of the total amount ultimately released was present on day 1, followed by sustained release of the remaining FITC over the next 28 days (Figure S4).

Effects of NF-κB Decoy NPs on Survival

Treatment with NF-κB decoy NPs 21 days after MCT injection significantly ($P<0.01$) improved the survival rate (Figure 6).

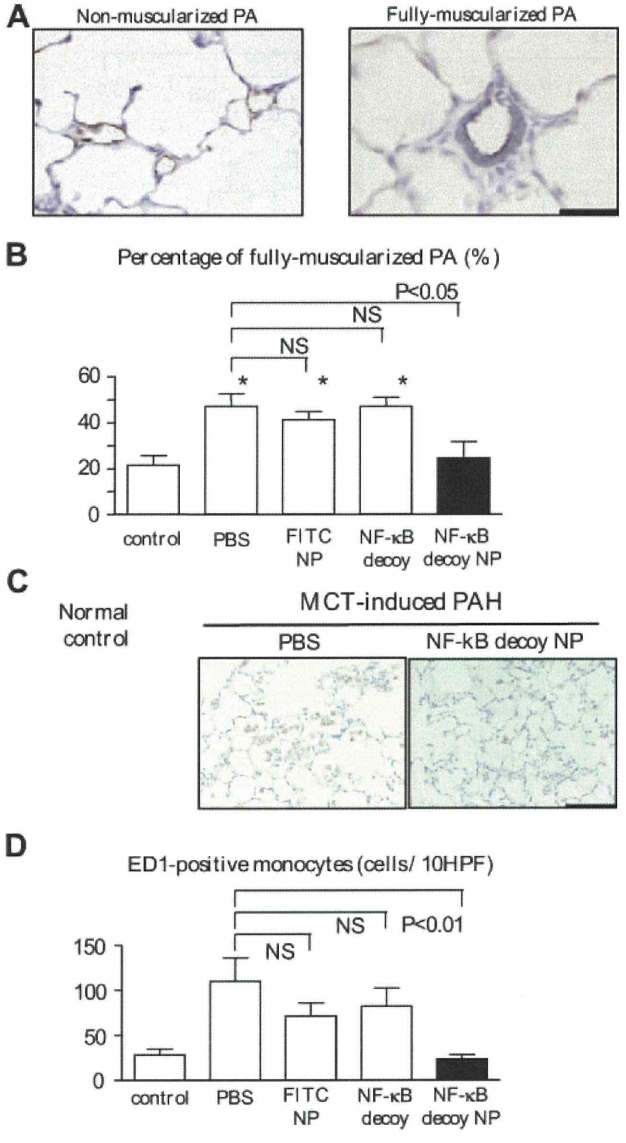

Figure 4. Effects of NF-κB decoy NPs on small pulmonary arterial remodeling and infiltration of monocytes. A, Representative micrographs of nonmuscularized and fully muscularized small pulmonary arteries stained immunohistochemically against the endothelial layer (brown) and medial smooth muscle cells (blue). Scale bar: 50 μm. B, The percentage of fully muscularized small pulmonary arteries in the different treatment groups. Data are mean±SEM (n=6 each). *$P<0.05$ vs normal control. C, Representative micrographs of pulmonary alveoli stained immunohistochemically for ED-1–positive monocytes. Scale bar: 50 μm. D, Infiltration of ED-1–positive monocytes into the lung (the number of positive cells per 10 high-power field cross sections). Data are mean±SEM (n=6 each). *$P<0.01$ vs normal control.

Discussion

The present study demonstrates for the first time that intratracheal instillation of PEG-PLGA NPs is an excellent system for drug delivery of NF-κB decoy to the lung. The FITC signals were detected not only in small bronchial tracts but also in alveolar macrophages and small pulmonary arteries for ≤14 days after a single instillation. After cellular uptake of NPs, NPs might slowly release encapsulated decoy into the cytoplasm as PLGA is hydrolyzed. This might protect the encapsulated decoy from intracellular degradation before its arrival at the nuclear target. Our in vitro studies in cultured human monocytes and pulmonary arterial smooth muscle cells support this notion. Therefore, this platform nanotechnology may represent a novel NP-mediated drug delivery system for treatment of severe lung diseases, including PAH.

The present study also reports a pivotal role of NF-κB in the pathogenesis of PAH. Recently, Sawada et al[19] and Huang et al[20] reported that systemic daily administration of pyrrolidine dithiocarbamate, a nonspecific inhibitor of NF-κB, attenuated the development of MCT-induced PAH. Pyrrolidine dithiocarbamate is known to be a low molecular weight thiol compound and has anti-inflammatory and antioxidant activity independent of the NF-κB pathway. Indeed, in a study by Huang et al,[20] pyrrolidine dithiocarbamate treatment had no effect on MCT-induced NF-κB activation. In contrast, we found in the present study that NF-κB is activated in alveolar macrophages and small pulmonary arteries associated with NF-κB–dependent inflammatory factors (eg, MCP-1, IL-1, and TNF-α) in patients with PAH and rats with MCT-induced PAH, and blockade of NF-κB activation by a single intratracheal instillation of NF-κB decoy NPs reduced inflammatory changes. These data suggest that NF-κB might be pivotal in mediating inflammatory changes seen in PAH.

We also found that intratracheal instillation of NF-κB decoy NPs prevented the development of PAH (increased RV pressure, RV hypertrophy, and pulmonary artery remodeling) in the prevention protocol. We and others have reported that blockade of MCP-1 reduces vascular pathology after vascular injury[9,21–25] and the development of PAH.[5,6] In addition, as we reported in human coronary artery smooth muscle cells in vitro,[12,26] we found that NF-κB decoy NPs attenuated proliferation of human PASMCs in vitro. Therefore, the beneficial effects of NF-κB decoy NPs can be attributable to inhibition of inflammation and smooth muscle cell proliferation resulting from reduced NF-κB activation.

