厚生労働科学研究研究費補助金

循環器疾患等総合研究事業

心筋微小血管造影装置の開発による糖尿病性心筋微小循環障害の可視化（臨床研究実施チームの整備）

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厚生労働科学研究費補助金（循環器疾患等総合研究事業）
（総括）研究報告書

心筋微小血管造影装置の開発による糖尿病性心筋微小循環障害の可視化に関する研究
（臨床研究実施チームの整備）

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微小血管の可視化を目的とし、病院設置型微小血管造影装置の開発とその臨床応用を進めている。ファントムを用いた検討では、理論上直径50μmまでの血管を描出することが可能であり、被検者の被曝量も、臨床上許容範囲にあることが判明した。血管新生療法を受ける患者を対象とし、治療前後における側副血行の発達を比較し、微小新生血管の描出を試みている。

A. 研究目的

難知性の重症末梢動脈閉塞症に対する血管新生療法の臨床応用が進められている。しかしながら、自覚症状の改善に比べ、血管造影等の諸検査では、有意な改善がが認められないことも少なくなく、血管新生療法の有効性に関する客観的な評価法の確立が望まれる。

糖尿病性微小血管障害に関しても、その病態把握や治療効果判定の手段は未だ確立されていないのが現状である。

本研究の目的は、病院設置型微小血管造影装置を開発、臨床応用することによって、上記のような微小循環障害の病態および治療効果の新しい評価法を確立することである。

B. 研究方法

新エネルギー産業技術総合開発機構(NEDO)の支援のもと、浜松ホトニクス(株)を中心に、NHKエンジニアリングサービス、国立循環器病センター研究所、東海大学医学部等が協力して、病院設置型の微小血管造影装置を開発した。装置は、高出力のCT用X線源とハイビジョンの高感度撮像系により構成されている。チャートを用いて、解像度を測定し、犬冠動脈のファントムで中核枝の評価およびウサギの虚血肢モデルでの再生血管の評価を行った。また、吸収線量および散乱線の測定を行い、安全性の検討をした。臨床応用では、末梢動脈閉塞症に対する血管新生療法前後に微小血管造影を施行し、虚血肢下腿の微小血管を評価した。

(倫理面への配慮)

倫理委員会の審議・承認を得、本検査の合併症・効能・不利益・利益を説明し、本人及び家族の同意の元に施行した。

C. 研究結果

一般の血管造影では250μmが解像度の限界であったが、病院設置型微小血管造影装置では50μmまで観察可能であった。ヒトに対する臨床応用として、血管新生療法を行う下肢末梢動脈閉塞症の患者を対象に、これまでに合計7回の微小血管造影を施行した。造影に伴う被曝線量は通常の血管造影と同レベルであることが判明した。微小血管造影によって通常の造影では描出困難な100μm以下の微小血管が鮮明に描出された。DSAに比較して少なくとも1−2分枝末梢側の血管が描出可能であった。1ヶ月から1年の間隔を置いて施行したフォローアップ造影における微小血管の再現性は良好であった。

D. 考察

病院設置型微小血管造影装置の1号機は、通常の血管造影と同等の安全性を有している。また、その微小血管描出能は通常装置に比し優れていることは明白で、ヒトの微小血管評価に用いることが可能な新しい検査法と言える。造影検査を繰り返して施行し得た症例における微小血管の再現性は良好であり、血管新生療法前後における新生血管の評価に関しても、本装置を用いて行うことが可能と思われた。ただし、現時点ではまだ症例数が少なく、新生血管出現の有無について結論するには至っていない。

E. 結論

本研究で開発された病院設置型微小循環造影装置は50μmの微小血管が観察可能であり、安全性や再現性にも問題なかった。末梢動脈閉塞症に対する血管新生療法によって、微小血管新生がどのように促進されているのか、その評価については、今後の症例の積み重ねが必要で

ある。

F. 健康危険情報
特になし。

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H. 知的財産権の出願・登録状況
特になし。

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別紙4

研究成果の刊行に関する一覧表

【原著】

発表者氏名	論文タイトル名		巻号	ページ	出版年
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A Long-Acting Prostacyclin Agonist with Thromboxane Inhibitory Activity for Pulmonary Hypertension

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Rationale: The balance between prostacyclin and thromboxane plays an important role in the regulation of pulmonary vascular tone. Recently, we developed ONO-1301, a novel, long-acting prostacyclin agonist with thromboxane synthase inhibitory activity.
Objectives: We investigated whether modulation of prostacyclin/thromboxane balance by ONO-1301 ameliorates monocrotaline-induced pulmonary hypertension in rats.
Methods: After subcutaneous injection of monocrotaline or vehicle, rats were randomized to receive repeated subcutaneous administration of ONO-1301 or vehicle twice per day for 3 wk.
Measurements and Main Results: There was significant development of pulmonary hypertension 3 wk after monocrotaline injection. Treatment with ONO-1301 significantly attenuated the increases in right ventricular systolic pressure and ratio of right ventricular weight to body weight in monocrotaline rats. Furthermore, ONO-1301 significantly attenuated the increase in medial wall thickness of peripheral pulmonary arteries in monocrotaline rats. The half-life of plasma ONO-1301 concentration after a single subcutaneous administration was approximately 5.6 h. A single administration of ONO-1301 increased plasma cyclic adenosine 3′, 5′-monophosphate level, which lasted at least up to 8 h. Treatment with ONO-1301 significantly decreased plasma 11-dehydro-thromboxane B_2, a metabolite of thromboxane, in monocrotaline rats. Finally, Kaplan-Meier survival curves demonstrated that repeated administration of ONO-1301 improved survival rate in monocrotaline rats compared with vehicle administration (80 vs. 30% in 6-wk survival).
Conclusions: Subcutaneous administration of a novel prostacyclin agonist (ONO-1301) markedly attenuated monocrotaline-induced pulmonary hypertension and improved survival in rats. The beneficial effects of ONO-1301 may occur through its long-lasting stimulation of cyclic adenosine 3′, 5′-monophosphate and inhibition of thromboxane synthase.

Keywords: cAMP; monocrotaline; hemodynamics; vascular remodeling

Pulmonary arterial hypertension is a rare but life-threatening disease (1, 2). The pathogenesis includes pulmonary vasoconstriction, endothelial cell proliferation, smooth muscle cell proliferation, and *in situ* thrombosis (3, 4). Prostacyclin, a metabolite of arachidonic acid, has vasoprotective effects, including vasodilation, antiplatelet aggregation, and inhibition of smooth muscle cell proliferation (5–8). Thus, continuous intravenous infusion of prostacyclin (epoprostenol) has become recognized as a therapeutic breakthrough for pulmonary arterial hypertension (9–16). The dramatic success of long-term intravenous prostacyclin has led to the development of prostacyclin analogs (oral beraprost, aerosolized iloprost, and subcutaneous treprostinil; Figure 1) (17–20). Nevertheless, treatment with prostacyclin or its analogs has some problems in the clinical setting. Their duration of acting is so short that they need to be continuously infused or frequently administered (9–20). In addition, these compounds failed to inhibit thromboxane synthesis during treatment (21).

We developed a new type of prostacyclin agonist, ONO-1301, which has long-lasting prostacyclin activity and thromboxane synthase inhibitory activity. Prostacyclin and its analogs are unstable because 15-hydroxyprostaglandin dehydrogenase metabolizes their prostanoid structures, including a five-membered ring and allylic alcohol. In contrast, ONO-1301 is chemically and biologically stable because of the absence of prostanoid structures. Interestingly, ONO-1301 has thromboxane synthase inhibitory activity because of the presence of a 3-pyridine radical. It has been reported that augmented release of thromboxane A_2, which is both a potent pulmonary vasoconstrictor and a procoagulant (22), occurs in patients with pulmonary hypertension (23, 24). The imbalance of thromboxane and prostacyclin is considered to contribute to the development of pulmonary arterial hypertension. These findings raise the possibility that administration of ONO-1301 may have beneficial effects on pulmonary hemodynamics.

Thus, the purposes of this study were (*1*) to investigate whether a single subcutaneous administration of ONO-1301 has long-lasting prostacyclin activity in rats, (*2*) to investigate whether subcutaneous administration of ONO-1301 inhibits thromboxane synthesis in monocrotaline (MCT)-induced pulmonary hypertension in rats, (*3*) to examine whether intermittent subcutaneous ONO-1301 improves pulmonary hemodynamics and survival in MCT rats, and (*4*) to elucidate the underlying mechanisms responsible for the beneficial effects of this compound.

METHODS

Animals

We used 95 male Wistar rats weighing 100 to 120 g. The rats were randomly given a subcutaneous injection of either 60 mg/kg MCT (MCT rats) or 0.9% saline (vehicle) and assigned to receive repeated subcutaneous injection of ONO-1301 (Ono Pharmaceutical Co., Ltd., Osaka, Japan) or vehicle. This protocol resulted in the creation of three groups: sham rats given vehicle (n = 10), MCT rats given vehicle (n = 10), and MCT rats treated with ONO-1301 (n = 13). In addition, 20 rats were studied to evaluate the effect of ONO-1301 on survival in MCT rats. Furthermore, 42 rats were studied to evaluate the effect of ONO-1301 on plasma cAMP level (n = 12) and 11-dehydro-thromboxane B_2 (TXB_2) level (n = 30).

