The next estrus day was considered to be d 0 of pregnancy. As reported previously, delivery usually occurs in our rat colony during the morning on d 21 of pregnancy (23). The average number (±SEM) of pups per mother at delivery was 13.10 ± 1.78 (n = 122). All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

RT-PCR for GHS-R 1a mRNA

Total RNA was extracted from fetal tissues on d 14, 15, and 19 of pregnancy using Trizol reagent (Invitrogen, Carlsbad, CA) as described previously (24). First-strand cDNA was synthesized from 2 μg of total RNA by random primer RT. The resulting cDNA was subjected to PCR amplification using sense and antisense primers specific for GHS-R1a (24). PCR products were electrophoresed on a 2% agarose gel. GAPDH was used as a control housekeeping gene.

Autoradiography for [^{125}I]acyl ghrelin

Fetuses [embryonic d 17 (E17)] were embedded in Tissue-Tec OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and frozen. Sections cut using a cryostat were mounted on gelatin-coated glass slides. Autoradiography was performed as described previously (14) with the following minor modifications. After preincubation for 30 min in incubation buffer at room temperature, sections were incubated for 12 h at 4 C in buffer containing 20 ng/ml rat [^{125}I-Tyr^{29}]acylated rat ghrelin. Nonspecific binding was determined in the presence of excess unlabeled acyl or des-acyl rat ghrelin (10 μg/ml). Sections were then exposed to an IP plate (Fuji Film, Tokyo, Japan) for 12 h and analyzed on BAS-5000 (Fuji Film).

Preparation of anti-GHS-R serum

The [Cys^{0}]-rat GHS-R [342–364] peptide was synthesized using the Fmoc solid-phase method on a peptide synthesizer (433A; Applied Biosystems, Foster City, CA), then purified by reverse phase-HPLC. The synthesized peptide (10 mg) was conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (6 mg) (mcKLH; Pierce, Rockford, IL) in conjugation buffer (Pierce). The conjugate was emulsified with an equal volume of Freund's complete adjuvant and was used to immunize New Zealand white rabbits by intracutaneous and sc injection. Animals were boosted every 2 wk and bled 7 d after each injection. The specificity of the antisera was confirmed by the immunoreactivity of GHS-R-expressing cells (CHO-GHSR62 cells) and lack thereof in control cells.

Immunohistochemistry for GHS-R

Immunohistochemical analyses for GHS-R were performed on frozen fetuses (E17 and 19) using a modification of a method that has been described previously (25). The fetuses were placed in fixative for 5 d at 4 C and then transferred to 0.1 M phosphate buffer containing 20% sucrose. They were cut into serial, 12-μm-thick sections at −20 C with a cryostat. The sections were incubated for 2 d with a rabbit-anti-GHS-R antibody at 4 C. Slides were then incubated with Alexa-546-labeled goat-antirabbit IgG antibody (Molecular Probes, Inc., Eugene, OR; dilution 1:400). Samples were observed with the aid of an Olympus AX-70 fluorescence microscope (Olympus, Tokyo, Japan). To examine the specificity of GHS-R antibody in tissue sections, the reaction was also performed using GHS-R antibody that had been preabsorbed with excess synthetic GHS-R (10 μg).

Measurement of acyl and des-acyl ghrelin, IGF-I, and corticosterone

Levels of acyl or des-acyl ghrelin were measured by specific ELISA kits for acyl or des-acyl ghrelin (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan). The ELISA can detect each acyl or des-acyl ghrelin using two specific antibodies recognizing only acyl ghrelin (octanoylation modification at [Ser^{3}]-ghrelin [1–11]) or only des-acyl ghrelin (nonoctanoylation modification at [Ser^{3}]-ghrelin [1–11]). Blood collected from pregnant rats and their fetuses was immediately put into chilled polypropylene tubes containing a protease inhibitor, aprotinin (Sigma-Aldrich, St. Louis, MO), and 2Na-EDTA and then centrifuged. We then added a 10% plasma volume of 0.1 N HCl. Maternal blood was taken at 0830 h (satiety phase) at 2-d intervals from d 11–21. Fetal blood and amniotic fluid were collected on d 17, 19, and 21.

To examine the transit of maternal acyl ghrelin to the fetal circulation, acyl ghrelin (0.2 and 20 nmol) or saline was injected into pregnant rats iv under light ether anesthesia on d 19 of pregnancy (n = 12 per group). Blood was then collected from both the mother and fetus at 5, 10, and 30 min after injection.