Furthermore, we found that a single intratracheal treatment of NF-κB decoy NPs 3 weeks after MCT injection improved survival rate in the treatment protocol, suggesting that this NP-mediated NF-κB decoy delivery may have significant therapeutic effects. We did not examine the therapeutic effects of repetitive intratracheal instillation of NF-κB decoy NPs, because it is technically difficult to perform multiple intratracheal instillation of this NP system in rats and other small animals. For translation of our present findings into clinical medicine, further studies are needed to investigate whether repetitive delivery of NPs into lungs produces greater therapeutic effects over time.

Several points are worth mentioning with regard to potential clinical applicability. First, from a toxicological point of view, no adverse reactions, eg, pulmonary inflammation, after exposure to a single intratracheal instillation of FITC-NPs (PEG-PLGA at 1 mg per body) or NF-κB decoy NPs (NF-κB decoy at 50 μg per body in rats weighing 250 to 300 g) were noted in the rat model, suggesting that the NPs used in this study may not cause an adverse reaction. However, the 3-week observation period for this NP system might be too short to determine its safety. Second, we reported recently that neither intravenous injection of the NF-κB decoy at 1 mg per body in monkeys nor deployment

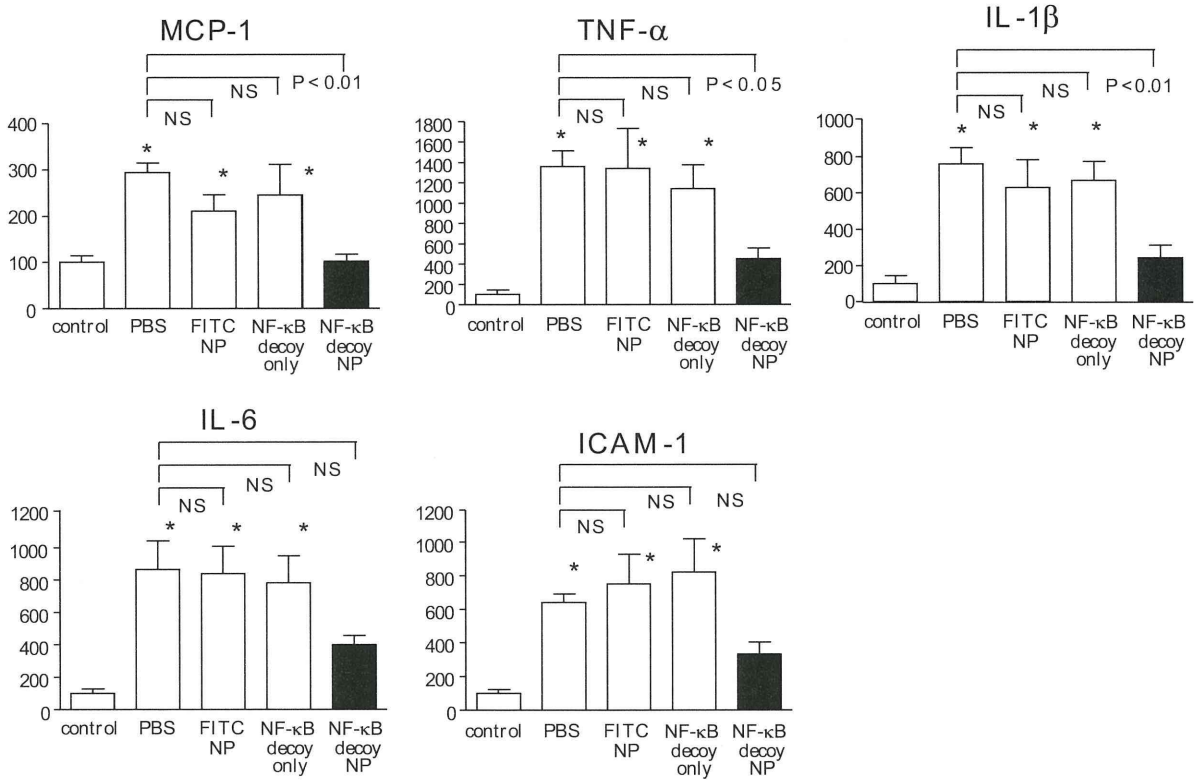

Figure 5. Effects of NF-κB decoy NPs on mRNA levels of various inflammatory and proliferative factors 21 days after MCT injection (n=5 each). *$P<0.01$ vs normal control.

of an NF-κB decoy-eluting stent (≈600 μg per stent) in rabbits showed systemic adverse effects.[12] More important are the findings of a clinical trial that we completed recently to test the feasibility and safety of the NF-κB decoy. The decoy was transfected into the stented coronary artery sites at doses of 1000, 2000, or 4000 μg per body via a channel balloon catheter immediately after successful percutaneous coronary intervention in 18 patients with flow-limiting coronary stenosis.[27] The patients showed low restenosis rates and no evidence of systemic adverse effects during the 6-month observation period. These data support the notion that NF-κB decoy can be applied in a clinical setting. Third, this NP system itself is not suitable for inhalant therapy, because it is known that most inhaled NPs are exhaled rather than being delivered into the lung.[28] In contrast, microparticles with aerodynamic diameters between 2 and 8 μm reach small bronchi. However, the microparticles are easily recognized and eliminated by the mucociliary clearance system and alveolar macrophages immediately after they reach the small bronchi.[28] In contrast, polymeric NPs escape the clearance system of the lung when they are delivered into small bronchi and are, thus, taken up by alveoli, macrophages, and pulmonary small vessels. Therefore, to use this NP system for inhalant therapy, we need to develop the nanocomposite microsized particles[28] that will decompose to NPs after reaching the small bronchi.