In Vivo Experimental Protocol

After rats were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg), they were given a subcutaneous injection of either

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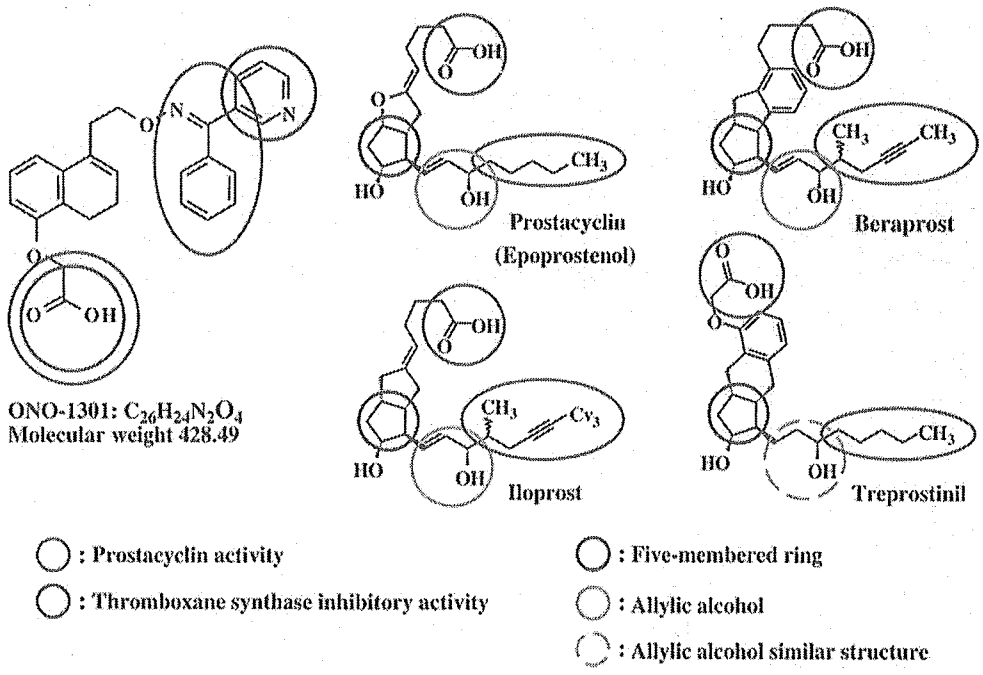

Figure 1. Molecular structures of ONO-1301, epoprostenol, and conventional prostacyclin analogs (beraprost, iloprost, and treprostinil). Epoprostenol and prostacyclin analogs share common characteristic prostanoid structures, including a five-membered ring and allylic alcohol (*blue and yellow circles*). In contrast, ONO-1301 has a carboxylic acid and a lipid-soluble functional group that activate the prostacyclin receptor (*green circles*), but does not have prostanoid structures, which allows long-lasting prostacyclin activity. Unlike epoprostenol and conventional prostacyclin analogs, ONO-1301 has thromboxane synthase inhibitory activity because of a 3-pyridine radical and carboxylic acid within its molecule (*red circles*).

60 mg/kg MCT or vehicle. Then, ONO-1301 (20 mg/kg/d) or vehicle was injected subcutaneously twice per day for 3 wk after MCT injection. Animals were maintained on standard rat chow. Hemodynamic studies were performed on Day 22. A polyethylene catheter (PE-50) was inserted into the right carotid artery to measure heart rate and mean arterial pressure. A polyethylene catheter (PE-50) was inserted through the right jugular vein into the right ventricle (RV) for measurement of RV pressure. Finally, cardiac arrest was induced by injection of 2 mmol/L potassium chloride through the catheter. The ventricles and lungs were excised, dissected free, and weighed. The ratio of RV weight to body weight (RV/BW), the ratio of left ventricular plus septal weight to body weight (LV + S/BW), and the ratio of RV weight to left ventricular plus septal weight (RV/LV + S) were calculated as indexes of ventricular hypertrophy, as reported previously (25). All protocols were performed in accordance with guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute.

Morphometric Analysis of Pulmonary Arteries

Paraffin sections 4-μm thick were obtained from the middle region of the right lung and stained with elastic van Gieson for examination by light microscopy. The external diameter and medial wall thickness of the pulmonary arteries were measured in 20 muscular arteries (ranging in size from 25 to 100 μm in external diameter) by two investigators who were blinded to treatment allocation. For each artery, the medial wall thickness was expressed as follows: % wall thickness = ([medial thickness × 2]/external diameter) × 100, as reported previously (26).

Assay for Plasma ONO-1301 Concentration

To estimate the half-life of ONO-1301, we measured plasma ONO-1301 concentration in rats after a subcutaneous injection (n = 4). Blood was drawn at 0.25, 0.5, 1, 2, 4, 8, and 24 h after a single subcutaneous administration of ONO-1301 (10 mg/kg). Plasma ONO-1301 concentration was measured by liquid chromatography tandem mass spectrometry assay.

Assay for Plasma cAMP Level

To investigate whether a single subcutaneous administration of ONO-1301 has long-lasting prostacyclin activity in rats, we measured plasma cAMP levels after ONO-1301 injection. Twelve rats were assigned to receive a single subcutaneous injection of ONO-1301 (10 mg/kg) or vehicle (n = 6 each). Blood was drawn from the right carotid artery at baseline and 1, 2, 4, 6, and 8 h after ONO-1301 injection. Blood was immediately transferred into a chilled glass tube containing disodium ethylenediaminetetraacetic acid (1 mg/ml) and aprotinin (500 U/ml) and centrifuged immediately. Plasma cAMP levels was measured with a radioimmunoassay kit (cAMP assay kit; Yamasa Shoyu, Chiba, Japan), as reported previously (27).

Assay for Plasma 11-dehydro-TXB_2 Level

To investigate the acute effect of ONO-1301 or prostacyclin (epoprostenol) on thromboxane synthesis in MCT rats, we measured plasma 11-dehydro-TXB_2, a metabolite of thromboxane A_2, after administration of ONO-1301 (10 mg/kg), epoprostenol, or vehicle (n = 5 each). Epoprostenol was infused via a polyethylene catheter (PE-50) inserted into the right jugular vein. Infusion of epoprostenol was begun at 10 ng/kg/min, increased gradually to 150 ng/kg/min over 30 min, escalated to 300 ng/kg/min over the next 30 min, and held at this dose for 1 h, as reported previously (21, 28, 29).

To investigate the chronic effect of ONO-1301 on thromboxane synthesis in MCT rats, we measured plasma 11-dehydro-TXB_2 after repeated subcutaneous injection of ONO-1301 (20 mg/kg/d) or vehicle twice per day for 3 wk (n = 5 each). Blood was drawn from the right carotid artery and plasma 11-dehydro-TXB_2 level was measured with an enzyme immunoassay kit (11-dehydro-TXB_2 assay kit; Cayman Chemical Co., Ann Arbor, MI), as reported previously (30).

Survival Analysis

To evaluate the effect of intermittent subcutaneous administration of ONO-1301 on survival in MCT rats, 20 rats received repeated injection of ONO-1301 or vehicle twice per day (n = 10 each). Survival was estimated from the date of MCT injection to the death of the rat or 6 wk after injection.

Statistical Analysis

All data were expressed as mean ± SEM. Comparisons of parameters among the three groups were made by one-way analysis of variance, followed by Newman-Keuls' test. Comparisons of the time course of parameters between the two groups were made by two-way analysis of variance for repeated measures, followed by Newman-Keuls' test. Survival curves were derived by the Kaplan-Meier method and compared by log-rank test. A value of $p < 0.05$ was considered statistically significant.

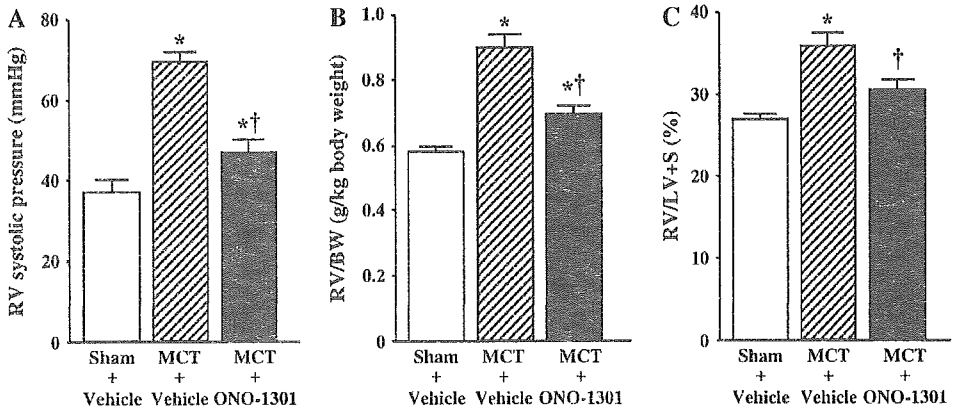

Figure 2. Effects of ONO-1301 on right ventricular (RV) systolic pressure (*A*), RV weight to body weight (RV/BW; *B*), and RV weight to left ventricular plus septal weight (RV/LV + S; *C*) in sham rats given vehicle (sham + vehicle), monocrotaline (MCT) rats given vehicle (MCT + vehicle), and MCT rats treated with ONO-1301 (MCT + ONO-1301). Data are mean ± SEM. *$p < 0.05$ versus sham + vehicle; †$p < 0.05$ versus MCT + vehicle.

