*Full Paper*

# A Novel Long-Acting Prostacyclin Agonist (ONO-1301) With an Angiogenic Effect: Promoting Synthesis of Hepatocyte Growth Factor and Increasing Cyclic AMP Concentration via IP-Receptor Signaling

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**Abstract.** The purpose of this study was to evaluate the angiogenic potency of ONO-1301, a novel prostacyclin agonist, using a murine sponge model. Solutions of ONO-1301 or hepatocyte growth factor (HGF), as a positive control, were injected into sponges in the backs of mice, daily for 14 days. Hemoglobin and HGF levels in the sponge were increased for up to 14 days on daily treatment with ONO-1301 while on HGF treatment, they peaked on day 7 and had decreased again by day 14. ONO-1301 also upregulated c-Met expression for 14 days in a dose-dependent manner. When the mice were pretreated with an antibody to HGF or the prostaglandin I (IP)-receptor antagonist CAY10441, the angiogenic effect of ONO-1301 was dramatically reduced. Plasma concentrations of cyclic adenosine monophosphate (cAMP) were increased in a dose-dependent manner by once daily treatment with ONO-1301 for 14 days. This effect was reduced by pretreatment with the IP-receptor antagonist. In conclusion, hemoglobin level was increased by repeated treatment with ONO-1301 for 14 days. It is suggested that ONO-1301 induced angiogenesis by promoting the synthesis of HGF and upregulated c-Met expression, followed by an increase in cAMP concentrations mediated by IP-receptor signaling.

*Keywords*: ONO-1301, hepatocyte growth factor (HGF), angiogenesis, sponge model, hemoglobin

## Introduction

ONO-1301 was developed as a novel long-acting prostacyclin agonist with thromboxane synthase inhibitory activity (1). Unlike prostacyclin, ONO-1301 does not possess a five-membered ring or allylic alcohol in its molecular structure; this accounts for the improved biological and chemical stability of this compound. Further, the presence of a 3-pyridine radical in ONO-1301 brings inhibitory activity for thromboxane synthase,

by which ONO-1301 escapes in vivo desensitization of activity. ONO-1301 has been reported to improve pulmonary hypertension (2, 3), pulmonary fibrosis (4), arterial obstructive disease, and cardiac infarction (5 – 7), due to its vasodilatory, antiplatelet, and bronchodilatory properties, respectively. ONO-1301 also improves nerve conduction velocity disorder in diabetic model rats (8) and has demonstrated a neuroprotective effect in a rat brain ischemic model (9, 10). It has also been reported that ONO-1301 suppressed acute hepatic injury by a synthetic prostacyclin agonist through hepatocyte growth factor (HGF) expression (11). It has been demonstrated that HGF up-regulation contributes to angiogenesis in mice (12). HGF, purified and cloned as a mitogenic

protein from hepatocytes (13 – 15), regulates their development, survival, and proliferation through the c-Met receptor. HGF has also been shown to exert an angiogenesis-promoting effect in human synovial fibroblasts through the c-Met receptor pathway (16).

Previous studies have reported that $PGE_2$ regulates angiogenesis (17, 18). Its receptor is EP3 (19, 20), and suppression of cAMP is caused by EP3 activation (21, 22). On the other hand, $PGI_2$ (prostacyclin) agonists such as lipoprost and trepostinil have a high binding affinity for prostaglandin I (IP) receptors (23), and stimulation of the IP receptor promotes cAMP formation (24). It has been known that the contribution of EP3 for angiogenesis is high compared with that of the IP receptor. However, there are few reports about the IP receptor in angiogenesis. A previous study reported that the prostacyclin receptor upregulates the expression of angiogenic genes in human endometrium via crosstalk with epidermal growth factor receptor and the extracellular signaling receptor kinase 1/2 pathway (25). Furthermore, recently it was reported that a prostacyclin agonist showed a new beneficial effect by protecting against airway hyper-responsiveness and remodeling in mice (26).

We have already demonstrated the angiogenic effects of ONO-1301-loaded PLGA microspheres in the murine sponge model (27). However, the angiogenic potency and the mechanism of action of ONO-1301 in vivo remain unclear.

In this study, we focused on the angiogenic effect of ONO-1301 in increasing HGF concentrations. We also considered the mechanism of angiogenesis stimulated by treatment with ONO-1301. The primary aim of the study was to evaluate the angiogenic potency of ONO-1301 in the murine sponge model. Sponges were implanted under the skin on the backs of mice. After healing, ONO-1301 or HGF solution (as a positive control) was injected into the sponge for 7 or 14 days, after which the sponges were removed, weighed, and hemoglobin or HGF levels in the sponges were measured using ELISA. The secondary aim was to clarify the mechanism of the angiogenic effect induced by ONO-1301 treatment. Hemoglobin, HGF, and plasma cAMP concentrations in the sponges were measured after pretreatment with anti-HGF antibody or an IP-receptor antagonist in murine sponge models.