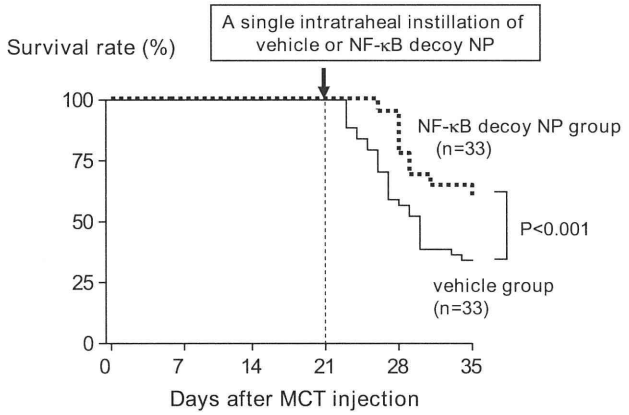

Figure 6. Effects of NF-κB decoy NPs on survival rate. Survival rate analyzed by the Kaplan–Meier method in vehicle and NF-κB decoy NP groups.

Perspectives

This study has shown that NF-κB is activated in pulmonary arterial lesions in patients with PAH and in rats with MCT-induced PAH, and blockade of NF-κB by NP-mediated NF-κB decoy delivery not only prevented the development of MCT-induced PAH in the prevention protocol but also improved survival rate in the treatment protocol. These data support the notion that NF-κB plays a pivotal role in the pathogenesis of PAH and, thus, represents a new therapeutic target for PAH. This nanotechnology platform may be developed as a more effective and less invasive nanomedicine in PAH therapy.

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Disclosures

K.E. and R.M. hold a patent on the results reported in this study. The remaining authors report no conflicts.

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ONLINE SUPPLEMENT

Nanoparticle-Mediated Delivery of NF-κB Decoy into Lungs Ameliorates Monocrotaline-Induced Pulmonary Arterial Hypertension

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Conflict of Interest: Drs. Egashira and Morishita hold a patent on the results reported in this study. The remaining authors report no conflicts.

Online supplement word count (excluding title page): 425

Expanded Materials and Methods

Histopathological and immunohistochemical examination of human lungs

Human lung tissue was obtained from autopsy specimens in 4 patients who died due to idiopathic pulmonary arterial hypertension (PAH) and 2 patients died due to non-lung disease (Figure S1). The lung tissues were isolated and fixed with formalin, which were dehydrated, embedded in paraffin, and cut into 5-μm thick slices. The slices were then stained with Hematoxylin and Eosin solution or immunostained with either an epitope (α-p65) on the p65 subunit of nuclear factor-κB (NF-κB) (α-p65, 1:100, Boehringer Mannheim, Roche Diagnostics, Basel, Switzerland), [1, 2] monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6 (all from R&D systems) or nonimmune mouse IgG (Dako). This study protocol was approved by the Committee on Ethics on Clinical Study, Kyushu University Faculty of Medicine.

Preparation of nanoparticles The NF-κB decoy oligodeoxynucleotides sequences are 5'-CCTTGAAGGGATTTCCCTCC-3' and 3'-GGAACTTCCCTAAAGGGAGG-5'. GGGATTTCCC is the consensus sequence for the NF-κB binding site. The decoy is directed against the NF-κB binding site in the promoter region that corresponds to NF-κB-responsive genes.[3, 4] The decoy works to inhibit binding of this transcription factor to the promoter region.[3, 4] The NF-κB decoy have been shown to bind to free NF-κB, preventing NF-κB transactivation of the cytokine genes. For trace experiments, fluorescein-isothiocyanate (FITC, Dojindo laboratories, Kumamoto, Japan)-labeled NF-κB decoy was also prepared.

A poly-(ethylene glycol)-*block*-lactide/glycolide copolymer (PEG-PLGA) with an average molecular weight of 22,900 and a PLGA copolymer ratio of lactide to glycolide of 75:25 (Absorbable Polymers International,USA) was used as a wall material for the NP.

PEG-PLGA nanoparticles (NP) encapsulated with FITC, NF-κB decoy or FITC-labeled NF-κB decoy were prepared by solvent diffusion method. [5, 6] Hydrophobic PLGA are dissolved in the ethylacetate. Hydrosoluble PEG are first dissolved in water and emulsified in the PLGA dissolving organic phase. An oil phase solution of PEG-PLGA was slowly poured into an aqueous solution containing PVA and emulsified using a microtip probe sonicator. The PEG-PLGA copolymer solution also contained 5 % (w/v) FITC as fluorescence marker and 5 % (w/v) NF-κB decoy in preparation of fluorescence marker and NF-κB decoy encapsulated PEG-PLGA nanoparticles, respectively. The resulted oil-in-water emulsion was then stirred at room temperature. The formed PEG-PLGA nanoparticles were collected by centrifugation and washed with Millipore water for 3 times to remove excessive emulsifier.

The mean particle size was analyzed by light scattering method (Microtrack UPA150; Nikkiso, Tokyo, Japan). A sample of nanoparticulate suspension in distilled water was used for particle size analysis. An average diameter of PEG-PLGA NP was 44 nm with a narrow size distribution (see Figure in this page).

