has been recognized as a major predictor of atherosclerotic disease[6, 7].

Adipose tissue is an important endocrine organ that secretes many biologically active molecules, such as leptin, adiponectin, tumor necrosis factor-$\alpha$ (TNF-$\alpha$), and monocyte chemoattractant protein 1 (MCP-1), which are collectively termed adipocytokines[8, 9]. Dysregulated production of proinflammatory and anti-inflammatory adipocytokines seen in visceral obesity is associated with metabolic syndrome[8, 10], suggesting that inflammatory changes in adipose tissue may contribute to the development of various aspects of metabolic syndrome and result in type 2 diabetes and atherosclerotic vascular disease.

Recent studies have demonstrated that adipose tissue in obese subjects is characterized by increased infiltration of macrophages, suggesting that they are important sources of inflammation in adipose tissue[11, 12]. C-C chemokine receptor-2 (CCR2), known as a receptor for MCP-1, plays a key role in monocyte/macrophage recruitment and macrophage-dependent inflammatory response and the development of atherosclerosis[13]. Mouse models have demonstrated that adipose tissue macrophage (ATM) accumulation via the MCP-1/CCR2 pathway is both necessary and sufficient for the development of insulin resistance related with obesity; for instance, mice with targeted deletions in the genes for MCP-1 or CCR2 have decreased ATM content, decreased inflammation in adipose tissue, and protection from high fat diet-induced insulin resistance[14, 15]. In contrast, mice overexpressing MCP-1 have increased numbers of ATMs along with increased insulin resistance[14, 16]. Recently, we demonstrated that a CCR2 inhibitor, propagermanium improves insulin resistance and hepatic steatosis in db/db mice, suggesting that postnatal inhibition of CCR2 is effective against obesity-related metabolic disorders[17]; however, db/db mice are of limited value as models of common human obesity. By comparison, C57BL6 mice maintain a normal weight on chow, but are genetically predisposed to weight gain on a high fat and high sugar (HF/HS) diet, thus resembling human obesity more closely than other mouse models[18]. High fat and high sugar feeding in C57BL6 mice is well established as a model of the insulin resistance and glucose intolerance that occur in human obesity[19]. Therefore, it is conceivable to use this model to show the effects of CCR2 inhibition on the development of diet-induced obesity (DIO)-related metabolic disorders.

In the present study we examined the preventive effect of propagermanium on the development of diet-induced obesity and metabolic disorders caused by an HF/HS diet in mice.

## Methods

### Materials

Propagermanium (3-oxygemylpropinic acid polymer) was kindly provided by Sanwa Kagaku Kenkyusho Co. (Nagoya, Japan).

### Animal Preparation and Experimental Design

Male C57BL/6J mice were obtained from Charles River (Yokohama, Japan) at 5 weeks of age. The mice were fed normal chow or a high fat/high sucrose (HF/HS) diet (55% fat, 28% carbohydrate in energy; Oriental Bio Service, Yokohama, Japan) without additional supplementation (non-treated group) or with chow supplemented with 0.005% or 0.05% propagermanium (propagermanium-treated groups) for 12 weeks from 6 weeks of age ($n=10$ in each group). This concentration of propagermanium in the feeding chow results in an approximate dose of 5 mg/kg and 50 mg/kg body weight per day in each mouse, respectively, adjusted for previous measurement of food intake. This dose had no significant effect on normal mice[20] and caused no deaths in this study. The IC50 value for propagermanium inhibition of CCR2 is 0.52 $\mu$g/mL[21]. Animals were provided with the diet and water ad libitum and were maintained on a 12-hour light/dark cycle. All animal experiments were conducted according to the Guidelines for Animal Experiments at Kyoto University School of Medicine.

### Body Fat Composition Analysis

Body fat composition was measured using a LaTheta (LCT-100M) experimental animal CT system (Aloka, Tokyo, Japan)[17].

### Quantitative Real-Time PCR

Total RNA was extracted from frozen adipose tissue (200 mg) and liver tissue (50 mg) using an RNeasy mini kit (Qiagen, Valencia, CA). The cDNA was synthesized from total RNA using Super Script III (Invitrogen). Real-time polymerase chain reaction was performed on an ABI PRISM 7700 using the SYBR GREEN polymerase chain reaction Master Mix (Applied Biosystems, Warrington, UK). Primer sets are shown in **Table 1**. The mRNA levels were normalized relative to the amount of $\beta$-actin mRNA and expressed in arbitrary units.

### Analysis of Metabolic Parameters

Plasma insulin concentration was measured with an insulin assay kit (Morinaga Institute of Biological

Table 1. Primers used for real-time PCR

| Primer | Sequence |
|---|---|
| adiponectin | |
| forward | 5'-GATGGCAGAGATGGCACTCC-3' |
| reverse | 5'-CTTGCCAGTGCTGCCGTCAT-3' |
| MCP-1 | |
| forward | 5'-CCACTCACCTGCTGCTACTCA-3' |
| reverse | 5'-TGGTGATCCTCTTGTAGCTCTCC-3' |
| Emr1 | |
| forward | 5'-CTTTGGCTATGGGCTTCCAGTC-3' |
| reverse | 5'-GCAAGGAGGACAGAGTTTATCGTG-3' |
| TNF-α | |
| forward | 5'-CCCAGACCCTCACACTCAGATC-3' |
| reverse | 5'-GCCACTCCAGCTGCTCCTC-3' |
| CD11c | |
| forward | 5'-CTGGATAGCCTTCTTCTGCTG-3' |
| reverse | 5'-GCACACTGTGTCCGAACTC-3' |
| MGL-1 | |
| forward | 5'-TGAGAAAGGCTTTAAGAACTGGG-3' |
| reverse | 5'-GACCACCTGTAGTGATGTGGG-3' |
| IL-10 | |
| forward | 5'-GCTCTTACTGACTGGCATGAG-3' |
| reverse | 5'-CGCAGCTCTAGGAGCATGTG-3' |
| SREBP-1c | |
| forward | 5'-GGAGCCATGGATTGCACATT-3' |
| reverse | 5'-GCTTCCAGAGAGGAGGCCAG-3' |
| β-actin | |
| forward | 5'-TACCACAGGCATTGTGATGG-3' |
| reverse | 5'-TTTGATGTCACGCACGATTT-3' |

Science, Yokohama, Japan). Cholesterol, triglyceride, and free fatty acid (FFA) were measured by Cholesterol E test, Triglyceride E test, NEFA C test (Wako Pure Chemical Industries Ltd., Osaka, Japan), respectively. The plasma adiponectin level was measured with an adiponectin ELISA kit (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan). The plasma leptin level was measured with a leptin ELISA kit (Morinaga Institute of Biological Science). For glucose tolerance tests (GTT), mice were deprived of food for 24 hours and then injected i.p. with glucose (1.5 g/kg body mass). For insulin tolerance tests (ITT), mice were injected i.p. with human regular insulin (1 U/kg; Eli Lilly and Co., Kobe, Japan). For GTT and ITT, blood samples were collected from the tail vein before and after injection, and plasma glucose concentration was measured with a Glutest Ace (Sanwa Kagaku Kenkyusho Co.). For measurement of other blood markers, a blood sample was collected from the retro-orbital plexus after 14h fasting using a heparin-coated micropipette.

### Measurement of Hepatic Triglyceride Content

Hepatic triglyceride contents were measured as previously described[17]. Tissue triglyceride contents were expressed as mg/mg protein.

### Statistical Analysis

All data are expressed as the means ± S.D. The statistical significance of differences was assessed by one-way ANOVA followed by Fisher's post-hoc test. Differences of $p<0.05$ were regarded as significant. All statistical analyses were performed using StatView version 5.0 software (SAS Institute, Cary, NC).

## Results

### Propagermanium Suppressed Body Weight Gain and Adiposity in DIO Mice

We first examined the effect of propagermanium on body weight gain and visceral fat accumulation in DIO mice. Propagermanium treatment slightly but significantly suppressed body weight gain in DIO mice (**Table 2**) without affecting food intake (data not shown), but a dose-dependent effect was not observed. Propagermanium treatment also decreased visceral fat accumulation in DIO mice measured by CT scan (**Table 2**).

### Effect of Propagermanium on Lipid and Glucose Metabolism in DIO Mice

To assess the effect of propagermanium on lipid and glucose metabolism, we then measured plasma lipid, glucose, and insulin levels. There was no difference in fasting plasma triglyceride (TG), FFA, and total cholesterol (T-Cho) levels between non-treated and propagermanium-treated DIO mice (**Table 2**) or in HbA1c; however, propagermanium treatment significantly decreased fasting blood glucose and plasma insulin concentrations in DIO mice (**Table 2**).