RESULTS

Effects of ONO-1301 on Pulmonary Hemodynamics and Vascular Remodeling

RV systolic pressure was significantly increased 3 wk after MCT injection (Figure 2A). However, the increase was significantly attenuated by subcutaneous administration of ONO-1301 (10 mg/kg twice per day). Similarly, the increases in RV/BW and RV/LV + S in MCT rats were significantly attenuated by treatment with ONO-1301 (Figures 2B and 2C). There were no significant differences in heart rate or mean arterial pressure among the three groups (Table 1).

Representative photomicrographs showed that hypertrophy of the pulmonary vessel wall after MCT injection was attenuated in MCT rats treated with ONO-1301 compared with those given vehicle (Figure 3A). Quantitative analysis demonstrated a significant increase in percent wall thickness after MCT injection, but this change was ameliorated by ONO-1301 (Figure 3B).

Long-Lasting Activity of ONO-1301

We measured plasma ONO-1301 concentrations after a single subcutaneous administration of ONO-1301. The increase in plasma ONO-1301 concentration reached a peak at 4 h, and the half-life of plasma ONO-1301 concentration was approximately 5.6 h (Figure 4). In addition, a single subcutaneous administration of ONO-1301 significantly increased plasma cAMP level in rats (Figure 5). The increase in plasma cAMP level reached a peak at 6 h and lasted at least up to 8 h after ONO-1301 injection. These results suggest that subcutaneous administration of ONO-1301 has long-lasting activity in rats.

Inhibitory Effect of ONO-1301 on Thromboxane Synthase

Although administration of prostacyclin (epoprostenol) markedly increased plasma 11-dehydro-TXB_2 level in MCT rats with established pulmonary hypertension, ONO-1301 did not significantly increase plasma 11-dehydro-TXB_2 level, even after bolus injection (Figure 6A). Plasma 11-dehydro-TXB_2 level was markedly elevated 3 wk after MCT injection (Figure 6B). However, 3-wk treatment with ONO-1301 significantly attenuated the increase in plasma 11-dehydro-TXB_2 level in MCT rats.

Survival Analysis

Kaplan-Meier survival curves demonstrated that MCT rats treated with ONO-1301 had a significantly higher survival rate than MCT rats given vehicle (80 vs. 30% in 6-wk survival; Figure 7).

DISCUSSION

In the present study, we demonstrated that (*1*) a novel prostacyclin agonist (ONO-1301) ameliorated the development of MCT-induced pulmonary hypertension and improved survival in MCT rats; (*2*) ONO-1301 had a long half-life of approximately 5.6 h, and a single administration of ONO-1301 caused a long-lasting increase in plasma cAMP level; and (*3*) ONO-1301 attenuated the increase in plasma 11-dehydro-TXB_2 level in MCT rats.

TABLE 1. PHYSIOLOGIC PROFILES OF EXPERIMENTAL GROUPS

	Sham + Vehicle	MCT + Vehicle	MCT + ONO-1301
No.	10	10	13
BW, g	210 ± 3	159 ± 13*	176 ± 2*
Heart rate, bpm	410 ± 7	400 ± 14	405 ± 16
MAP, mm Hg	122 ± 3	114 ± 5	113 ± 3
RV systolic pressure, mm Hg	36 ± 2	68 ± 4*	47 ± 3*†
RV/BW, g/kg BW	0.58 ± 0.01	0.90 ± 0.05*	0.69 ± 0.03*†
RV/LV + S	0.27 ± 0.01	0.36 ± 0.02*	0.30 ± 0.01†
LV + S/BW, g/kg BW	2.15 ± 0.04	2.52 ± 0.12*	2.26 ± 0.04†

Definition of abbreviations: bpm = beats/minute; BW = body weight; LV + S/BW = ratio of left ventricular plus septal weight to body weight; MAP = mean arterial pressure; MCT = monocrotaline; MCT + ONO-1301, MCT rats treated with ONO-1301; MCT + Vehicle, MCT rats given vehicle; RV = right ventricular; RV/BW = ratio of RV weight to body weight; RV/LV + S = ratio of RV weight to left ventricular plus septal weight; sham + vehicle, sham rats given vehicle.

Data are mean ± SEM.

* $p < 0.05$ versus sham + vehicle.

† $p < 0.05$ versus MCT + vehicle.

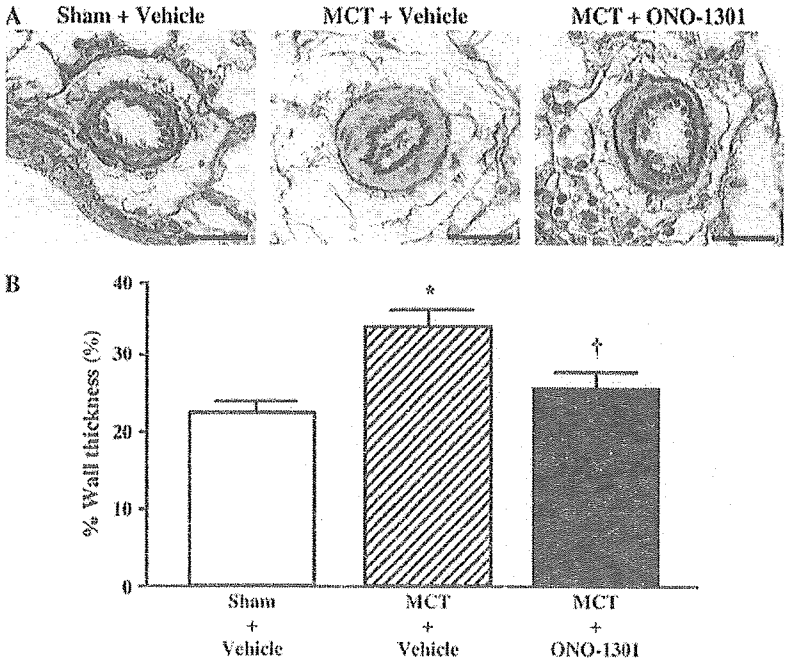

Figure 3. (*A*) Representative photomicrographs of peripheral pulmonary arteries 3 wk after MCT injection. *Scale bars* = 20 μm. (*B*) Quantitative analysis of percent wall thickness in peripheral pulmonary arteries. Data are mean ± SEM. *$p < 0.05$ versus sham + vehicle; †$p < 0.05$ versus MCT + vehicle.

Conventional prostacyclin and its analogs need continuous infusion or frequent administration because of their short duration of acting. Epoprostenol has a very short half-life (< 6 min), iloprost has a serum half-life of 20 to 25 min, and the elimination half-life of beraprost is 35 to 40 min after oral administration (31). Treprostinil sodium, a stable prostacyclin analog, has been reported to have a half-life of 4.6 h after cessation of continuous subcutaneous infusion (32). With regard to cAMP, a second messenger of prostacyclin and its analogs, it has been reported that plasma cAMP levels remained increased at 4 h and normalized at 6 h after inhalation of iloprost (33), and that plasma cAMP levels reached a peak at 30 min and subsequently returned to baseline levels at 2 h after administration of oral beraprost (27). In our results, the half-life of plasma ONO-1301 concentration was approximately 5.6 h, and a single subcutaneous administration of ONO-1301 increased plasma cAMP level at least up to 8 h. Because the method for administration was different between ONO-1301 and conventional prostacyclin analogs (compare a subcutaneous single shot of ONO-1301, continuous intravenous infusion of epoprostenol, continuous subcutaneous infusion of treprostinil, inhalation of iloprost, and oral administration of beraprost), it is difficult to directly compare the lasting effects of prostacyclin activity of ONO-1301 with that of conventional prostacyclin analogs. Nevertheless, the long half-life of ONO-1301 and long-lasting increases in plasma cAMP levels indicate that ONO-1301 exhibits chemical and biologic stability comparable to that of conventional prostacyclin and its analogs. ONO-1301 does not contain prostanoid structures such as a five-membered ring and allylic alcohol, which are subject to metabolism by 15-hydroxyprostaglandin dehydrogenase. These may be the reason for the long-lasting activity of ONO-1301. The present study also demonstrated that repeated administration of ONO-1301 twice per day markedly attenuated the development of MCT-induced pulmonary hypertension, as indicated by significant decreases in RV systolic pressure and RV weight. Thus, intermittent subcutaneous administration of ONO-1301 may be sufficient for the treatment of pulmonary hypertension.