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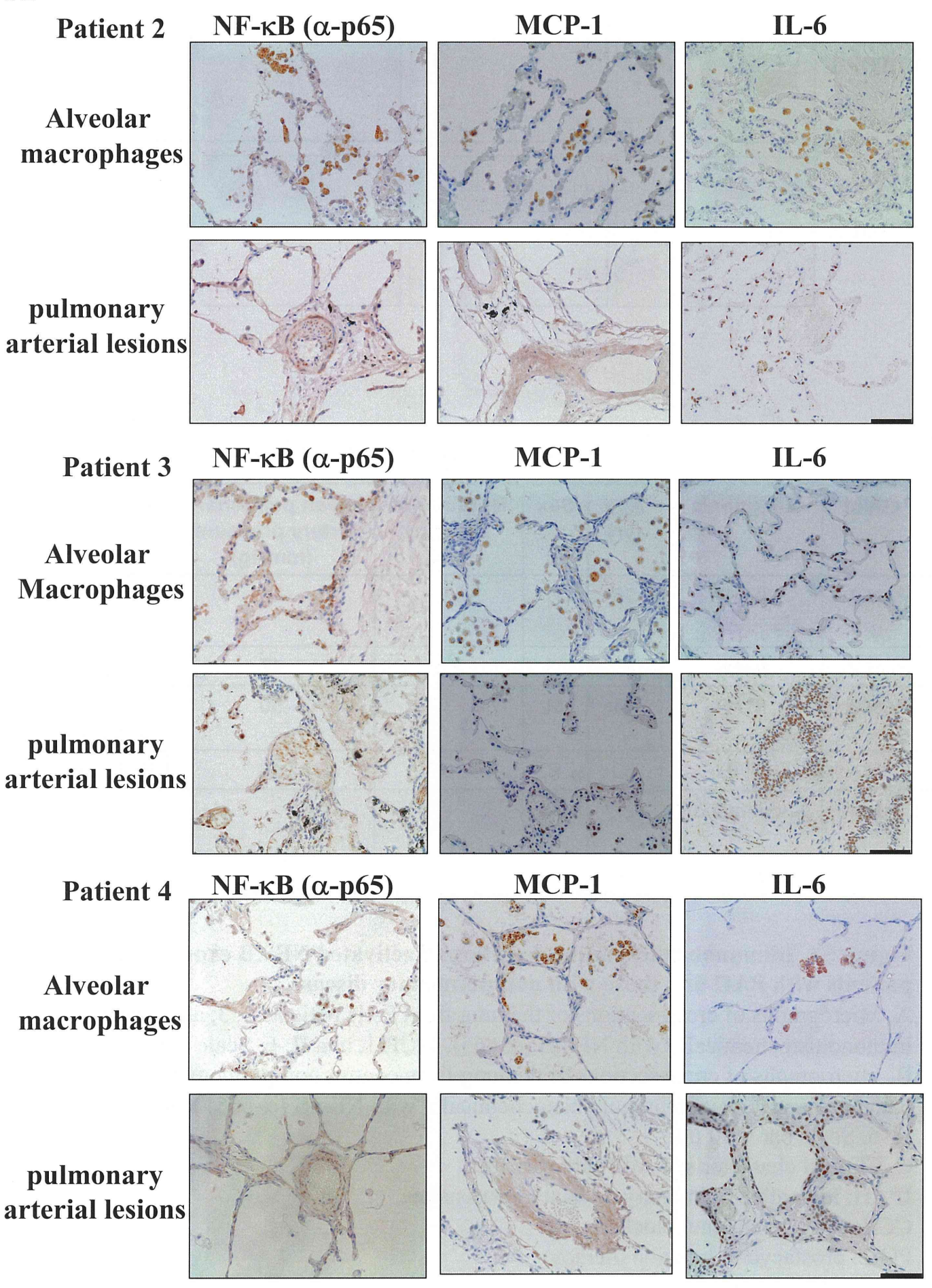

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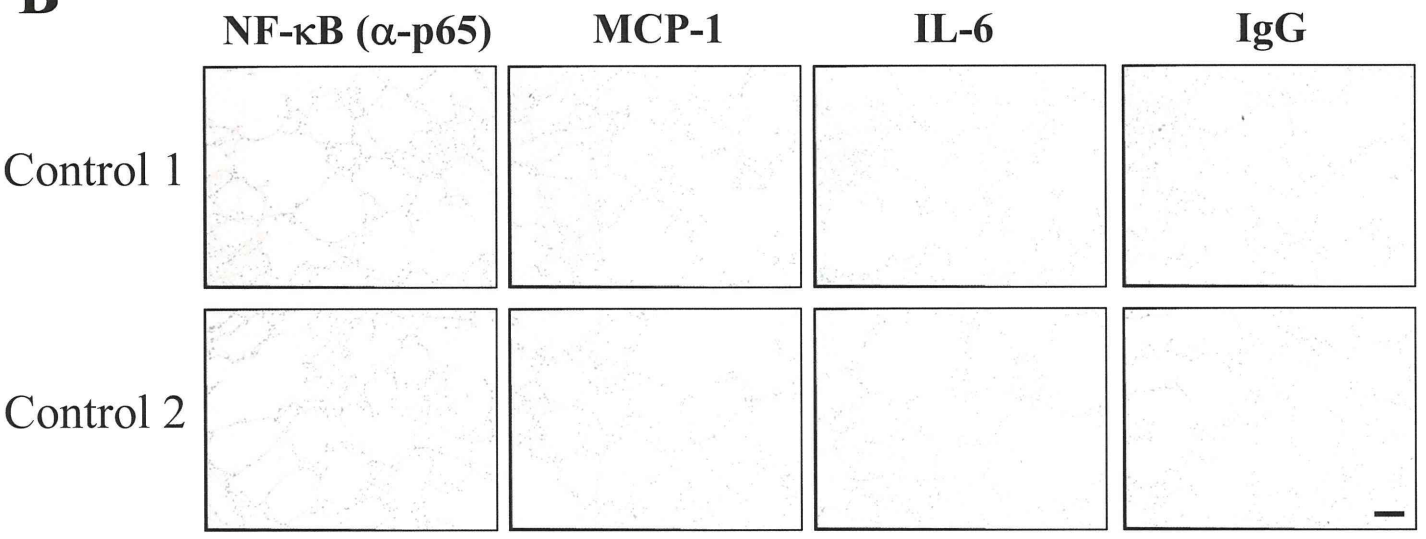

