phantoms containing detailed grey matter and bone structures for nuclear medicine imaging SNM 56th Annual Meeting. Toronto, Canada, 2009

4. Iida H, Zeniya T, Maruno H, Imabayashi E, Matsuda H, Ishida K, Nakazawa M, Watabe H, Akamatsu T. Physical Performance Evaluation of Quantitative SPECT Reconstruction Package - QSPECT SNM 56th Annual Meeting. Toronto, Canada, 2009
5. Iida H. Three-dimensional realistic brain phantom containing bone and grey matter structures for evaluating PET/SPECT imaging techniques BRAIN09. CHICAGO, 2009
6. 飯田秀博. 脳神経核医学画像における部分容積効果の影響と補正のあり方について 第 10 回日本脳神経核医学研究会　シンポジウム. 旭川市, 2009
7. 飯田秀博. 新たな画像診断システム：脳梗塞治療特区から 第１２回日本栓子検出と治療学会大会　シンポジウム５. 大阪国際会議場, 2009
8. Iida H. Ultra-fast CMRO2/CBF with 15O-PET - As an application of multiple tracer approach Tianjin International Radiology Conference on Medical Imaging Progress. Tianjin,China, 2009
9. 飯田秀博. 脳血流量 SPECT 検査の標準化（司会の言葉） 第４９回日本核医学会学術総会. 旭川市, 2009

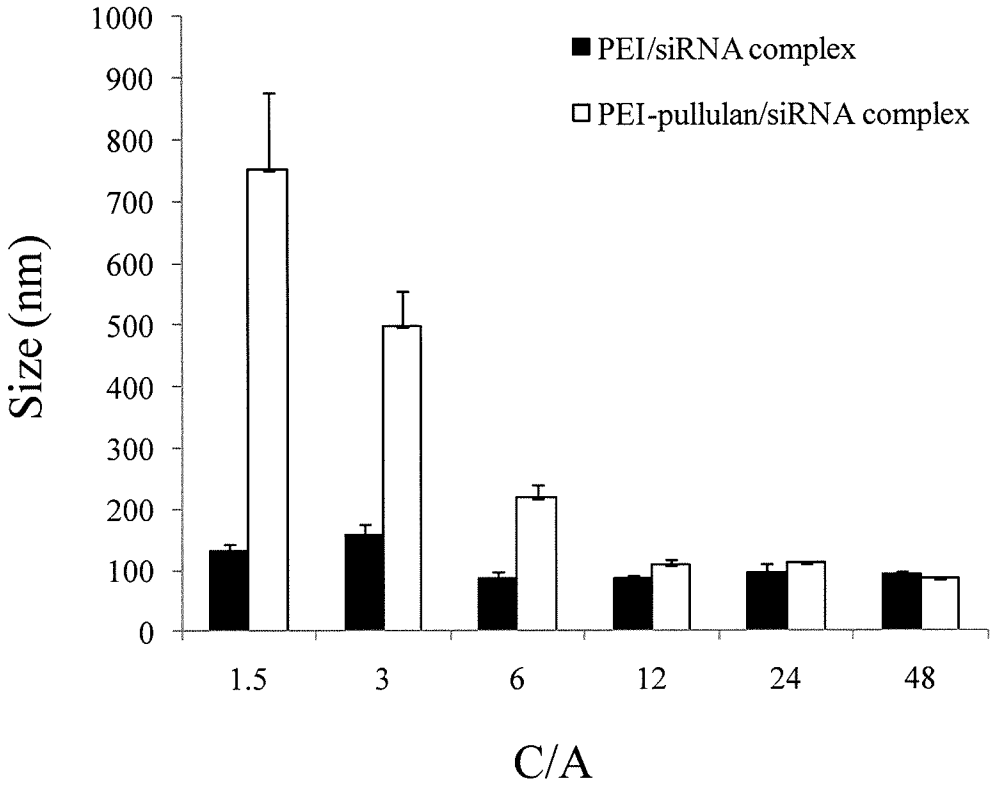

Figure 1. Diameter of the PEI/siRNA or PEI-pullulan/siRNA complexes. Polymer and siRNA complexes were simply prepared by incubating siRNA and polymer in water. The diameters of the complexes were determined using a Zetasizer.

Table 1. Mortality of mice after injection of complexes.

C/A ratio	3	6	12	24	48
PEI/siRNA complex	0/3	2/3	3/3	2/2	2/2
PEI-pullulan/siRNA complex	0/3	0/3	0/3	0/3	0/3

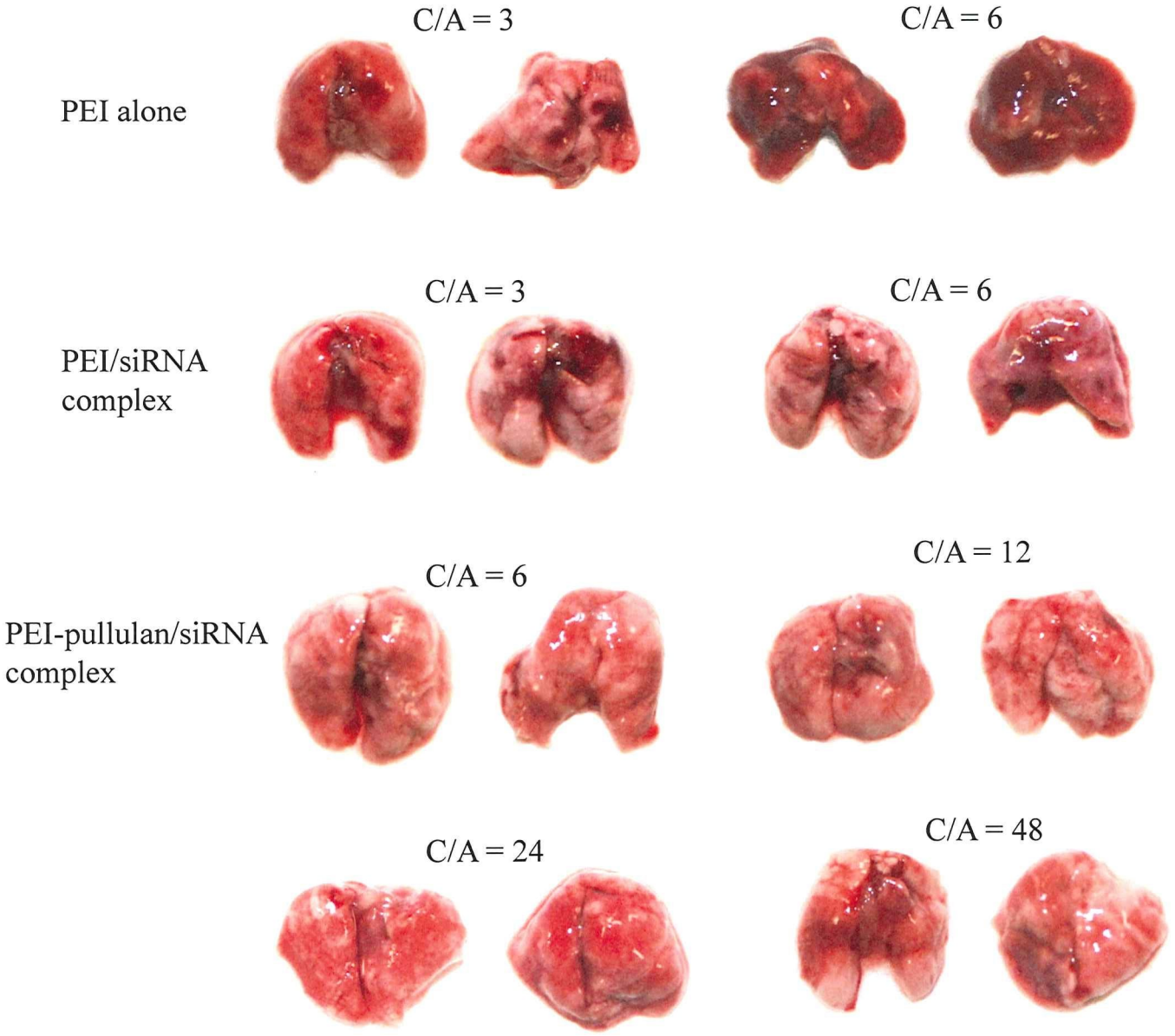

Figure 2. Delivery of PEI alone or polymer/siRNA complexes into mice.

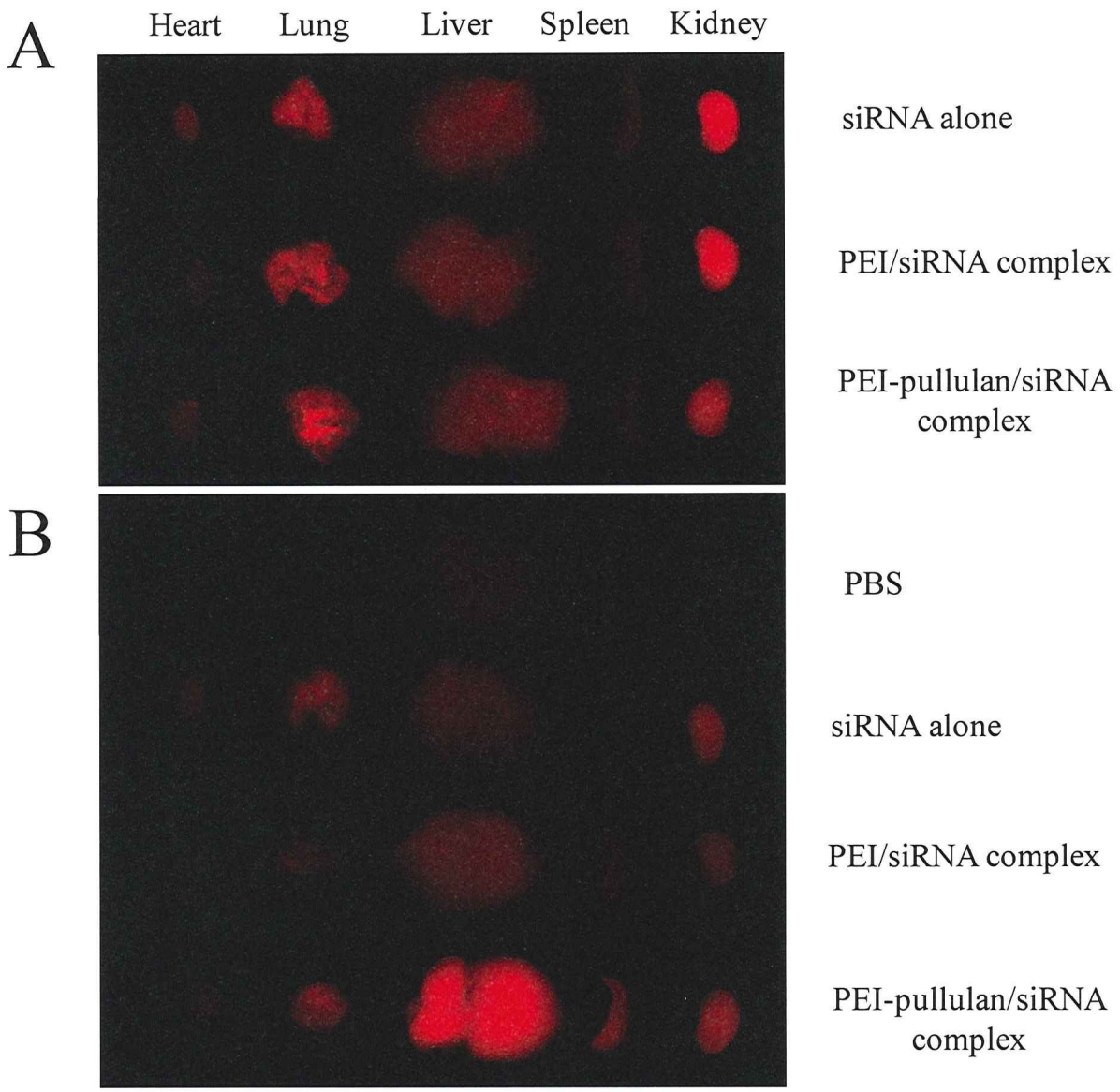

Figure 3. Biodistribution after injection of PBS, siRNA alone, or polymer/fluorescein-labeled siRNA complexes. The siRNA was bound with PEI at a C/A ratio of 3 and with PEI-pullulan at a C/A ratio of 48. The fluorescence in each tissue (heart, lung, liver, spleen, and kidney) was detected (A) 1 or (B) 3 h after the injection.