Thromboxane, produced by endothelial cells and platelets, has a potent vasoconstrictor effect, smooth muscle mitogenic property, and platelet aggregation effect (22). Earlier studies

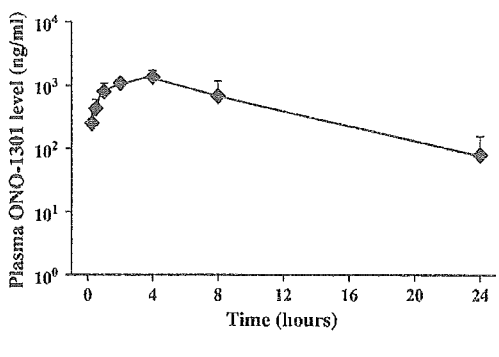

Figure 4. Plasma ONO-1301 concentration after a subcutaneous injection of this compound.

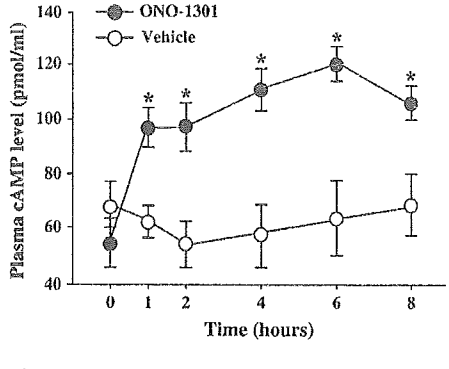

Figure 5. Changes in plasma cAMP level after a single subcutaneous administration of ONO-1301 (*solid circles*) or vehicle (*open circles*). Data are mean ± SEM. *$p < 0.05$ versus vehicle.

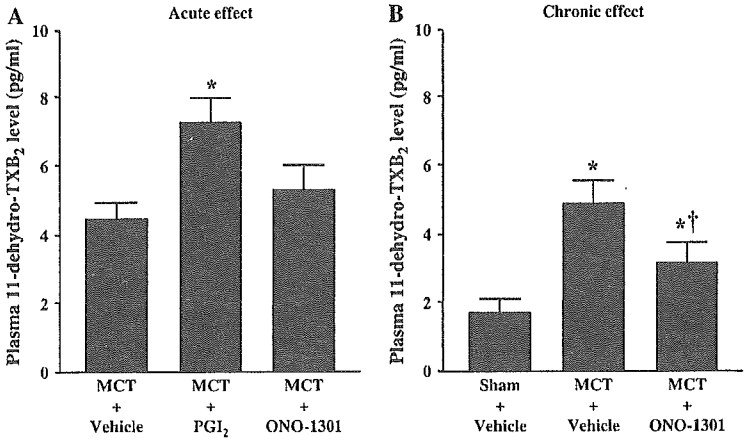

Figure 6. (*A*) Acute effects of ONO-1301 and epoprostenol (PGI_2) on plasma 11-dehydro-thromboxane B_2 (TXB_2), a metabolite of thromboxane A_2, in MCT rats. Data are mean ± SEM. *p < 0.05 versus MCT + vehicle. (*B*) Chronic effects of 3-wk treatment with ONO-1301 on plasma 11-dehydro-TXB_2 level. Data are mean ± SEM. *p < 0.05 versus sham + vehicle; †p < 0.05 versus MCT + vehicle.

have demonstrated impaired prostacyclin synthesis and increased thromboxane production in patients with pulmonary arterial hypertension, suggesting that imbalance of the release of thromboxane and prostacyclin plays an important role in the development of pulmonary hypertension (23, 24). Furthermore, thromboxane-receptor density is increased in the RV of patients with pulmonary hypertension (34). Rich and colleagues have shown that inhibition of thromboxane synthase modestly improves pulmonary hemodynamics in patients with pulmonary arterial hypertension (35). ONO-1301 has a 3-pyridine radical, which is known to inhibit thromboxane synthase through interaction with carboxylic acid via a hydrogen bond. In the present study, plasma 11-dehydro-TXB_2 level was markedly elevated in MCT rats. However, treatment with ONO-1301 greatly diminished its level. Furthermore, in the acute phase, plasma 11-dehydro-TXB_2 level in MCT rats did not significantly increase after administration of ONO-1301, although the plasma level markedly increased after epoprostenol infusion, which is consistent with the earlier study of Cuiper and colleagues (21). Therefore, it is possible that ONO-1301 attenuates MCT-induced pulmonary hypertension partly via improvement of prostacyclin/thromboxane imbalance.

In the present study, ONO-1301 also attenuated the increase in medial wall thickness of peripheral pulmonary arteries. Activation of prostacyclin receptors has been shown to suppress the growth of vascular smooth muscle cells through a cAMP-dependent pathway. Thus, ONO-1301 may attenuate the development of pulmonary vascular remodeling at least in part via a cAMP-dependent pathway. Importantly, repeated administration of ONO-1301 improved survival in MCT rats compared with vehicle administration. The increased survival rate in MCT rats is considered to be associated with the amelioration of pulmonary hypertension. Thus, intermittent subcutaneous administration of ONO-1301 may be an alternative approach for severe pulmonary hypertension refractory to conventional therapy.

In conclusion, subcutaneous administration of a novel prostacyclin agonist (ONO-1301) markedly attenuated MCT-induced pulmonary hypertension and improved survival in rats. The beneficial effects of ONO-1301 may occur through its long-lasting stimulation of cAMP and inhibition of thromboxane synthase. Thus, administration of this compound may be a promising therapeutic strategy for the treatment of pulmonary arterial hypertension.

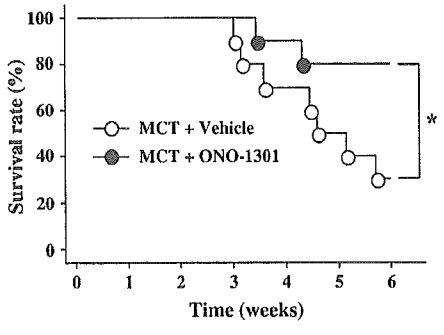

Figure 7. Kaplan-Meier survival curves showing significantly higher survival rate in MCT + ONO-1301 (*solid circles*) than in MCT + vehicle (*open circles*; log-rank test, *p < 0.05).

Conflict of Interest Statement: None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Prostacyclin agonist with thromboxane synthase inhibitory activity (ONO-1301) attenuates bleomycin-induced pulmonary fibrosis in mice

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[1]*Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, Osaka;* [2]*Second Department of Internal Medicine, Nara Medical University, Nara;* [3]*Department of Internal Medicine, National Cardiovascular Center, Osaka;* [4]*Department of Cardiac Physiology, National Cardiovascular Center Research Institute, Osaka;* [5]*Ono Pharmaceutical Co., Ltd. Research Headquarters, Osaka; and* [6]*Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan*

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Murakami, Shinsuke, Noritoshi Nagaya, Takefumi Itoh, Masaharu Kataoka, Takashi Iwase, Takeshi Horio, Yoshinori Miyahara, Yoshiki Sakai, Kenji Kangawa, and Hiroshi Kimura. Prostacyclin agonist with thromboxane synthase inhibitory activity (ONO-1301) attenuates bleomycin-induced pulmonary fibrosis in mice. *Am J Physiol Lung Cell Mol Physiol* 290: L59–L65, 2006. First published September 9, 2005; doi:10.1152/ajplung.00042.2005.—The balance between prostacyclin and thromboxane A_2 (TXA_2) plays an important role in pulmonary homeostasis. However, little information is available regarding the therapeutic potency of these prostanoids for pulmonary fibrosis. We have recently developed ONO-1301, a novel long-acting prostacyclin agonist with thromboxane synthase inhibitory activity. Thus we investigated whether repeated administration of ONO-1301 attenuates bleomycin-induced pulmonary fibrosis in mice. After intratracheal injection of bleomycin or saline, mice were randomized to receive repeated subcutaneous administration of ONO-1301 or vehicle. Bronchoalveolar lavage (BAL) and histological analyses were performed at 3, 7, and 14 days after bleomycin injection. In vitro studies using mouse lung fibroblasts were also performed. ONO-1301 significantly attenuated the development of bleomycin-induced pulmonary fibrosis, as indicated by significant decreases in Ashcroft score and lung hydroxyproline content. ONO-1301 significantly reduced total cell count, neutrophil count, and total protein level in BAL fluid in association with a marked reduction of TXB_2. A single administration of ONO-1301 significantly increased plasma cAMP level for >2 h. In vitro, ONO-1301 and a cAMP analog dose-dependently reduced cell proliferation in mouse lung fibroblasts. The reduction in cell proliferation by ONO-1301 was attenuated by a protein kinase A (PKA) inhibitor. Furthermore, bleomycin mice treated with ONO-1301 had a significantly higher survival rate than those given vehicle. These results suggest that repeated administration of ONO-1301 attenuates the development of bleomycin-induced pulmonary fibrosis and improves survival in bleomycin mice, at least in part by inhibition of TXA_2 synthesis and activation of the cAMP/PKA pathway.

adenosine 3′,5′-cyclic monophosphate; fibroblast; neutrophil; survival

IDIOPATHIC PULMONARY FIBROSIS (IPF) is a life-threatening disease characterized by progressive dyspnea and worsening of pulmonary function (6, 22). The pathological features of IPF are fibroblast proliferation with increased amounts of extracellular matrix and varying degrees of persistent inflammation of the alveolar septa (22). Thus a novel therapeutic strategy against these abnormalities may be effective for the treatment of IPF.