C

Clinical characteristics of patients

Patient	Diagnosis	Age (yr)	Sex	Duration of Disease (yr)	mean pulmonary artery pressure (mmHg)	treatments
PAH 1	IPAH	35	F	2	48	Beraprost, Bosentan
PAH 2	IPAH	22	M	2.5	47	Beraprost
PAH 3	IPAH	14	M	3	50	Beraprost, CCB, HOT
PAH 4	IPAH	50	F	3	46	PGI2, HOT
Control1	Sudden death	27	F	-	-	no
Control2	peritonitis	29	F	-	-	no

Figure S1. Immunohistochemical detection of activated NF-κB expression in patients with PAH and those with non-pulmonary disease.
A, Micrographs of cross-sections of the lung from PAH patients 2, 3, and 4 stained immunohistochemically with NF-κB (α-p65), MCP-1, and IL-6. Scale bar = 50 μm
B, Micrographs of cross-sections of the lung from control non-pulmonary disease patients 1 and 2 stained immunohistochemically with NF-κB (α-p65)), MCP-1, and IL-6. Scale bar = 50 μm.
C, Clinical characteristics of study patients.
IPAH: idiopathic pulmonary arterial hypertension,
CCB: calcium channel blocker,
PGI2: prostacyclin infusion therapy,
HOT: home oxygen therapy.

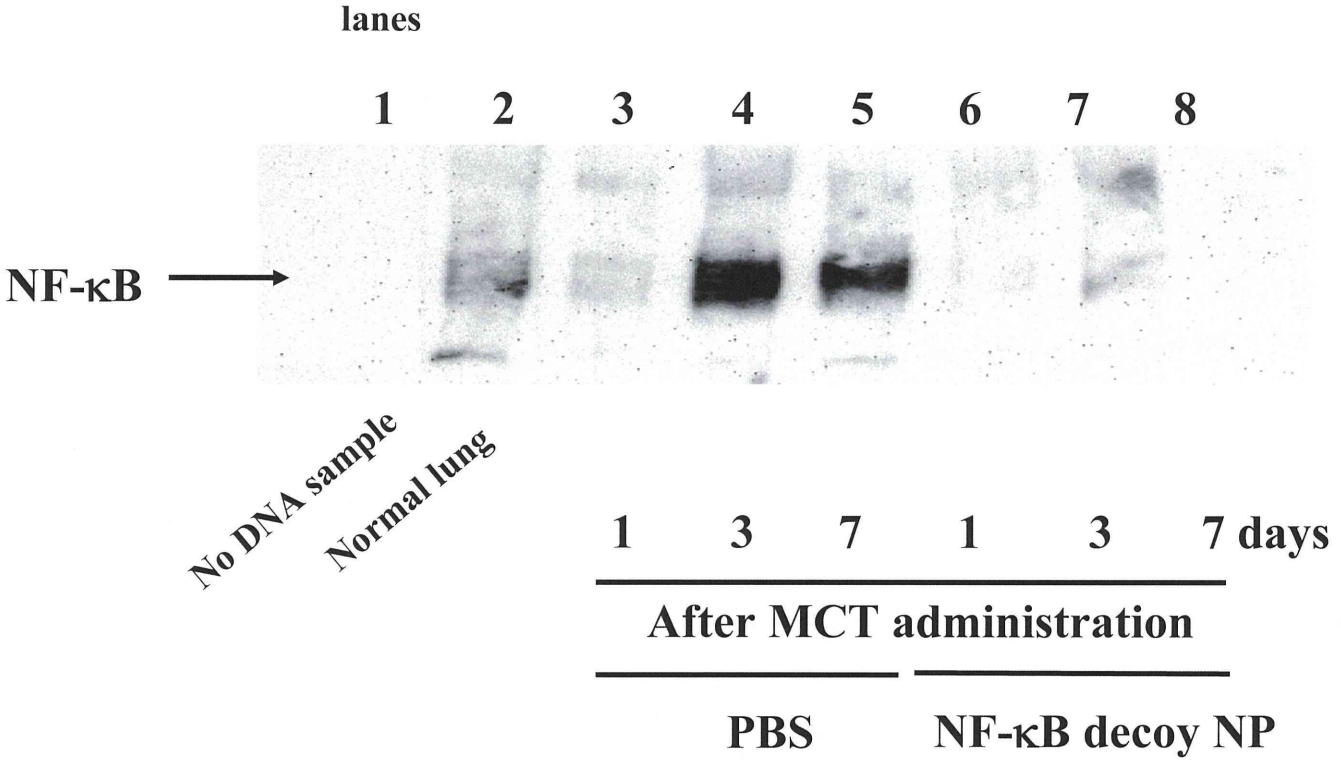

Figure S2. Assessment of DNA binding activity of NF-κB by electrophoretic mobility shift assay.

Lane 1: Labeled probe with no DNA sample. lane 2: Labeled probe with normal lung DNA. Lanes 3, 4, and 5: Labeled probe with lung DNA from PBS-treated animals 1, 3, and 7 days after MCT injection. Lanes 6, 7, and 8: Labeled probe with lung DNA from animals treated with NF-κB decoy NP 1, 3, and 7 days after MCT injection.
These DNA binding assay experiments were repeated three times; results from all three trials were similar and representative results are shown.