To further evaluate the effect of propagermanium on glucose metabolism, we next conducted an intraperitoneal insulin tolerance test (IPITT) and glucose tolerance test (IPGTT). HF/HS diet feeding impaired insulin sensitivity and glucose tolerance in C57BL/6 mice (**Fig. 1A, B**); however, propagermanium-treated DIO mice showed a significant decrease in plasma glucose after insulin stimulation compared with non-treated DIO mice (**Fig. 1A**). Although propagermanium-treated DIO mice showed a slight decrease in plasma glucose compared with non-treated DIO mice only 60 minutes after intraperitoneal injection of glucose (**Fig. 1B**), plasma insulin levels during IPGTT were markedly decreased in propagermanium-treated DIO mice (**Fig. 1C**). These data indicate that propa-

**Table 2.** Characteristics of control and HF/HSD mice treated with or without propagermanium

| | Normal diet | HF/HSD | HF/HSD + Pro (5 mg) | HFD/HSD + Pro (50 mg) |
|---|---|---|---|---|
| Body weight (g) | 32.1 ± 1.0 | 48.2 ± 1.0 | 45.9 ± 2.3* | 46.2 ± 1.5* |
| Visceral fat (g) | 2.0 ± 0.2 | 5.9 ± 0.4 | 5.2 ± 0.5* | 5.3 ± 0.1* |
| Blood glucose (mg/dL) | 66.6 ± 9.9 | 158.7 ± 15.0 | 128.2 ± 16.1** | 135.0 ± 10.6** |
| HbA1c (%) | ND | 3.0 ± 0.2 | 2.9 ± 0.3 | 2.8 ± 0.3 |
| Plasma insulin (ng/mL) | 0.5 ± 0.1 | 2.6 ± 1.0 | 1.5 ± 0.6** | 1.1 ± 0.3** |
| Plasma TG (mg/dL) | 87.2 ± 9.9 | 105.9 ± 22.6 | 111.1 ± 16.3 | 118.2 ± 13.6 |
| Plasma T-Cho (mg/dL) | 100.2 ± 10.9 | 172.2 ± 21.9 | 162.5 ± 12.2 | 164.2 ± 11.5 |
| Plasma FFA (mEq/L) | 1.4 ± 0.3 | 1.9 ± 0.3 | 1.9 ± 0.1 | 2.0 ± 0.2 |

Results are expressed as the mean ± S.D. *$p < 0.05$, **$p < 0.01$ vs untreated HF/HSD mice. ($n = 10$ per group) HF/HSD, high fat and high sucrose diet, ND, not determined; TG, triglyceride; T-Cho, total cholesterol; FFA, free fatty acid.

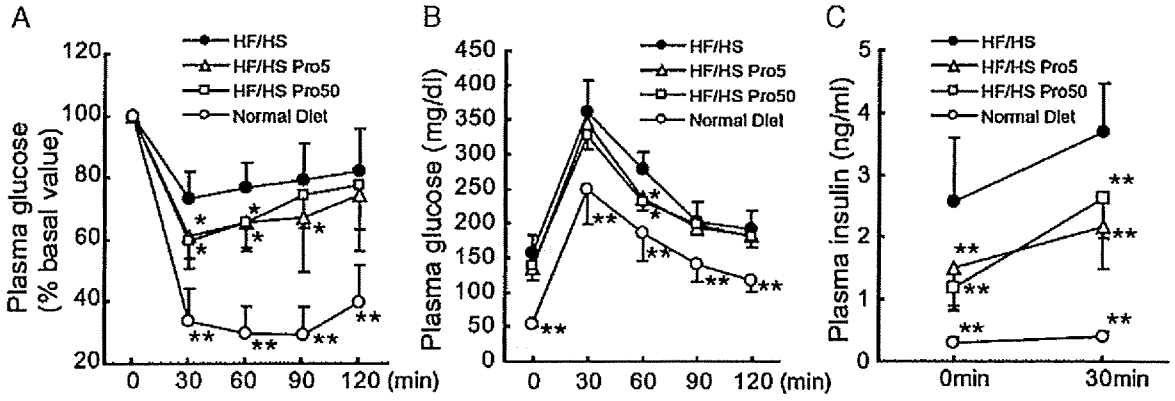


**Fig. 1.** Effect of propagerumanium treatment on insulin sensitivity and glucose tolerance.

The response of plasma glucose and insulin to single intraperitoneal injection of insulin (A) or glucose (B, C) in C57BL6 mice fed a normal diet (open circle), HF/HS diet (closed circle), or HF/HS diet with propagermanium treatment with doses of 5 mg/kg (open triangle) and 50 mg/kg (open square). *$p < 0.05$; **$p < 0.01$; vs. HF/HS diet group ($n = 10$ per group).

germanium improves insulin resistance in DIO mice.

### Effect of Propagermanium Treatment on Macrophage Accumulation in Adipose Tissue and Adipocytokine Expression in DIO Mice

We next determined the effect of propagermanium on adipose tissue inflammation and adipocytokine expression in adipose tissue and plasma. ATM accumulation was markedly increased in DIO mice compared with lean control mice, as shown by the increase in F4/80 mRNA expression (**Fig. 2A**); however, propagermanium treatment significantly decreased F4/80 mRNA expression in DIO mice by 50%, indicating that propagermanium reduced ATM accumulation in DIO mice (**Fig. 2A**). Furthermore, propagermanium decreased the expression of the pro-inflammatory (M1) macrophage specific gene, CD11c, whereas propagermanium increased the expression of the anti-inflammatory (M2) macrophage specific gene, macrophage galactose N-acetyl-galactosamine-specific lectin 1 (MGL-1), IL-10, in adipose tissue, suggesting that CCR2 inhibition shifted macrophage polarization from the M1 to M2 state in adipose tissue (**Fig. 2B, C, D**). In accordance with these results, MCP-1 and TNF-$\alpha$ mRNA expressions in adipose tissue were significantly reduced in propagermanium-treated DIO mice compared with non-treated DIO mice (**Fig. 2E, F**), indicating that propagermanium treatment suppressed adipose tissue inflammation in DIO mice. Adipose tissue expression of adiponectin mRNA was markedly decreased in DIO by 35% compared with lean control mice (**Fig. 3A**); however, propagermanium treatment increased adipose tissue adiponectin expression 2-fold in DIO mice. Similar to the gene expression, propagermanium-treated DIO mice showed higher plasma adiponectin than non-treated DIO mice (**Fig. 3B**). Furthermore, propagermanium reduced plasma leptin levels in DIO mice (**Fig. 3C**).

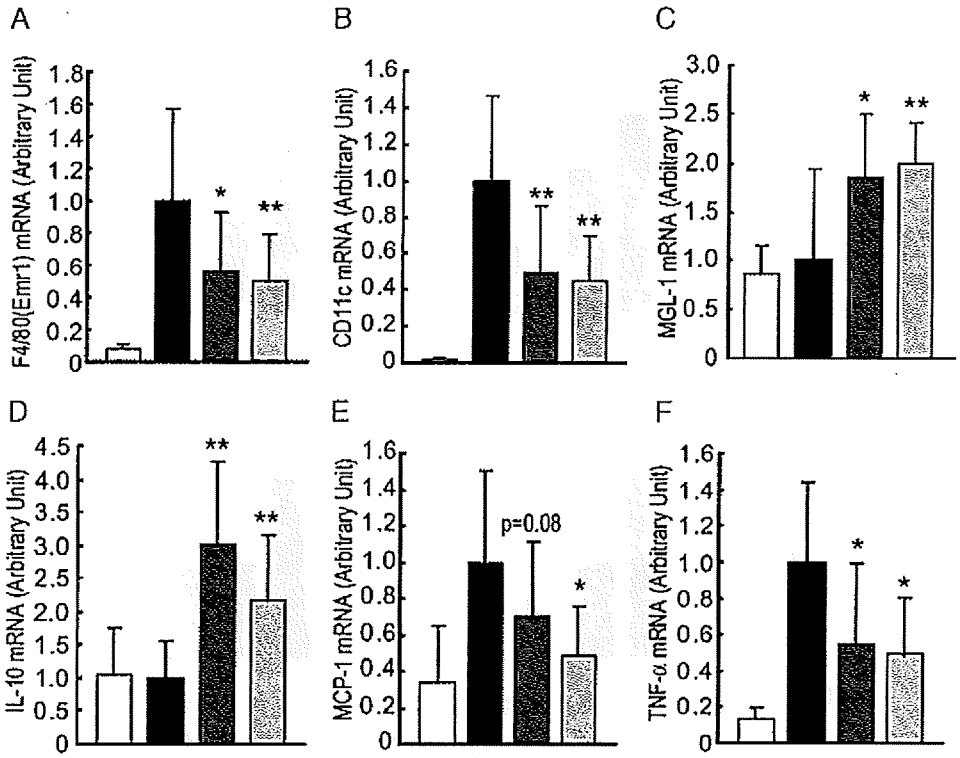


**Fig. 2.** Effect of propagermanium treatment on ATM accumulation and adipose tissue inflammation.

Expressions of Emr1 (F4/80) mRNA (A), CD11c mRNA (B), MGL-1 mRNA (C), IL-10 mRNA (D), MCP-1 mRNA (E) and TNF-$\alpha$ mRNA (F) in eWAT from C57BL6 mice fed a normal diet (white bar), HF/HS diet (black bar), or HF/HS diet with propagermanium treatment at doses of 5 mg/kg (dark gray bar) and 50 mg/kg (light gray bar) $^{*}p<0.05$; $^{**}p<0.01$; vs. HF/HS diet group ($n=10$ per group).