To determine the effect of maternal treatment with acyl ghrelin on plasma IGF-I and corticosterone levels in the fetal circulation, fetal plasma IGF-I and corticosterone levels were measured by enzyme immunoassay kit (Funakoshi, Tokyo, Japan) and [^{125}I]corticosterone RIA kit (ICN Biomedicals, Costa Mesa, CA), respectively. The limit of assay sensitivity was 5 ng/ml for IGF-I and 20 ng/ml for corticosterone. The intra and interassay coefficients of variation were 5 and 16%, respectively, for IGF-I, and 6 and 12%, respectively, for corticosterone.

Ghrelin administration and neonatal body weights

We sc injected either saline, acyl ghrelin (1.5 or 3.0 nmol), or des-acyl ghrelin (3.0 nmol) three times a day (at 0830, 1330, and 1830 h) from d 14 to delivery, or continuously infused vehicle, acyl ghrelin (0.125 or 0.5 nmol/h) or des-acyl ghrelin (0.5 nmol/h) through an osmotic minipump implanted sc from d 15 until delivery (n = 10 per group) (11, 26). We also injected 3 nmol acyl ghrelin three times a day from d 14 to delivery into pair-fed pregnant rats and the effect was compared with saline-treated pregnant rats. Neonatal body weights were measured on the day of delivery. If the pups numbered more than 15 or less than 11 they were excluded from the analyses.

Passive immunization for acyl ghrelin

Rat acyl ghrelin (3 mg) was conjugated to a carrier protein, mcKLH (3 mg), in conjugation buffer (Pierce) (7). Each conjugate was emulsified with an equal volume of Freund's adjuvant. Immunization, initiated by intradermal injection in 44-d-old female rats, was repeated six times at 2-wk intervals. As a control antigen, carrier protein alone without ghrelin was administered. Rats were mated on d 114 after the fifth immunization. The antibody titers were verified in diluted plasma every 10 d after immunization using [^{125}I]ghrelin binding capacity.

Quantitative RT-PCR of GH mRNA in fetal pituitary

The pituitary gland and blood were collected from E19 and E20 fetuses, isolated from the mothers' implanted osmotic minipump (acyl ghrelin 0.5 nmol/h and saline). GH mRNA expression was measured by real-time quantitative PCR as described previously (25). Experiments contrasted the relative levels of both GH and GAPDH transcripts in every sample. The total RNA from each tissue was extracted using an RNeasy Micro kit (Qiagen, Valencia, CA) and synthesized into first-strand cDNA using an iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). An aliquot of the first-strand cDNA (40–100 ng tissue equivalent) was quantified on an iCycler (Bio-Rad Laboratory) using iQ SYBR Green Supermix (Bio-Rad Laboratory) with primers to amplify GAPDH (25) and GH specifically (26).

Incorporation of [^{3}H]thymidine or 5-bromo-2′-deoxyuridine (BrdU) into cultured cells

We assessed the effect of acyl and des-acyl ghrelin administration on the proliferation of fetal skin cells by measuring the incorporation of [^{3}H]thymidine (2 μCi/ml) or BrdU (10 μM). Dispersed fetal skin cells were prepared from E17 fetuses by sequential collagenase treatment, papain digestion, and mechanical desegregation. Dispersed cells were then suspended in MCDB15^{3}HAA medium (F-Peptide Co., Ltd., Yamagata, Japan) containing 2% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 5 ng/ml epidermal growth factor. Cells were seeded in polyethylenimine-coated 48- and 96-well dishes at densities of 5×10^5 per well and 3×10^4 per well for the [^{3}H]thymidine and BrdU experiments, respectively. BrdU was detected using a Cell Proliferation ELISA Kit (Roche Diagnostic GmbH, Mannheim, Germany), as reported by Kusunoki *et al.* (27).

Statistics

Values are given as means ± SEM. Comparisons between two groups were made by ANOVA with the *post hoc* Fisher test. Differences at $P < 0.05$ were accepted as statistically significant.

Results

Expression of GHS-Rs in fetal tissue

GHS-R1a mRNA expression was detected in various fetal tissues with a high density in the spinal cord from E14 until birth (Fig. 1A). GHS-R mRNA expression in the fetal pituitary was also detected at E19. To confirm the expression of GHS-R in fetal tissues at the protein level, we performed immunohistochemistry on E17 fetuses using an antibody specific for GHS-R. Positive cells were distributed extensively in fetal tissues; the skin, bone, intestine, tongue, and muscle being stained particularly strongly (Fig. 1, C–G). Immunoreactivity was not detected in sections that were incubated with GHS-R antiserum that had been preabsorbed with excess synthetic GHS-R (Fig. 1, D-2 and F-2). Although RT-PCR analysis demonstrated the expression of GHS-R mRNA in sections of the brain, pituitary, stomach, and lung, only relatively weak staining was observed in these organs.

