Inhibitory Effects of RAGE-aptamer on Development of Monocrotaline-induced Pulmonary Arterial Hypertension in Rats

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Abstract

Background: The receptor for advanced glycation end products (RAGE), a transmembrane receptor belonging to the immunoglobulin superfamily, is overexpressed in pulmonary artery smooth muscle cells (PASMCs) in patients with pulmonary arterial hypertension (PAH) and is implicated in the etiology of PAH. Recently, we reported that RAGE-aptamer, a short and single-stranded DNA directed against RAGE, inhibited an inappropriate increase in cultured PASMCs in PAH. The aim of this study was to determine the efficacy of RAGE-aptamer in monocrotaline-induced PAH in rats.

Methods and Results: Rats were assigned to either an untreated control group, a group that received continuous subcutaneous administration of RAGE-aptamer immediately after monocrotaline injection, or a group that received control-aptamer immediately after monocrotaline injection. All rats survived 21 days after injection of monocrotaline and control-aptamer or RAGE-aptamer. Injection of monocrotaline with continuous subcutaneous delivery of control-aptamer resulted in higher right ventricular systolic pressure compared with controls. This increase was attenuated by continuous subcutaneous delivery of RAGE-aptamer. The proportion of small pulmonary arteries with full muscularization was greater in the monocrotaline and control-aptamer group than in the control group. Continuous subcutaneous delivery of RAGE-aptamer significantly reduced the percentage of small pulmonary arteries with full muscularization

Conclusions: Continuous subcutaneous delivery of RAGE-aptamer suppresses development of monocrotaline-induced PAH in rats. Inhibition of RAGE ameliorates muscularization of

small pulmonary arteries. Treatment with RAGE-aptamer might be a new therapeutic option for PAH.

Introduction

Pulmonary arterial hypertension (PAH) is a progressive disease characterized by elevated pulmonary artery pressure and pulmonary vascular resistance, which are caused by pulmonary vasoconstriction and by vascular remodeling [1-3]. Pulmonary vascular remodeling is induced by an inappropriate increase in vascular cells, including pulmonary artery smooth muscle cells (PASMCs) [4]. To achieve reverse remodeling of pulmonary vessels, anti-proliferative agents for PASMCs are required.

The receptor for advanced glycation end products (RAGE) is a type I single-pass transmembrane protein belonging to the immunoglobulin superfamily [5]. RAGE is thought to be involved in a broad range of inflammatory, degenerative, and hyperproliferative diseases, including rheumatoid arthritis, diabetic nephropathy, atherosclerosis, and cancer [6]. RAGE and S100A8 and A9, which are ligands of RAGE, are overexpressed in PASMCs of patients with PAH and implicated in the etiology of PAH [7, 8]. RAGE signal inhibition decreases proliferation of PASMCs in patients with PAH [7, 8].

Recently, we reported that a DNA aptamer, a short and single-stranded DNA, directed against RAGE (RAGE-aptamer) inhibited the binding of AGE to RAGE and attenuated the development and progression of streptozotocin-induced diabetic nephropathy in rats and deoxycorticosterone acetate/salt-induced renal injury in mice, which were associated with the reduction of oxidative stress [9, 10]. We also reported that RAGE-aptamer inhibited an inappropriate increase in cultured PASMCs in PAH [7]. Therefore, we investigated the efficacy of RAGE-aptamer in monocrotaline-induced PAH in rats.

Materials and Methods

Preparation of RAGE-aptamer

RAGE-aptamer was obtained by an *in vitro* selection process (SELEX method) from a pool of approximately 10^{15} different nucleic acid sequences as previously described [7, 9-11].

Sequences directed against RAGE were obtained and sequences of the selected RAGE and control aptamers were cATTcTTAgATTTTTgTcTcAcTTAggTgTAgATggTgAT and aTcgAccTggAgggcgAgcAgcTcggATccAgTcgcgTgAg, respectively. These aptamers were modified with phosphorothioate for protection against degradation by nuclease. Phosphorothioate nucleotides are indicated by capital letters.