厚生労働科学研究費補助金
（創薬基盤推進研究事業：ヒトゲノムテーラーメード研究）

分担研究報告書

創薬ターゲットとしての mPGES-1

分担研究者　笹栗　俊之　九州大学大学院　医学研究院　臨床薬理学分野　教授

研究要旨　マクロファージに発現している誘導型プロスタグランジン E 合成酵素 microsomal-prostaglandin E synthase-1（mPGES-1）が、粥状硬化巣の炎症を抑制して粥腫を安定化させる新たな創薬ターゲット分子となりうるか否かを検討した。ヒト骨髄性白血病細胞株 THP-1 をリポ多糖（LPS）で刺激すると、mPGE-1 の発現が誘導されるが、細胞性粘菌由来分化誘導因子 differentiation-inducing factor（DIF）は、この mPGES-1 の誘導を強力に抑制することを見出した。臨床応用も視野に入れ、そのメカニズムの検討を現在行っている。

A. 研究目的

血管壁の炎症は、粥状硬化巣の病変形成において、中心的な役割を果たすと考えられている。なかでも、マクロファージの活性化は粥腫を不安定化し、粥腫の破裂や血栓の形成を誘導することにより、急性冠症候群の発症に寄与すると考えられている。

プロスタグランジン E_2（PGE_2）は炎症反応の重要なメディエーターであり、マクロファージは炎症性反応の一つとして PGE_2 を産生する。PGE_2 の産生酵素としては、microsomal prostaglandin E synthase（mPGES-1、mPGES-2）と cytosolic prostaglandin E synthase（cPGES）の 3 種類が知られている。このうち mPGES-1 は炎症性刺激によりその発現が上昇し、炎症の進展に関与することが示唆されており、創薬のターゲットになる可能性がある。

そこで我々は、炎症性刺激に対するマクロファージの反応過程における mPGES 発現と PGE_2 産生への関与について、ヒト骨髄性白血病細胞株 U937 および THP-1 を用いて検討した。さらに、細胞性粘菌由来分化誘導因子 differentiation-inducing factor（DIF）の新規抗炎症薬としての可能性について検討した。

B. 研究方法

1　細胞培養

THP-1 の培養には 10%ウシ胎仔血清を加えた RPMI1640 培地を用いた。また、phorbol 12-myristate 13-acetate（PMA、100nM）にて分化誘導した後、リポ多糖（LPS）および DIF-1 を用いて刺激した。ヒト由来大腸がん HCT-116 細胞の培養には 10%ウシ胎仔血清を加えた DMEM 培地を用いた。

2. ウェスタンブロット

刺激した細胞を回収し、SDS-PAGE にてタンパク質を分離した。タンパク質を転写したメンブレンを一次抗体（COX-2、mPGES、GAPDH）と反応させ、抗体と結合したタンパク質を検出した。

3　ヒト mPGES-1 プロモーター活性測定

24 穴培養用プレートに 1×10^5 個のヒト大腸がん由来 HCT-116 細胞を撒き、24 時間後、ヒト mPGES-1 プロモーター（-35/-1068 bp）組み込んだホタルルシフェラーゼレポーターベクター（pGL3-Basic）と導入効率の適正化のためのウミシイタケルシフェラーゼレポーターベクター(pRL-SV40)を細胞に導入した。24 時間後、DIF-1（10 または 30 μ M）で 6、12、及び 24 時間処理した。処理後の HCT-116 細胞におけるルシフェラーゼ活性を Promega 社のキット Dual Luciferase Assay System を用いて測定した。

C. 研究結果

1 DIFファミリーによるmPGES-1のタンパク質発現抑制

PMAを24時間作用させてTHP-1細胞に分化を誘導し、DIF-1(30μM)にて3時間刺激した後、LPS(1または10μg/ml)で24時間刺激した。回収したサンプルのCOX-2およびmPGES-1のタンパク質発現について検討したところ、DIF-1での前処置によりこれらの酵素の発現が抑制されていた(図1A)。

次に、DIF-1によるmPGES-1発現抑制の時間依存性の検討を行った。PMAで分化誘導を行ったTHP-1細胞をLPS(10μg/ml)で24時間刺激した後DIF-1(30μM)を添加し1、3、6、12、24時間後にサンプルを回収した。図1Bに示すように、LPSで24時間刺激することにより mPGES-1 の発現は明らかに上昇し、時間経過とともにさらに発現が上昇していく。一方 LPS で 24 時間刺激後にDIF-1 を添加した群では、時間経過にしたがって若干の発現上昇が認められるものの、コントロール群と比較して明らかな発現抑制効果が認められた。

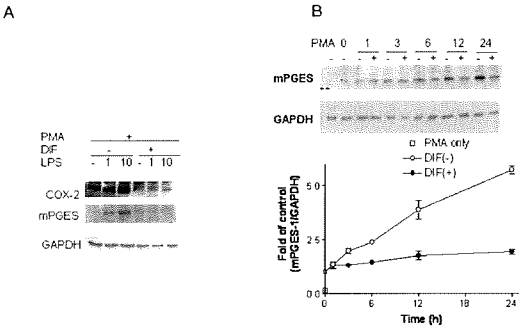

図1 DIFの炎症関連酵素に及ぼす影響

次にDIF-1の濃度を変化させて検討を行ったところ、図2Aに示すような濃度依存性が認められた。また、図2BはDIF-1のアナログの1つであるDIF-3の効果について検討したものであるが、DIF-3にもDIF-1と同様にmPGES-1発現抑制作用が認められた。この結果から、mPGES-1の発現抑制はDIFファミリーに共通した作用である可能性が示唆された。

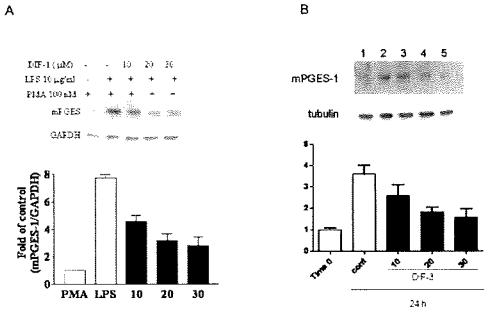

図2 DIFファミリーによるmPGES-1のタンパク質発現抑制

2 DIF-1によるmPGES-1 mRNA発現抑制

PMAで分化誘導を行ったTHP-1細胞をLPS(10μg/ml)で24時間刺激した後DIF-1(30μM)を添加し24時間後にサンプルを回収し総RNAを抽出した。これを用いてリアルタイムPCR法にてmRNAの発現量の変化を検討した。図3に示すようにDIF-1の添加により明らかなmPGES-1のmRNAの発現量の低下が認められた。この結果から、DIF-1がmRNAの発現量を低下させることにより、mPGES-1のタンパク質発現量を減少させていることが示唆された。

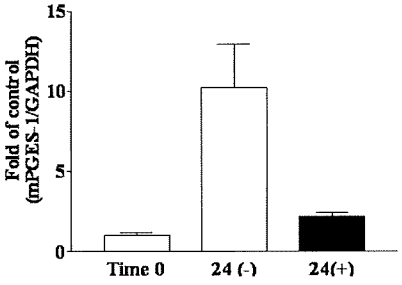

図3 mPGES-1のmRNA発現に及ぼすDIF-1の効果

また、ヒトのmPGES-1プロモーター領域をクローニングし、その活性に及ぼすDIF-1の効果を検討した。これにより、DIFはプロモーター活性を低下させることにより、mRNAの発現を抑制していることが示唆された(図4)。

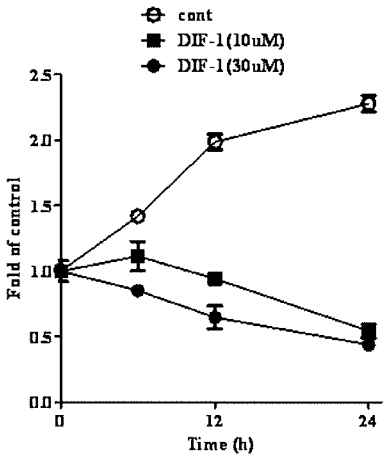

図 4 mPGES-1 プロモーター活性に及ぼす DIF-1 の効果

3 DIF-1 の他 PGES への影響

PGES には、mPGES-1 の他にも数種類存在するので、他の PGES に及ぼす DIF-1 の効果を検討したが、特に変化はみられなかった(図 5)。

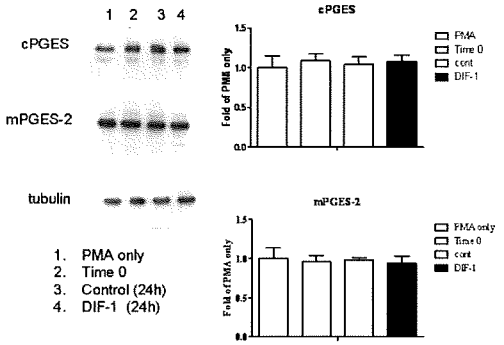

図 5 DIF-1 による他の PGES への影響

D. 考察

粥腫の炎症を抑制する治療標的分子を探索する目的で、マクロファージの活性化と炎症反応のメディエーターである PGE_2 の産生系に着目して検討を行った。

既存の抗炎症薬である非ステロイド性抗炎症薬(NSAIDs)は、シクロオキシゲナーゼ-2(COX-2)を抑制することにより下流の PGE_2 の産生を抑制するが、NSAIDs は COX-1 をも抑制するため、生理機能の維持に必要なエイコサノイドの産生も低下させてしまう。この問題を解決するため、炎症時に誘導される COX-2 に特異的な阻害薬(セレコキシブなど)が開発され、すでに臨床使用されているが、これらの薬剤は、プロスタサイクリンの産生減少によると思われる心血管イベントの増加を引き起こすことが報告され、その使用が制限されている。

そこで、炎症を増悪させる PGE_2 の産生を特異的に抑制する方法を開発するため、炎症性刺激下における直接的な PGE_2 産生酵素である mPGES の抑制を試みた。

ヒト骨髄性白血病細胞株 THP-1 を、PMA で処置することによりマクロファージ様細胞に分化させ、これに炎症性刺激を加えると、mPGES-1 タンパク質の誘導が認められるが、細胞性粘菌が産生する分化誘導因 DIF-1 が、炎症性刺激により誘導される mPGES-1 の発現を抑制した。さらに、DIF は mPGES-1 のプロモーター活性を抑制することにより、mPGES-1 の発現を抑制していることが示唆された。この結果は、DIF が新しい機序の抗炎症作用を有する可能性を示すものである。

E. 結論

mPGES-1 が有望な抗炎症薬の創薬ターゲットであり、DIF が抗炎症薬として機能する可能性が示唆された。

F. 健康危険情報

本研究では現在のところ健康に危険を及ぼす可能性はない。

G. 研究発表

1. Matsuda T, Takahashi-Yanaga F, Yoshihara T, Maenaka K, Watanabe Y, Miwa Y, Morimoto S, Kubohara Y, Hirata M, Sasaguri T (2010) *Dictyostelium* differentiation-inducing factor-1 binds to mitochondrial malate dehydrogenase and inhibits its activity. J Pharmacol Sci. (in press)