Prostanoids, which are metabolites of arachidonic acid, are important regulators of pulmonary homeostasis. Prostacyclin inhibits migration, proliferation, and collagen synthesis in fibroblasts (14, 29). Conversely, thromboxane A_2 (TXA_2) promotes fibroblast proliferation, increases pulmonary vascular permeability, and induces lung inflammation (18, 21, 24). Interestingly, a recent study has demonstrated that the decreased ratio of prostacyclin synthesis to thromboxane synthesis is associated with the development of pulmonary fibrosis (4). Thus we hypothesized that compounds that regulate the balance between prostacyclin and TXA_2 may have beneficial effects in IPF.

Recently, we have developed a novel nonprostanoid long-acting prostacyclin agonist possessing a potent inhibitory activity against thromboxane synthase, named ONO-1301. Unlike prostacyclin, ONO-1301 does not possess a five-membered ring and allylic alcohol in its molecule, which contributes to the biological and chemical stability of this compound. As a result, this compound can be given by subcutaneous administration twice a day. Its inhibitory effect on thromboxane synthesis is mediated by binding of thromboxane synthase to 3-pyridine moiety and a carboxylic acid group in ONO-1301 (30).

Thus the purpose of this study was to investigate whether modulation of prostacyclin/TXA_2 balance by ONO-1301 attenuates bleomycin-induced pulmonary fibrosis and improves survival in bleomycin-injected mice.

METHODS

Animals. We used specific pathogen-free female C57BL/6 mice weighing 18–20 g. The mice were randomly given intratracheal injection of either bleomycin (Nippon Kayaku, Tokyo, Japan) or saline, and assigned to receive repeated administration of ONO-1301 or vehicle. This protocol resulted in the creation of three groups: sham mice given vehicle (Sham group, $n = 34$), bleomycin mice given vehicle (Vehicle group, $n = 34$), and bleomycin mice treated with ONO-1301 (ONO-1301 group, $n = 34$). Twenty-four additional mice were studied to evaluate the effect of ONO-1301 administration on survival in bleomycin mice. Twenty-five mice were studied to examine the effect of ONO-1301 on plasma cAMP. Finally, 15 mice were

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used to examine the effects of ONO-1301 on established pulmonary fibrosis. All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute.

ONO-1301. We have recently developed a novel nonprostanoid long-acting prostacyclin agonist possessing a potent inhibitory activity against thromboxane synthase, {7,8-dihydro-5-[(E)-2-[(α-(3-pyridyl)benzylidene)amino-oxy]ethyl]-1-naphtyl-oxy} acetic acid, named ONO-1301 (Fig. 1). This compound has two interesting structural features. First, as stated above, unlike prostacyclin, ONO-1301 does not possess a five-membered ring and allylic alcohol in its molecule. This structural feature contributes to the biological and chemical stability of this compound. Second, this compound possesses a 3-pyridine moiety at one end of the molecule and a carboxylic acid group at the other. The inhibitory effect of ONO-1301 on thromboxane synthesis is mediated by binding of thromboxane synthase to 3-pyridine moiety and a carboxylic acid group in ONO-1301.

In vivo experimental protocol. After the mice were anesthetized by intraperitoneal injection of pentobarbital sodium, they were given intratracheal injection of either bleomycin (0.02 or 0.03 units/mouse) dissolved in 50 μl of saline or saline alone. Then, ONO-1301 (6 $mg \cdot kg^{-1} \cdot day^{-1}$) dissolved in 100 μl of saline, or saline was administered by subcutaneous injection twice a day throughout the experiment. This dose was determined to obtain maximum effects, based on dose-response experiments. Systolic blood pressure in the conscious state was measured by the indirect tail-cuff method with a blood pressure monitor (MK-2000; Muromachi Kikai, Tokyo, Japan) 0, 30, 60, 120, and 360 min after administration. ONO-1301 at a dose of 6 $mg \cdot kg^{-1} \cdot day^{-1}$ did not influence blood pressure. The mice were maintained under standard conditions with free access to food and water.

The effects of ONO-1301 on pulmonary fibrosis were analyzed by the following parameters: *1*) the severity of pulmonary fibrosis such as histological examination using the Ashcroft score and lung hydroxyproline content on *day 14* (0.02 units/mouse), 2) the severity of acute lung injury such as that reflected in total cell count, differential cell count, and total protein level in bronchoalveolar lavage (BAL) fluid on *day 3* and *day 7* (0.02 units/mouse), *3*) TXB_2 and active transforming growth factor-β1 (TGF-β1) levels in BAL fluid on *day 3* and *day 7* (0.02 units/mouse), *4*) intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression in whole lung tissue on *days 3* and *7* (0.02 units/mouse), and *5*) the survival rate on *day 21* (0.03 units/mouse).

Finally, to investigate the effects of ONO-1301 on established pulmonary fibrosis, 15 mice were randomly given an intratracheal injection of either bleomycin or saline. ONO-1301 or saline was administered from *day 14* to *28* (Sham, Vehicle, and ONO-1301 groups, $n = 5$ each). These mice were evaluated on *day 28*.

BAL analysis. Total and differential cell counts in BAL fluid were determined as described previously ($n = 5$ each) (20). The supernatant of BAL fluid was used for the measurement of total protein, TXB_2, which is a stable metabolite of TXA_2, and active TGF-β1 levels. The total protein level was measured by Bradford assay (Bio-Rad, Tokyo, Japan). The TXB_2 level was measured with an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). The active TGF-β1 level was measured with a mouse TGF-β1 ELISA kit (R&D Systems, Minneapolis, MN).

Histological examination. The right lung was fixed by inflation with 4% paraformaldehyde and embedded in paraffin ($n = 5$ each). Sections 4 μm thick were stained with hematoxylin-eosin. The Ashcroft score was used for semiquantitative assessment of fibrotic changes (1). The severity of fibrotic changes in each histological section of the lung was assessed as the mean score of severity from observed microscopic fields. Thirty fields in each section were analyzed. Grading was performed in a blinded fashion by three observers, and the mean was taken as the fibrosis score.

Measurement of hydroxyproline content. To quantify lung collagen content as an indicator of pulmonary fibrosis, the hydroxyproline content in the lung was measured in each group according to the previously described method ($n = 5$ each) (20). The left lung was quick-frozen and kept at −80°C until the assay. After the lung was homogenized, the suspension was hydrolyzed with 0.5 ml of 12 N hydrochloric acid for 20 h at 100°C. After neutralization, a 0.1 ml aliquot of supernatant was mixed in 1.5 ml of 0.3 N lithium hydroxide solution. The hydroxyproline content was analyzed by high-performance chromatography.

Quantification of ICAM-1 and VCAM-1 expression by ELISA. We investigated the effect of ONO-1301 on ICAM-1 and VCAM-1, which are key molecules in leukocyte migration into lung tissues, expression in the bleomycin-treated lung. The treated lungs were quick-frozen and kept at −80°C until the assay. The lungs were homogenized in 1.5 ml of saline. The homogenates were centrifuged at 2,000 *g* for 10 min at 4°C, and the supernatants were assayed for ICAM-1 and VCAM-1 concentrations by ELISA kits (R&D Systems).

Measurement of cAMP level. To evaluate the effect of ONO-1301 on plasma cAMP, normal mice were assigned to receive a single administration of ONO-1301 (3 mg/kg). Blood samples were obtained 0, 30, 60, 120, and 360 min after administration and were immediately transferred into a chilled glass tube containing disodium EDTA (1 mg/ml) and aprotinin (500 U/ml) and centrifuged immediately at 4°C

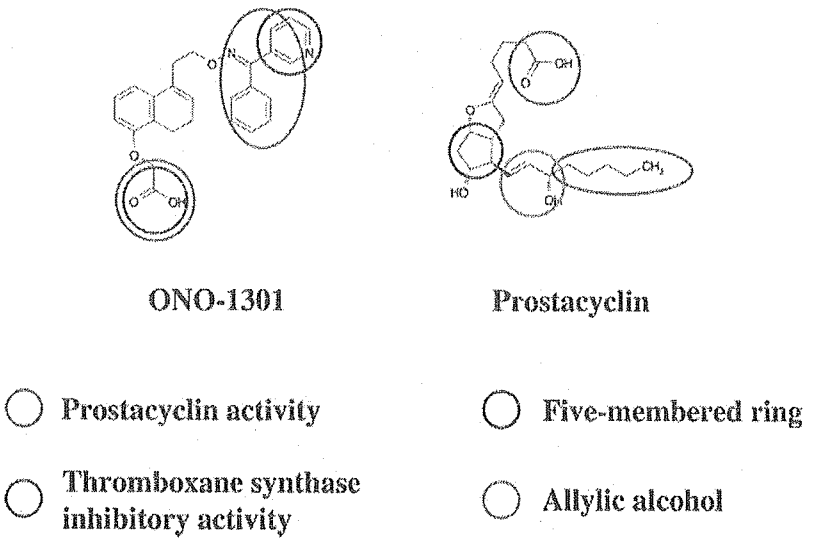

Fig. 1. Molecular structures of ONO-1301 and prostacyclin. Unlike prostacyclin, ONO-1301 does not possess a 5-membered ring and allylic alcohol, which contributes to the biological and chemical stability of this compound. ONO-1301 possesses a 3-pyridine moiety at 1 end of the molecule and a carboxylic acid group at the other, which contribute to inhibition of thromboxane synthesis. Green circle indicates prostacyclin activity; red circle indicates thromboxane synthase inhibitory activity; blue circle indicates 5-membered ring; orange circle indicates allylic alcohol.