Protocols for Animal Experiments

Adult male Wistar rats (Charles River, Yokohama, Japan: 250–300 g in body weight) received a single subcutaneous injection of 60 mg/kg monocrotaline (Sigma-Aldrich, St. Louis, MO) as previously described [12, 13]. Rats were assigned to either an untreated control group ($n = 8$), a group that received continuous subcutaneous administration of RAGE-aptamer (1.5 $\mu\text{g/day}$) immediately after monocrotaline injection ($n = 5$), or a group that received control-aptamer (1.5 $\mu\text{g/day}$) immediately after monocrotaline injection ($n = 9$). Continuous subcutaneous delivery of RAGE or control-aptamer was achieved by implanting an osmotic minipump (model 1004; 0.11 $\mu\text{L/h}$) (Alzet, Cupertino, CA) subcutaneously in the neck. Three weeks after treatment, hemodynamics and lung tissues were examined as previously

described[12, 13]. All surgery was performed under inhalation of isoflurane (1.8% vol/vol) anesthesia and efforts were made to minimize pain. Experimental protocols were approved by and conducted in accordance with the recommendations of the Okayama University Animal Care and Use Committee (permit number OKU-2020567).

Assessment of right ventricular Pressure and RV hypertrophy

The rats were anesthetized with inhalation of isoflurane, and a high-fidelity 1.4F Millar catheter (Millar Instruments Inc) was inserted into the right ventricle (RV) through the right jugular vein as previously described [13]. RV systolic pressure (RVSP) was measured with the PowerLab System using Chart 5.0 software. After hemodynamics had been recorded, rats were anesthetized with isoflurane. their lungs and heart were isolated. The RV wall was dissected from the left ventricle (LV) and ventricular septum (VS). The wet weight of the RV and LV+VS was determined, and RV hypertrophy was expressed as RV weight/(LV+VS) weight.

Histological analysis

The lung was fixed for histology in 10% neural buffered formalin. For paraffin embedding, the lungs were dissected in tissue blocks from all lobes. Sectioning at 3 μ m was performed from all paraffin-embedded blocks.

To confirm the expression of RAGE in sections of lungs, immunohistochemical analysis was performed using a DAKO LSAB System (DakoCytomation, Kyoto) according to

the manufacturer's instructions, as previously described [14]. Briefly, slides of lung sections embedded in paraffin were deparaffinized, rehydrated, and then subjected to antigen retrieval by autoclave treatment (15 minutes, 121 °C) in 10 mmol/L citrate buffer at a pH of 6.0. Following antigen retrieval tissues were blocked in bovine serum albumin. Rabbit polyclonal anti-RAGE antibody (1:20 dilution) (ab3611, abcam) was added, and the sections were incubated at 4°C overnight. Peroxidase activity was visualized with diaminobenzidine tetrahydrochloride solution.

To assess the type of remodeling in the muscular pulmonary arteries, Elastica van Gieson staining was performed according to common histopathological procedures. In each rat, 30–40 intra-acinar arteries were categorized as fully muscular (those with a complete medial coat of muscle), partially muscular (those with only a crescent of muscle), or nonmuscular (those with no apparent muscle), counted, and averaged with a range of diameters from 25 to 50 μ m as previously described [13, 15].

Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD). For comparison between different treatment groups, statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test. Values of $P < 0.05$ were considered to be significant.

Results

Effects of RAGE-aptamer on right ventricular systolic pressure and RV hypertrophy

All rats survived 21 days after injection of monocrotaline and control-aptamer or RAGE-aptamer. Body weight was significantly decreased in the monocrotaline + control-aptamer group compared with that in the control group (404 ± 32 versus 442 ± 15 g; $P < 0.05$). There was no significant difference in body weight between the monocrotaline + RAGE-aptamer group and control group (monocrotaline + RAGE-aptamer: 410 ± 12 g versus control; $P = 0.110$).