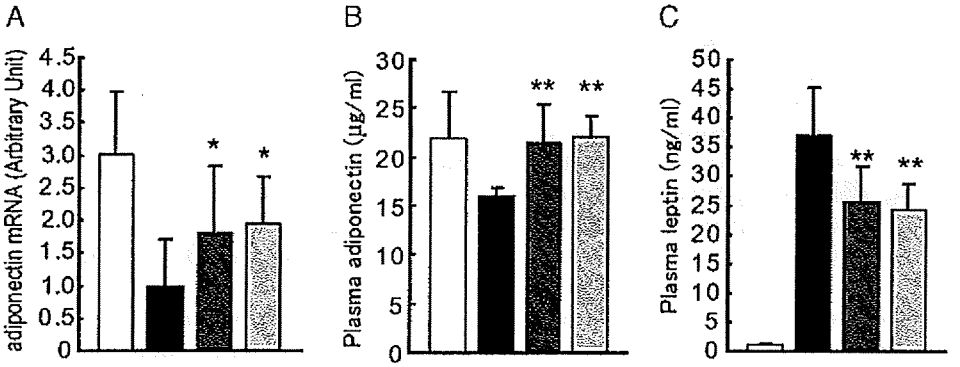


**Fig. 3.** Effect of propagermanium treatment on adipokine expression and secretion.

Expression of adiponectin mRNA (A) in eWAT, and plasma level of adiponectin (B) and leptin (C) in C57BL6 mice fed a normal diet (white bar), HF/HS diet (black bar), or HF/HS diet with propagermanium treatment at doses of 5 mg/kg (dark gray bar) and 50 mg/kg (light gray bar) $^{*}p<0.05$; $^{**}p<0.01$; vs. HF/HS diet group ($n=10$ per group).

These data indicate that propagermanium treatment improves adipocytokine expression in obese adipose tissue of DIO mice.

### Effect of Propagermanium on Hepatic Steatosis and Inflammation in Obese Mice

Finally, we assessed the effect of propagermanium

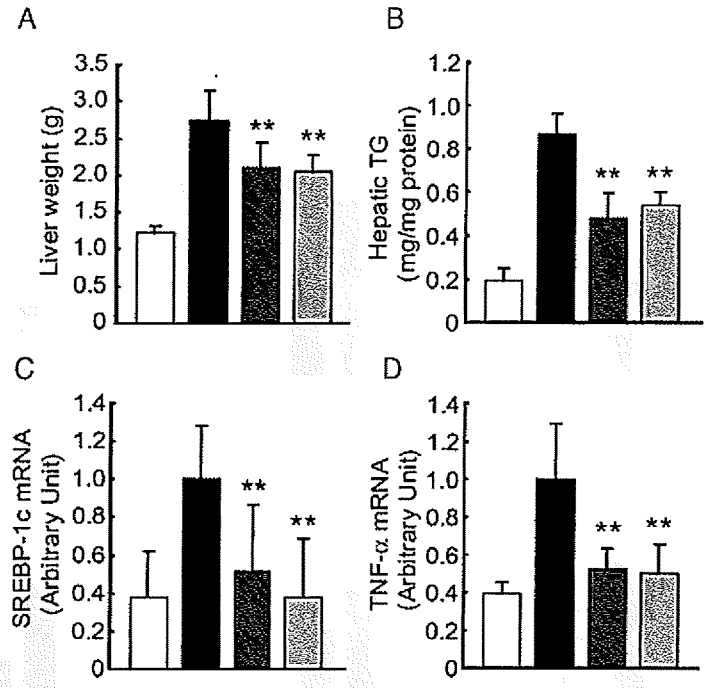


**Fig. 4.** Effect of propagermanium on hepatic steatosis and inflammation.

Liver weight (A), hepatic TG contents (B), expression of SREBP-1c mRNA (C), and TNF-$\alpha$ mRNA (D) in C57BL6 mice fed a normal diet (white bar), HF/HS diet alone (black bar), or HF/HS diet with propagermanium treatment with 5 mg/kg (dark gray bar) and 50 mg/kg (light gray bar) $^{*}p<0.05$; $^{**}p<0.01$; vs. HF/HS diet group ($n=10$ per group).

on hepatic steatosis and hepatic inflammation. Liver weight increased 2-fold in non-treated DIO mice compared with lean control mice (**Fig. 4A**); however, the liver weight of propagermanium-treated DIO mice was reduced compared with that of non-treated DIO mice (**Fig. 4A**). Similarly, non-treated DIO mice had more than 4-fold higher hepatic TG contents than lean control mice (**Fig. 4B**). Propagermanium-treated DIO mice showed 45% lower hepatic TG contents than non-treated DIO mice. Consistently, the expression of sterol regulatory element binding protein-1c (SREBP-1c), a transcription factor that regulates the expression of genes important in lipid synthesis, was decreased in propagermanium-treated DIO mice, suggesting that reduced hepatic TG content by propagermanium treatment is caused by a decrease in hepatic lipogenesis (**Fig. 4C**).

Expression of TNF-$\alpha$ mRNA in the liver was increased in DIO mice compared with the lean control, and its increase was inhibited by propagermanium treatment (**Fig. 4D**), suggesting that propagermanium also suppresses liver tissue inflammation.

## Discussion

In this study, we have clearly shown that treatment with propagermanium improved insulin resistance and hepatic steatosis by inhibiting macrophage infiltration into adipose tissue through the suppression of CCR2 function during the development of diet-induced obesity in C57BL/6 mice.

Propagermanium has been used as a therapeutic agent against hepatitis B virus-induced chronic hepatitis in Japan[22]. Recently, Yokochi *et al.* demonstrated that this drug suppressed monocyte migration *in vitro* and macrophage infiltration *in vivo* by inhibiting CCR2 function. The molecular mechanism of the action of propagermanium has been explained by its targeting glycosylphosphatidylinositol-anchored proteins that are closely associated with CCR2 and the resulting blockade of MCP-1/CCR2-mediated signaling[21]. Many animal studies have shown that propagermanium improves liver injury[20, 23], atherosclerosis[24] and diabetic nephropathy[25, 26] by reducing monocyte/macrophage infiltration into the lesion. In this study we demonstrated that inhibition of CCR2

function by propagermanium treatment reduced macrophage infiltration into adipose tissue in DIO mice as well as db/db mice[17]. Many reports suggests that macrophage infiltration triggeris the inflammatory response in adipose tissue through the secretion of pro-inflammatory cytokine and increases the inflammatory vicious cycle by interactions between macrophages and adipocytes in obesity[11, 12, 27, 28]. Furthermore, Lumeng *et al.* demonstrated the importance of the macrophage phenotype in inflammatory responses in adipose tissue[29] and found a phenotypic switch from anti-inflammatory (M2) macrophages in lean mice to pro-inflammatory (M1) macrophages in mice with diet-induced obesity. Obesity thus led to a change in the polarization of ATMs to a state that favors inflammation; however, ccr2−/− ATMs were polarized to the M2 type, even when the mice were fed a high-fat diet. In our study, CCR2 inhibition decreased the expression of M1 macrophage-specific gene (CD11c) and increased the expression of M2 macrophage-specific gene (MGL1 and IL-10) in adipose tissue of DIO mice, suggesting that propagermanium treatment shifted ATMs polarization from M1 to M2. We also found that propagermanium treatment suppressed adipose tissue inflammation, decreasing TNF-$\alpha$ and MCP-1 expressions in DIO mice. Therefore, in the present study, we provided further evidence that macrophage infiltration, (especially, M1 macrophage infiltration) via CCR2 plays a pivotal role in the induced inflammatory changes in adipose tissue during the development of obesity.

Recent investigation suggests that adipose tissue inflammatory changes contribute to the development of insulin resistance. It has been shown that adipose tissue-derived proinflammatory cytokines, such as TNF-$\alpha$, can actually cause insulin resistance in experimental models[9, 30]. Further, TNF-$\alpha$ reduces the expression of adiponectin in adipocytes by suppressing its promoter activity in a dose-dependent manner[31]. Adiponectin works as an insulin-sensitizing agent[19, 32] and hence a decrease in plasma adiponectin is related to insulin resistance in obesity. In this study, we observed that adiponectin expression in adipose tissue and the plasma adiponectin level decreased in DIO mice compared with lean control mice, as well as impaired insulin sensitivity and glucose tolerance. However, we could improve insulin resistance by propagermanium treatment, possibly by suppressing adipose tissue inflammation and increasing the plasma adiponectin level. These results strongly suggest that dysregulated adipokine expression and secretion induced by pro-inflammatory macrophage infiltration through CCR2 causes insulin resistance in diet-induced obesity.

In the present study, we successfully ameliorated hepatic steatosis by propagermanium treatment in DIO mice. Propagermanium did not affect plasma FFA levels in DIO mice, suggesting that propagermanium has no effect on hydrolysis of TG in the liver. Hepatic fat accumulation is mainly caused by increased lipogenesis through stimulating lipogenic enzymes via SREBP-1c by hyperinsulinemia or inflammatory cytokine and increased FFA influx from visceral adipose tissue[33-35]. Thus, the decreased expression of SREBP-1c along with improved hyperinsulinemia and suppression of hepatic inflammation by propagermanium treatment could be involved in the amelioration of hepatic steatosis.

We found that inhibition of CCR2 by propagermanium treatment slightly suppressed body weight gain, mainly by reducing visceral fat mass and liver weight without affecting food intake in DIO mice. Fruebis *et al.* reported that exogenous administration of globular adiponectin induced weight loss by increasing lipid oxidation in the muscles of obese mice[32]. We previously demonstrated that propagermanium treatment increased the enzymes associated with lipid oxidation including carnithine palmitoyltransferase (CPT) and acyl-CoA oxidase (ACOX) in the muscle and liver of db/db mice[17]. In the present study, we confirmed that propagermanium increased the expression of ACOX and uncoupling protein 3 (UCP3), which contributes to energy expenditure[36, 37], in muscle (supplemental figure). Thus, an increase in plasma adiponectin, lipid oxidation, and energy expenditure in muscle by propagermanium treatment might suppress obesity in DIO mice. Further, we also detected a decreased plasma leptin level in propagermanium-treated DIO mice. Leptin is an adipocyte-derived hormone and cytokine that regulates energy balance, and increased circulating leptin, a marker of leptin resistance, is common in obesity in both experimental animals and humans[38]. Thus, amelioration of leptin resistance by propagrmanium treatment might also lead to the suppression of diet-induced obesity.