2. Ohmine T, Miwa Y, Takahashi-Yanaga F, Morimoto S, Maehara Y, & Sasaguri T (2009) The involvement of aldosterone in cyclic stretch-mediated activation of NADPH

oxidase in vascular smooth muscle cells. Hypertens Res. 32(8), 690-699

3. Zhan D-Y, Morimoto S, Du C-K, Wang Y-Y, Lu Q-W, Tanaka A, Ide T, Miwa Y, Takahashi-Yanaga F, & Sasaguri T (2009) Therapeutic effect of β-adrenoceptor blockers using a mouse model of dilated cardiomyopathy with a troponin mutation. Cardiovasc Res. 84(1), 64-71

4. Tadano N, Morimoto S, Takahashi-Yanaga F, Miwa Y, Ohtsuki I, & Sasaguri T (2009) Propyl gallate, a strong antioxidant, increases the Ca^{2+} sensitivity of cardiac myofilament. J Pharmacol Sci. 109(3), 456-458

5. Tanaka R, Miwa Y, Mou K, Tomikawa M, Eguchi N, Urade Y, Takahashi-Yanaga F, Morimoto S, Wake N, & Sasaguri T (2009) Knockout of the *l-pgds* gene aggravates obesity and atherosclerosis in mice. Biochem Biophys Res Commun. 378(4), 851-856

6. 笹栗俊之 (2009) GSK-3βと創薬. 医学のあゆみ 229(4), 293-294

7. Takahashi-Yanaga F & Sasaguri T (2009) Drug development targeting the glycogen synthase kinase-3β (GSK-3β)-mediated signal transduction pathway: Inhibitors of the Wnt/β-catenin signaling pathway as novel anticancer drugs. J Pharmacol Sci. 109(2), 179-183

出願中特許
・出願番号 特願 2009-238935、出願日 2009-10-16
・発明の名称 分化誘導因子又はその類縁体を含有する抗炎症剤

Intratracheal Gene Transfer of Adrenomedullin Using Polyplex Nanomicelles Attenuates Monocrotaline-induced Pulmonary Hypertension in Rats

Mariko Harada-Shiba[1], Itaru Takamisawa[1], Kanjiro Miyata[2,3], Takehiko Ishii[2,3], Nobuhiro Nishiyama[3,4], Keiji Itaka[3,4], Kenji Kangawa[5], Fumiki Yoshihara[6], Yujiro Asada[7], Kinta Hatakeyama[7], Noriya Nagaya[8] and Kazunori Kataoka[3,4,9]

[1]Department of Bioscience, National Cardiovascular Center Research Institute, Suita, Japan; [2]Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, Tokyo, Japan; [3]Center for NanoBio Integration, The University of Tokyo, Tokyo, Japan; [4]Division of Clinical Biotechnology; Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; [5]National Cardiovascular Center Research Institute, Suita, Japan; [6]Division of Hypertension and Nephrology, National Cardiovascular Center, Suita, Japan; [7]Department of Pathology, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan; [8]Department of Regenerative Medicine, National Cardiovascular Center Research Institute, Suita, Japan; [9]Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, Tokyo, Japan

Pulmonary arterial hypertension (PAH) is a life-threatening disease characterized by progressive PAH and right ventricular failure. Despite recent advances in therapeutic approaches using prostanoids, endothelin antagonists, and so on, PAH remains a challenging condition. To develop a novel therapeutic approach, we have established a nonviral gene delivery system of poly(ethylene glycol) (PEG)-based block catiomers, which form a polyplex nanomicelle with a nanoscaled core–shell structure in the presence of DNA. The polyplex nanomicelle from PEG-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide} (PEG-*b*-P[Asp(DET)]), having ethylenediamine units at the side chain, showed ~100-fold increase in luciferase transgene expression activity in mouse lung via intratracheal administration with a minimal toxicity compared with the polyplex from linear poly(ethylenimine) (LPEI). The transfection activity was highest on day 3 after administration and remained detectable until day 14. PEG-*b*-P[Asp(DET)] polyplex nanomicelles were formulated with a therapeutic plasmid bearing the human adrenomedullin (*AM*) gene and intratracheally administered to rats with monocrotaline-induced pulmonary hypertension. The right ventricular pressure significantly decreased 3 days after administration as confirmed by a notable increase of pulmonary human AM mRNA levels. Intratracheal administration of PEG-*b*-P[Asp-(DET)] polyplex nanomicelles showed remarkable therapeutic efficacy with PAH animal models without compromising biocompatibility.

Received 29 July 2008; accepted 9 March 2009; published online 31 March 2009. doi:10.1038/mt.2009.63

INTRODUCTION

Idiopathic pulmonary arterial hypertension (PAH) is a rare disease characterized by a progressive increase in pulmonary vascular resistance, leading to right heart failure and death.[1] Recent advances in therapeutic approaches to PAH show promising targeting pathways believed to play critical pathogenic or pathophysiologic roles;[2] however, despite these findings, PAH remains a challenging condition.[3]

Adrenomedullin (AM), a peptide isolated from human pheochromocytoma,[4] has multiple beneficial effects on cardiovascular tissues, including a powerful hypotensive effect.[5] Moreover, AM is indicated for PAH because of its prodilatory effects and the abundance of AM receptors in the lung.[6] Inhalation of AM was reported to ameliorate PAH in animal models[7] as well as in PAH patients without inducing systemic hypotension, but this effect was transient.[8] To overcome these barriers, a new, efficacious, and long-lasting AM therapy for PAH is warranted.

Gene therapy is one of the strategic approaches to continuously supply therapeutic peptides or proteins to target tissues.[6] Gene delivery to the lung via inhalation can avoid many problems associated with intravenous delivery, such as immediate nuclease degradation in the blood stream and the difficulty associated with penetrating endothelial barriers. In this regard, AM-based gene therapy through intratracheal route for PAH may have a promise.[7] Successful gene delivery via inhalation strongly depends on the development of advanced gene vectors to protect the therapeutic plasmid, provide site-specific targeting, and effectively release these plasmids for the desired pharmacological effect.

To develop a novel gene therapy system, we utilized our established polymeric library consisting of poly(ethylene glycol) (PEG)-based block catiomers, which form core–shell polyplex nanomicelles with core sequestration of the therapeutic plasmid.[9,10]

Correspondence: *Mariko Harada-Shiba, Department of Bioscience, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. E-mail: mshiba@ri.ncvc.go.jp*

These polyplex nanomicelles are well dispersed even in aqueous media containing serum proteins and protect plasmid DNA from degradation by nuclease *in vivo*.[11-13] We recently developed P[Asp(DET)], a poly(aspartamide) derivative bearing an *N*-(2-aminoethyl)aminoethyl group as the side chain, that showed improved transfection efficiency and biocompatibility compared to linear poly(ethylenimine) (LPEI).[14] The PEG-based block catiomer with P[Asp(DET)] was applied *in vivo* to deliver therapeutic plasmids for a murine, skull bone defect model and a rabbit carotid artery with neointima model; its successful therapeutic efficacy with these mammalian studies provided the impetus for expanded application into the treatment of intractable diseases suited for gene therapy.[15,16]

In this paper, we report advanced, pulmonary transfection efficiencies using intratracheally inhaled PEG-*b*-P[Asp(DET)] polymeric nanomicelles without compromising biocompatibility. The intratracheal administration of the *AM* gene by PEG-*b*-P[Asp-(DET)] polyplex nanomicelles reduced right ventricular pressure in PAH animal models without inducing inflammation, suggesting its suitability as a vector for translational research.

RESULTS

Reporter gene transfer using PEG-*b*-P[Asp(DET)] via intratracheal administration

Plasmids bearing the luciferase reporter gene were formulated with PEG-*b*-P[Asp(DET)] (N/P = 80) and LPEI (N/P = 6) and were sprayed intratracheally into ICR mice. Here, N/P ratio refers to the unit molar ratio of the amino group in the polymer to the phosphate group in the plasmid DNA. After 1 day, the mice were killed and the pulmonary tissues were harvested to quantify luciferase activity. PEG-*b*-P[Asp(DET)] polyplex nanomicelles showed nearly a 100-fold increase in luciferase levels than the LPEI controls (**Figure 1**). **Figure 2** shows the time-dependent changes of luciferase gene expression in the pulmonary tissue with PEG-*b*-P[Asp(DET)] polyplex nanomicelles. Luciferase activity was highest on day 3, and remained detectable until day 14. To elucidate the effect of PEG-*b*-P([Asp(DET)]/pLuc N/P ratios on luciferase gene expression, a series of the nanomicelles formulated under the varying N/P ratios (20, 40, and 80) were also examined. **Figure 3** shows an ~50-fold increase in luciferase expression from N/P = 20 to N/P = 80 over a 3-day period. Next, PEG-*b*-P[Asp(DET)] polyplex nanomicelles loaded with plasmid DNA bearing the yellow fluorescence protein (YFP) gene (N/P = 80) or LPEI/pYFP polyplexes (N/P = 6) were sprayed intratracheally in ICR mice. After 1 day, the pulmonary tissue was harvested and the YFP gene expression was visualized by fluorescence microscopy (**Figure 4**). Significantly higher fluorescence intensity was clearly seen in the lungs treated with the PEG-*b*-P[Asp(DET)] polyplex nanomicelle than the LPEI polyplexes; moreover, YFP fluorescence activity was distinctly visible for the animals treated with PEG-*b*-P[Asp(DET)] polyplex micelles in the secondary bronchi and lower pulmonary generations. To evaluate the toxicity, immunohistochemistry was conducted on the lung tissues after the transfection of LPEI/pLuc controls (N/P = 6) (**Figure 5a–c**) or PEG-*b*-P[Asp(DET)]/pLuc (N/P = 80) (**Figure 5d–f**). The lung administered with LPEI/pLuc showed moderate infiltration of neutrophils at the

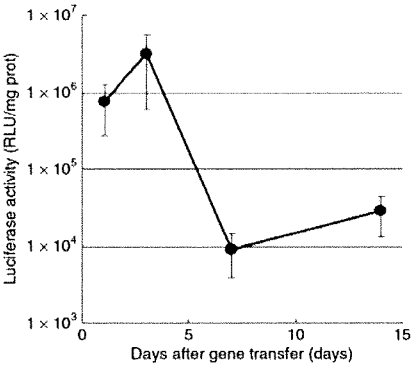

Figure 2 Time-dependent changes in gene expression after intratracheal administration of PEG-*b*-P[Asp(DET)] polyplex nanomicelle loaded with luciferase gene. The mice (five mice per group) were anesthetized and the polyplex nanomicelle was administered intratracheally. After the indicated time, the lung tissues were harvested, homogenized, and measured for luciferase activity (mean ± SEM, *N* = 5). PEG-*b*-P[Asp(DET)], PEG-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide}.

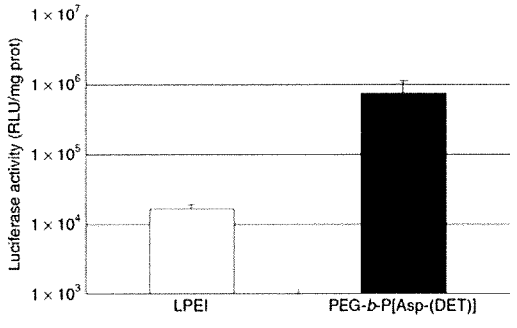

Figure 1 Luciferase gene expression by intratracheal administration of LPEI polyplex (N/P = 6) or PEG-*b*-P[Asp(DET)] polyplex nanomicelle (N/P = 80). Samples of the polyplex and the polyplex nanomicelle were prepared before the administration and left for 1 day. The mice (five mice per group) were anesthetized and the polyplex or the polyplex nanomicelle was administered intratracheally. At 24 hour postadministration, the lung tissues were harvested, homogenized, and measured for luciferase activity (mean ± SEM, *N* = 5). LPEI, linear poly(ethylenimine); PEG-*b*-P[Asp(DET)], PEG-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide}.