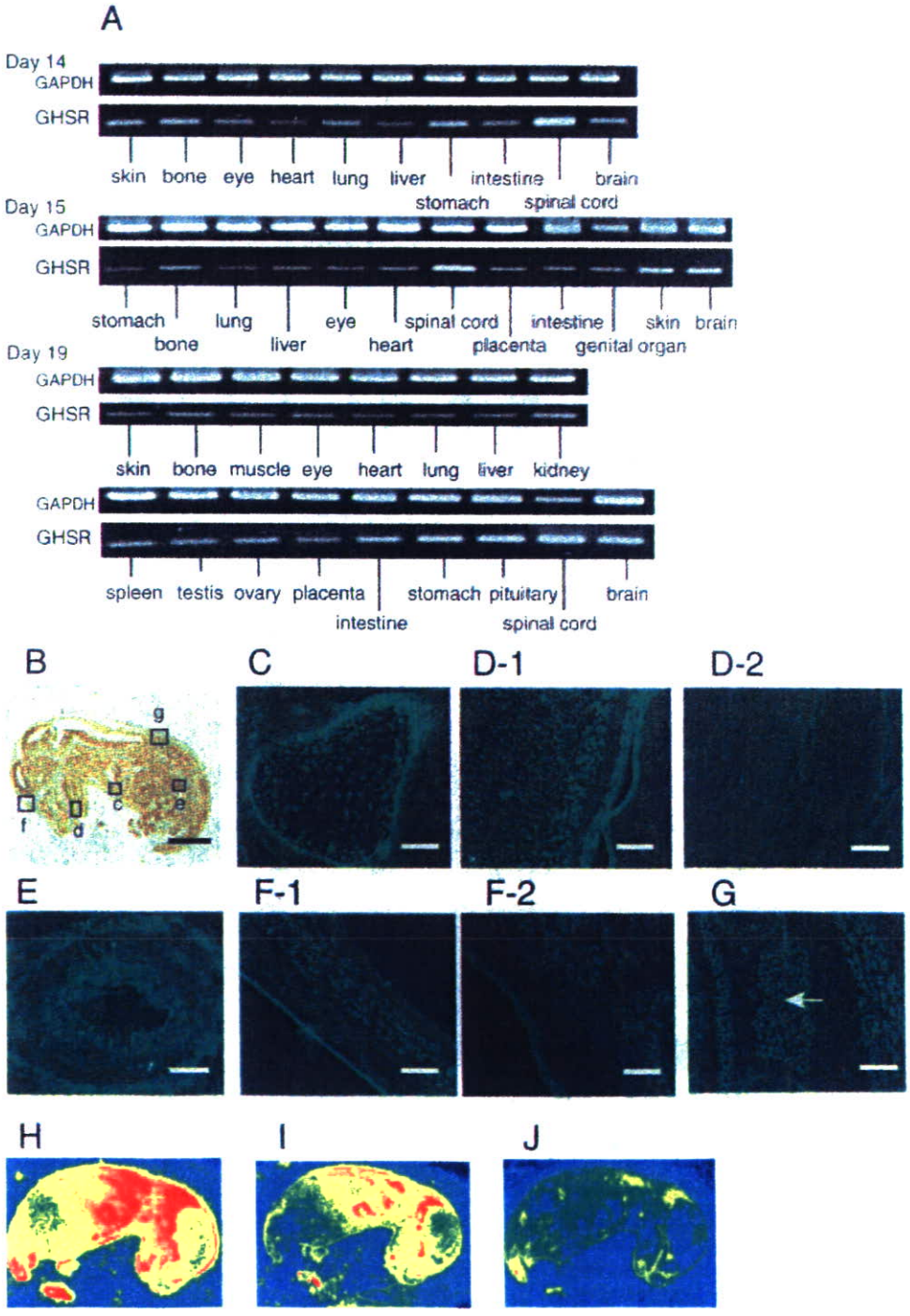

FIG. 1. A, GHS-R 1a mRNA expression in various fetal tissues. Fetal (E14, 15, 19) tissue cDNA fragments were amplified by PCR in the presence of oligonucleotide primers specific for GHS-R 1a. B, Sagittal section of E17 fetuses immunostained for GHS-R by avidin-biotin-peroxidase complexes (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA) using a diaminobenzidine substrate kit (Vector Laboratories). C–G, Immunofluorescence staining for GHS-R in fetal ribs (C), tongue (D), intestine (E), skin (F), and the muscle between the two transverse processes of a thoracic vertebra (G). D-2 and F-2 represent the immunostaining using absorbed antiserum with excess of synthetic GHS-R. *Bar* scales are: 3.7 mm (B); 75 μm (C); 65 μm (D); 75 μm (E); 100 μm (F); 75 μm (G). H–J, [^{125}I]acyl ghrelin autoradiograph of a sagittally sectioned fetus (E17) (H). Replacement was examined in the presence of excess unlabeled acyl (I) or des-acyl (J) ghrelin. The *red color* indicates the highest binding state with [^{125}I]acyl ghrelin (high binding order: *red* > *yellow* > *green* > *blue colors*).

[^{125}I]Acyl ghrelin autoradiography revealed dense binding to bone, skin, heart, and tongue (Fig. 1H); similar to the immunohistochemistry, the brain and digestive tract bound the isotope only weakly. In addition, excess unlabeled acyl ghrelin (Fig. 1I) and des-acyl ghrelin (Fig. 1J) could displace with [^{125}I]acyl ghrelin binding. More potent replacement was observed in excess unlabeled des-acyl ghrelin treatment (Fig. 1J).

Circulating ghrelin levels during late pregnancy

We measured the circulating levels of acyl and des-acyl ghrelin in pregnant rats and their fetuses, respectively. The levels of acyl ghrelin in maternal plasma exhibited a gradual but not significant decline in late pregnancy (Fig. 2A). In contrast, des-acyl ghrelin increased significantly during late pregnancy (Fig. 2A). Both ghrelin forms, acyl and des-acyl ghrelin, were also present in the fetal circulation; these levels decreased gradually as the time for delivery approached (Fig. 2B). We noticed a significant difference in des-acyl ghrelin levels when compared between the maternal and fetal plasma: the fetal levels of des-acyl ghrelin were 5- to 10-fold higher than the maternal levels (Fig. 2, A and B). On d 17 and 19 of pregnancy, we detected a large quantity of des-acyl ghrelin in the amniotic fluid (Fig. 2C). Acyl ghrelin levels increased rapidly in fetal blood within 5 min of administration of either 0.2 or 20 nmol acyl ghrelin (iv) into the mother (Fig. 2D). In the case of the 20-nmol dose, although maternal trunk ghrelin levels declined 30 min after injection, fetal trunk ghrelin levels were still increased at the sampling time.