In summary, we have shown that blockade of CCR2 function ameliorated both insulin resistance and hepatic steatosis along with decreased pro-inflammatory macrophage infiltration into adipose tissue in diet-induced obese mice, which are more similar to human obesity than leptin-receptor deficient mice. Our results thus strongly suggest that CCR2 plays an important role in the pathogenesis of metabolic syndrome and propagermanium may be a beneficial drug for the treatment of metabolic syndrome in human.

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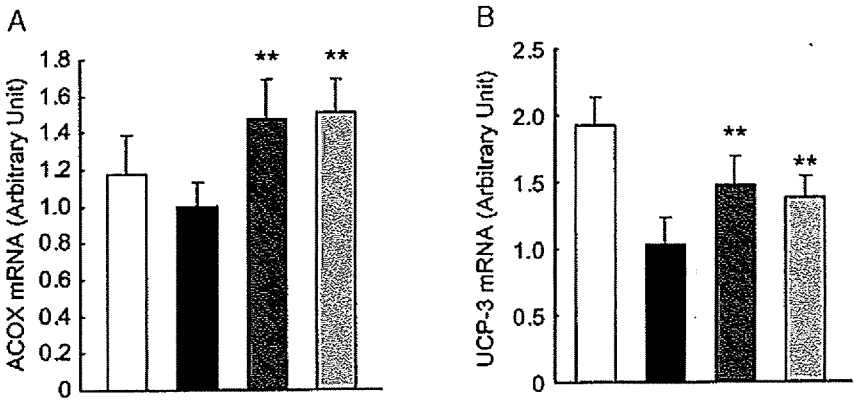


**Supplementary Fig.**

Expression of ACOx mRNA (A) and UCP3 mRNA (B) in muscle from C57BL6 mice fed a normal diet (white bar), HF/HS diet (black bar), or HF/HS diet with propagermanium treatment at doses of 5 mg/kg (dark gray bar) and 50 mg/kg (light gray bar) $^{*}p<0.05$; $^{**}p<0.01$; vs. HF/HS diet group ($n=10$ per group).

## Supplementary Method

### Quantitative Real-Time PCR

Total RNA was extracted from frozen muscle tissue (50 mg) using an RNeasy mini kit (Qiagen, Valencia, CA). The cDNA was synthesized from total RNA using Super Script III (Invitrogen). Real-time polymerase chain reaction was performed on an ABI PRISM 7700 using the SYBR GREEN polymerase chain reaction Master Mix (Applied Biosystems, Warrington, UK). Primer sets were as follows: acyl CoA oxidase forward: GCCAGGACTATCGCATGATT, reverse: GCCCAACTGTGACTTCCATC. The mRNA levels were normalized relative to the amount of β-actin mRNA and expressed in arbitrary units.

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# Transitional change in rat fetal cell proliferation in response to ghrelin and des-acyl ghrelin during the last stage of pregnancy

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ABSTRACT

Expression of mRNA for the ghrelin receptor, GHS-R1a, was detected in various peripheral and central tissues of fetal rats, including skin, bone, heart, liver, gut, brain and spinal cord, on embryonic day (ED)15 and ED17. However, its expression in skin, bone, heart and liver, but not in gut, brain and spinal cord, became relatively weak on ED19 and disappeared after birth (ND2). Ghrelin and des-acyl ghrelin facilitated the proliferation of cultured fetal (ED17, 19), but not neonatal (ND2), skin cells. On the other hand, with regard to cells from the spinal cord and hypothalamus, the proliferative effect of ghrelin continued after birth, whereas the effect of des-acyl ghrelin on neurogenesis in these tissues was lost at the ED19 fetal and ND2 neonatal stages. Immunohistochemistry revealed that the cells in the hypothalamus induced to proliferate by ghrelin at the ND2 stage were positive for nestin and glial fibrillary acidic protein. These results suggest that in the period immediately prior to, and after birth, rat fetal cells showing proliferation in response to ghrelin and des-acyl ghrelin are at a transitional stage characterized by alteration of the expression of GHS-R1a and an undefined des-acyl ghrelin receptor, their responsiveness varying among different tissues.



Ghrelin was first identified in 1999 in rat and human stomach as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R), through which ghrelin stimulates release of GH from the pituitary [1]. Two types of GHS-R, types 1a and 1b (GHS-R1a and 1b, respectively), have so far been characterized, and only the former is able to activate signal transduction of the receptor linking downstream to phospholipase C, resulting in an increase of intracellular calcium [2,3]. Ghrelin consists of 28 amino acids and is characterized by the presence of octanoic acid on the serine 3 residue, recognized by *O*-acyltransferase [4]. This octanoylation is essential for the binding and activation of GHS-R1a [4].

In addition to its GH-stimulating action, many physiological functions of ghrelin have been revealed during the last decade, such as regulation of food intake and energy metabolism, gastrointestinal coordination, facilitation of cell proliferation, survival and/or inhibition of apoptosis, regulation of the cardiovascular system, and the secretion of insulin and gut hormones [5–14]. We have previously demonstrated in rats that fetal growth is increased by treatment of the mother with exogenous ghrelin, and that the effect of ghrelin on fetal growth is diminished by immunization against ghrelin *in vivo* [15]. Ghrelin also stimulates the proliferation of cultured cells from fetal skin and spinal cord at E17 *in vitro* [15,16]. In addition, we have found that amniotic fluid contains a large quantity of des-acyl ghrelin, and that proliferation of cells from rat fetal skin and spinal cord at E17 is also stimulated by treatment with des-acyl ghrelin *in vitro* through a new receptor subtype with higher affinity for des-acyl ghrelin than for ghrelin [15,16]. Subsequently, in binding studies using $^{125}$I labeling des-acyl ghrelin, we clarified that des-acyl ghrelin has at least one binding site in the membrane fraction from fetal spinal cord [16]. These results suggest that ghrelin and des-acyl ghrelin can facilitate neurogenesis in the rat fetal spinal cord through both the GHS-R1a and also an unidentified GHS-R-independent alternative pathway. Although this specific receptor for des-acyl ghrelin has not yet been identified, there are some important related issues to be resolved. The proliferation of fetal tissues, including the spinal cord and skin, may be facilitated by maternally derived ghrelin and des-acyl ghrelin. If this is the case, after birth it is questionable whether the proliferation of neonatal tissues is also facilitated by these two molecules in the same way as that during fetal life. In the present study, therefore, we examined the expression of GHS-R1a mRNA in fetal and neonatal tissues, and compared the cell proliferation response to ghrelin and des-acyl ghrelin in fetal and neonatal skin, spinal cord and hypothalamus.

## Materials and methods

*Animals.* Wistar rats were housed under controlled temperature (23 ± 1 °C) and 12:12 h light–dark conditions (lights on at 0700 h). Female rats were mated on the day of proestrus at approximately 3

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months of age. The next estrus day was considered to be day 0 of pregnancy. Delivery usually occurred in our rat colony during the morning on day 21 of pregnancy. All procedures were performed in accordance with the guidelines for animal care stipulated by the Japanese Physiological Society.

*RT-PCR.* Total RNA was isolated from the various tissues, including skin, bone, lung, heart, liver, intestine, stomach, brain and spinal cord, of fetuses at embryonic day (ED) 15, 17 and 19, neonates at neonatal day (ND) 2, and also the pituitary on ED19 and ND2, using Trizol Reagent (Life Technologies, Inc., Gaithersburg, MD). Single-stranded DNA was generated from 1 μg of total RNA with the use of Superscript 3 preamplification reagent (Life Technologies, Inc., Bethesda, MD) in accordance with the manufacturer's instructions. PCR was carried out using a BD advantage™ 2 PCR Enzyme System (BD Science, CA). The PCR primers specific for GHS-R1a were 5′-GATACCTCTTTTCCAAGTCCTTC GAGCC-3′ for sense and 5′-TTGAACACTGCCACCCGGTACTTCT-3′ for antisense (nucleotides 842–869 and 1001–1025; accession No. AB001982, GenBank), and those specific for GAPDH were 5′-CGGCAAGTTC AACGGCACA-3′ for sense and 5′-AGACGCCAGTAGACTCCACGACA-3′ for antisense (nucleotides 1002–1020 and 1125–1147; Accession No. AF106860, GenBank).

*Primary culture of cells isolated from fetal and neonatal skin, spinal cord and hypothalamus.* Skin, spinal cord and hypothalamus were harvested from fetuses at ED17 and 19 and neonates at ND2. The uterus usually contained 10–14 embryos, 10 of which were utilized for primary culture. Dissociation was performed mechanically and enzymatically with a combination of collagenase and papain for skin, or papain only for spinal cord and hypothalamus. Dispersed skin cells were then suspended in MCDB153HAA medium (F-Peptide Co., Ltd., Yamagata, Japan) containing 1% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 5 ng/ml epidermal growth factor. For the BrdU experiments, skin cells were seeded in polyethylenimine-coated 96-well dishes at a density of $3 \times 10^4$/well. On the other hand, spinal cord and hypothalamic cells were suspended in DMEM with $NaHCO_3$, penicillin (100 U/ml) and 2% fetal calf serum, followed by plating onto L-ornithine- and laminin-coated 96-well plates at $1 \times 10^4$ cells per well for the BrdU experiments, and onto L-ornithine and laminin-coated 4-well culture slides at $3 \times 10^4$ cells per well for immunohistochemistry.