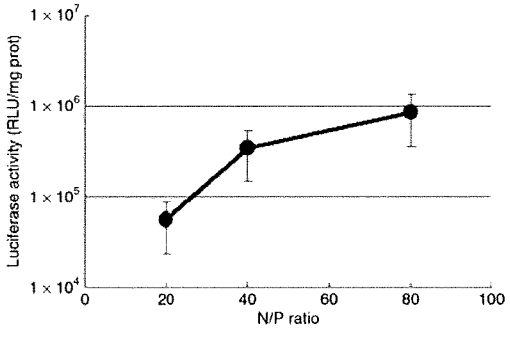

Figure 3 Charge-ratio-dependent changes in gene expression after intratracheal administration of the PEG-*b*-P[Asp(DET)] polyplex nanomicelle loaded with luciferase gene. The mice (five mice per group) were anesthetized and the polyplex nanomicelle was administered intratracheally. At 3 days postadministration, the lung tissues were harvested, homogenized, and measured for luciferase activity (mean ± SEM, *N* = 5). PEG-*b*-P[Asp(DET)], PEG-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide}.

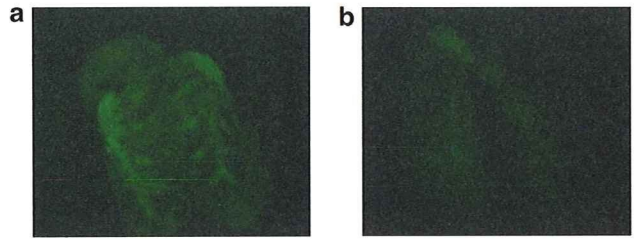

Figure 4 Fluorescence photographs of lungs transfected by intratracheal administration of YFP gene using PEG-*b*-P[Asp(DET)] polyplex nanomicelle or LPEI polyplex. (**a**) PEG-*b*-P[Asp(DET)] polyplex nanomicelle (N/P = 80), (**b**) LPEI polyplex (N/P = 6). The lung specimens were observed under a fluorescence microscope (SZX12; Olympus). LPEI, linear poly(ethylenimine); PEG-*b*-P[Asp(DET)], PEG-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide}.

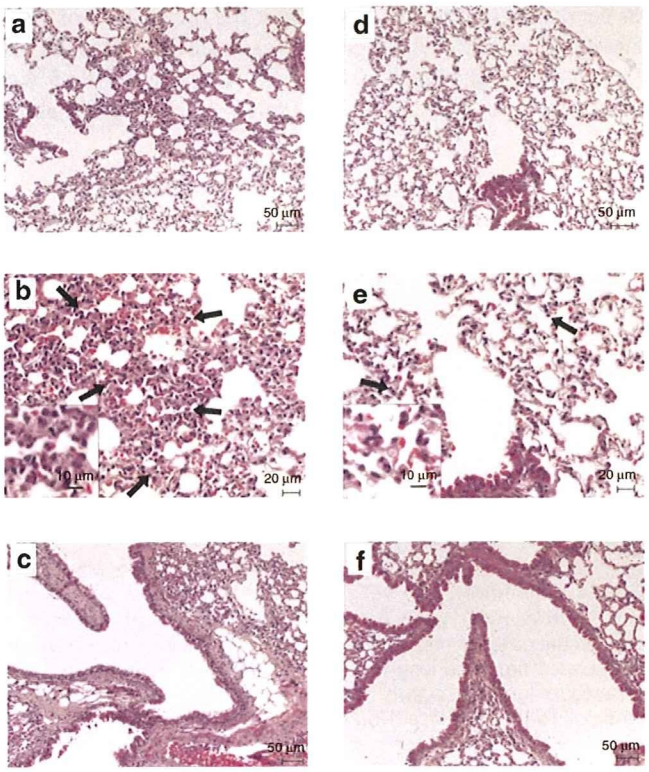

Figure 5 Representative photomicrographs of lung tissues 7 days postintratracheal administration of LPEI/pLuc (N/P = 6) (**a**–**c**) or PEG-*b*-P[Asp(DET)]/pLuc (N/P = 80) (**d**–**f**). The terminal bronchiole and alveoli of the lungs administered with LPEI polyplex (**a**,**b**) and PEG-*b*-P[Asp(DET)] polyplex nanomicelle (**d**,**e**) are shown. The neutrophil infiltration is indicated with arrows in the photomicrographs with higher magnification (**b**,**e**). Each inset is the picture with higher magnification. The bronchus of the lungs administered with LPEI polyplex (**c**) and PEG-*b*-P[Asp(DET)] polyplex nanomicelle (**f**) are also shown. LPEI, linear poly(ethylenimine); PEG-*b*-P[Asp(DET)], PEG-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide}.

terminal bronchiole and alveoli as indicated (Figure 5a,b). However, the lung administered with PEG-*b*-P[Asp(DET)]/pLuc, neutrophilic infiltration was scattered and minimal or absent (Figure 5d,e). No apparent inflammatory infiltrate was observed in the bronchus of the both groups (Figure 5c,f). The findings thereby supported increased biocompatibility with the PEG-*b*-P[Asp(DET)] polyplex micelle. To further evaluate the toxicity of the gene carrier systems, mRNA levels of inflammatory cytokines in the pulmonary tissue were measured using real-time reverse transcriptase (RT)-PCR. A nontreated cohort was used as a control. Proinflammatory cytokine mRNA levels did not increase for intratracheally administered naked pLuc in saline or the PEG-*b*-P[Asp(DET)] polyplex micelles; however, LPEI/pLuc polyplexes revealed a twofold increase in TNF-α, interleukin (IL)-6, IL-10, and Cox-2 compared to the control (Figure 6a–d). Notably, PEG-*b*-P[Asp(DET)]/pLuc proinflammatory levels were statistically similar to the negative control cohorts in TNF-α, IL-6, IL-10, and Cox-2 mRNA levels.

Effect of *AM* gene transfer by PEG-*b*-P[Asp(DET)] polyplex nanomicelle in a rat model of PAH

After 4 weeks of monocrotaline injection, right ventricular pressure was increased to twice of the normal value (Figure 7). Notably, right ventricular pressure was decreased significantly by an intratracheal spray of the PEG-*b*-P[Asp(DET)] polyplex nanomicelle loaded with the expression vector of AM (N/P = 40). On the other hand, right ventricular pressure did not change significantly after administration of naked plasmid encoding the *AM* gene in saline or the LPEI polyplex loaded with the *AM* gene, or the polyplex nanomicelle loaded with the luciferase gene. The mRNA levels of human AM in the lung were measured by real-time RT-PCR (Figure 8). The lung transfected with the polyplex nanomicelle loaded with the expression vector of AM had high levels of AM mRNA. Alternatively, the levels were much lower in the lung transfected with the LPEI polyplex loaded with the expression vector of AM. The lung transfected with the polyplex nanomicelle loaded with the luciferase gene or with the naked *AM* gene in saline showed no expression of human AM.

DISCUSSION

The large number of human diseases presenting poor prognoses and limited efficacy with current therapeutic regimens necessitates the advent of alternative approaches. PAH is such a disease without a highly efficacious therapeutic regimen.[17] PAH patients are currently treated with a variety of drugs including prostacyclin, prostacyclin analogues, calcium channel blockers, nitric-oxide inhalation, angiotensin-converting enzyme inhibitors, endothelin receptor antagonists, and phosphodiesterase type 5 inhibitors; in severe cases lung transplantation and subsequent immunosuppression are necessary.[17] However, promising alternative therapies for PAH have been recently reported. For example, Champion *et al.* reported that adenoviral gene transfer of endothelial nitric-oxide synthase to the lungs of endothelial nitric-oxide synthase knockout mice ameliorates the symptoms of PAH.[18] Champion *et al.* also reported that adenoviral gene transfer of calcium gene-related peptide attenuates the symptoms of PAH.[19] Nagaya *et al.* reported that transfection of human prostacyclin synthase using hemagglutinating virus of Japan–liposomes ameliorates monocrotaline-induced PAH.[20] However, in these attempts, viral or viral-related vectors were used for the delivery of therapeutic genes and these gene carriers have the potential for immunogenicity and inflammatory response. In diseases where a single dose can cure or provide palliative care, viral vectors may be suitable; however, PAH therapy requires repeated

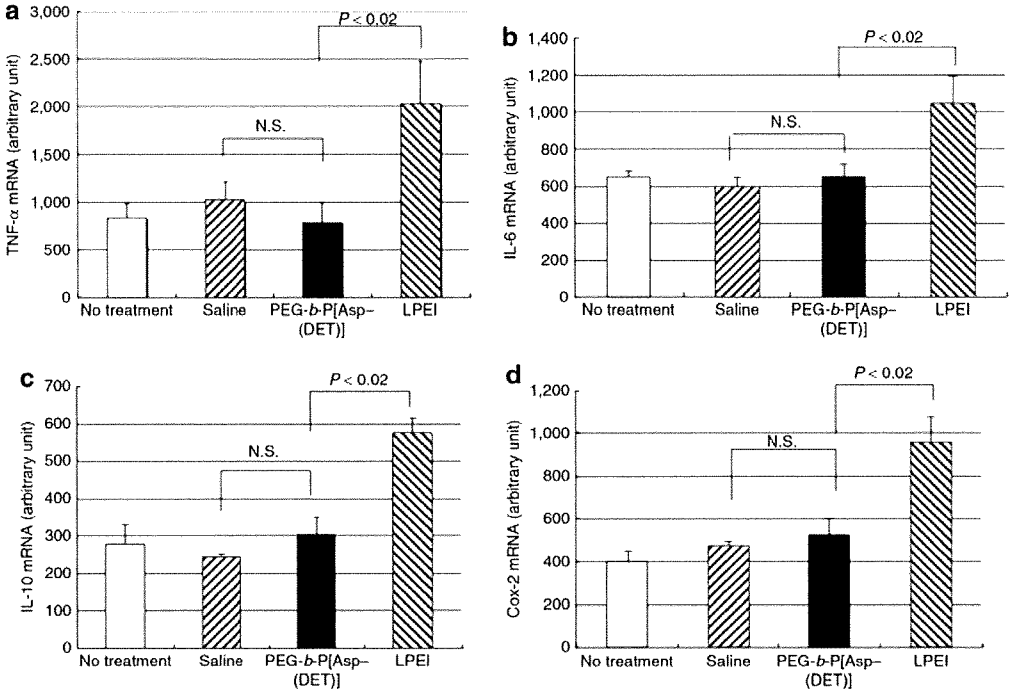

Figure 6 mRNA expression of inflammatory cytokines in lung tissues 7 days postintratracheal administration of luciferase gene in saline; LPEI polyplex with luciferase gene (N/P = 6); or PEG-*b*-P[Asp(DET)] polyplex nanomicelle with luciferase gene (N/P = 80) (mean ± SEM, $N = 4$). (**a**) TNF-α, (**b**) IL-6, (**c**) IL-10, (**d**) Cox-2. IL, interleukin; LPEI, linear poly(ethylenimine); TNF, tumor necrosis factor; PEG-*b*-P[Asp(DET)], PEG-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide}.