Table 1. *Physiological profiles of three experimental groups*

	Sham	Vehicle	ONO-1301
n	8	8	8
Body weight, g	22.1±0.2	19.3±0.6*	21.8±0.4†
Lung weight/body weight, mg/g	5.8±0.1	14.8±1.7*	9.4±0.5*†

Data are means ± SE. These measurements were performed at 14 days after bleomycin injection. Sham, sham mice given vehicle; Vehicle, bleomycin mice given vehicle; ONO-1301, bleomycin mice treated with ONO-1301; $*P < 0.05$ vs. Sham group; $\dagger P < 0.05$ vs. Vehicle group.

($n = 5$ each). The plasma cAMP level was measured with a radioimmunoassay kit (Yamasa Shoyu, Chiba, Japan) as described previously (13).

Survival analysis. To evaluate the effect of ONO-1301 on survival in bleomycin-injected mice, 24 mice received repeated administration of ONO-1301 ($n = 12$) or vehicle ($n = 12$) for 21 days. Survival was estimated from the date of bleomycin injection to the death of the mouse or 21 days after injection.

In vitro study. Mouse lung fibroblasts were isolated from lung tissue by mincing and enzymatic digestion with collagenase type III (10 mg/lung; Worthington Biochemical, Lakewood, NJ) for 80 min at 37°C, as reported previously (11). Cell suspension was filtered through 70-μm filters (BD Biosciences, Mountain View, CA). Then, these cells were centrifuged, washed, and cultured in complete medium composed of DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS, Invitrogen) and 1% penicillin-streptomycin (Invitrogen). Fibroblasts were used after the first cell passage. To evaluate the effect of ONO-1301 on intracellular cAMP, mouse lung fibroblasts grown in 24-well plates were incubated with various concentrations of ONO-1301 in the presence of 5×10^{-4} M 3-isobutyl-1-methylxanthine (Nacalai Tesque, Kyoto, Japan), a phosphodiesterase inhibitor ($n = 8$ each), for 10 min. The intracellular cAMP level was measured with a radioimmunoassay kit as described previously (10). The effects of ONO-1301 and 8-bromo cAMP (Sigma, St. Louis, MO), a cAMP analog, on cell proliferation were assessed using a CellTiter 96 aqueous one solution cell proliferation assay kit (Promega, Madison, WI) according to the manufacturer's directions ($n = 8$ each). Cells were treated for 48 h with fresh medium containing 2.5% FCS along with various concentrations of ONO-1301 or 8-bromo cAMP. The effect of ONO-1301 (10^{-6} M) on cell proliferation in the presence of a myristoylated protein kinase A (PKA) inhibitor (10^{-6} M) (Protein Kinase A Inhibitor 14–22 Amide, Cell-permeable, Myristoylated; Calbiochem, Cambridge, MA) was also evaluated. Finally, to investigate the underlying mechanism responsible for regulation of cell proliferation, mouse lung-derived fibroblasts were treated with various concentrations of imidazole (Wako Pure Chemical Industries, Osaka, Japan), a thromboxane synthesis inhibitor, or beraprost sodium (Cayman Chemical), a stable prostacyclin analog.

Statistical analysis. All data are expressed as means ± SE unless otherwise indicated. Comparisons were made by one-way ANOVA followed by Newman-Keuls test. Survival curves were derived by the Kaplan-Meier method and compared by log-rank test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Physiological profiles. The physiological profiles of the three experimental groups are shown in Table 1. Body weight was significantly lower in bleomycin mice given vehicle (Vehicle group) than in normal mice given vehicle (Sham group). However, a significant decrease in body weight was not observed in bleomycin mice treated with ONO-1301 (ONO-1301 group). Bleomycin injection significantly increased wet lung weight to body weight ratio. However, the increase was significantly attenuated by treatment with ONO-1301.

Inhibition of pulmonary fibrosis. The normal alveolar structure was maintained in the Sham group (Fig. 2*A*). Fourteen days after bleomycin injection, the alveolar walls were thickened and the air spaces were collapsed in the Vehicle group. In contrast to the findings in mice treated with bleomycin alone, pulmonary fibrosis was less severe in the ONO-1301 group. Semiquantitative assessment by the Ashcroft score demonstrated that the degree of pulmonary fibrosis in the ONO-1301 group was significantly lower than that in the Vehicle group (Fig. 2*B*). The hydroxyproline content in the lung was significantly increased after bleomycin injection (Fig. 2*C*). However,

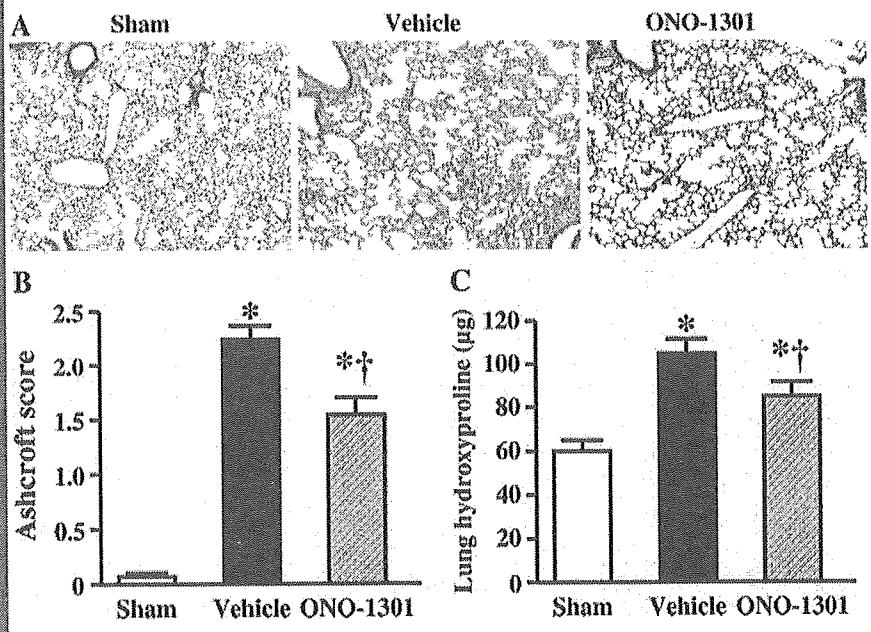

Fig. 2. *A*: representative photomicrographs of lung tissue at 14 days after bleomycin injection. Bleomycin-induced pulmonary fibrosis was attenuated by treatment with ONO-1301. Hematoxylin-eosin stain; magnification ×100. *B*: semiquantitative analyses of lung tissue using the Ashcroft score, a marker for pulmonary fibrosis. *C*: effect of ONO-1301 administration on hydroxyproline content in left lung of bleomycin-injected mice. Data are means ± SE. $*P < 0.05$ vs. Sham group; $\dagger P < 0.05$ vs. Vehicle group.

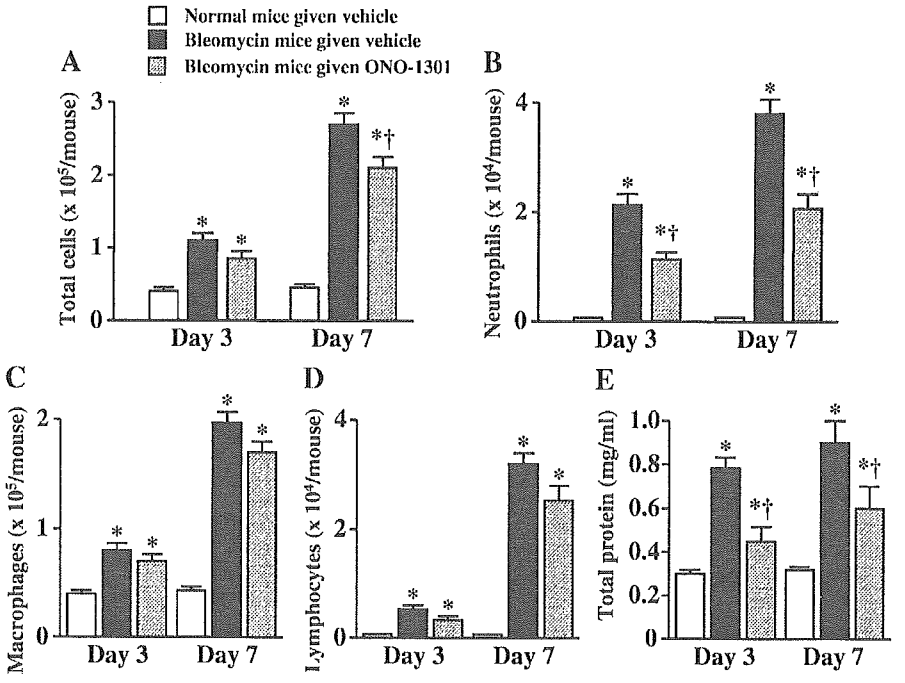

Fig. 3. Effects of ONO-1301 administration on total and differential cell counts (macrophages, *C*; lymphocytes, *D*) in bronchoalveolar lavage (BAL) fluid at 3 and 7 days after bleomycin injection. ONO-1301 significantly inhibited the increases in total cell count at 7 days (*A*) and neutrophil count at 3 and 7 days (*B*) after bleomycin injection. *E*: effect of ONO-1301 administration on total protein level in BAL fluid at 3 and 7 days after bleomycin injection. ONO-1301 significantly inhibited the increase in total protein level in BAL fluid. Data are means ± SE. $*P < 0.05$ vs. Sham group, $\dagger P < 0.05$ vs. Vehicle group.