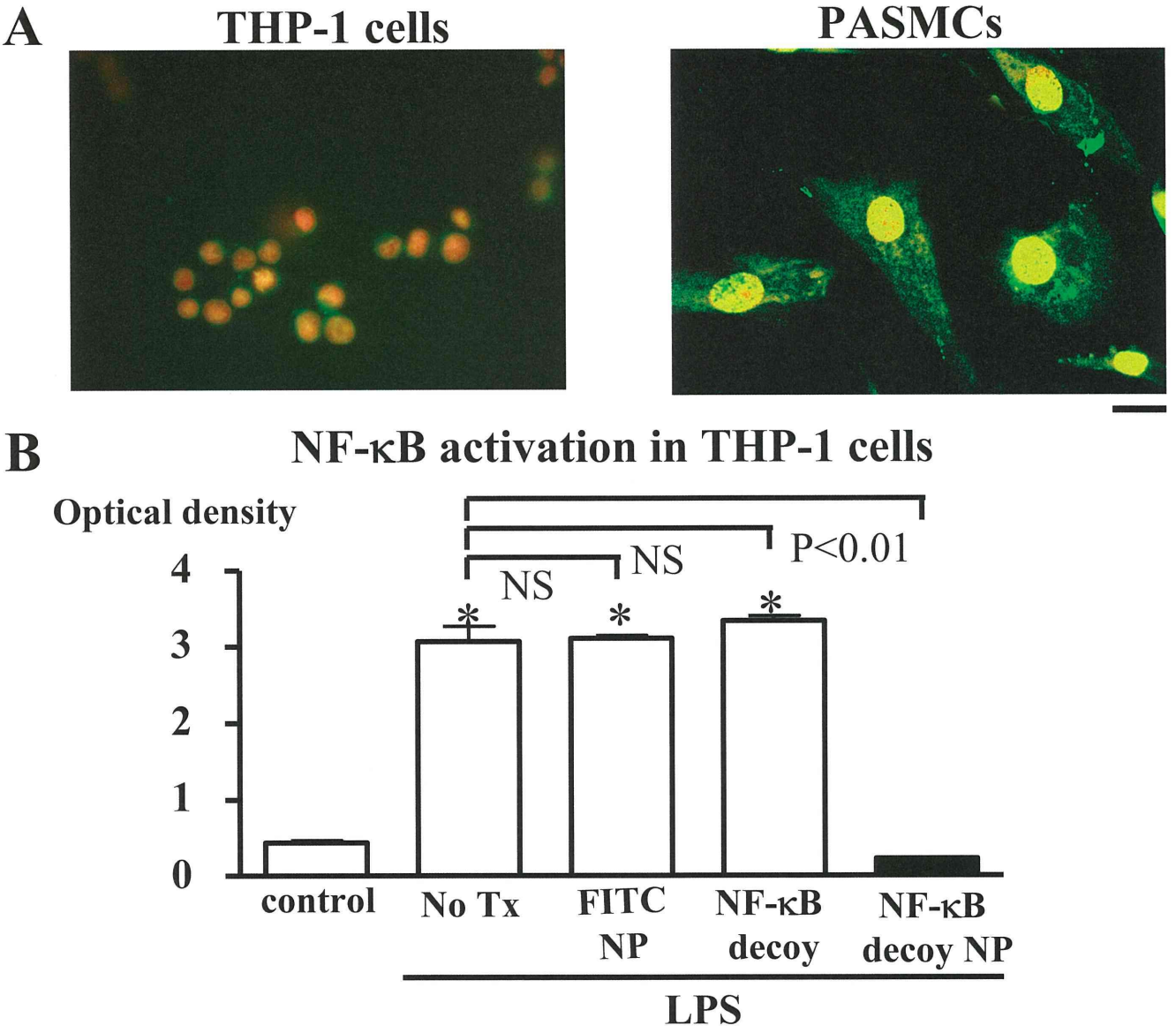

Figure S3. *In vitro* cellular uptake of FITC-labeled NF-κB decoy NP and inhibitory effect of NF-κB decoy NP on NF-κB activation of human monocyte cell line (THP-1 cells) and proliferation of human pulmonary artery smooth muscle cells (hPASMC).

A, Fluorescence microscopic pictures of human monocyte cell line (THP-1 cells) and hPASMC incubated with FITC-labeled NF-κB decoy-NP for 60 minutes. Nuclei were counterstained with propidium iodide. Scale bar = 20 μm.

B, Effects of NF-κB decoy-NP on LPS-stimulated activation of NF-κB (ELISA-based DNA binding assay against NF-κB p65 subunit: arbitrary unit) in THP-1 cells. Data are mean ± SEM ($n = 6$ each). *$P < 0.01$ versus normal control.

C, Effects of NF-κB decoy NP on FBS-stimulated proliferation of hPASMC (BrdU incorporation index: arbitrary unit). Data are mean ± SEM ($n = 6$ each). *$P < 0.01$ versus normal control. Data are percent changes from control (100 %).

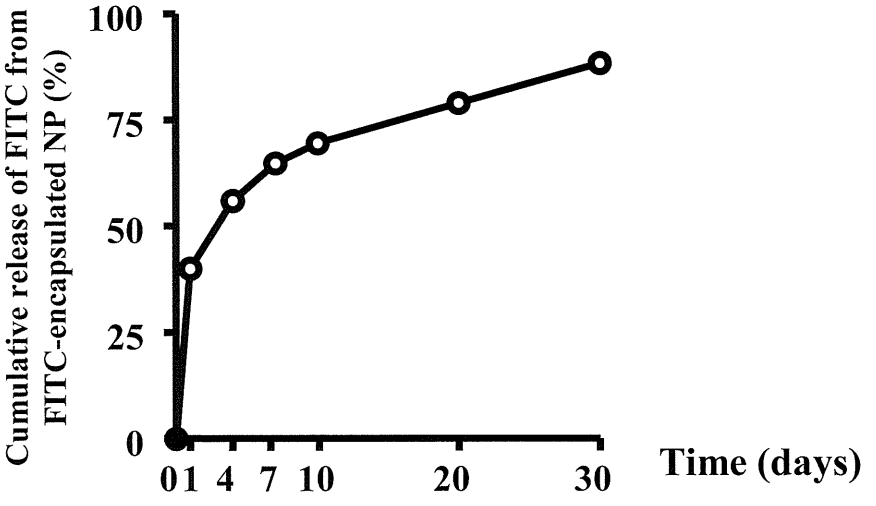

Figure S4. In vitro time course of cumulative FITC release from the FITC-incorporated NP (n = 8 each). The percentage of incremental quantities of released FITC was plotted against time.