In the present study, hemoglobin levels in the ONO-1301-treatment group were significantly increased for 14 days compared with the vehicle-treated group. It was suggested that increase of hemoglobin level was caused by the HGF synthesis–promoting effect of ONO-1301. ONO-1301 also significantly upregulated c-Met expression compared with vehicle for 14 days, even though upregulation of c-Met expression by treatment with HGF

was not maintained for 14 days. The benefits of ONO-1301 disappeared by pretreatment with the IP-receptor antagonist CAY-10441 or anti-HGF antibody. These results clarified the mechanism through which ONO-1301 increased angiogenesis: ONO-1301 increases cAMP concentrations via IP-receptor signaling; the increased cAMP promoted synthesis of HGF, which then caused angiogenesis.

## Materials and Methods

### Materials

ONO-1301 was generously supplied by ONO Pharmaceutical Co., Ltd. (Osaka). Recombinant human HGF was obtained from Pepro Tech., Inc. (Rocky Hill, NJ, USA). Normal IgG control was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). CAY-10441 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Methods

*Preparation of ONO-1301 and HGF solutions*: ONO-1301 solution was prepared by suspension in 1% (v/v) ethanol. HGF was dissolved in phosphate-buffered saline (PBS, pH 7).

*Preparation of antibodies to HGF, normal IgG control, and CAY-10441 solutions*: The antibodies against HGF were prepared as described in our previous paper (11). Recombinant rat HGF was expressed in Chinese hamster ovary cells and purified from the culture supernatant, as described elsewhere (28, 29). The purity of the rat HGF exceeded 98%, as determined by SDS-PAGE (Bio-Rad, Hercules, CA, USA) and protein staining with Coomassie brilliant blue. Female Japanese White rabbits (Japan SLC, Inc., Hamamatsu) weighing approximately 2 kg were immunized by subcutaneous (s.c.) injection of recombinant rat HGF (50 $\mu$g/rabbit) in complete Freund's adjuvant. Booster injections were given once or twice at 2-week intervals. The antiserum titer was monitored using ELISA. IgGs from anti-rat HGF serum were purified using Protein A-Sepharose Fast Flow (GE Healthcare Japan, Tokyo). Normal IgG control and IP-receptor antagonist CAY-10441 were dissolved in PBS (pH 7).

### Murine sponge model

Male ddY mice weighing 20 – 30 g (Japan SLC, Inc.) were housed in groups of 6 – 10 animals per cage in a temperature-controlled room (23°C ± 2°C) with a relative humidity of 60% ± 10%, and free access to food (CRF-1; Oriental Yeast Co., Ltd., Tokyo) and water. The lights were on from 07:00 to 19:00 h. Experiments were conducted between 09:00 and 17:00. All procedures

regarding animal care and use were carried out according to the regulations of the Experimental Animal Care and Use Committee of Mukogawa Women's University. All experiments conformed to the guidelines on the ethical use of animals of the Japanese Government Notification, and all efforts were made to minimize both the number of animals used for experiments and their suffering.

*i) Preparation of sponge discs and implantation*: Sponge discs were implanted according to the previous method (30). Polyurethane sponge discs, 5-mm-thick and 13-mm diameter, were used as matrices in monitoring for angiogenesis. Animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.; Tokyo Kagaku Kogyo Co., Ltd., Fushimi) and then the dorsal hair shaved and skin wiped with 70% ethanol. The sponge discs were implanted aseptically with curved artery forceps through a 1-cm-long dorsal mid-line incision into an s.c. pouch. The incision wound after sponge implantation was secured by a 5-0 silk suture, followed by glue (AronAlphaA 'Sankyo'; Daiichi-Sankyo Co., Ltd., Tokyo). Postoperatively, the animals were monitored for any signs of infection at the operation site, discomfort, or distress; any animal showing such signs was immediately sacrificed. After sponge implantation, the mice were divided into groups. The volume of injection was 0.05 mL per animal in all groups. The amount of ONO-1301 solution or control HGF solution per animal was treated in sponge in order to evaluate the angiogenesis effect for treatment by topical administration. In the previous murine sponge model study, the unit of reagent per animal was employed (31). The unit of CAY10441 per body weight was employed according to previous methods (32).

*ii) Treatment*: Treatment with ONO-1301 or HGF solution by injection into the sponge discs was started immediately after implantation of the discs. ONO-1301 solution (1, 10, or 30 $\mu$g/animal) or HGF solution [0.08, 0.17, or 0.33 $\mu$g/animal, dosages based on the results of a previous study (33)] were injected into the sponges in the backs of the mice once a day for 7 or 14 days. The data of mice, in which bleeding from the neovascularity when ONO-1301 or HGF solution was treated, was not used in this study.

In the inhibition studies, antibody to HGF, normal IgG control (8 mg/kg), or CAY-10441 solution (1, 3, or 10 mg/kg) was administered by intraperitoneal (i.p.) injection once every 3 days for 14 days, 1 h before the ONO-1301 or HGF injection. The first treatment of antibody to HGF, normal IgG control, or CAY-10441 was given 1 h before implantation of the sponge discs.