*Incorporation of BrdU into cultured cells.* Cell proliferation was measured by Cell Proliferation ELISA with BrdU (Roche Diagnostic GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions with some optimization for the present cell culture conditions as follows. Briefly, after incubation for 1 day, the cells were treated with ghrelin or des-acyl ghrelin at a final concentration of 0.003–0.3 or 3 nM for 24 h. Subsequently, BrdU was added to the cells to label newly synthesized DNA, followed by further incubation for 12 h. After incubation, the cells were fixed and denatured, and incubated with anti-BrdU antibody for 90 min. Each well was washed out three times and reacted with substrate solution until color development. The absorbance of the reaction was measured by an immunoreader. Comparisons between control and treated groups were made by ANOVA with the *post hoc* Fisher test. Differences at $P < 0.05$ were accepted as statistically significant.

*Immunohistochemistry.* Cultured cells from neonatal hypothalamus at ND2 on the poly-L-ornithine- and laminin-coated 4-well culture slides, which had incorporated BrdU after incubation for 1 day during the ELISA preparation procedure, were fixed with 4% paraformaldehyde in 0.1 M PBS for 30 min. After DNA denaturation with 2 M HCl and blocking with 2% normal goat serum in PBS

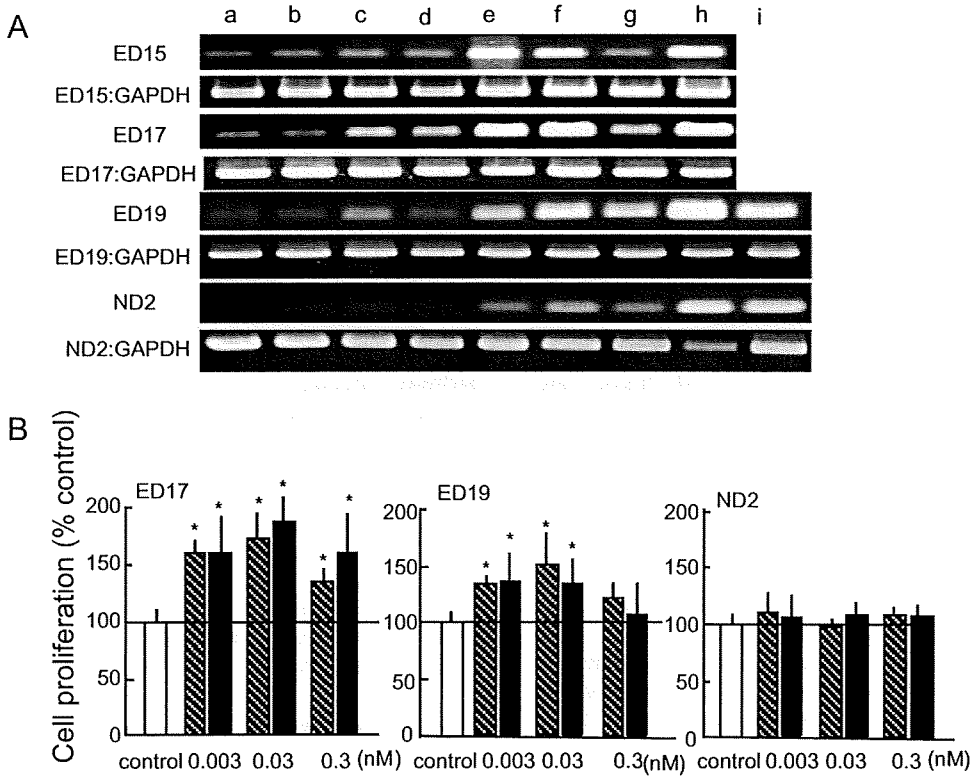


**Fig. 1.** (A) Detection of GHS-R1a mRNA in various tissues by RT-PCR. The PCR product amplified with primers specific for GHS-R1a was detected from embryonic day (ED) 15, 17 and 19 to neonatal day (ND) 2. GAPDH mRNA was also run as an internal control. Lane a, skin; b, bone; c, heart; d, liver; e, intestine; f, stomach; g, brain; h, spinal cord; i, pituitary. (B) Cell proliferation effects of ghrelin (slash bar) and des-acyl ghrelin (black bar) on cultured fetal (ED17, 19) and neonatal (ND2) skin cells. Proliferating cells were quantified by BrdU ELISA assay, and the data were expressed as values relative to the control. Values are presented as the mean + SEM ($n = 16$). Asterisks indicate significant differences vs. control ($P < 0.05$).

for 30 min at room temperature, the fixed cells were incubated overnight at 4 °C with either anti-mouse microtubule-associated protein 2 (MAP2; 1:2000, Chemicon International), anti-nestin (1:2000, Chemicon International) or anti-glial fibrillary acidic protein (GFAP; 1:2000, IMGENEX) as the primary antibody. The cells were then washed with PBS and incubated with Alexa Fluor 488-conjugated donkey anti-mouse IgG. When polyclonal rabbit anti-GHS-R was used as the primary antibody [15], the cells were incubated with Alexa Fluor 555-conjugated donkey anti-rabbit IgG as the secondary antibody. After the washing step, the cells were further incubated with rat anti-BrdU monoclonal antibody (1:500, Abcam, Cambridge, UK) as the primary antibody for double staining, followed by incubation with Cy™3-conjugated donkey anti-rat IgG polyclonal antibody (1:1000, Jackson Immuno Research Laboratories, Inc., PA) as the secondary antibody. For double staining of the GHS-R for either Map2, nestin or GFAP, cells fixed with 4% paraformaldehyde in 0.1 M phosphate buffer were first incubated with either mouse anti-Map2, anti-nestin or anti-GFAP primary antibody, and then with rabbit anti-GHS-R antibody.

## Results and discussion

Expression of GHS-R1a mRNA was detected in various fetal peripheral and central tissues including skin, bone, heart, liver, gut, brain and spinal cord on ED15 and ED17, with high intensity in the gut, brain and spinal cord (Fig. 1A), and was also recognized in the pituitary on ED19. These results were agreement with our previous study [15]. However, GHS-R1a mRNA expression in skin, bone, heart and liver became relatively weak on ED19 and disappeared after birth (ND2). In addition, we confirmed the disappearance of GHS-R1a mRNA expression in neonatal skin on ND2 and ND10 by quantitative analysis of mRNA (data not shown). In contrast, expression of GHS-R1a mRNA was continuously detected in gut, brain, spinal cord and pituitary on ND2 as well as in fetuses on ED17 and 19 (Fig. 1A).

The disappearance of GHS-R1a mRNA expression on ND2 may indicate that the effect of ghrelin on skin cell proliferation via GHS-R1a may be lost after birth. To investigate this possibility, we examined the incorporation of BrdU into cultured skin cells from fetuses at ED17 and 19 and neonates at ND2. Ghrelin facilitated the incorporation of BrdU in a dose-dependent manner in cultured skin cells prepared from fetuses at ED17 and 19 (Fig. 1B). On the other hand, no significant increase of BrdU incorporation was observed in cultured skin cells prepared from neonates at ND2 (Fig. 1B). These results supported the earlier prediction that the effect of ghrelin on skin cell proliferation via GHS-R1a is lost after birth. Also, as was the case with ghrelin, the effects of des-acyl ghrelin on cell proliferation differed between fetal and neonatal skin (Fig. 1B). Des-acyl ghrelin also did not increase the incorporation of BrdU into cultured skin cells prepared from ND2 neonates, unlike the situation for fetuses at ED17 and 19. In skin, bone, heart and liver, therefore, expression of the undefined des-acyl ghrelin receptor also might disappear after birth.

Next, we compared the effects of ghrelin and des-acyl ghrelin on neurogenesis using cultured cells prepared from spinal cord