Nanoparticle-Mediated Delivery of Pitavastatin Into Lungs Ameliorates the Development and Induces Regression of Monocrotaline-Induced Pulmonary Artery Hypertension

Ling Chen, Kaku Nakano, Satoshi Kimura, Tetsuya Matoba, Eiko Iwata, Miho Miyagawa, Hiroyuki Tsujimoto, Kazuhiro Nagaoka, Junji Kishimoto, Kenji Sunagawa, Kensuke Egashira

Abstract—Pulmonary artery hypertension (PAH) is an intractable disease of the small PAs in which multiple pathogenic factors are involved. Statins are known to mitigate endothelial injury and inhibit vascular remodeling and inflammation, all of which play crucial roles in the pathogenesis of PAH. We tested the hypothesis that nanoparticle (NP)-mediated delivery of pitavastatin into the lungs can be a novel therapeutic approach for the treatment of PAH. Among the marketed statins, pitavastatin was found to have the most potent effects on proliferation of PA smooth muscle cells in vitro. We formulated pitavastatin-NP and found that pitavastatin-NP was more effective than pitavastatin alone in inhibiting cellular proliferation and inflammation in vitro. In a rat model of monocrotaline-induced PAH, a single intratracheal instillation of NP resulted in the delivery of NP into alveolar macrophages and small PAs for up to 14 days after instillation. Intratracheal treatment with pitavastatin-NP, but not with pitavastatin, attenuated the development of PAH and was associated with a reduction of inflammation and PA remodeling. NP-mediated pitavastatin delivery was more effective than systemic administration of pitavastatin in attenuating the development of PAH. Importantly, treatment with pitavastatin-NP 3 weeks after monocrotaline injection induced regression of PAH and improved survival rate. This mode of NP-mediated pitavastatin delivery into the lungs is effective in attenuating the development of PAH and inducing regression of established PAH, suggesting potential clinical significance for developing a new treatment for PAH. (***Hypertension.* 2011;57:343-350.**) • **Online Data Supplement**

Key Words: pulmonary hypertension ■ nanotechnology ■ pitavastatin ■ inflammation ■ leukocytes

Pulmonary artery hypertension (PAH) is an intractable disease of the small PAs resulting in progressive increases in pulmonary vascular resistance, right ventricular (RV) failure, and ultimately premature death.[1,2] Mortality from PAH remains high, even after introduction of vasodilator therapies such as prostacyclin infusion, endothelin receptor antagonists, and phosphodiesterase inhibitors (which have raised the 5-year survival rate to ≈50%). Although these drugs were originally developed for non-PAH vascular diseases, they were introduced into treatment for clinical PAH on the basis of the vasodilator hypothesis. Therefore, a new idea that might lead to a breakthrough curative treatment for PAH is urgently needed.

In addition to vasoconstriction, other multiple factors (endothelial injury/apoptosis, obstructive vascular remodeling, proliferation, and inflammation) play an important role in the mechanism of PAH.[1,2] Therefore, we hypothesized that a controlled, local delivery system targeting a battery of those pathogenic factors intrinsic to PAH pathology would be a favorable therapeutic approach with high translational potential to clinical medicine. In this respect, we focused on the vasculoprotective effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, the so-called statins. Statins are known to increase expression and activity of endothelial nitric oxide synthase (eNOS) and thus ameliorate endothelial injury.[3–6] Prior studies have reported that systemic administration of statins attenuates monocrotaline (MCT)-induced and hypoxia-induced PAH in animals.[7–9] These beneficial therapeutic effects of statins on PAH, however, were observed after daily administration of high doses of statins, a regimen that could lead to serious adverse side effects in the clinical setting. However, not all studies have reported beneficial effects of statins with regard to PAH in animal models.[10,11] We recently reported that (1) intratracheal administration of bioabsorbable polymeric nanoparticles (NPs) represented a novel drug delivery system into the lung; and (2) NP-mediated delivery of a nuclear factor (NF)-κB decoy into the lungs effectively inhibited NF-κB–mediated inflammation and thus, attenuated the development and progression of PAH in a rat model of MCT-induced PAH.[12] This

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nanotechnology platform may optimize the efficacy and minimize the potential side effects of drugs.

Therefore, the primary aim of this study was to test the hypothesis that NP-mediated local delivery of statins to the lung is an innovative therapeutic approach for PAH. Pitavastatin was selected as the nanoparticulation compound because this drug has shown the most potent beneficial effects on human endothelial and smooth muscle cells in vitro compared with other statins.[13,14] We then used a rat model of MCT-induced PAH and examined (1) whether this NP-mediated delivery of pitavastatin into the lung is more effective than intratracheal or systemic administration of pitavastatin in attenuating the development of PAH and (2) whether this NP-mediated delivery system induces regression of established PAH.

Materials and Methods

Human PA Smooth Muscle Cell Proliferation Assay

Human PA smooth muscle cells (PASMCs) were seeded on 96-well culture plates at 10^4 cells per well in SmBM. After 24 hours of starvation, 10% fetal bovine serum was added for cell stimulation. In addition, various concentrations of statins (simvastatin, pitavastatin, atorvastatin, losuvastatin, fluvastatin, and pravastatin) or vehicle were added (n=6 per group). Statins were purchased, extracted from products, and purified. Cells were incubated for another 24 hours after addition of 5′-bromo-2′-deoxyuridine, and 5′-bromo-2′-deoxyuridine incorporation was evaluated by an ELISA kit from Calbiochem.