*iii) Quantification of angiogenesis by hemoglobin assay*: On days 7 or 14 after implantation of the sponge disc, animals were anesthetized and killed by cervical dislocation and the sponge implants carefully excised and weighed. Each implant was homogenized (T10 basic ULTRA-TURRAX; IKA® Japan K.K., Osaka) in a 2-fold volume of distilled water for sponge weight and centrifuged at 12, 000 × *g* for 30 min. The supernatants were used for the assessment of hemoglobin. The hemoglobin was quantified colorimetrically at 540 nm in a microplate reader (Sunrise Remote R; Tecan Japan Co., Ltd., Kawasaki). The concentration of hemoglobin was assayed in parallel and expressed as mg Hb/g wet tissue. Previous studies have shown that hemoglobin level correlates well with other methods for the detection and quantification of angiogenesis in tissue (34, 35).

*iv) Quantification of HGF in sponge*: HGF concentration was measured in the sponge discs after 7 or 14 days administration of ONO-1301 or control HGF solution. Sponge discs were carefully excised and weighed prior to homogenization (T10 basic ULTRA-TURRAX) in a 2-fold volume of an HGF extract solution (Institute of Immunology Co., Ltd., Tokyo) for sponge weight and centrifuged at 12, 000 × *g* for 30 min. The supernatants were used for the assessment of HGF. HGF concentrations were measured using a commercially available ELISA kit (Institute of Immunology Co., Ltd.) according to the protocol supplied by the manufacturer. The minimum detectable level of HGF using the ELISA kits was found to be 0.2 ng/ml. All the samples were assayed at least in duplicate.

*v) Quantification of cAMP in plasma*: Blood samples were obtained from the inferior vena cava at 14th day after repeated treatment with ONO-1301 or control HGF solution once a day. Blood collection was performed after 24 h from the final treatment of ONO-1301 or control HGF solution. Blood samples were collected in pyrogen-free tubes containing heparin and centrifuged at 12,000 × *g* for 10 min. The supernatants were used for the assessment of cAMP. cAMP concentrations were measured using a commercially available ELISA kit (DetectX® Cyclic AMP Immunoassay kit; Arbor Assays, Ann Arbor, MI, USA) (36) according to the protocol supplied by the manufacturer. All samples were assayed at least in duplicate.

*vi) Western blotting*: Samples for measurement of HGF concentration in sponge were used in western blotting. The total protein concentration was measured using a Bradford method. For the western blotting assay was subjected to SDS-PAGE (Bio-Rad) and the proteins were then transferred onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with non-fat dried milk in TBS containing 0.1% Tween 20 (TBST) for 2 h at 4°C, and blots were then incubated at 4°C overnight with Met Antibody (Cell Signaling Technology, Inc., Beverly, MA, USA),

GAPDH (FL-335) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) with 1:1000 dilution. After washing 3 times, the membranes were incubated for 1 h at room temperature with secondary antibody (horseradish peroxidase–conjugated species-specific antibody). Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA).

### Statistical analyses

The data on sponge weights, hemoglobin, and HGF concentrations and c-Met/GAPDH in the implanted sponge discs and plasma cAMP concentrations were expressed as means ± S.E.M. and were evaluated for statistical significance using the Bonferroni test for differences between the groups. While the overall significance was determined in repeated measures of one-way ANOVA. A $P$-value of < 0.05 was considered to be statistically significant.

## Results

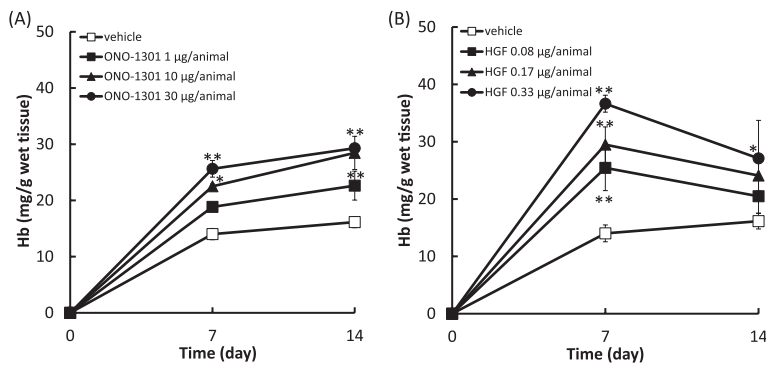### Effects of 14-day repeated ONO-1301 or HGF treatment on angiogenesis in the murine sponge model