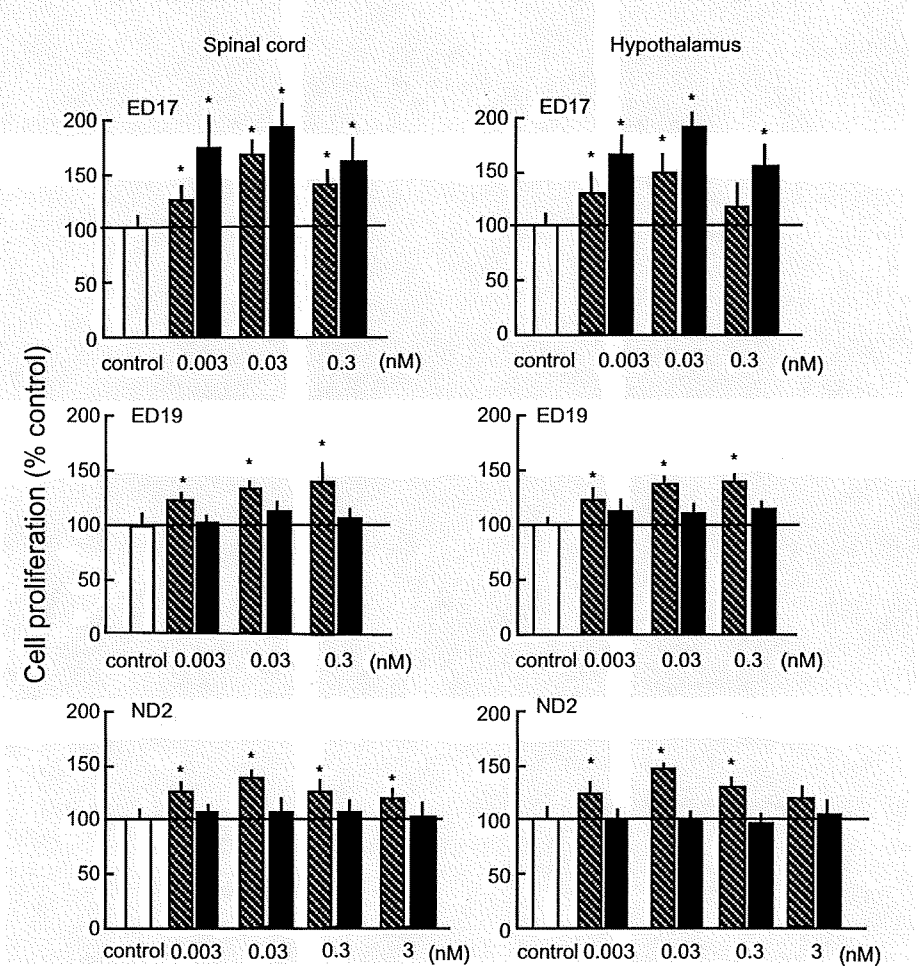


**Fig. 2.** Cell proliferation effects of ghrelin (slash bar) and des-acyl ghrelin (black bar) on cultured cells from fetal and neonatal spinal cord (left column) and hypothalamus (right column). Spinal cord and hypothalamus samples were collected from fetuses at embryonic day 17 and 19 (ED17, ED19) and neonates at the 2 days of age (ND2). Proliferative cells were quantified by BrdU ELISA assay, and the data were expressed as values relative to the control. Values are presented as the means + SEM ($n = 16$). Asterisks indicate significant differences vs. control ($P < 0.05$).

and hypothalamus on ED17 and 19 and ND2. The hypothalamus is well known to be the direct target of appetite stimulation by ghrelin and des-acyl ghrelin in adult rats [6,17]. Treatment with ghrelin facilitated the incorporation of BrdU into cultured cells of both spinal cord and hypothalamus on ED17 and 19 and on ND2 (Fig. 2). These continuously facilitating effects of ghrelin on proliferation of cells from neonatal spinal cord and hypothalamus are consistent with the expression of GHS-R1a mRNA in those tissues at the same stage. On the other hand, des-acyl ghrelin had no significant effect on these tissues at ED19 and ND2 (Fig. 2). Even when we increased the dose of des-acyl ghrelin to 3 nM, no facilitation of BrdU incorporation was observed (Fig. 2). In spinal cord and hypothalamus, therefore, although the expression of GHS-R1a appears to be maintained, the undefined des-acyl ghrelin receptor may disappear around the time of birth and thereafter. These results suggest that ghrelin, but not des-acyl ghrelin, plays an important role in neurogenesis in the spinal cord and hypothalamus of neonatal as well as fetal rats.

Immunofluorescence double staining of cultured cells treated with ghrelin that had incorporated BrdU into their DNA was performed to identify proliferating cells among cultured neonatal hypothalamic cells. Cells showing positivity for nestin and GFAP also had nuclear BrdU positivity, i.e. neuronal precursor cells and glial cells (mainly astrocyte), respectively (Fig. 3A). Cells with Map2 positivity showed a typical neuron-like shape with extended dendrites, and did not show nuclear BrdU positivity (data not shown). Immunofluorescence double staining with both anti-GHS-R and anti-nestin, anti-Map2 or anti-GFAP antibody showed that a proportion of nestin- and GFAP-positive cells expressed GHS-R immunoreactivity (Fig. 3B). These results indicated that ghrelin may play an important role in neurogenesis, acting on neuronal precursor cells and glial cells in neonatal hypothalamus.

Our present findings suggest that the fetal cell proliferation response to ghrelin and des-acyl ghrelin is a transitional one dependent on the type of tissue and developmental stage. Although the reason for the disappearance of the effect of ghrelin and des-acyl ghrelin on proliferation of skin or neuronal cells after birth is currently unclear, it may be associated with loss of their receptors, since disappearance or continuation of the proliferative effect of ghrelin on skin and spinal cord cells was consistent with changes

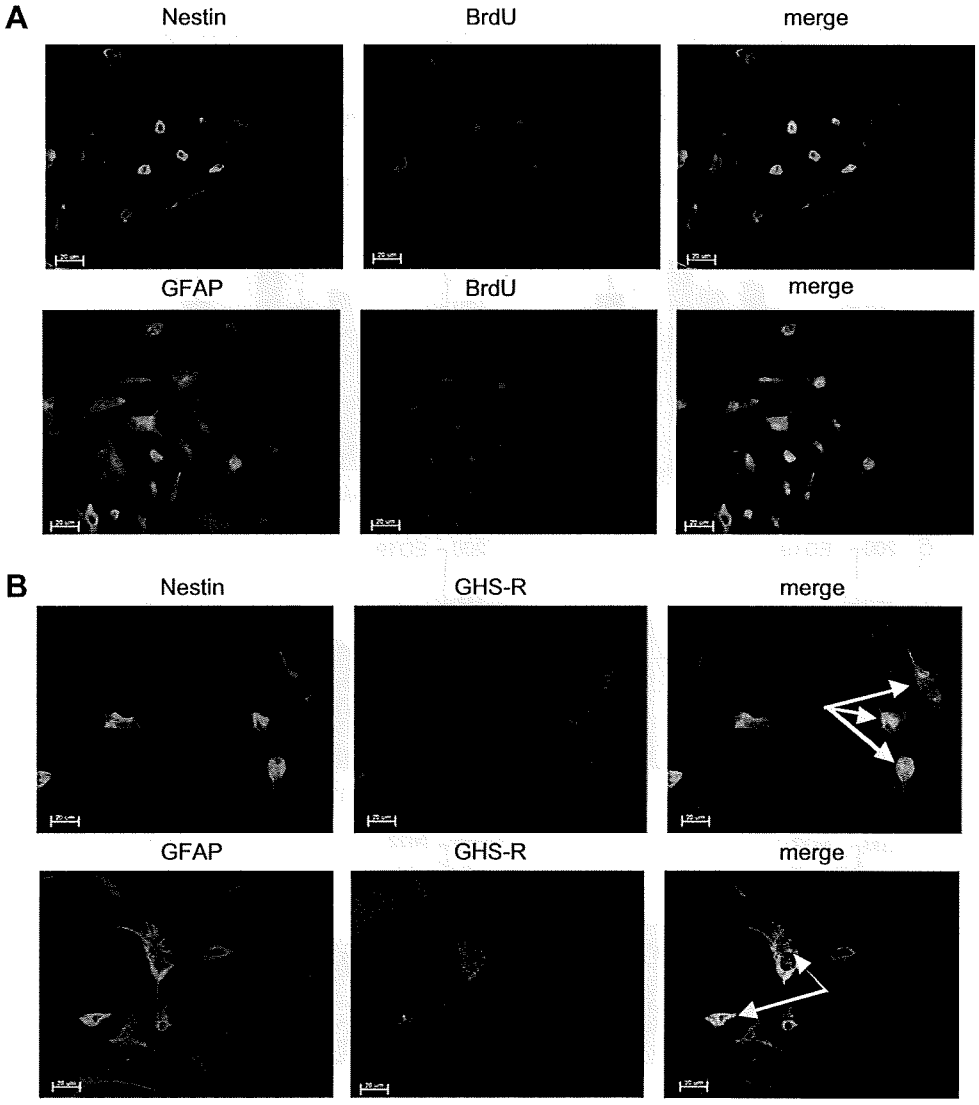


**Fig. 3.** Identification of proliferating cells in the rat neonatal hypothalamus. (A) Double immunofluorescence staining using anti-nestin or anti-GFAP antiserum together with anti-BrdU antiserum demonstrates co-localization of both nestin and BrdU or both GFAP and BrdU in the same cells. (B) Double immunofluorescence staining using anti-nestin or anti-GFAP antiserum together with anti-GHS-R antiserum demonstrates that GHS-R is localized in a proportion of cells positive for nestin or GFAP (indicated by arrow). Each scale bar is 20 μm.

in the expression of ghrelin receptor GHS-R1A mRNA in the same tissues. Recently, it has been shown that down-regulation or disappearance of hormonal receptor mRNA is involved in DNA methylation [18,19]. For example, leptin resistance (a decrease of leptin action) may be associated with DNA methylation involving the leptin receptor and/or anorexia hormone (α-melanocortin) receptor [20,21]. Therefore, DNA methylation of either GHS-R1A or some related transcriptional region might occur in skin cells close to the time of birth.

We have previously demonstrated that rat fetal growth was increased by treatment of the pregnant dams with exogenous ghrelin, and that the effect of ghrelin on fetal growth was diminished by immunization against ghrelin *in vivo* [15]. In addition, we have found that amniotic fluid contains a large quantity of des-acyl ghrelin, and that proliferation of cells from rat fetal skin is also stimulated by treatment with both ghrelin and des-acyl ghrelin *in vitro* [15]. On the basis of these findings, we speculate that both maternal ghrelin and des-acyl ghrelin play a crucial role in fetal growth by directly stimulating the proliferation of fetal cells. The disappearance of GHS-R1a mRNA coincides with the start of growth hormone (GH) secretion from the fetal pituitary [15]. Therefore it is likely that the growth of some peripheral tissues, such as skin, bone, heart, and liver, may become dependent on fetal pituitary GH, which is secreted in response to ghrelin and des-acyl ghrelin from the mother as parturition approaches.