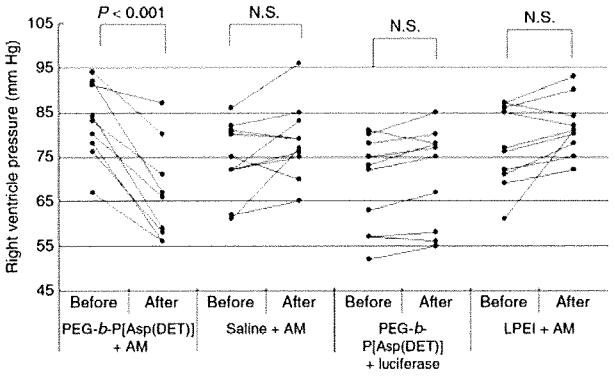

Figure 7 Effect of gene transfer on the right ventricle pressure in the PAH rat model. At 4 weeks after subcutaneous monocrotaline injection, a hemodynamic study was performed to measure the RV pressure indicated as "Before". The PEG-*b*-P[Asp(DET)] polyplex nanomicelle loaded with AM expression vector; AM expression vector in saline; PEG-*b*-P[Asp(DET)] polyplex micelle loaded with luciferase gene; or the LPEI polyplex loaded with AM expression vector were sprayed intratracheally. Three days later, a hemodynamic study was performed again to measure the RV pressure indicated as "After". AM, adrenomedullin; LPEI, linear poly(ethylenimine); PAH, pulmonary arterial hypertension; PEG-*b*-P[Asp(DET)], PEG-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide}; RV, right ventricular.

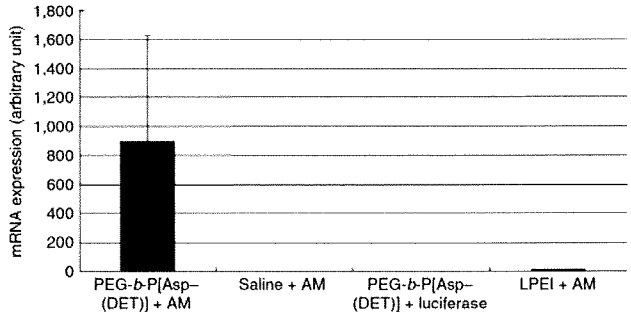

Figure 8 mRNA expression of human AM in the PAH rat model postintratracheal administration of PEG-*b*-P[Asp(DET)] polyplex nanomicelles loaded with the AM expression vector; AM expression vector in saline; PEG-*b*-P[Asp(DET)] polyplex nanomicelles loaded with the luciferase gene; or LPEI polyplexes loaded with the AM expression vector. Rats were transfected intratracheally at 4 weeks after subcutaneous monocrotaline injection. Three days later, lungs were harvested, homogenized, and measured for AM mRNA using real-time RT-PCR (mean ± SEM, $N = 4$). AM, adrenomedullin; LPEI, linear poly(ethylenimine); PAH, pulmonary arterial hypertension; PEG-*b*-P[Asp(DET)], PEG-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide}.

administrations for efficacy, hence the utility of viral or viral-based gene therapy is contraindicated.

Alternatively, nonviral gene carriers have been recognized with several advantages over viral vectors in terms of safety, immunogenicity, and ease of manufacture. To develop a method of gene therapy suitable for clinical translation, four primary factors must be clearly addressed: (i) the gene carrier, (ii) the therapeutic gene, (iii) the route of administration, and (iv) patient compliance. In this study, we chose to further explore the promising (i) PEG-*b*-P[Asp(DET)] polyplex nanomicelle nonviral gene carrier system based upon our previous findings. Next, we chose (ii) *AM* as the therapeutic gene because of its reported effectiveness in the transient treatment of PAH. For the route of administration, we selected (iii) intratracheal administration to avoid the rapid propensity of nuclease degradation in the blood compartment and also that we might exploit the lung, based upon its enormous surface area, for

use as a therapeutic bioreactor for AM production. Pulmonary administration is a promising therapeutic route of administration in the clinic for its (iv) high patient compliance with utilization of an inhaler or nebulizer.

Recently, we have demonstrated that PEG-*b*-P[Asp(DET)] polyplex nanomicelles achieved amplified *in vitro* and *in vivo* transfection activity with minimal cytotoxicity.[14–16,21] With regard to the transfection mechanism, P[Asp(DET)] possesses the ethylenediamine side chain, which undergoes two-step protonation from the mono-protonated gauche form at physiological pH to di-protonated anti form at acidic pH, thereby exhibiting an effective buffering function in the acidic endosomal compartment.[22] Also, we revealed the membrane destabilization effect of P[Asp(DET)] responding to acidic endosomal pH conditions by hemolysis, leakage of cytoplasmic enzyme (lactate dehydrogenase assay), and confocal laser scanning microscopic observation.[22] Consequently, we observed the facilitated transport of Cy5-labeled plasmid DNA by the P[Asp(DET)] polyplexes from endo/lysosomal compartment into cytoplasm directly under the confocal laser scanning microscope in single cellular level.[22] Therefore, the increased transgene expression in **Figure 3** may be a result of the facilitated translocation of the polyplex nanomicelles from the endosome to the cytoplasm based on the buffering capacity (proton sponge effect) and/or endosomal membrane–destabilizing effect of P[Asp(DET)] segment. The reason why a relatively high N/P ratio was required for the efficient transfection (**Figure 3**) may be because the membrane-destabilizing effect of P[Asp(DET)] is dependent on the polymer concentration as previously reported. Nevertheless, the PEG-*b*-P[Asp(DET)] polyplex nanomicelles displayed minimal cytotoxicity even at a high N/P ratio, which may be due to the pH-sensitive properties of P[Asp(DET)] segment.[22] The highly transfectable but less cytotoxic properties of PEG-*b*-P[Asp(DET)] polyplex nanomicelles motivated us to apply them to the gene therapy of PAH animal models through the intratracheal administration in this study.

A number of nonviral vectors including polyplex and lipoplex have been applied for *in vivo* intratracheal transfection. Special notice for the pulmonary gene delivery via airways is that the lung has the features critically influencing the transfection efficiency, such as the presence of surfactant, alveolar macrophages, and mucocilliary clearance mechanisms. In the early 1990s, lipoplex was used by aerosol delivery or intratracheal instillation. However, cationic lipids were shown to have a decreased transfection efficiency due to the interaction with lung surfactant compared to cationic polymer like PEI.[23,24] To overcome the surfactant barriers, cationic emulsion was used and showed much higher transfection activity compared with lipoplexes, such as lipofectin, lipofectamine, and DMRIE/c.[25] However, even for the cationic emulsion, the luciferase activity was limited to 55 pg/mg protein, which is significantly lower than the value attained by the polyplex nanomicelles loaded with PEG-*b*-P[Asp(DET)] [3,000,000 relative light unit/mg protein (135 ng/mg protein)] as reported here. Polyplexes made from cationic polymer was reported to show higher transfection efficiency compared with cationic lipoplexes for pulmonary gene delivery via airways.[23,24] PEI or modified PEI has been shown to be one of the most effective agents for constructing gene delivery systems available today with high levels of pulmonary gene transfer by airways.[26,27] Intratracheal injection of polyplex loaded with 22 kd of LPEI (ExGen 500) showed up to 20,000–40,000 relative light unit/mg protein of luciferase activity in the lung by adjusting N/P ratio. Worth noting is that the nanomicelles achieved two orders of magnitude higher value in luciferase gene expression compared to the LPEI polyplex. Furthermore, no induction of cytokine responses is appealing for the nanomicelles over LPEI polyplex (**Figure 6**), which was reported to induce the activation of $CD8^+$ and $CD4^+$ T cells, and Fas ligand–mediated antigen-induced cell death.[28,29]

To ameliorate the symptoms of PAH in animal models, several genes have been identified including: endothelial nitric-oxide synthase, inducible nitric-oxide synthase, prostacyclin synthase, calcium gene-related peptide, vascular endothelial growth factor, and hepatocyte growth factor.[18–20,30–36] We used AM as a therapeutic gene because of its high potency and long-term effectiveness as a vasodilator in the pulmonary vascular bed.[37] The effect of pulmonary vasodilation is mediated by cyclic adenosine monophosphate–dependent and nitric oxide–dependent mechanisms.[38] PAH patients have elevated plasma AM concentrations, which increase in step with the disease's severity[39,40] often resulting in pulmonary hypotension. In previous studies, intravenous administration[41] and inhalation[8] of the AM peptide showed acute hemodynamic and hormonal efficacy in PAH patients. However, despite alternative routes of delivery, the small AM peptide was rapidly degraded *in vivo* displaying a poor pharmacokinetic profile with a temporal window of only 30–45 minutes. For the treatment of PAH, sustained effect of AM is required. In this report, the therapeutic indicator for successful nonviral AM delivery was a decrease in the right ventricular pressure. Indeed, for the polyplex nanomicelle/pAM formulation, the right ventricular pressure did decrease, but more importantly the persistence of *AM* gene expression continued within a therapeutic range for a minimum of 3 days. The results indicate that the therapeutic approach using the polyplex nanomicelle as a vector does not require chronic infusion or very frequent inhalation, which will make the therapy more clinically applicable.

In this study, we succeeded in delivering DNA to the lung via intratracheal administration using the polyplex nanomicelles, resulting in extremely improved transfection efficiency with concomitant high biocompatibility. More specifically, the lung transfected with the polyplex nanomicelle had much lower toxicity than that transfected with the LPEI polyplex, according to the histological findings and measurement of the mRNA levels of inflammatory cytokines (**Figure 6a–d**). We also developed an effective treatment for the PAH rat model by delivering the therapeutic *AM* gene with polyplex nanomicelles from PEG-*b*-P[Asp(DET)]. These results showed a significant increase in transfection efficiently *in vivo* with intratracheal administration. The PEG-*b*-P[Asp(DET)] polyplex nanomicelle delivery system clearly showed promising *in vivo* results of transgene and therapeutic AM expression, when coupled with the clear visual, localization of the polyplex nanomicelles in the pulmonary tissue and the lack of proinflammatory responses. We posit that the PEG-*b*-P[Asp(DET)] nonviral gene carrier clearly shows those characteristics requisite for novel and advanced therapeutic systems ideally suited for translational research.

MATERIALS AND METHODS

Materials. An expression vector for YFP (RIKEN, Tokyo, Japan) was amplified in competent HB101 *Escherichia coli* and purified by Plasmid Giga Kits (Qiagen, Hilden, Germany). An expression vector for luciferase with a CAG promoter was provided by RIKEN. An expression vector for human AM was constructed as follows. The *EcoRI*/*Xho*I fragment of the full-length human AM complementary DNA (cDNA)[42] was ligated into the *EcoRI*/*Xho*I site of pcDNA1 (Invitrogen, Carlsbad, CA). The restriction maps of expression vector for luciferase and human AM cDNA are listed in **Supplementary Figure S1a,b**, respectively. To confirm that pcDNA/AM encodes AM, pcDNA/AM was transfected into Chinese hamster ovary cells, and the medium and the cells were collected for the measurement of immunoreactive AM using an AM radioimmunoassay Shionogi (Cosmic, Tokyo, Japan).

Animals. Male ICR mice weighing 25–30 g were administered the reporter gene intratracheally. Male Wistar rats weighing 100–120 g were used to make a model of PAH. All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute (Osaka, Japan).