Representative photographs of sponges removed from the backs of mice in each group are shown in Fig. 1. The red color becomes deeper as the ONO-1301 or HGF dosages increased. As shown in Fig. 2A, repeated administration of ONO-1301 solution (1, 10, or 30 $\mu$g/animal) into the sponge implants for 14 days caused a marked dose-dependent increase in angiogenesis as evidenced by increasing hemoglobin levels in sponge granuloma tissues. Hemoglobin levels obtained after once daily HGF treatment (0.08, 0.17, or 0.33 $\mu$g/animal) in the sponge reached their peak at 7th day, and the effect had decreased somewhat by the 14th day (Fig. 2B). Repeated treatment with ONO-1301 therefore increased hemoglobin levels for longer than HGF.
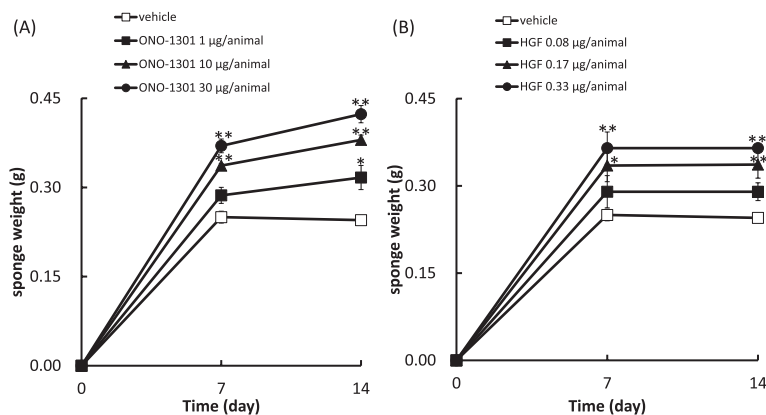
Sponge weights after repeated treatment with ONO-1301 (1, 10, or 30 $\mu$g/animal) or HGF solution (0.08, 0.17, or 0.33 $\mu$g/animal) are shown in Fig. 3, A and B, respectively. Sponge weights after repeated treatment with ONO-1301 were increased in a dose-dependent manner due to an increase in granuloma tissue, in comparison with those treated with control (Fig. 3A). Sponge weights after repeated treatment with HGF had also increased by the 7th day, but had not increased any further by the 14th day (Fig. 3B). Repeated treatment with ONO-1301 therefore increased the amount of granuloma tissue in the sponges more than treatment with HGF.
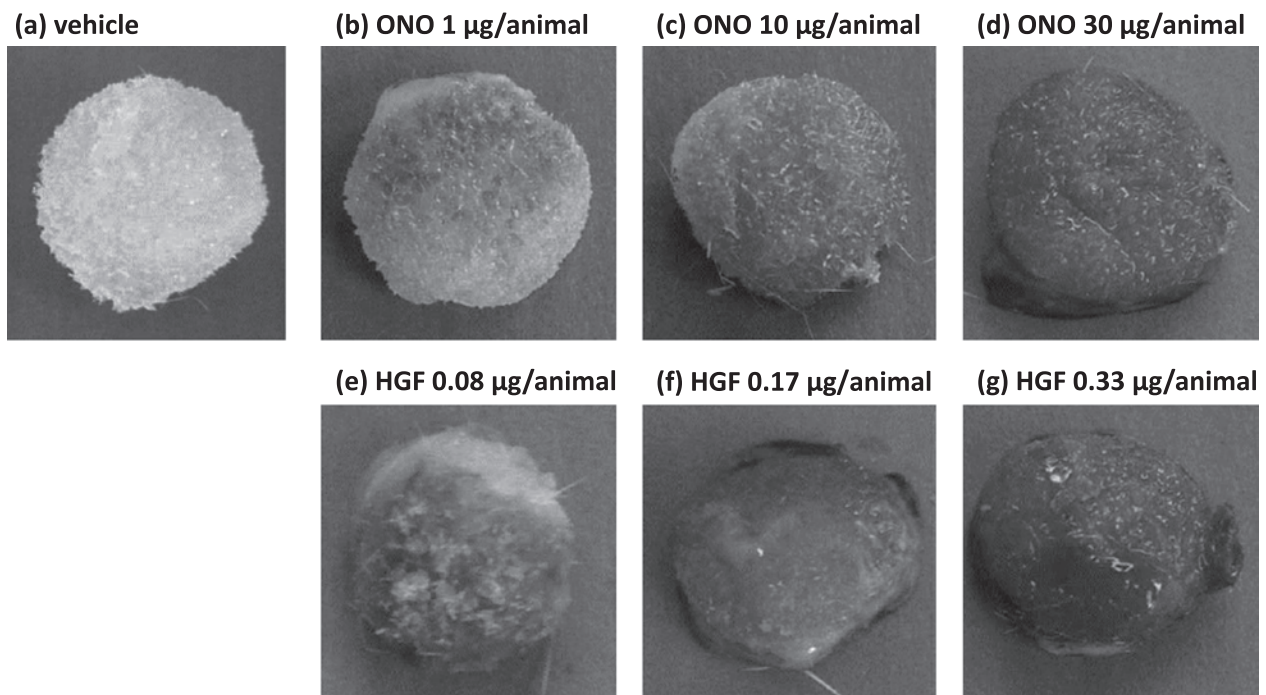


**Fig. 1.** Photographs of sponges implanted in the backs of mice from the ONO-1301 or HGF 7-day repeated treatment groups. A sponge treated with vehicle by injection once a day for 7 days is shown in (a). Sponges treated with ONO-1301 (1, 10, and 30 $\mu$g/animal) by injection once a day for 7 days are shown in (b), (c), and (d), respectively. Sponges treated with HGF (0.08, 0.17, 0.33 $\mu$g/animal) by injection once a day for 7 days are shown in (e), (f), and (g), respectively.