Our previous binding studies using [$^{125}$I] des-acyl ghrelin indicated the presence of a specific binding site for des-acyl ghrelin in fetal spinal cord at ED17, and confirmed that the binding was displaced by unlabeled des-acyl ghrelin [16]. The present study showed that des-acyl ghrelin, but not ghrelin, did not increase BrdU incorporation into cultured cells from spinal cord and hypothalamus at ED19 and ND2, suggesting disappearance of the undefined des-acyl ghrelin receptor after birth. In adult rats, however, des-acyl ghrelin stimulates appetite by acting on the lateral hypothalamus and stimulates peristaltic motion of the large bowel by acting on the spinal cord [17,22]. These observations indicate that the undefined des-acyl ghrelin receptor may be present in the spinal cord and hypothalamus of adult rats. Therefore, it is likely that although the undefined des-acyl ghrelin receptor exists only in neuronal precursor cells of the fetal spinal cord and hypothalamus up to ED17, it may be expressed in neurons, but not neuronal precursor cells, of the adult spinal cord and hypothalamus.

In the present study, on the other hand, ghrelin continuously facilitated cell proliferation in the spinal cord and hypothalamus of ND2 neonates, suggesting that ghrelin may still play an important role in neurogenesis after birth. This possibility is supported by another study indicating that ghrelin promoted the proliferation of neural cells isolated from the hippocampus of adult rats [23]. An increase of BrdU incorporation in response to ghrelin was observed in not only neuronal progenitor cells but also glial cells (mainly astrocyte) of the hypothalamus. As astrocyte is well known to play many important roles in neuronal differentiation, neurogenesis, storage and release of glutamic acid, and nutrients support [24–26], stimulation of astrocyte proliferation by ghrelin may be significantly important. Although GHS-R-knockout mouse fetuses show no evidence of any abnormality [27], the proliferative effect of ghrelin may be compensated by the actions of growth factors such as nerve growth factor.

In summary, we have demonstrated that the ghrelin receptor GHS-R1a or the undefined des-acyl ghrelin receptor disappear in some tissues, such as skin, close to and just after birth, accompanied by disappearance of the effects of ghrelin and des-acyl ghrelin on cell proliferation. On the other hand, facilitation of neurogenesis in the spinal cord or hypothalamus by ghrelin, but not by des-acyl ghrelin, continues after birth. These results suggest that the fetal cell proliferation response to ghrelin and des-acyl ghrelin is transitional, and occurs through alterations in the expression of GHS-R 1a and the undefined des-acyl ghrelin receptor, depending on the tissue and the stage of development.

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# A survey of attitudes toward clinical research among physicians at Kyoto University Hospital

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## Abstract

**Background:** In Japan, only clinical research related to investigational new drug trials must be notified to regulatory bodies, and this lack of a uniform standard for clinical research has caused a number of difficulties. The objective of this study was to assess the willingness of physicians to participate in clinical research and to identify effective methods to promote and enhance clinical research.

**Methods:** We conducted a cross-sectional survey by administrating questionnaires to physicians in 31 departments in Kyoto University Hospital from October through November 2007.

**Results:** A total of 51.5% (310 of 602) of physicians completed the questionnaire. More than two-thirds of them reported currently participating in clinical research, and nearly all believed that clinical research is necessary for physicians. Less than 20% of respondents had specific training regarding clinical research, and most reported a need to acquire concepts and skills regarding clinical research, especially those related to statistics. "Paperwork was complicated and onerous" was the most frequently cited obstacle in conducting clinical research, followed by "few eligible patients" and "lack of time". Previous participation in and prospective participation in clinical research, previous writing a research protocol were positively associated with current participation in clinical research.

**Conclusions:** Physicians in university hospitals need more training regarding clinical research, particularly in biostatistics. They also require administrative assistance. Our findings indicate that the quality of clinical research could be improved if training in clinical research methodology and biostatistics were provided, and if greater assistance in the preparation of study documents requested by the institutional Independent Ethics Committee were available.

## Background

Good Clinical Practice (GCP) should be used for designing, conducting, recording, and reporting trials that involve the participation of human subjects[1]. This guideline should be followed when generating clinical trial data that are intended to be submitted to regulatory authorities. In the United States, many research sites conduct clinical trials in compliance with GCP standards [2], and the European Clinical Trial Directive made GCP mandatory for all clinical drug trials [3].

In Japan, clinical trials of new drugs can be classified into two categories: investigational new drug (IND) application trials, and studies that do not seek marketing approval (non-notified trials). The former are strictly regulated by the Pharmaceutical Affairs Law[4] and by the Ministry of Health, Labour and Welfare (MHLW) Ordinance on GCP[5], which was adopted in Japan in 1997 and is based on the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) E6 Guidelines[1]. In striking contrast to other countries, Japanese researchers can conduct clinical trials without notifying or applying to the authorities, unless they require new drug approval (NDA). In fact, there is little legal regulation of non-notified trials in Japan. The only guidance provided by the MHLW is Ethical Guidelines for Clinical Studies [6], which was published in 2003 and has no legal implications. The main difficulty in conducting non-notified trials is that the policies of Japanese ethics committees vary by medical institution or hospital. Thus, a trial that is disallowed by one institution might be approved by another, perhaps without sufficient discussion of its ethical or scientific implications.

Before 2003, applications for IND trials were only submitted by the company responsible for manufacturing and marketing the drug. After revision of the Pharmaceutical Affairs Law, an investigator can now initiate and notify the relevant authorities of an IND trial, which involves strict observance of GCP. As of 2007, the number of investigator-initiated IND trials has been very small, and notified by certain university hospitals, including Kyoto University Hospital (KUH), and the Japan Medical Association. However, many non-notified trials, undertaken in observance of the Ethical Guidelines, have been conducted by KUH and other hospitals.

KUH is one of the seven largest university hospitals in Japan, and a total of 348 faculty, 176 senior residents, and 125 junior residents were employed there as of October 2007. Since 2007, it has been one of seven distinguished research sites chosen by the Ministry of Education, Culture, Sports, Science and Technology to participate in the Coordinating Support and Training Program for Translational Research, which seeks to promote quality in clinical research.

The objective of the present study was to investigate both the willingness of physicians to participate in clinical research and their attitudes toward such research. In addition, we aimed to identify methods of support and training that might assist physicians in conducting research. We aimed that our findings may foster future academic clinical research both at KUH and other university hospitals in Japan.

## Methods

### *Respondents and survey administration*

From October through November 2007 we conducted a cross-sectional survey of 31 departments in KUH. We initially contacted the directors of each of the 34 departments in KUH to explain the study and to ask for their participation in the study. Thirty-one departments consented to participate. The person in charge of each department distributed the study description and questionnaire by hand or by mail to the physicians belonging to the department, and later collected them. Residents, faculty, and doctoral students (physicians) with medical degree were invited to participate. It was not necessary to obtain ethical approval for this survey, as this survey was out of jurisdiction of Ethical Guidelines for Epidemiological Research[7], which shall be applied to studies on etiology of human disease and diagnostic or therapeutic procedures.

### *Questionnaire*

An initial questionnaire was prepared to gain a better understanding of the current state of clinical research and to guide development of activities at the Translational Research Center, Kyoto University Graduate School of Medicine. To prepare the questionnaire, we modified and added questions to a questionnaire from a similar study conducted in Tokushima University Hospital [8].

The questionnaire inquired about demographic data--including age range and employment status--and attitudes regarding clinical research, clinical research training, and submission of articles on clinical research. Since ICH E6 guidelines for GCP, an international standard for research ethics, is based on and consistent with the principles of the Declaration of Helsinki, we queried the respondents' knowledge of the World Medical Association Declaration of Helsinki [9].

Using multiple-choice questions, respondents were asked about (1) the benefits of conducting clinical research and desired lecture topics on clinical research; (2) the difficulties of conducting research, among physicians who had participated in such research; and (3) the content of reviewers' comments, among physicians who had submitted a clinical research article. The questionnaire was anonymous, and included a separate form to state freely their name or additional opinions for those physicians who wished to collaborate on any further research project.

### *Statistical Analysis*

Descriptive analysis was used to examine respondents' perception of the benefits and difficulties of clinical research. Answers to multiple-choice questions were summed and listed in order of frequency. The chi-square test was used to compare the age range and the proportion

of physicians employed by internal medicine departments (ie, internal medicine, pediatrics, psychiatry and radiology) among respondents with those among both nonrespondents and national physicians.

Bivariate analyses were performed to identify factors that might be associated with current participation in clinical research. We used chi-square tests for categorical variables and *t* test for continuous variables. The continuous variables in this dataset were age range (decade) and knowledge of Helsinki. Correlation analyses were performed to test for multicollinearity between 5 sets of factors we hypothesized might be highly correlated (age range and status, past participation in clinical research and past submission for publication of a manuscript on clinical research, past participation in clinical research and past writing of a research protocol, past participation in clinical research and prospective participation in clinical research, and past submission for publication of a manuscript on clinical research and past writing of a research protocol). Decisions to include factors in the multiple logistic regression analysis were based on the strength of correlated factors ($r < 0.75$) or a $P$ value < .05 on bivariate analyses. We performed multiple logistic regression analysis to identify factors that were correlated with participation in clinical research.