Synthesis and characterization of PEG-b-P[Asp(DET)]. PEG-*b*-P[Asp(DET)] was prepared as previously described.[14] Briefly, PEG-poly(β-benzyl-L-aspartate) (PEG-PBLA) diblock copolymer was synthesized by the ring-opening polymerization of β-benzyl-L-aspartate *N*-carboxyanhydride from the terminal primary amino group of α-methoxy-ω-amino PEG (M_w: 12,000; Nippon Oil and Fats, Tokyo, Japan). Gel-permeation chromatography confirmed that the copolymer was unimodal with a narrow molecular weight distribution (M_w/M_n: 1.23), and the number of benzyl-L-aspartate repeating units was calculated to be 68 by ^{1}H-NMR. The N-terminal amino group of PEG-PBLA was then acetylated using acetic anhydride in dichloromethane solution to obtain PEG-PBLA-Ac. The obtained polymer was dissolved in distilled *N,N*-dimethylformamide (Wako Pure Chemical Industries, Osaka, Japan) and reacted with diethylenetriamine (Tokyo Kasei Kogyo, Tokyo, Japan) for 24 hours at 40 °C in a dry argon atmosphere to undergo aminolysis of the benzyl side chain. After 24 hours, the solution was slowly dripped into a 10% acetic acid solution and dialyzed (Spectra/Por Membrane, 3,500 molecular weight cutoff; Spectrum Laboratories, Rancho Dominguez, CA) against 0.01 N HCl and subsequently against distilled water. The final solution was lyophilized to obtain PEG-*b*-P[Asp(DET)] as the hydrochloride salt form. ^{1}H-NMR confirmed the complete substitution of benzyl ester of the polymer with diethylenetriamine through the aminolysis reaction, as well as the chemical structure of the obtained PEG-*b*-P[Asp(DET)] block copolymer.

Preparation of polyplex nanomicelles. The PEG-*b*-P[Asp(DET)] block copolymer and plasmid DNA were separately dissolved in 10 mmol/l HEPES buffer (pH 7.4). Both solutions were mixed at the indicated nitrogen/phosphate ratios [=(total amines in cationic segment)/(phosphates in plasmid DNA)] and incubated overnight at room temperature to make PEG-*b*-P[Asp-(DET)] polyplex nanomicelle. LPEI (ExGen; Cosmo Bio, Tokyo, Japan) polyplexes were prepared by mixing plasmid DNA and LPEI according to the manufacturer's protocol.

In vivo *gene delivery by intratracheal administration.* ICR mice were anesthetized by intraperitoneal administration of pentobarbital (30 mg/kg) (Dainippon Sumitomo Pharma, Osaka, Japan). Tracheostomies were performed under sterile conditions for PEG-*b*-P[Asp-(DET)] polyplex nanomicelle or LPEI polyplex (10 μg of DNA for each mouse) in a 50 μl of solution administration by a microsprayer Model IA-1C (Penn Century, Philadelphia, PA). After the indicated time, the mice were killed by cervical dislocation and the pulmonary tissues harvested. To measure luciferase activity, the pulmonary tissues were homogenized in a lysis buffer using a polytron. The lysate was then centrifuged at 14,000*g* for 10 minutes at 4 °C, and 20 μl of the supernatant was analyzed for luciferase activity by a Luminous CT-9000D luminometer (Dia-Iatron, Tokyo, Japan), according to a previously described method.[43] Background of luciferase activity in the lung was measured from the lung of mice after administration of saline, which was <3% of the total activity of day 14. All the data of luciferase activity were obtained by subtraction of background data. To detect YFP expression, mice were killed by cervical dislocation and the lungs harvested. Frozen sections (5-μm thick) of the lung specimens were visualized by a fluorescence microscope (SZX12; Olympus, Tokyo, Japan). To examine the histological features of the lung tissue, the specimens were also fixed in 4% paraformaldehyde and embedded in paraffin. Sections (3-μm thick) were stained with hematoxylin.

Isolation of RNA and cDNA synthesis. Total RNA was extracted using the Trizol method (Gibco BRL Life Technologies, Breda, Netherlands) according to the protocol provided by the manufacturer. The RNA was dissolved in RNAse-free water and quantified by a spectrophotometer. The cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

Real-time RT-PCR. mRNA expression levels of TNF-α, IL-6, IL-10, Cox-2 and human AM were measured by quantitative real-time RT-PCR based on TaqMan chemistry (Applied Biosystems) using an ABI PRISM 7700 sequence detector (Applied Biosystems). The reaction mixture contained 0.5 μl of 5 μmol/l probe (final concentration, 100 nmol/l); 1 μl of 10 μmol/l forward primer and 1 μl of 10 μmol/l reverse primer (400 nmol/l final concentration of each primer); 12.5 μl of TaqMan Universal Mastermix, 5 μl of diethyl pyrocarbonate–treated water, and 5 μl of a cDNA sample. Assay controls were performed in the same TaqMan plate with no-template controls to test for the contamination of any assay reagents. The thermocycling conditions were initiated at 50 °C for 2 minutes with an enzyme activation step of 95 °C for 10 minutes followed by 40 PCR cycles of denaturation at 95 °C for 15 seconds, and anneal/extension at 60 °C for 1 minute.

Hemodynamic studies. Hemodynamic studies were performed 4 weeks after gene transfer. Rats were anesthetized with intraperitoneal pentobarbital (30 mg/kg) and placed on a heating pad to maintain body temperature 37–38 °C throughout the study. Under sterile conditions, a polyethylene catheter (PE-50; BD Biosciences, San Jose, CA) was inserted through the right jugular vein into the right ventricle to measure right ventricular pressure by a hemodynamic transducer (PowerLab 8/30; ADInstruments, Colorado Springs, CO).

Evaluation of gene transfer effect in a PAH rat model. Monocrotaline (60 mg/kg) was subcutaneously injected into male Wistar rats and left for 4 weeks to make a model of PAH. After 4 weeks, a hemodynamic study was performed to introduce a catheter into the right ventricle through the right jugular vein. The PEG-*b*-P[Asp(DET)] polyplex nanomicelle loaded with the AM expression vector (200 μg of DNA for each rat) in a 200 μl of solution was sprayed intratracheally. Three days later, a hemodynamic study was performed again and the gene transfer effect was evaluated. Pulmonary tissue specimens were frozen to measure *AM* gene expression by real-time RT-PCR.

Statistical analysis. All data are expressed as means ± SEM unless otherwise indicated. Comparisons of parameters among four groups were made by one-way analysis of variance, followed by Scheffe's multiple-comparison test. Paired *t*-test was applied for the comparison of the values before and after the gene transfection (**Figure 7**).

SUPPLEMENTARY MATERIAL

Figure S1. The restriction maps of expression vector for luciferase and human adrenomedullin cDNA.

ACKNOWLEDGMENTS

This work was supported by the Core Research Program for Evolutional Science and Technology from the Japan Science and Technology

Corporation, by Grants-in-Aid for Scientific Research from the Japanese Ministry of Health, Labor, and Welfare (H19-Nano-012 and H20-Genomu-Ippan-008), by the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan, and by the Takeda Science Foundation. We thank Keiko Jinno, Shoko Obora, Hiroko Miyata, Moto Ohira, and Eri Abe and Mutsumi Goda (National Cardiovascular Center Research Institute) for their excellent technical assistance, including animal care. We also thank Hisayuki Matsuo and Hitonobu Tomoike for their helpful discussion and advice, Darin Y. Furgeson (University of Wisconsin–Madison) for proofreading of this manuscript.