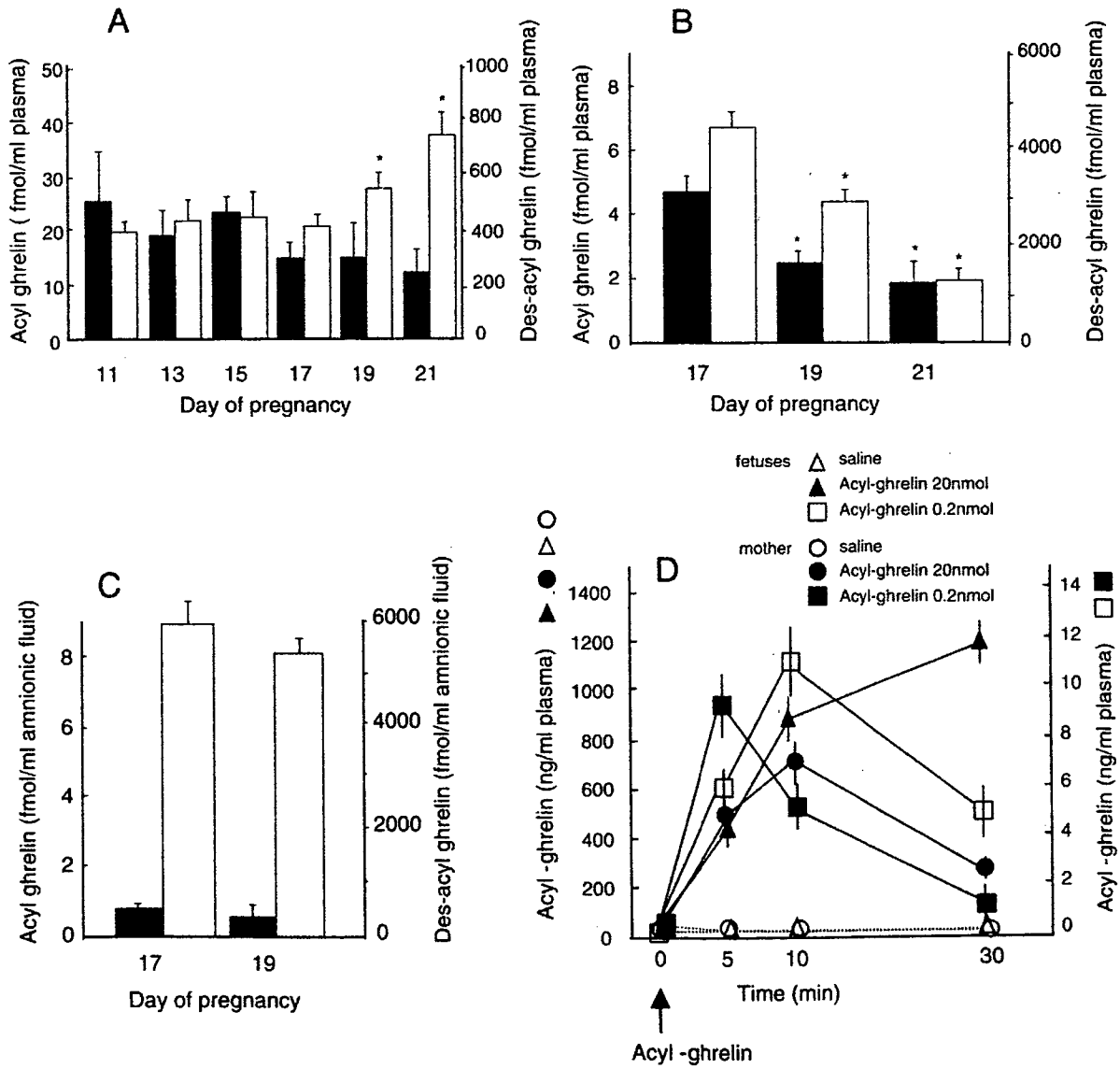

FIG. 2. A, Plasma acyl and des-acyl ghrelin levels in pregnant rats during late pregnancy. B, Fetal plasma levels of acyl and des-acyl ghrelin. C, Acyl and des-acyl ghrelin levels in amniotic fluid. All *black and white bars* represent the levels of acyl and des-acyl ghrelin, respectively. Each *bar* and *vertical line* represent the mean ± SEM (n = 8). *Asterisks* indicate significant differences (A, $P < 0.05$ *vs.* E11; B, $P < 0.05$ *vs.* E17). D, Maternal and fetal levels of circulating acyl ghrelin after iv injection of saline or 0.2 and 20 nmol acyl ghrelin into mothers at d 19 of pregnancy. The *right and left vertical scales* correspond to 0.2 and 20 nmol acyl ghrelin treatments, respectively.

Effect of chronic ghrelin treatment on fetal body weight at birth

We examined the effect of prolonged maternal treatment with ghrelin, beginning at d 14 or 15 of pregnancy and lasting until delivery, on neonatal body weight at birth. Chronic treatment with acyl ghrelin, either by injection three times per day (Fig. 3A) or constant infusion through an osmotic mini-pump (Fig. 3B), significantly increased the average neonatal body weight at birth in comparison to that of neonates delivered by a saline-treated group. We observed more than a 10% body weight gain, and the increase was dose-dependent. No significant changes were observed after treatment with des-acyl ghrelin.

We investigated the effect of acyl ghrelin injection on food intake of pregnant females. Daily treatment with acyl ghrelin significantly increased daily maternal food intake (Fig. 3C). However, a paired feeding study demonstrated that even when pregnant females treated with acyl ghrelin consumed the same amount of food as saline-treated pregnant females, neonatal body weight was significantly greater in the ghrelin-treated group (Fig. 3D).

To examine the effect of endogenous maternal ghrelin on fetal development, we compared the birth weight of pups born to mothers passive-immunized against a complex of acyl ghrelin and mcKLH (carrier protein) with that of pups born to mothers passive-immunized against mcKLH. After six immunizations at 2-wk intervals beginning at 44 d after birth, rats were mated when the relative ghrelin binding titer was maximally increased (Fig. 3E). Although body weight gain was temporarily lower, it was not significantly so. The body weights of ghrelin-immunized females recovered gradually to normal levels at 104 d of age (Fig. 3E). The body weights of neonates born to mothers passive-immunized against acyl ghrelin were lower than those of neonates born to saline-treated mothers (Fig. 3F).