A $P$ value of less than 0.05 was considered to be statistically significant. Analysis was performed using STAT View (SAS Institute Inc, Cary, NC).

## Results

### *Characteristics of respondents*

Among 602 physicians from the 31 departments who received the questionnaire, a total of 51.5% (310 of 602) completed the questionnaire. A total of 175 faculty and 58 residents responded; 173 faculty and 243 residents did not respond ($P < 0.001$). As to age range, 47.8% of nonrespondents were aged 20 to 29, 16.8% of nonrespondents were aged 30 to 39, and 24.2% of nonrespondents were 40 to 49. Table 1 provided age range of respondents. There were statistically significant difference between respondents and nonrespondents on age range ($P < 0.001$). The survey respondents were not representative of all physicians at KUH: Faculty was more likely to complete survey than were residents, possibly because many junior residents did not receive the questionnaire. As junior residents rotate through various specialties, some of the person in charge of each department hesitated to distribute the questionnaire to junior residents. A total of 96 faculty and residents employed in internal medicine departments responded to the questionnaire, and 137 faculty and residents in surgical or other departments responded to the questionnaire. There were 164 nonrespondents in internal medicine departments and 252 nonrespondents in surgical or other departments ($P$ =

**Table 1: Characteristics of the 304 respondents**

| Characteristic | Percent* | | |
|---|---|---|---|
| | resident or doctoral student (n = 129) | faculty (n = 175) | total (n = 304) |
| Age range | | | |
| <= 29 | 15.5 | 0.6 | 6.9 |
| 30-39 | 82.2 | 31.4 | 53.0 |
| 40-49 | 2.3 | 53.1 | 31.6 |
| >= 50 | 0.0 | 14.9 | 8.6 |
| Internal medicine departments | 50.4 | 36.0 | 42.1 |
| Current participation in clinical research | 48.8 | 82.3 | 68.1 |
| Past participation in clinical research | 53.5 | 89.1 | 74.0 |
| Prospective participation in clinical research | 61.2 | 89.7 | 77.6 |
| Previous training course in clinical research | 11.6 | 18.9 | 15.8 |
| Do you consider it is necessary for physicians to conduct clinical research? | | | |
| yes | 96.1 | 97.1 | 96.7 |
| Have you ever written research protocol? | | | |
| yes | 14.0 | 51.4 | 35.5 |
| Have you submitted for publication of a manuscript on clinical research? | | | |
| yes | 24.8 | 50.9 | 39.8 |
| Do you know "World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects"? | | | |
| I know very well | 10.9 | 28.0 | 20.7 |
| I know to some degree | 81.4 | 68.0 | 73.7 |
| I don't know | 5.4 | 2.9 | 3.9 |

* Percent values were expressed as ratio in respondents of each age range. Percentage may not total 100% due to missing or blank data.

0.657 vs respondents). In comparison, there were 77358 physicians in internal medicine departments and 90969 physicians in surgical or other departments in hospitals in Japan in December 2006 [10] ($P = 0.146$ vs respondents). Respondents did not differ from nonrespondents and national physicians in the proportion of physicians who belonged to internal medicine departments.

Table 1 lists the respondents' characteristics by status: resident or doctoral student vs faculty. Six respondents with other status or with blank data for status were deleted. Among respondents, 68% of physicians reported current participation in clinical research; 74% reported past participation in clinical research. More faculty than resident or doctoral student reported past participation in, current participation in and prospective participation in clinical research. Most physicians (97%) believed that it is necessary for physicians to conduct clinical research. More than half of faculty had written a research protocol and reported submitting for publication of a manuscript on clinical research, whereas 14% of counterpart had written a research protocol and 25% of counterpart reported submitting for publication of a manuscript on clinical research. However, only 16% had taken a training course in clinical research offered by either the Japan Clinical Oncology Group (9), Kyoto University Graduate School of Medicine (9), other domestic universities and scientific societies (9), or foreign institutions (2). Most physicians (94%) were aware of the World Medical Association Declaration of Helsinki; 4% were not.

### *Attitudes*

Respondents were queried regarding the benefits of conducting clinical research. Obtaining a better understanding of disease was the most frequently cited benefit, and was mentioned by 255 physicians (47.3%). Enhanced standing in society or the hospital was the second most frequently cited benefit, and was mentioned by 150 physicians (27.8%), followed by obtaining research grants or awards. Eleven respondents (2.0%) felt that there was no benefit (Table 2).

**Table 2: Attitude towards clinical research**

| Question | Percent*(%) |
|---|---|
| What benefits do you think are brought to physicians of conducting clinical research? | |
| Physicians can obtain a better understanding of disease | 47.3 |
| Physicians will enhance standing in society or in hospital | 27.8 |
| Physicians will obtain research grants or awards | 12.8 |
| Physicians will obtain credits to be board certified doctor | 4.1 |
| There is no benefit to physicians | 2.0 |
| Which lecture topics related to clinical research are interesting or useful? | |
| Statistical analysis | 25.3 |
| How to write a protocol | 20.7 |
| Paperwork and procedures† | 13.2 |
| Cost management for clinical research | 12.7 |
| Informed consent form to patients | 10.5 |
| Compensation | 9.2 |
| Medical ethics | 8.0 |
| What were the criticisms of reviewers when you submitted for publication a manuscript on clinical research ? | |
| Statistical analysis | 36.9 |
| Selection of patients | 21.0 |
| Aim or meaning of research | 19.1 |
| Definition of the technical terms | 10.2 |
| Ethical problems | 5.7 |
| What difficulties did you meet of conducting clinical research? | |
| The paperwork was complicated and onerous | 26.2 |
| Eligible patients were very few | 18.9 |
| Lack time | 17.6 |
| Too many examinations were scheduled | 11.5 |
| There was no benefit to patients | 8.6 |
| I could not continue clinical research because of transfer of physicians | 6.3 |
| Patients missed appointments | 5.4 |
| Patients didn't consent to take placebo | 3.6 |
| Doctor-patient relationships were damaged by offering clinical research | 0.8 |

* Percent values were expressed as ratio in total answers. Percentage may not total 100% due to missing or blank data.
†Paperwork and procedures mean production and management of study documents regarding submission to institutional review board and completion of case report form.

Most physicians (93.2%) wanted to attend lectures or seminars on one or more topics related to clinical research. The most frequently cited desired lecture topics were statistical analysis, how to write a protocol, paperwork and procedures (production and management of study documents regarding submission to institutional review board and completion of case report form), and cost management in clinical research (Table 2).

Respondents who had submitted research papers for publication were asked to indicate the criticisms of reviewers. Statistical analysis was the most frequent reviewer criticism, followed by selection of patients, aim or meaning of research, and definition of technical terms (Table 2).

Regarding the difficulties of conducting clinical research, respondents indicated that the "paperwork was complicated and onerous", that there were "few eligible patients", and that the respondents "lack time" (Table 2).

### *Factors associated with current participation in clinical research*

Age range had moderate correlation with status (r = 0.635), as did past participation in clinical research with prospective participation in clinical research (r = 0.505). Past participation in clinical research had some correlation with past submission for publication of a manuscript on clinical research (r = 0.413), as did past submission for publication of a manuscript on clinical research with past writing of a research protocol (r = 0.311) and past participation in clinical research with past writing of a research protocol (r = 0.282).

In bivariate analyses, current participation had statistically significant correlation with status, age range, past participation in clinical research, prospective participation in clinical research, past submission for publication a manuscript on clinical research, training course in clinical research, past writing a research protocol and knowledge of the World medical Association Declaration of Helsinki. A multivariable logistic regression model was developed including all these correlated factors as variables. Current participation was positively associated with past participation in, prospective participation in clinical research and past writing of a research protocol (Table 3). Age range of 30-39 was negatively associated with current participation in clinical research: Respondents aged 30 to 39 were less than quarter (odds ratio, 0.24; 95% confidence interval,

**Table 3: Effect of status, age range, and attitudes to current participation in clinical research**

| Characteristic | Odds ratio (95% CI) | *P* value* |
|---|---|---|
| Status | | |
| resident or doctoral student | reference | |
| faculty | 1.416(0.568-3.531) | 0.4554 |
| Age range, y | | |
| <=29 | reference | |
| 30-39 | 0.240(0.064-0.907) | 0.0353 |
| 40-49 | 0.354(0.069-1.822) | 0.2142 |
| >=50 | 0.218(0.028-1.684) | 0.1442 |
| Past participation in clinical research | | |
| yes | 5.680(2.40-13.441) | < 0.0001 |
| no | reference | |
| Prospective participation in clinical research | | |
| yes | 5.756(2.508-13.212) | < 0.0001 |
| no | reference | |
| Previous training course in clinical research | | |
| yes | 2.081(0.678-6.389) | 0.2002 |
| no | reference | |
| Previous writing of a research protocol | | |
| yes | 2.631(1.130-6.125) | 0.0249 |
| no | reference | |
| Previous submission for publication of a manuscript on clinical research | | |
| yes | 1.798(0.815-3.967) | 0.1464 |
| no | reference | |
| Do you know "WORLD MEDICAL ASSOCIATION DECLARATION OF HELSINKI Ethical Principles for Medical Research Involving Human Subjects"? | | |
| I know very well | 4.219(0.561-31.728) | 0.1619 |
| I know to some degree | 2.457(0.413-14.623) | 0.3233 |
| I don't know | reference | |

* P < 0.05 is considered statistically significant.
The R2 value was 0.378. CI, confidence intervals