Injection of monocrotaline with continuous subcutaneous delivery of control-aptamer resulted in higher right ventricular systolic pressure compared with controls (53 ± 17 versus 21 ± 5 mmHg; $P < 0.01$; Fig. 1A and B). This increase was attenuated by continuous subcutaneous delivery of RAGE-aptamer (23 ± 6 mmHg; $P < 0.01$ versus monocrotaline + control-aptamer group; Fig. 1A and B).

The RV/(LV+VS) ratio was significantly increased in the monocrotaline + control-aptamer group compared with that in the control group (0.54 ± 0.05 versus 0.24 ± 0.03 ; $P < 0.0001$). Administration of RAGE-aptamer tended to ameliorate the RV/(LV+VS) ratio (monocrotaline + RAGE-aptamer: 0.46 ± 0.07 versus monocrotaline + control-aptamer; $P = 0.061$).

Effects of RAGE-aptamer on small pulmonary arterial remodeling

Immunohistochemical analysis revealed that RAGE was overexpressed in vascular media of distal pulmonary arteries of the monocrotaline + control-aptamer group (Fig. 2A, middle

image), but RAGE was not expressed in those of the control and monocrotaline + RAGE-aptamer groups (Fig. 2A, right and left images).

The proportion of small pulmonary arteries with full muscularization was greater in the monocrotaline + control-aptamer group than in the control group ($78\% \pm 25\%$ versus $9\% \pm 13\%$; $P < 0.01$; Fig. 2A and B). Continuous subcutaneous delivery of RAGE-aptamer significantly reduced the percentage of small pulmonary arteries with full muscularization ($42\% \pm 18\%$; $P < 0.01$ versus the monocrotaline + control-aptamer group; Fig. 2A and B). There were no significant differences in the proportion of small pulmonary arteries with partial muscularization between the three groups (control: $20\% \pm 18\%$, monocrotaline + control-aptamer: $13\% \pm 16\%$ and monocrotaline + RAGE-aptamer: $22\% \pm 24\%$).

Discussion

The major findings of this study were that continuous subcutaneous administration of RAGE-aptamer significantly decreased right ventricular systolic pressure and pulmonary artery muscularization in monocrotaline-induced PAH model rats. Delivery of RAGE-aptamer suppressed development of monocrotaline-induced PAH in rats.

RAGE is overexpressed in PASMCs of patients with PAH and lungs of monocrotaline- and sugen-induced PAH rats [7, 8]. The present study also showed by immunohistochemical analysis that RAGE was overexpressed in vascular media of small pulmonary arteries of monocrotaline-induced PAH rats and that RAGE-aptamer administration reduced the

expression of RAGE. The effect of RAGE-aptamer administration might contribute to improvement of PAH.

RAGE plays an important role in the inappropriate increase of PASMCs in PAH, and its inhibition may be a new therapeutic strategy for PAH [7, 8]. We previously reported that RAGE-aptamer inhibited an inappropriate increase in cultured PASMCs in PAH [7]. The present study also showed that RAGE-aptamer significantly reduced the percentage of small pulmonary artery muscularization in monocrotaline-induced PAH in rats.

RAGE-aptamer inhibits binding of advanced glycation end products to RAGE. RAGE-aptamer also attenuates development and progression of streptozotocin-induced diabetic nephropathy in rats and deoxycorticosterone acetate/salt-induced renal injury in mice [9, 10]. With regard to ligands for RAGE in PAH, Greenway et al studied patients with pulmonary hypertension secondary to congenital heart disease [16]. They reported that S100A4/Mts1 was expressed in smooth muscle cells of lesions that showed neointimal formation and increased intensity in vessels with an occlusive neointima and plexiform lesions. Meloche et al reported that activation of RAGE by S100A4 decreased bone morphogenetic protein receptor type II (BMPRII) expression in human PASMCs [8]. We previously showed that S100A8 and A9 were overexpressed in PASMCs in idiopathic PAH and heritable PAH [7]. S100A8/A9 induces phosphorylation of RAGE and leads to activation of diverse signal effectors, such as Rac1 and nuclear factor- κ B in various cells [6, 17]. Therefore, the S100A8/A9-RAGE signal may play an important role in the pathogenesis of PAH. Further studies are required to clarify this possibility.