**Fig. 2.** Effect of ONO-1301 or HGF treatment for 14 days on angiogenesis in the murine sponge model. The change of hemoglobin levels in the sponge during repeated treatment with ONO-1301 (A) or HGF (B) for 14 days. Values are the mean ± S.E.M. from groups of 5 animals. *$P < 0.05$, **$P < 0.01$ vs. vehicle group (Bonferroni test / ANOVA).



**Fig. 3.** Sponge weights after treatment with ONO-1301 or HGF treatment for 14 days on angiogenesis in the murine sponge model. The changes in the weights of sponges removed from the backs of mice following repeated treatment with ONO-1301 (A) or HGF (B) for 14 days. Values are the mean ± S.E.M. from groups of 5 animals. *$P < 0.05$, **$P < 0.01$ vs. vehicle group (Bonferroni test / ANOVA).
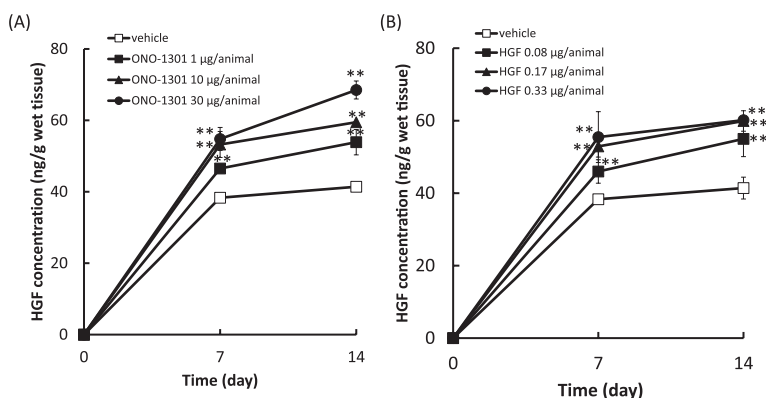


**Fig. 4.** HGF concentrations in the sponges after repeated treatment with ONO-1301 or HGF for 14 days in the murine sponge model. Values are the mean ± S.E.M. from groups of 5 animals. *$P < 0.05$, **$P < 0.01$ vs. vehicle group (Bonferroni test / ANOVA).

*HGF concentrations in sponge after 14-day repeated treatment with ONO-1301 or HGF*

HGF concentrations after repeated treatment with ONO-1301 (1, 10, or 30 μg/animal) or HGF (0.08, 0.17, or 0.33 μg/animal) are shown in Fig. 4, A and B, respectively. HGF concentrations were increased dose-dependently during treatment with ONO-1301 for 14 days compared with the control (Fig. 4A), while in the HGF treatment groups, although HGF concentrations were increased on the 7th day, there was no further increase at the 14th (Fig. 4B). In this study, a correlation

between the increases in hemoglobin and HGF levels was observed. This correlation is in agreement with results described in previous reports (37).

*Comparison of c-Met expressions between repeated treatment of ONO-1301 and control HGF solution for 14 days*

Expressions of C-Met known as the receptor of HGF determined by western blotting are shown in Fig. 5. C-Met expressions in animals treated with ONO-1301 solution or HGF solution are shown in Fig. 5, A and B,
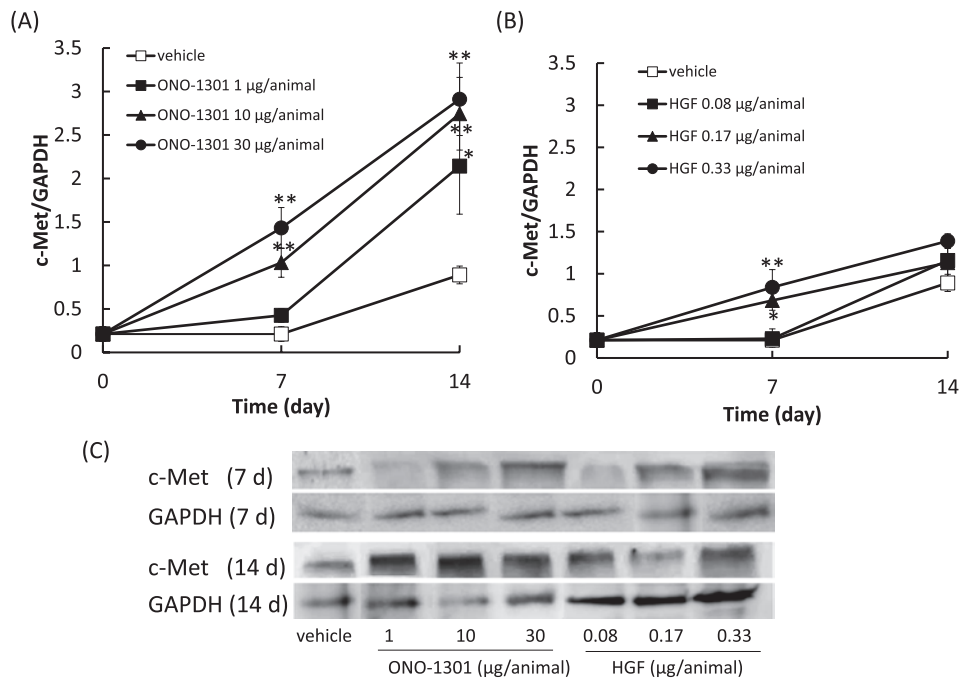
**Fig. 5.** Investigation of c-Met expression by western blotting. A: The ratio of c-Met/GAPDH expression by treatment with ONO-1301 for 14 days. B: The ratio of c-Met/GAPDH expression by treatment with HGF for 14 days. Values are the mean ± S.E.M. from groups of 3 animals. *$P < 0.05$, **$P < 0.01$ vs. vehicle group (Bonferroni test / ANOVA). C: Representative photographs of immunoblots for c-Met and GAPDH in each group at day 7 and 14.