subcutaneous administration of ONO-1301 significantly decreased the hydroxyproline content in bleomycin-injected mice.

Attenuation of lung inflammation. The recovery rate of BAL fluid was >85% in all groups. Total and differential cell counts were increased at 3 and 7 days after bleomycin injection (Fig. 3, *A–D*). However, subcutaneous administration of ONO-1301 significantly attenuated the increases in total cell count at 7 days and neutrophil count at 3 and 7 days after bleomycin injection. ONO-1301 administration tended to attenuate the increases in macrophage and lymphocyte counts, although these changes did not reach statistical significance. The total protein level was significantly increased at 3 and 7 days after bleomycin injection (Fig. 3*E*). However, the increase was significantly inhibited by ONO-1301.

Inhibition of thromboxane synthesis. The TXB_2 level in BAL fluid was significantly increased at 3 and 7 days after bleomycin injection (Fig. 4*A*), suggesting a pathological role of thromboxane in bleomycin-injected mice. Subcutaneous administration of ONO-1301 markedly inhibited the increase in TXB_2 level. The active TGF-β1 level in BAL fluid was significantly increased at 7 days after bleomycin injection (Sham group, 148 ± 6; Vehicle group, 251 ± 8; ONO-1301 group, 238 ± 9 pg/ml). ONO-1301 did not significantly alter the active TGF-β1 level in BAL fluid.

Inhibitory effect of ONO-1301 on ICAM-1 and VCAM-1 expression. The lung ICAM-1 and VCAM-1 levels were significantly increased at 3 and 7 days after bleomycin injection (Fig. 4, *B* and *C*). ONO-1301 administration sig-

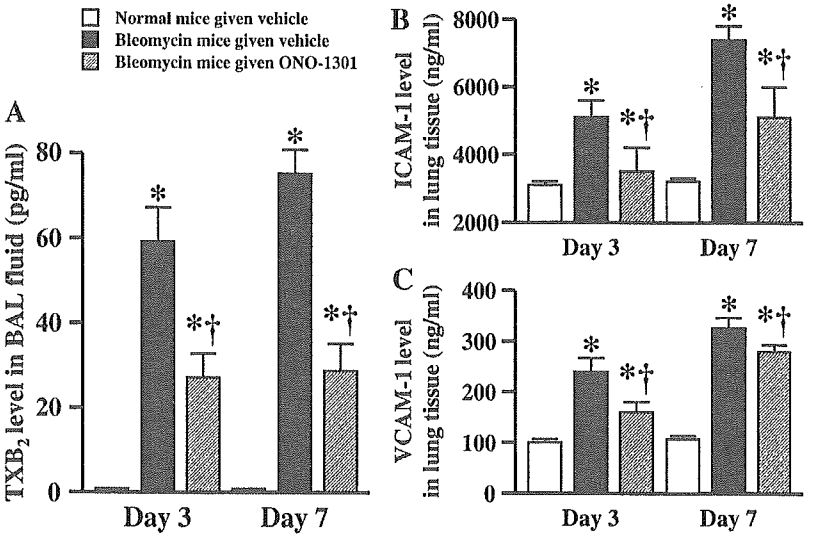

Fig. 4. *A*: effect of ONO-1301 administration on thromboxane B_2 (TXB_2) level in BAL fluid at 3 and 7 days after bleomycin injection. ONO-1301 markedly inhibited the increase in TXB_2 level in BAL fluid. Data are means ± SE. Effects of ONO-1301 administration on ICAM-1 (*B*) and VCAM-1 (*C*) levels in lung tissue at 3 and 7 days after bleomycin injection. $*P < 0.05$ vs. Sham group, $\dagger P < 0.05$ vs. Vehicle group.

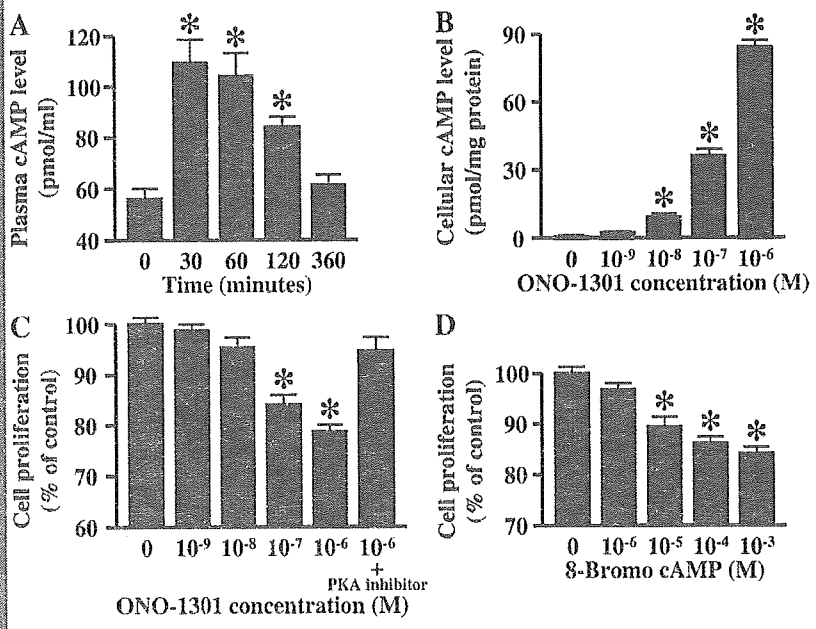

Fig. 5. *A*: time course of plasma cAMP level after a single subcutaneous administration of ONO-1301. *B*: dose-dependent effects of ONO-1301 on intracellular cAMP level. *C*: effect of ONO-1301 on fibroblast proliferation. ONO-1301 significantly reduced cell proliferation at concentrations of 10^{-7} M or greater, and this inhibitory effect was attenuated by a protein kinase A (PKA) inhibitor. *D*: effect of 8-bromo cAMP on fibroblast proliferation. Data are means ± SE. $*P < 0.05$ vs. Control.

nificantly attenuated the increases in ICAM-1 and VCAM-1 levels.

Activation of the cAMP/PKA pathway. A single subcutaneous administration of ONO-1301 significantly increased plasma cAMP level (Fig. 5*A*). The increase lasted longer than 2 h. In vitro, ONO-1301 dose-dependently increased intracellular cAMP level in mouse lung fibroblasts (Fig. 5*B*). ONO-1301 significantly reduced proliferation of mouse lung fibroblasts at concentrations of 10^{-7} M or greater, and this inhibitory effect was attenuated by a PKA inhibitor (Fig. 5*C*). The reduction in cell proliferation by ONO-1301 was reproduced by 8-bromo cAMP (Fig. 5*D*). Beraprost sodium (10^{-7} M) significantly reduced fibroblast proliferation (86% of control). However, imidazole at different concentrations (10^{-6}-10^{-9} M) did not significantly regulate cell proliferation.

Survival analysis. Kaplan-Meier survival curves demonstrated that bleomycin mice treated with ONO-1301 had a significantly higher survival rate than those given vehicle (75 vs. 33% 21-day survival, log-rank test, $P < 0.05$, Fig. 6).

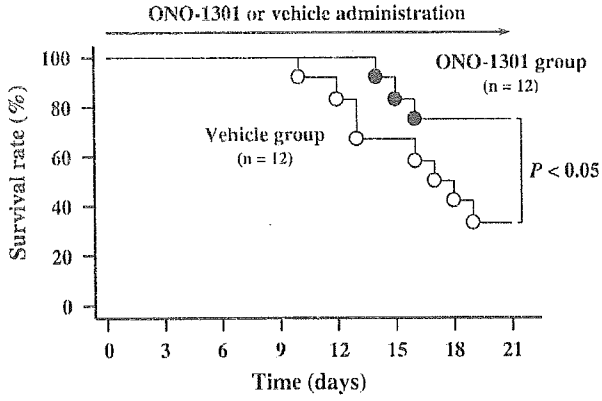

Fig. 6. Kaplan-Meier survival curves. Bleomycin mice treated with ONO-1301 (●) had a significantly higher survival rate than those given vehicle (○) (log-rank test, $P < 0.05$).