In another set of experiments, a 1.0-mL suspension of pitavastatin at 5 mg/mL, fluorescein isothiocyanate (FITC)–NP (1 mg/mL lactide/glycolide copolymer [PLGA]), pitavastatin-NP containing 1.0 mg/mL PLGA and 5 mg/mL pitavastatin, or vehicle was added to each well (n=6 per group). Cells were incubated for another 4 days, and the cells were fixed with methanol and stained with Diff-Quick staining solution. A single observer counted the number of cells per plate.

Preparation of PLGA-NP

A PLGA with an average molecular weight of 20 000 and a copolymer ratio of lactide to glycolide of 75:25 (Wako Pure Chemical Industries, Osaka, Japan) was used as wall material for the NP. PLGA-NP incorporated with FITC or pitavastatin (Kowa Pharmaceutical Co Ltd, Tokyo, Japan) was prepared by a previously reported emulsion solvent diffusion method in purified water.[15,16] PLGA was dissolved in a mixture of acetone and methanol. Then, FITC or pitavastatin was added to this solution. The resultant PLGA-FITC or PLGA-statin solution was emulsified in a polyvinyl alcohol solution with stirring at 400 rpm by using a propeller-type agitator with 3 blades (Heidon 600G, Shinto Scientific, Tokyo, Japan). After the system was agitated for 2 hours under reduced pressure at 40°C, the entire suspension was centrifuged (20 000*g* for 20 minutes at −20°C). After the supernatant was removed, purified water was added and mixed with the sediment. The wet mixture was then centrifuged again to remove the excess polyvinyl alcohol and the unencapsulated reagent that could not adsorb onto the surfaces of the NPs. After this process was repeated, the resultant dispersion was freeze-dried under the same conditions. The FITC- and pitavastatin-loaded PLGA-NP contained 13% (wt/vol) FITC and 13% (wt/vol) pitavastatin, respectively. A sample of NP suspension in distilled water was used for particle size analysis. The diameter of NPs was 196±29 nm. Surface charge (zeta potential) was also analyzed by Zetasizer Nano (Sysmex, Hyogo, Japan) and was anionic (−15±10 mV at pH 4.4).

Experimental Animal Models

All experiments were reviewed and approved by the committee on ethics on animal experiments, Kyushu University Faculty of Medicine, and were conducted according to the guidelines of the American Physiological Society. Adult male Sprague-Dawley rats (Charles River, Yokohama, Japan; 250 to 300 g body weight) were injected subcutaneously with 60 mg/kg MCT (Wako), which induces severe PAH in 3 weeks.[12,17]

In a prevention protocol, animals were divided into 4 groups that received intratracheal instillation of phosphate-buffered saline (PBS), pitavastatin only (100 μg), FITC-NP (1 mg PLGA), or pitavastatin-NP (100 μg pitavastatin per mg PLGA) immediately after MCT injection. For intratracheal instillation, a 0.1-mL suspension of pitavastatin, FITC-NP, or pitavastatin-NP was injected gently into the trachea of animals, accompanied by an equal volume of air. This dose of pitavastatin was selected because we examined the effects of intratracheal instillation of various concentrations and volumes of pitavastatin suspension (10, 30, 100, or 300 μg per animal in 0.05, 0.1, and 0.2 mL PBS) and confirmed that a 0.1-mL suspension of pitavastatin containing 100 μg pitavastatin was an optimal dose in our experiments. In a treatment protocol, rats were divided into 4 groups that received intratracheal instillation of PBS, pitavastatin only (100 μg), FITC-NP (1 mg PLGA), or pitavastatin-NP (100 μg pitavastatin per mg PLGA) 21 days after MCT injection when severe PAH had already been established. In another set of experiments, 3 other groups received systemic daily oral pitavastatin at doses of 0.3, 1.0, 3.0, and 10 mg/kg, dissolved in 0.5% carboxymethyl cellulose, by gavage from the day of MCT injection until the mice were euthanized on day 21.

Biodistribution of FITC-NP After Intratracheal Administration Into the Lung

Biodistribution of FITC in the lung was examined in rats that received intratracheal instillation of FITC-NP. Animals were euthanized and the tracheas were exposed. The lungs were inflated with a solution of 10% phosphate-buffered formalin (pH 7.4) by using a catheter inserted into the trachea. The lungs were then removed en bloc and placed into 10% phosphate-buffered formalin for a further 12 to 18 hours. After light and fluorescence stereoscopic photographs of the lungs were taken, the tissues were processed and embedded in OCT compound, and cross sections of 5-μm thickness were prepared for detecting NP distribution by fluorescence photomicroscopy. The tissue specimens were also processed and embedded in paraffin according to standard procedures, and 5-μm sections were cut. Sections were further examined to detect NP distribution by immunostaining.

Direct RV Pressure Measurements

Three weeks after MCT administration, the animals were anesthetized with sodium pentobarbital, and polyethylene catheters were then inserted into the right ventricle through the jugular vein and carotid artery for hemodynamic measurements. RV systolic pressure and systemic blood pressure were measured with a polygraph system (AP-601G, Nihon Kohden).[12,17]

Echocardiographic Measurements of RV and PA Hemodynamics

Transthoracic echocardiographic measurements (Vevo 2100 ultrasound system; Primetech Inc) were performed as described previously.[18] Additional details are provided in the online-only Data Supplement (available at http://hyper.ahajournals.org).

Assessment of Right Heart Hypertrophy and PA Remodeling

After systemic arterial and RV pressures were recorded, the animals were euthanized and the lungs and heart were isolated. The RV wall was dissected from the left ventricle and ventricular septum. Wet weight of the right ventricle and of the left ventricle plus ventricular