respectively. Representative photograph of western blotting for C-Met and GAPDH at the 7th and 14th day are shown in Fig. 5C. ONO-1301 significantly up-regulated c-Met expression compared to the vehicle group for 14 days in a dose-dependent manner. While up-regulation of c-Met expression in the HGF-treatment group was not observed for 14 days. These results suggested that ONO-1301 might promote not only HGF synthesis but also upregulation c-Met expression.

*Hemoglobin levels in sponges after pretreatment with anti-HGF antibody, normal IgG control, or IP-receptor antagonist*

The increases in hemoglobin levels by treatment with HGF or ONO-1301 were suppressed by pretreatment with anti-HGF antibodies (Fig. 6). While the increase in hemoglobin levels by treatment with HGF or ONO-1301 was not suppressed by pretreatment with normal IgG control. Furthermore, pretreatment with CAY-10441, a specific IP-receptor antagonist, also reversed the increasing effect on hemoglobin levels by treatment with ONO-1301 in a dose-dependent manner.
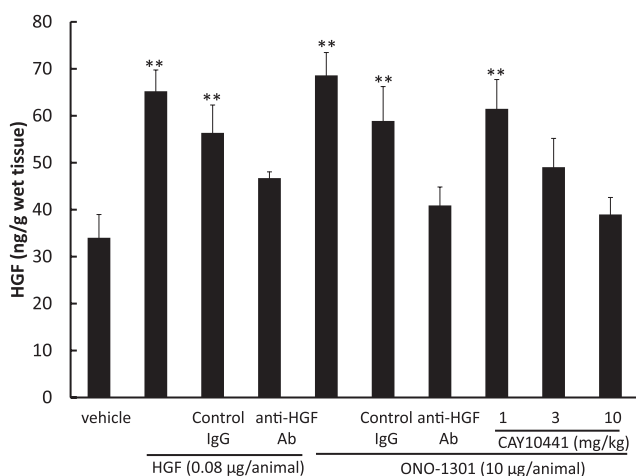
*HGF concentrations in sponges after pretreatment with anti-HGF antibody or IP-receptor antagonist*

The increases in HGF concentrations were suppressed by pretreatment with anti-HGF antibody and IP-receptor
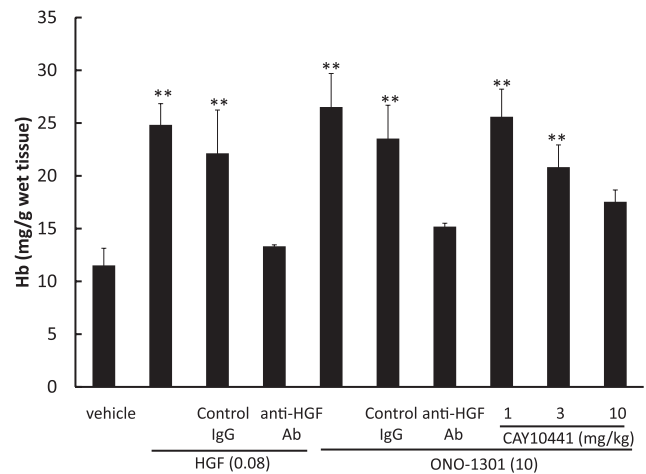


**Fig. 6.** Hemoglobin levels on the 14th day after treatment with anti-HGF antibody or IP receptor antagonist. Values are the mean ± S.E.M. from groups of 4 animals. **$P < 0.01$ vs. vehicle group (Bonferroni test / ANOVA).

antagonist in a dose-dependent manner (Fig. 7). Anti-HGF antibody neutralized both the exogenous HGF injected and the endogenous HGF increase stimulated by treatment with ONO-1301. IP-receptor antagonist also suppressed the increase in HGF synthesis stimulated by treatment with ONO-1301.