REFERENCES

1. Rubin, LJ (2006). Pulmonary arterial hypertension. *Proc Am Thorac Soc* **3**: 111–115.
2. Badesch, DB, Abman, SH, Ahearn, GS, Barst, RJ, McCrory, DC, Simonneau, G *et al.* (2004). Medical therapy for pulmonary arterial hypertension: ACCP evidence-based clinical practice guidelines. *Chest* **126**(1 Suppl): 35S–62S.
3. D'Alonzo, GE, Barst, RJ, Ayres, SM, Bergofsky, EH, Brundage, BH, Detre, KM *et al.* (1991). Survival in patients with primary pulmonary hypertension. Results from a national prospective registry. *Ann Intern Med* **115**: 343–349.
4. Kitamura, K, Kangawa, K, Kawamoto, M, Ichiki, Y, Nakamura, S, Matsuo, H *et al.* (1993). Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun* **192**: 553–560.
5. Kitamura, K, Kangawa, K and Eto, T (2002). Adrenomedullin and PAMP: discovery, structures, and cardiovascular functions. *Microsc Res Tech* **57**: 3–13.
6. Nagaya, N, Mori, H, Murakami, S, Kangawa, K and Kitamura, S (2005). Adrenomedullin: angiogenesis and gene therapy. *Am J Physiol Regul Integr Comp Physiol* **288**: R1432–R1437.
7. Nagaya, N, Okumura, H, Uematsu, M, Shimizu, W, Ono, F, Shirai, M *et al.* (2003). Repeated inhalation of adrenomedullin ameliorates pulmonary hypertension and survival in monocrotaline rats. *Am J Physiol Heart Circ Physiol* **285**: H2125–H2131.
8. Nagaya, N, Kyotani, S, Uematsu, M, Ueno, K, Oya, H, Nakanishi, N *et al.* (2004). Effects of adrenomedullin inhalation on hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension. *Circulation* **109**: 351–356.
9. Katayose, S and Kataoka, K (1997). Water-soluble polyion complex associates of DNA and poly(ethylene glycol)-poly(L-lysine) block copolymer. *Bioconjug Chem* **8**: 702–707.
10. Kakizawa, Y and Kataoka, K (2002). Block copolymer micelles for delivery of gene and related compounds. *Adv Drug Deliv Rev* **54**: 203–222.
11. Katayose, S and Kataoka, K (1998). Remarkable increase in nuclease resistance of plasmid DNA through supramolecular assembly with poly(ethylene glycol)-poly(L-lysine) block copolymer. *J Pharm Sci* **87**: 160–163.
12. Itaka, K, Yamauchi, K, Harada, A, Nakamura, K, Kawaguchi, H and Kataoka, K (2003). Polyion complex micelles from plasmid DNA and poly(ethylene glycol)-poly(L-lysine) block copolymer as serum-tolerable polyplex system: physicochemical properties of micelles relevant to gene transfection efficiency. *Biomaterials* **24**: 4495–4506.
13. Harada-Shiba, M, Yamauchi, K, Harada, A, Takamisawa, I, Shimokado, K and Kataoka, K (2002). Polyion complex micelles as vectors in gene therapy—pharmacokinetics and *in vivo* gene transfer. *Gene Ther* **9**: 407–414.
14. Kanayama, N, Fukushima, S, Nishiyama, N, Itaka, K, Jang, WD, Miyata, K *et al.* (2006). A PEG-based biocompatible block catiomer with high buffering capacity for the construction of polyplex micelles showing efficient gene transfer toward primary cells. *ChemMedChem* **1**: 439–444.
15. Itaka, K, Ohba, S, Miyata, K, Kawaguchi, H, Nakamura, K, Takato, T *et al.* (2007). Bone regeneration by regulated *in vivo* gene transfer using biocompatible polyplex nanomicelles. *Mol Ther* **15**: 1655–1662.
16. Akagi, D, Oba, M, Koyama, H, Nishiyama, N, Fukushima, S, Miyata, T *et al.* (2007). Biocompatible micellar nanovectors achieve efficient gene transfer to vascular lesions without cytotoxicity and thrombus formation. *Gene Ther* **14**: 1029–1038.
17. Nossaman, BD, Gur, S and Kadowitz, PJ (2007). Gene and stem cell therapy in the treatment of erectile dysfunction and pulmonary hypertension; potential treatments for the common problem of endothelial dysfunction. *Curr Gene Ther* **7**: 131–153.
18. Champion, HC, Bivalacqua, TJ, Greenberg, SS, Giles, TD, Hyman, AL, Kadowitz, PJ (2002). Adenoviral gene transfer of endothelial nitric-oxide synthase (eNOS) partially restores normal pulmonary arterial pressure in eNOS-deficient mice. *Proc Natl Acad Sci USA* **99**: 13248–13253.
19. Champion, HC, Bivalacqua, TJ, Toyoda, K, Heistad, DD, Hyman, AL and Kadowitz, PJ (2000). *In vivo* gene transfer of prepro-calcitonin gene-related peptide to the lung attenuates chronic hypoxia-induced pulmonary hypertension in the mouse. *Circulation* **101**: 923–930.
20. Nagaya, N, Yokoyama, C, Kyotani, S, Shimonishi, M, Morishita, R, Uematsu, M *et al.* (2000). Gene transfer of human prostacyclin synthase ameliorates monocrotaline-induced pulmonary hypertension in rats. *Circulation* **102**: 2005–2010.
21. Masago, K, Itaka, K, Nishiyama, N, Chung, UI and Kataoka, K (2007). Gene delivery with biocompatible cationic polymer: Pharmacogenomic analysis on cell bioactivity. *Biomaterials* **28**: 5169–5175.
22. Miyata, K, Oba, M, Nakanishi, M, Fukushima, S, Yamasaki, Y, Koyama, H *et al.* (2008). Polyplexes from poly(aspartamide) bearing 1,2-diaminoethane side chains induce pH-selective, endosomal membrane destabilization with amplified transfection and negligible cytotoxicity. *J Am Chem Soc* **130**: 16287–16294.
23. Bragonzi, A, Dina, G, Villa, A, Calori, G, Biffi, A, Bordignon, C *et al.* (2000). Biodistribution and transgene expression with nonviral cationic vector/DNA complexes in the lungs. *Gene Ther* **7**: 1753–1760.
24. Wiseman, JW, Goddard, CA, McLelland, D and Colledge, WH (2003). A comparison of linear and branched polyethylenimine (PEI) with DCChol/DOPE liposomes for gene delivery to epithelial cells *in vitro* and *in vivo*. *Gene Ther* **10**: 1654–1662.
25. Kim, TW, Chung, H, Kwon, IC, Sung, HC, Shin, BC and Jeong, SY (2005). Airway gene transfer using cationic emulsion as a mucosal gene carrier. *J Gene Med* **7**: 749–758.
26. Densmore, CL (2006). Advances in noninvasive pulmonary gene therapy. *Curr Drug Deliv* **3**: 55–63.
27. Furgeson, DY, Chan, WS, Yockman, JW and Kim, SW (2003). Modified linear polyethylenimine-cholesterol conjugates for DNA complexation. *Bioconjug Chem* **14**: 840–847.
28. Gautam, A, Densmore, CL and Waldrep, JC (2001). Pulmonary cytokine responses associated with PEI-DNA aerosol gene therapy. *Gene Ther* **8**: 254–257.
29. Regnstrom, K, Ragnarsson, EG, Koping-Hoggard, M, Torstensson, E, Nyblom, H and Artursson, P (2003). PEI—a potent, but not harmless, mucosal immuno-stimulator of mixed T-helper cell response and FasL-mediated cell death in mice. *Gene Ther* **10**: 1575–1583.
30. Budts, W, Pokreisz, P, Nong, Z, Van Pelt, N, Gillijns, H, Gerard, R *et al.* (2000). Aerosol gene transfer with inducible nitric oxide synthase reduces hypoxic pulmonary hypertension and pulmonary vascular remodeling in rats. *Circulation* **102**: 2880–2885.
31. Chicoine, LG, Tzeng, E, Bryan, R, Saenz, S, Paffett, ML, Jones, J *et al.* (2004). Intratracheal adenoviral-mediated delivery of iNOS decreases pulmonary vasoconstrictor responses in rats. *J Appl Physiol* **97**: 1814–1822.
32. Campbell, AI, Zhao, Y, Sandhu, R and Stewart, DJ (2001). Cell-based gene transfer of vascular endothelial growth factor attenuates monocrotaline-induced pulmonary hypertension. *Circulation* **104**: 2242–2248.
33. Gong, F, Tang, H, Lin, Y, Gu, W, Wang, W and Kang, M (2005). Gene transfer of vascular endothelial growth factor reduces bleomycin-induced pulmonary hypertension in immature rabbits. *Pediatr Int* **47**: 242–247.
34. Suhara, H, Sawa, Y, Fukushima, N, Kagisaki, K, Yokoyama, C, Tanabe, T *et al.* (2002). Gene transfer of human prostacyclin synthase into the liver is effective for the treatment of pulmonary hypertension in rats. *J Thorac Cardiovasc Surg* **123**: 855–861.
35. Ono, M, Sawa, Y, Fukushima, N, Suhara, H, Nakamura, T, Yokoyama, C *et al.* (2004). Gene transfer of hepatocyte growth factor with prostacyclin synthase in severe pulmonary hypertension of rats. *Eur J Cardiothorac Surg* **26**: 1092–1097.
36. Ono, M, Sawa, Y, Mizuno, S, Fukushima, N, Ichikawa, H, Bessho, K *et al.* (2004). Hepatocyte growth factor suppresses vascular medial hyperplasia and matrix accumulation in advanced pulmonary hypertension of rats. *Circulation* **110**: 2896–2902.
37. Lippton, H, Chang, JK, Hao, Q, Summer, W and Hyman, AL (1994). Adrenomedullin dilates the pulmonary vascular bed *in vivo*. *J Appl Physiol* **76**: 2154–2156.
38. Ishizaka, Y, Ishizaka, Y, Tanaka, M, Kitamura, K, Kangawa, K, Minamino, N *et al.* (1994). Adrenomedullin stimulates cyclic AMP formation in rat vascular smooth muscle cells. *Biochem Biophys Res Commun* **200**: 642–646.
39. Kakishita, M, Nishikimi, T, Okano, Y, Satoh, T, Kyotani, S, Nagaya, N *et al.* (1999). Increased plasma levels of adrenomedullin in patients with pulmonary hypertension. *Clin Sci (Lond)* **96**: 33–39.
40. Yoshibayashi, M, Kamiya, T, Kitamura, K, Saito, Y, Kangawa, K, Nishikimi, T *et al.* (1997). Plasma levels of adrenomedullin in primary and secondary pulmonary hypertension in patients <20 years of age. *Am J Cardiol* **79**: 1556–1558.
41. Nagaya, N, Nishikimi, T, Uematsu, M, Satoh, T, Oya, H, Kyotani, S *et al.* (2000). Haemodynamic and hormonal effects of adrenomedullin in patients with pulmonary hypertension. *Heart* **84**: 653–658.
42. Kitamura, K, Sakata, J, Kangawa, K, Kojima, M, Matsuo, H and Eto, T (1993). Cloning and characterization of cDNA encoding a precursor for human adrenomedullin. *Biochem Biophys Res Commun* **194**: 720–725.
43. de Wet, JR, Wood, KV, DeLuca, M, Helinski, DR and Subramani, S (1987). Firefly luciferase gene: structure and expression in mammalian cells. *Mol Cell Biol* **7**: 725–737.

Original Article

Impact of Statin Treatment on the Clinical Fate of Heterozygous Familial Hypercholesterolemia

Mariko Harada-Shiba[1], Takako Sugisawa[2], Hisashi Makino[2], Mitsuru Abe[3], Motoo Tsushima[2], Yasunao Yoshimasa[2], Takahiro Yamashita[2], Yoshihiro Miyamoto[2], Akira Yamamoto[4], Hitonobu Tomoike[5], Shinji Yokoyama[6]

[1]Department of Bioscience, National Cardiovascular Center Research Institute
[2]Division of Atherosclerosis and Diabetes, National Cardiovascular Center
[3]Division of Cardiology, National Cardiovascular Center
[4]Emeritus Scientist, National Cardiovascular Center Research Institute
[5]Hospital, National Cardiovascular Center
[6]Department of Biochemistry, Nagoya City University Graduate School of Medical Sciences

Aim: Familial hypercholesterolemia (FH) patients are at particular risk for premature coronary artery disease (CAD) caused by high levels of low density lipoprotein (LDL). Administration of statins enabled us to reduce LDL-C levels in heterozygous FH patients. To evaluate the impact of statins on the clinical fate of heterozygous FH, a retrospective study was performed.
Methods: We analyzed the clinical influence of statins on age at the first clinical onset of CAD in 329 consecutive FH patients referred to the lipid clinic of the National Cardiovascular Center. Among 329 heterozygous FH patients, the onset of CAD was identified in 101.
Results: The age at onset of CAD was 58.8 ± 12.5 years in the 25 patients on statins at onset, significantly higher than that in the 76 patients not on statins (47.6 ± 10.5 years) ($p<0.001$). The average age at CAD onset was significantly higher after widespread use of statins (54.2 ± 13.2 years in 48 patients, Group 1) compared to before October 1989 when statins were approved in Japan (46.9 ± 9.6 years in 53 patients; Group 2, $p=0.002$). A significant difference was seen between Groups 1 and 2 in the variables, including sex, prevalence of smoking habit, LDL-C, and the use of statins, aspirin and probucol. After adjusting for these variables, only statin use was independently associated with the difference in age at CAD onset by multivariable analysis.
Conclusion: Statins have improved the clinical course of patients with heterozygous FH.

J Atheroscler Thromb, 2010; 17:000-000.

Key words; Familial hypercholesterolemia, Statin, Coronary artery disease, LDL cholesterol

Introduction

Familial hypercholesterolemia (FH) is a heritable disease of high prevalence with an autosomal-dominant mode of transmission and is linked to mutations in the low-density lipoprotein (LDL) receptor gene. It is characterized by phenotypes of the elevation of plasma LDL, cutaneous and tendenous xanthomas, arcus corneae, and coronary artery disease (CAD) due to premature atherosclerosis[1]. The earliest clinical sign of heterozygous FH is an elevation of plasma LDL-cholesterol (LDL-C), noted as early as at birth[2]. All other clinical manifestations seem due to an increase of LDL-C in plasma. CAD is the most serious clinical manifestation and determines the prognosis of FH. According to a previous report, Japanese FH heterozygotes generally develop the first CAD event in their 40s or later for men and 50s or later for women[3].

Address for correspondence: Mariko Harada-Shiba, Department of Bioscience, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565 Japan.
E-mail: mshiba@ri.ncvc.go.jp
Received: October 28, 2009
Accepted for publication: December 4, 2009

To reduce plasma LDL-C in FH heterozygotes, bile acid-sequestering resins have been used since the 1970s to upregulate the LDL receptor, but their effect is limited to a 10 to 20% decline because of the concomitant induction of hepatic cholesterol synthesis[4]. Statins, competitive inhibitors of a rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl (HMG) CoA reductase, were introduced onto the market in the late 1980s. Pravastatin, the first approved statin in Japan, became commercially available at the beginning of October 1989 and simvastatin one year later[5]. Synthetic analogues became available in the late 1990s, including several "strong" statins, which lower the level of LDL-C by more than 40%[6]. Many large-scale clinical trials of statins worldwide, including Japan, showed that they reduced the risk of cardiac events or stroke in hypercholesterolemic populations[7-10]. Effective reduction of LDL-C by statins was also shown in FH heterozygotes[11, 12]; however, their clinical benefits in FH patients have not been clearly demonstrated with fixed clinical endpoints. This is partly because of the extremely high risk for CAD in FH patients, thus making controlled clinical trials of sufficient size to yield significant outcomes unethical.

Aim

Substantial numbers of FH patients have been referred to and regularly treated at the lipid clinic of the National Cardiovascular Center (NCVC) since it was founded in 1977. We therefore retrospectively analyzed the clinical records of these patients to assess the impact of the introduction of statins on the clinical prognosis of FH heterozygous patients, using patient age at the development of CAD. This parameter is specific and solid for each patient and the analysis is less influenced or biased by other factors. In addition, Mabuchi and colleagues used the same parameter in their study of Japanese FH reported before statin availability[13].