Delayed therapy. There were no significant differences in Ashcroft score and lung hydroxyproline content between the Vehicle group and ONO-1301 group at 28 days after bleomycin injection (data not shown).

DISCUSSION

In the present study, we demonstrated that *1*) repeated subcutaneous administration of ONO-1301 attenuated the development of bleomycin-induced pulmonary fibrosis, as indicated by decreases in Ashcroft score and lung hydroxyproline content, *2*) ONO-1301 attenuated the increases in total cell count, neutrophil count, and total protein level in BAL fluid, and *3*) ONO-1301 increased the survival rate in bleomycin-injected mice. We also demonstrated that *4*) ONO-1301 decreased the TXB_2 level in BAL fluid and inhibited ICAM-1 and VCAM-1 expression in the bleomycin-treated lung, and *5*) ONO-1301 inhibited lung fibroblast proliferation through activation of the cAMP/PKA pathway.

The balance between prostacyclin and TXA_2 plays an important role in pulmonary homeostasis. However, little information is available regarding the therapeutic potency of these prostanoids for pulmonary fibrosis. Recently, we have developed ONO-1301, a novel nonprostanoid long-acting prostacyclin agonist possessing a potent inhibitory activity against thromboxane synthase. Thus this compound was administered by subcutaneous injection twice a day.

Bleomycin induces lung inflammation followed by fibrosis when intratracheally injected in experimental animals (27). In the present study, subcutaneous administration of ONO-1301 significantly attenuated bleomycin-induced increases in total cell counts, neutrophil counts, and total protein level in BAL fluid. Earlier studies have shown that TXA_2 acts as a proinflammatory mediator via enhancement of pulmonary vascular permeability (18, 24) and neutrophil adhesion (33). In the present study, TXB_2, a stable metabolite of TXA_2, was significantly increased after bleomycin injection, which is consistent with previous studies (2, 8). However, ONO-1301 markedly

inhibited the increase in TXB_2 level in BAL fluid. Thus one of the possible mechanisms by which ONO-1301 attenuates lung inflammation may be mediated by inhibition of TXA_2 synthesis. A recent study has shown that a TXA_2 receptor agonist enhances the expression of adhesion molecules by human vascular endothelial cells (12). In the present study, ONO-1301 significantly inhibited ICAM-1 and VCAM-1 expression in the bleomycin-treated lung. Adhesion molecules including ICAM-1 and VCAM-1 have been shown to contribute to bleomycin-induced pulmonary fibrosis by mediating the accumulation of leukocytes (9, 16, 19, 23). These findings suggest that ONO-1301 may attenuate lung inflammation at least in part through inhibition of ICAM-1 and VCAM-1 expression.

Lung fibroblasts play an important role in the development of fibrosis in the lung (22, 25, 31). Prostaglandins are known to have various functions on lung fibroblasts via an elevation of intracellular cAMP level (3, 14–17). In the present study, a single subcutaneous administration of ONO-1301 significantly increased plasma cAMP level in mice. In vitro studies demonstrated that ONO-1301 dose-dependently increased intracellular cAMP level in mouse lung fibroblasts and that this compound dose-dependently inhibited fibroblast proliferation. The inhibitory effect of ONO-1301 was reproduced by 8-bromo cAMP, a cAMP analog, and attenuated by a PKA inhibitor. These results suggest that ONO-1301 directly inhibits fibroblast proliferation at least in part through activation of the cAMP/PKA pathway. Dussaubat et al. (5) have demonstrated that imidazole, a thromboxane synthesis inhibitor, decreases bleomycin-induced acute lung inflammation, but it does not affect pulmonary fibrosis at later points. In the present study, a prostacyclin analog, but not a thromboxane synthesis inhibitor, significantly reduced fibroblast proliferation. These results suggest that the inhibitory effect of ONO-1301 on fibroblast proliferation may be mediated mainly by its prostacyclin-like activity. TGF-β, especially TGF-β1 plays an important role in the pathogenesis of pulmonary fibrosis (7, 26, 32). In the present study, ONO-1301 did not significantly alter the active TGF-β1 level in BAL fluid. Previous studies have shown that a prostacyclin agonist suppresses TGF-β-induced connective tissue growth factor, a potent profibrotic mediator, expression in part through activation of the cAMP/PKA pathway (28, 29). Thus it is interesting to speculate that ONO-1301 attenuated the development of pulmonary fibrosis through suppression of connective tissue growth factor.

In the present study, ONO-1301 significantly improved survival in bleomycin-injected mice. ONO-1301 inhibited lung inflammation and lung fibroblast proliferation. As a result, ONO-1301 may have beneficial effects on survival in bleomycin-injected mice. Unfortunately, we could not observe significant differences in fibrotic changes between the Vehicle group and ONO-1301 group when we administered ONO-1301 after fibrosis was established. These results imply that ONO-1301 may be insufficient to reverse established pulmonary fibrosis.

In conclusion, subcutaneous administration of ONO-1301, a novel long-lasting prostacyclin agonist, attenuated the development of bleomycin-induced pulmonary fibrosis and improved survival in mice. The beneficial effects were mediated at least in part by inhibition of TXA_2 synthesis and activation of the cAMP/PKA pathway. Thus administration of this compound may be a new therapeutic strategy for the treatment of pulmonary fibrosis.

ACKNOWLEDGMENTS

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Transplantation of Mesenchymal Stem Cells Improves Cardiac Function in a Rat Model of Dilated Cardiomyopathy

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Background—Pluripotent mesenchymal stem cells (MSCs) differentiate into a variety of cells, including cardiomyocytes and vascular endothelial cells. However, little information is available about the therapeutic potency of MSC transplantation in cases of dilated cardiomyopathy (DCM), an important cause of heart failure.

Methods and Results—We investigated whether transplanted MSCs induce myogenesis and angiogenesis and improve cardiac function in a rat model of DCM. MSCs were isolated from bone marrow aspirates of isogenic adult rats and expanded ex vivo. Cultured MSCs secreted large amounts of the angiogenic, antiapoptotic, and mitogenic factors vascular endothelial growth factor, hepatocyte growth factor, adrenomedullin, and insulin-like growth factor-1. Five weeks after immunization, MSCs or vehicle was injected into the myocardium. Some engrafted MSCs were positive for the cardiac markers desmin, cardiac troponin T, and connexin-43, whereas others formed vascular structures and were positive for von Willebrand factor or smooth muscle actin. Compared with vehicle injection, MSC transplantation significantly increased capillary density and decreased the collagen volume fraction in the myocardium, resulting in decreased left ventricular end-diastolic pressure (11 ± 1 versus 16 ± 1 mm Hg, $P<0.05$) and increased left ventricular maximum *dP/dt* (6767 ± 323 versus 5138 ± 280 mm Hg/s, $P<0.05$).

Conclusions—MSC transplantation improved cardiac function in a rat model of DCM, possibly through induction of myogenesis and angiogenesis, as well as by inhibition of myocardial fibrosis. The beneficial effects of MSCs might be mediated not only by their differentiation into cardiomyocytes and vascular cells but also by their ability to supply large amounts of angiogenic, antiapoptotic, and mitogenic factors. (***Circulation.* 2005;112:1128-1135.**)

Key Words: myocytes ■ angiogenesis ■ heart failure ■ growth substances ■ transplantation

Despite advances in medical and surgical procedures, congestive heart failure remains a leading cause of cardiovascular morbidity and mortality.[1] Idiopathic dilated cardiomyopathy (DCM), a primary myocardial disease of unknown etiology characterized by a loss of cardiomyocytes and an increase in fibroblasts, is an important cause of heart failure.[2] Although myocyte mitosis and the presence of cardiac precursor cells in adult hearts have recently been reported,[3] the death of large numbers of cardiomyocytes results in the development of heart failure. Thus, restoring lost myocardium would be desirable for the treatment of DCM.

Mesenchymal stem cells (MSCs) are pluripotent, adult stem cells residing within the bone marrow microenvironment.[4] In contrast to their hematopoietic counterparts, MSCs are adherent and can be expanded in culture. MSCs can differentiate not only into osteoblasts, chondrocytes, neurons, and skeletal muscle cells but also into vascular endothelial cells[5] and cardiomyocytes.[6,7] In vitro, MSCs can be induced to differentiate into beating cardiomyocytes by 5-azacytidine treatment.[8] In vivo, MSCs directly injected into an infarcted heart have been shown to induce myocardial regeneration and improve cardiac function.[9] In addition, MSC implantation induces therapeutic angiogenesis in a rat model of hindlimb ischemia through vascular endothelial growth factor (VEGF) production by MSCs.[10,11] Myocardial blood flow abnormalities, even in the presence of angiographically normal coronary arteries, have been documented in patients with DCM.[12]

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