**Fig. 7.** HGF concentrations on the 14th day after treatment with anti-HGF antibody or IP receptor antagonist. Values are the mean ± S.E.M. from groups of 4 animals. **$P < 0.01$ vs. vehicle group (Bonferroni test / ANOVA).



**Fig. 8.** Plasma cAMP concentrations on the 14th day after treatment with ONO-1301. Values are the mean ± S.E.M. from groups of 4 animals. **$P < 0.01$ vs. vehicle group (Bonferroni test / ANOVA).

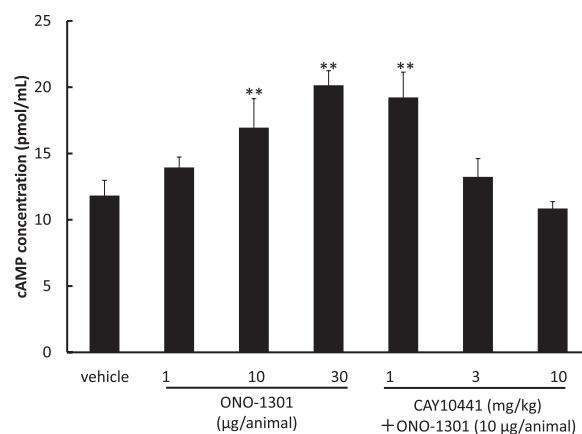*Plasma cAMP concentrations after repeated treatment with ONO-1301*

In order to determine the relationship between IP receptor and ONO-1301, plasma cAMP concentrations were measured after repeated treatment with ONO-1301. Repeated treatment with ONO-1301 increased cAMP concentrations dose-dependently. This increase of cAMP concentration was dose-dependently suppressed by pretreatment with the IP-receptor antagonist CAY10441 (Fig. 8).

## Discussion

Repeated treatment with ONO-1301 increased the hemoglobin levels, the amount of granuloma tissue in the sponges, and HGF concentrations in a dose-dependent manner more than repeated treatment with HGF.

HGF and its receptor c-Met have become the focus of intense scrutiny since its discovery in the late 1980s (38), especially regarding its role in cancer. HGF is now known to be a potent morphogen that can regulate tissue and organ regeneration and modulate cell morphology; it is a motogen that can stimulate cell motility and migration and is also a mitogen, able to regulate cell growth and death, as well as being an angiogenic factor (39). A recent study has indicated that HGF and the c-Met signaling complex may be targets in a new form of cancer therapy (40).

In this study, ONO-1301 also up-regulated c-Met expression compared with vehicle for 14 days significantly, although up-regulation of c-Met expression by treatment with HGF was not maintained for 14 days. These results suggested that the loss of increasing

hemoglobin level in the HGF treatment group at the 14th was caused by the differences of c-Met expression between the ONO-1301 and HGF treatment group. We also suggest that there may be some differences in the response to increasing endogenous HGF (by treatment with ONO-1301) and increasing exogenous HGF (by injection of HGF) during the 14-day experimental period.

In the previous article describing iloprost-induced desensitization of human prostacyclin (IP) receptor using isolated rabbit lungs, rapid desensitization could be acquired after 3-h infusion, and it seemed to be due to co-stimulation of EPI receptor and IP receptor (41). When we consider the half-life of prostacyclin agonist, it has been reported that the in vivo $T_{1/2}$ for epoprostenol, iloprost, beraprost, treprostinil, and ONO-1301 was 6 min, 20 – 25 min, 1.1 h, 1 – 4.6 h, and 5.6 h, respectively (42). In previous studies, long-term exposure of the IP receptor to PGI$_2$ analogs in vitro caused rapid and severe desensitization of the receptor (43 – 45). As described in those papers, the longest $T_{1/2}$ of ONO-1301 might be advantageous for acquiring desensitization.

The inhibition constant ($K_i$) values for the IP receptor of cicaprost, iloprost, carbacyclin, isocarbacyclin, beraprost, and ONO-1301 in the previous study using Chinese hamster ovary cells was 10, 11, 110, 15, 16, and 47 nM, respectively (46). ONO-1301 might have an ability to stimulate EPI receptors and IP receptors and then induce desensitization. Thus ONO-1301 has the possibility to cause desensitization. However, as shown in Fig. 8, desensitization of IP receptor following ONO-1301 administration was not observed judging from the cAMP level. Dose-dependent downregulation or desensitization by repeated treatment of ONO-1301 was not observed on the 14th day.