Methods

Subjects

Of the patients referred to the lipid clinic at NCVC from 1969 to 2007, 329 consecutive patients (139 men, 190 women) were diagnosed as FH heterozygotes using the criteria previously described[14]. Most of the FH patients analyzed in the present paper were referred to our lipid clinic by their general practitioner because of hypercholesterolemia. The medical records of patients were examined according to the analysis protocol approved by our institutional ethics committee (ID#M20-25-2). Of the 329 FH patients, 101 were identified as having CAD, specifically, coronary artery stenosis (less than 75%) on angiography, including 53 patients who had CAD at the first clinic visit. The other 228 patients did not have clinical or angiographic evidence of CAD. For each patient, the age at onset of CAD was determined by the first sign, ascertained by a standardized questionnaire, which included fixed clinical endpoints of CAD, administered by attending physicians at the clinic. The compliance with statins was evaluated from the medical records.

Clinical Risk Factors

Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared (kg/m^2). Hypertension was defined as the use of antihypertensive drugs or a blood pressure level higher than 140 mmHg systolic or 90 mmHg diastolic or both at the first clinic visit (the criteria for hypertension of the Japanese Society of Hypertension Guidelines)[15]. Diabetes mellitus was defined according to the 2002 Guideline for the Treatment of Diabetes Mellitus of the Japan Diabetes Society[16]. A family history of CAD was identified by the standardized questionnaire. Smoking was identified from patients' self-reporting. Achilles tendon thickness was measured as previously described[17].

Analysis of Serum Lipids

Fasting plasma lipid concentration was measured before any lipid-lowering treatment. Total cholesterol (TC), triglycerides (TG), and HDL cholesterol (HDL-C) levels were measured enzymatically using an automated system in the clinical laboratory of the NCVC. LDL-C level was calculated by the Friedewald formula when the TG level was less than 400 mg/dL; three patients with 400 mg/dL were omitted from this particular analysis. TG values were expressed as the median, (range), and logarithmically transformed before analysis.

Statistical Analysis

Statistical analysis was performed using the SPSS 15.0 (SPSS Inc., Chicago, IL) program. Parametric values are expressed as the mean ± standard deviation (SD). The statistical significance of differences in continuous variables was evaluated by Student's t test for unpaired data or ANOVA. The Pearson's χ^2 test was used to assess differences in the distribution of categorical traits.

Results

Patient Background

The baseline clinical characteristics of the 329

Table 1. Clinical characteristics of heterozygous FH patients with or without coronary artery disease (CAD) at first visit to our center.

	Total subjects	CAD (+)	CAD (−)	*p value*
n	329	101	228	
Age (years)	43.8 ± 16.0	48.9 ± 10.2	41.6 ± 17.6	<0.001
Sex				
Men	139 (42.2%)	66 (65.3%)	73 (32.0%)	<0.001
BMI (kg/m^2)	22.0±3.2	23.0 ± 2.7	22.6 ± 3.3	<0.001
Total cholesterol (mg/dL)	319 ± 70	333 ± 85	313 ± 61	0.039
Triglyceride (mg/dL)	(114) 80–176	(147) 96–193	(109) 76–162	0.263
HDL cholesterol (mg/dL)	50 ± 17	42 ± 14	54 ± 17	<0.001
LDL cholesterol (mg/dL)	241 ± 72	259 ± 84	232 ± 65	<0.001
Hypertension (*n*, %)	54 (16.4%)	33 (32.7%)	21 (9.2%)	<0.001
Diabetes Mellitus (*n*, %)	13 (4%)	8 (7.9%)	5 (2.2%)	0.014
Family history of CAD (*n*, %)	121 (36.8%)	46 (45.5%)	75 (32.9%)	0.028
Smoking habits (*n*, %)	127 (38.6%)	72 (71.3%)	55 (24.1%)	<0.001
Achilles tendon thickness (mm)	13.5 ± 5.4	16.2 ± 5.7	12.1 ± 4.6	<0.001
CAD present at first visit (*n*, %)	53 (16.1)	53 (52.5)	0 (0)	<0.001
Statin treatment at first clinic visit	39 (11.9)	18 (17.8)	21 (9.2)	0.541

Values are shown as the mean ± SD except for triglyceride. For triglyceride, the median (range) is shown.
BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; CAD, coronary artery disease

heterozygous FH patients analyzed in this study are shown in **Table 1**. Their plasma lipid and lipoprotein profiles are similar to patients in previous reports of Japanese FH[3, 18]. Patients with CAD were older, had higher levels of BMI, TC, and LDL-C, lower HDL-C, and a higher incidence of diabetes mellitus, hypertension, a family history of CAD, and smoking habit, compared to patients without CAD.

Onset of CAD

In the 101 patients with CAD, age by decade at the first onset of CAD is illustrated in **Fig. 1**. The average age was 45.8±10.6 years in men and 59.0±9.5 years in women, and this is consistent with a previous report of Japanese FH patients[13]. Analysis of CAD onset in relation to the presence (+) or absence (−) of statin treatment showed that in the 66 FH men with CAD, 13 did and 53 did not have statin treatment, and in the 35 FH women with CAD, 12 did and 23 did not have statin treatment. The age distribution at the first onset of CAD in statin (+) or statin (−) patients is shown in **Fig. 2**. The peak was at an older age in statin (+) men and women (Panels A and B, respectively) compared to statin (−). The lipid profile at the time of first onset of CAD in statin (+) and statin (−) patients is shown in **Table 2**. Statin (+) patients were older when CAD was identified and had lower TC and LDL-C levels than statin (−) patients.

To identify the factors that may influence the age

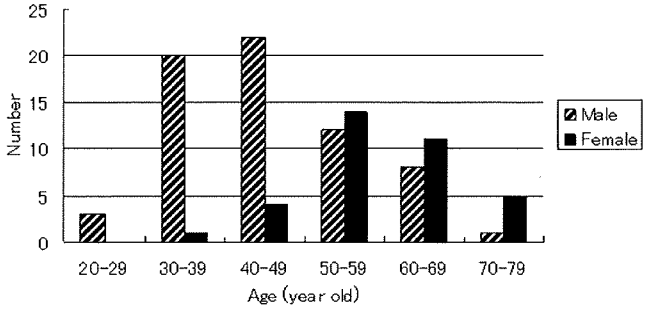

Fig. 1. Distribution of age when CAD was first identified in 101 men and women with heterozygous familial hypercholesterolemia (FH) and coronary artery disease (CAD), for the study period of 1969 to June 2007

at which CAD developed in statin (+) and statin (−) patients, we analyzed covariates (ANCOVA; **Table 3**), which included sex, smoking, BMI, hypertension, diabetes mellitus, family history of CAD, thickness of Achilles tendon, LDL-C levels, and the use of aspirin, probucol, and cholestyramine. We found that statin (+) patients were older when CAD developed, about 10 years older for each variable compared to statin (−) patients, which may be due to the use of statins and the reduction of LDL-C.

To determine the impact of statin treatment on the age at which CAD developed, we analyzed the same data for the pre- and post-statin eras. Pravastatin

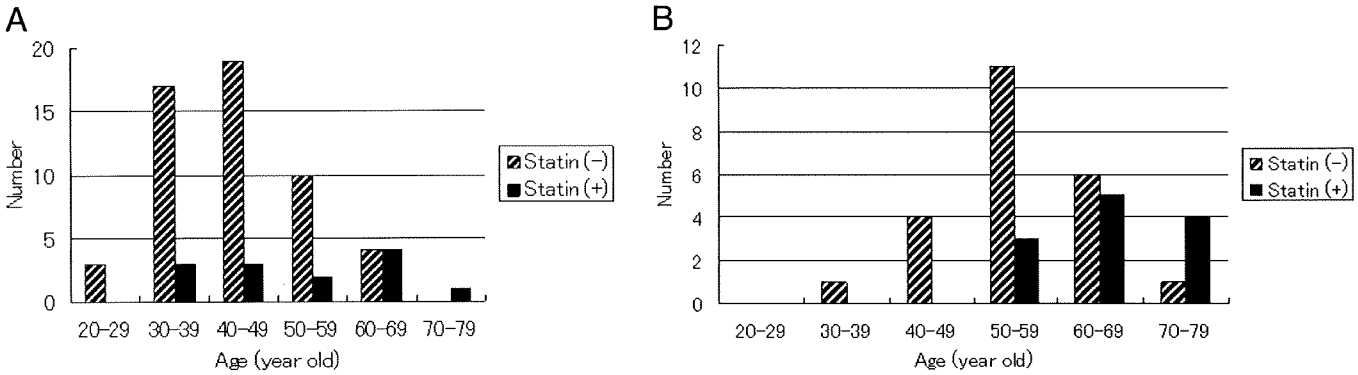

Fig. 2. Distribution of age when CAD was first identified in men (Panel A) and women (Panel B) with CAD taking a statin (+) or not (−)

Table 2. Age, lipid and lipoprotein profiles of FH at the onset of CAD in relation to statin use.

	Statin (+)	Statin (−)	*p value*
n	25	76	
Age of onset of CAD	57.8 ± 12.5	47.6 ± 10.5	<0.001
Lipid and lipoprotein profile at the event			
Total cholesterol (mg/dL)	242 ± 55	315 ± 108	<0.001
Triglycerides (mg/dL)	(127) 93-171	(115) 91-153	0.922
HDL cholesterol (mg/dL)	40 ± 12	38 ± 13	0.569
LDL cholesterol	167 ± 35	250 ± 108	<0.001

Values are shown as the mean ± SD except for triglyceride. For triglyceride, the median (range) is shown.

Table 3. Onset age of CAD adjusted by each variable.

Variables	Age (95% CI) in Statin (+)	Age (95% CI) in Statin (−)	*p value*
Overall	57.8 (55.3-60.3)	47.6 (46.4-48.8)	<0.001
Smoking habit	58.2 (54.1-62.3)	47.3 (44.8-49.7)	<0.001
Sex	57.2 (53.3-61.0)	48.1 (45.9-50.3)	<0.001
BMI	58.9(54.4-63.3)	47.5 (45.0-50.1)	<0.001
Hypertension	59.4 (54.8-64.4)	47.4 (44.8-49.9)	<0.001
Diabetes mellitus	58.7 (54.3-63.1)	47.7 (45.2-50.3)	<0.001
Family history of CAD	58.8 (54.4-63.2)	47.1 (44.6-49.7)	<0.001
Achilles tendon thickness	58.7 (54.3-63.2)	46.7 (44.0-49.4)	<0.001
LDL cholesterol	58.4 (53.9-63.0)	47.6 (45.0-50.3)	<0.001
Aspirin	57.2 (52.9-61.5)	48.2 (45.7-50.7)	0.001
Probucol	56.0 (51.0-61.0)	48.6 (46.0-51.3)	0.017
Cholestyramine	58.2 (53.0-63.3)	47.9 (45.2-50.6)	0.001

was the first statin approved in Japan. Patients were divided into two groups: Group 1 developed CAD before the end of September 1989 (n=53) and Group 2 developed CAD from October 1989 (to June 2007; n=48). Of the 66 men with CAD, 39 were in Group 1 and 27 in Group 2, and of the 35 women with CAD, 14 were in Group 1 and 21 in Group 2. The men and women whose CAD developed after the beginning of October 1989 were older than those who developed CAD before that date (**Fig. 3A, B**). At the first clinic visit, no clinical differences were seen in these patients in average age, BMI, plasma lipid and lipoprotein profile, Achilles tendon thickness and the incidence of hypertension, diabetes mellitus, and fam-