Meloche et al reported that RAGE inhibition by siRNA in monocrotaline-induced PAH model rats demonstrates therapeutic effects characterized by decreases in pulmonary artery pressure and hypertrophy [8]. We also showed that RAGE inhibition by RAGE-aptamer significantly decreased right ventricular systolic pressure and pulmonary artery muscularization in monocrotaline-induced PAH model rats. The precise mechanism of the therapeutic effects of RAGE inhibition is unknown. Since monocrotaline-induced PAH in rats is considered to be an "inflammatory" model of PAH, changes of proinflammatory cytokines, such as IL-6 and TNF- α , might be involved in the mechanism. RAGE also increases the formation of intracellular reactive oxygen species (ROS), which leads to oxidative stress. The production of ROS might be involved in the mechanism.

As for safety concerns of RAGE-aptamer, all rats survived 21 days after injection of control-aptamer or RAGE-aptamer. Body weight was significantly decreased in the monocrotaline + control-aptamer group compared with that in the control group, but RAGE-aptamer administration did not change body weight compared with that in the control group. We previously reported that RAGE-aptamer attenuated the development and progression of streptozotocin-induced diabetic nephropathy in rats and deoxycorticosterone acetate/salt-induced renal injury in mice [9, 10], indicating that RAGE-aptamer has protective effects on renal injury. We also reported that continuous infusion of RAGE-aptamer for 4 weeks did not affect blood pressure levels, lipid parameters, aspartate transaminase (AST), alanine aminotransferase (ALT) and liver weight in streptozotocin-induced diabetic model rats [9]. It is believed that aptamers are less immunogenic than

protein, but it is difficult to predict that they are free from a toxic effect [18]. Further studies are needed to clarify these points.

This study has several limitations. First, the sample size is small. Second, systemic blood pressure was not measured. Third, this study was performed using a preventative protocol for development of monocrotaline-induced PAH. Studies of treatment protocols aimed to investigate reversal effects of RAGE-aptamer in PAH are required in the future. Further studies are needed to obtain precise data.

In conclusion, continuous subcutaneous delivery of RAGE-aptamer suppresses development of monocrotaline-induced PAH in rats. Inhibition of RAGE ameliorates muscularization of small pulmonary arteries. Treatment with RAGE-aptamer might be a new therapeutic option for PAH.

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Declaration of conflicting interests

The Authors declare that there is no conflict of interest.

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Figure legends

Figure 1. Effects of RAGE-aptamer on right ventricular systolic pressure.

A: Representative images of right ventricular pressure in rats of the control (left), monocrotaline + control-aptamer (middle), and monocrotaline + RAGE-aptamer (right) groups. B: Right ventricular systolic pressure in each group. Data are mean $\pm$ SD. Control group: n = 8, monocrotaline + control-aptamer group; n = 9, and monocrotaline + RAGE-aptamer group: n = 5.

Figure 2. Effects of RAGE-aptamer on small pulmonary arterial remodeling 21 days after monocrotaline injection.

A: Representative immunohistochemical staining (brown) of RAGE in each group. RAGE is expressed in vascular media of a distal pulmonary artery of the monocrotaline + control-aptamer group (middle), but RAGE is not expressed in those of the control (left) and monocrotaline + RAGE-aptamer (right) groups. Bar = 100 μ m. B: Representative images of small pulmonary arteries by Elastica van Gieson staining in each group. Images of the control (left) and monocrotaline + RAGE-aptamer (right) groups show non-muscularized pulmonary arteries. An image of the monocrotaline + control-aptamer group (middle) shows a fully muscularized small pulmonary artery. Bar = 100 μ m. C: Percentage of fully muscularized small pulmonary arteries (PAs) in each group. Data are mean $\pm$ SD. Control group: n = 8, monocrotaline + control-aptamer group; n = 9, and monocrotaline + RAGE-aptamer group: n = 5.

Figure 1

Figure 2