It has been reported that the pharmacological profiles
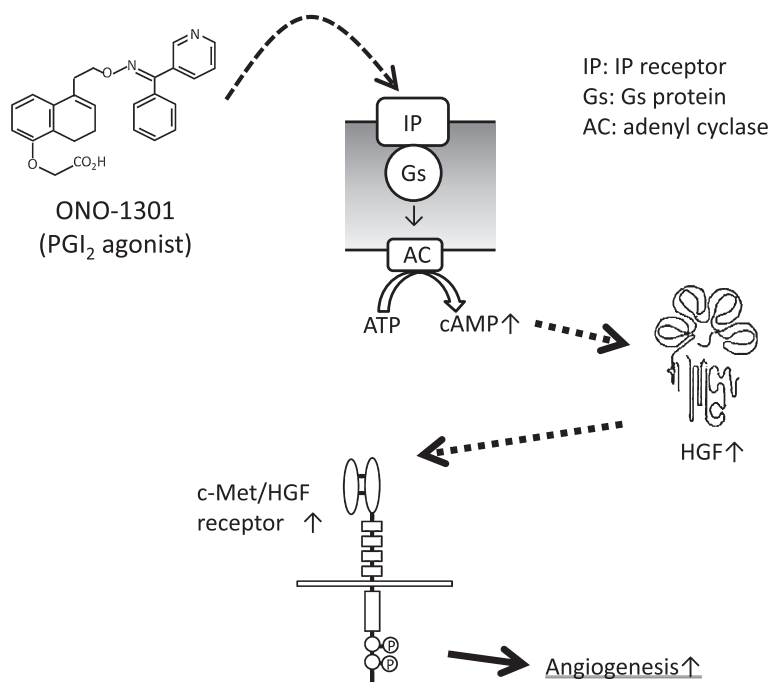
**Fig. 9.** Schematic representation of mechanism of angiogenesis following treatment with a novel long-acting prostacyclin agonist, ONO-1301.

of nonprostanoid PGI$_2$ agonists and PGI$_2$ analogs are difference in the activation of signal transduction, such as the phospholipase C pathway (47). The nonprostanoid PGI$_2$ agonist MRE-269 did not activate Ca$^{2+}$ signaling, but the PGI$_2$ analogs beraprost and iloprost did (48). The difference in pharmacological characteristics between ONO-1301 as a non-prostanoid PGI$_2$ agonist and PGI$_2$ analogs may be attributable to some differences in the activation of signal transduction.

ONO-1301 was developed as a prostacyclin agonist with thromboxane synthase inhibitory activity. The characterization with thromboxane synthase inhibitory activity might explain the above phenomena.

Pretreatment with anti-HGF antibodies and CAY-10441, a specific IP-receptor antagonist, reversed the increasing effect on hemoglobin levels by ONO-1301 treatment. These results indicate that ONO-1301 causes angiogenesis by promoting the synthesis of HGF and IP-receptor activation.

Repeated treatment with ONO-1301 increased cAMP concentrations dose-dependently. This increase of cAMP concentration was dose-dependently suppressed by pretreatment with the IP-receptor antagonist CAY10441. In a previous study, PGI$_2$ analogs (iloprost and treprorpstinil) have been shown to increase cAMP concentrations (49). Our data demonstrated that ONO-1301 also increased cAMP concentrations via the IP receptor and therefore behaved as a prostacyclin agonist in the murine

sponge model.

Previous studies reported that 3T3-L1 adipocytes in mice secreted HGF (50) and obesity in humans was associated with a marked increase in circulating HGF levels (51). A previous study also reported that immunoreactive hHGF secretion by MRC-5 cells and human gingival fibroblasts, GF-5, was markedly enhanced by interleukin-1$\alpha$ (IL-1$\alpha$) and -1$\beta$ (IL-1$\beta$) and tumor necrosis factor-$\alpha$ (TNF-$\alpha$). Furthermore, their results also showed that IL-1$\alpha$ and TNF-$\alpha$ increased the level of hHGF mRNA in the cells by increasing the transcriptional activity (52). HGF secration and expression in human skin fibroblast cells was markedly stimulated by c-AMP-elevating agents, forskolin, cholera toxin, prostaglandin E$_2$ (PGE$_2$), and 3-isobutyl-1-methylxanthine, as well as by the membrane-permeable cAMP analogue dibutyryl cAMP (53). From these previous results, ONO-1301 may be able to stimulate HGF secretion by cells such as fibroblasts via activation of a cAMP-mediated pathway.

These results suggested that ONO-1301 increases cAMP concentrations via the IP receptor, thereby acting as a PGI$_2$ agonist. Increased cAMP levels promote production of endogenous HGF without down-regulation and thereby stimulate angiogenesis in the murine sponge model. A schematic representation of the mechanism of angiogenesis stimulated by treatment with ONO-1301 is shown in Fig. 9.

In conclusion, hemoglobin levels in the ONO-1301-treatment group were significantly increased for 14 days compared with vehicle. It was suggested that the increase of hemoglobin level was caused by the HGF-synthesis promoting effect of ONO-1301. ONO-1301 also significantly upregulated c-Met expression compared with vehicle for 14 days, even though upregulation of c-Met expression by treatment with HGF was not maintained for 14 days. In addition, angiogenesis caused by ONO-1301 was due to increased cAMP concentrations via IP-receptor signaling. Increasing of cAMP promoted synthesis of HGF, causing angiogenesis.

## Conflicts of Interest

Yoshiki Sakai is an employee of ONO Pharmaceutical Co., Ltd. There are no financial or other relations that could lead to a conflict of interest.

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