Effect of ghrelin on GH mRNA levels in fetal pituitary tissue, and IGF-I and corticosterone levels in fetal plasma

If GH, prolactin, or corticosterone secretions from fetal pituitary or adrenal tissues were stimulated by maternal ghrelin, the released hormone might stimulate fetal development. We examined the effect on fetal pituitary GH mRNA levels and fetal plasma IGF-I or corticosterone levels by administering acyl ghrelin to pregnant females. However, pituitary GH mRNA at E19 and E20 was not affected by this treatment (Fig. 4A). In addition, fetal plasma IGF-I and corticosterone concentrations at E19 and E20 were not affected by maternal ghrelin treatment (Fig. 4, B and C). We found no significant change in fetal prolactin levels (data not shown).

Effect of ghrelin on proliferation of cultured fetal skin cells

To examine a possibility of direct effect of circulating ghrelin on fetal development, we examined the fetal cell proliferation by ghrelin using [^{3}H]thymidine and BrdU incorporation. We used primary cultured fetal skin cells at E17, because abundant cells at this stage were easy to collect. Both [^{3}H]thymidine (Fig. 5A) and BrdU (Fig. 5, B–E) incorporation increased significantly after treatment with acyl ghrelin in a dose-dependent or time-dependent manner. Des-acyl ghrelin was more potent than acyl ghrelin at stimulating the proliferation of fetal skin cells (Fig. 5E). The GHS-R antagonist [D-Lys^3]-GHRP-6 inhibited acyl ghrelin- and des-acyl ghrelin-stimulated cell proliferation (Fig. 5E).

Calcium-imaging analysis revealed two types of fetal skin cells (Fig. 5F): one type responding to des-acyl ghrelin, but not to acyl ghrelin, and the other responding to acyl ghrelin, but not to des-acyl ghrelin. No. 21 and 23 cells were shown as examples, respectively.

Discussion

The present study clearly demonstrated that maternal ghrelin would play an important role in fetal development during pregnancy; first, exogenous chronic treatment of the mother with ghrelin increased fetal body weight at birth; second, mothers immunized against ghrelin delivered fetuses with a lower body weight; and third, proliferation of cultured fetal skin cells was stimulated by ghrelin. Both GHS-R1a mRNA expression and GHS-R protein were detected in various fetal tissues. Autoradiography using [^{125}I]acyl ghrelin also demonstrated dense binding to the bone, skin, heart, and tongue. This distribution of functional GHS-R throughout peripheral fetal tissues suggests that ghrelin acts on such fetal peripheral tissues. Surprisingly, excess unlabeled des-acyl ghrelin could displace completely with [^{125}I]acyl ghrelin binding, suggesting that the acyl modification is dispensable for ghrelin function in binding site of fetal tissues. Because des-acyl ghrelin does not bind to GHS-R (1), we presume that fetal tissues may express a GHS-R subtype for des-acyl ghrelin. In support of this supposition (28, 29), it has been shown that the increases in plasma glucose and decreases in insulin, but not increases in GH secretion, induced by acyl ghrelin administration can be counteracted by coadministration of des-acyl ghrelin (28). In addition, ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and phosphatidylinositol 3-kinase/AKTJ (30).

Plasma total ghrelin levels have been measured in pregnant women, rats, and human fetuses (31–34). In pregnant rats, plasma total ghrelin, determined with an antibody recognizing the C-terminal region, was shown to decrease at around the middle to late stage of pregnancy (31). Total ghrelin increases at around mid-gestation in human pregnancy (32, 33). Human fetuses exhibit levels of total ghrelin in umbilical venous blood that are not correlated with either gestational age or maternal ghrelin levels (34). In addition, ghrelin mRNA expression has been observed in the placenta and ovary of pregnant rats, and in the fetal pancreas (3, 35, 36). It has also been reported that ghrelin might play an important role in the regulation of blood pressure and the development of preimplantation embryos (37, 38). In the present study, both acyl and des-acyl ghrelin were present in the maternal and fetal circulations during the last half of pregnancy, and there was a significant difference in des-acyl ghrelin levels between the maternal and fetal plasma. The fetal levels of plasma des-acyl ghrelin were 5- to 10-fold higher than the maternal levels. In addition, we detected a large quantity of des-acyl ghrelin in the amniotic fluid. As

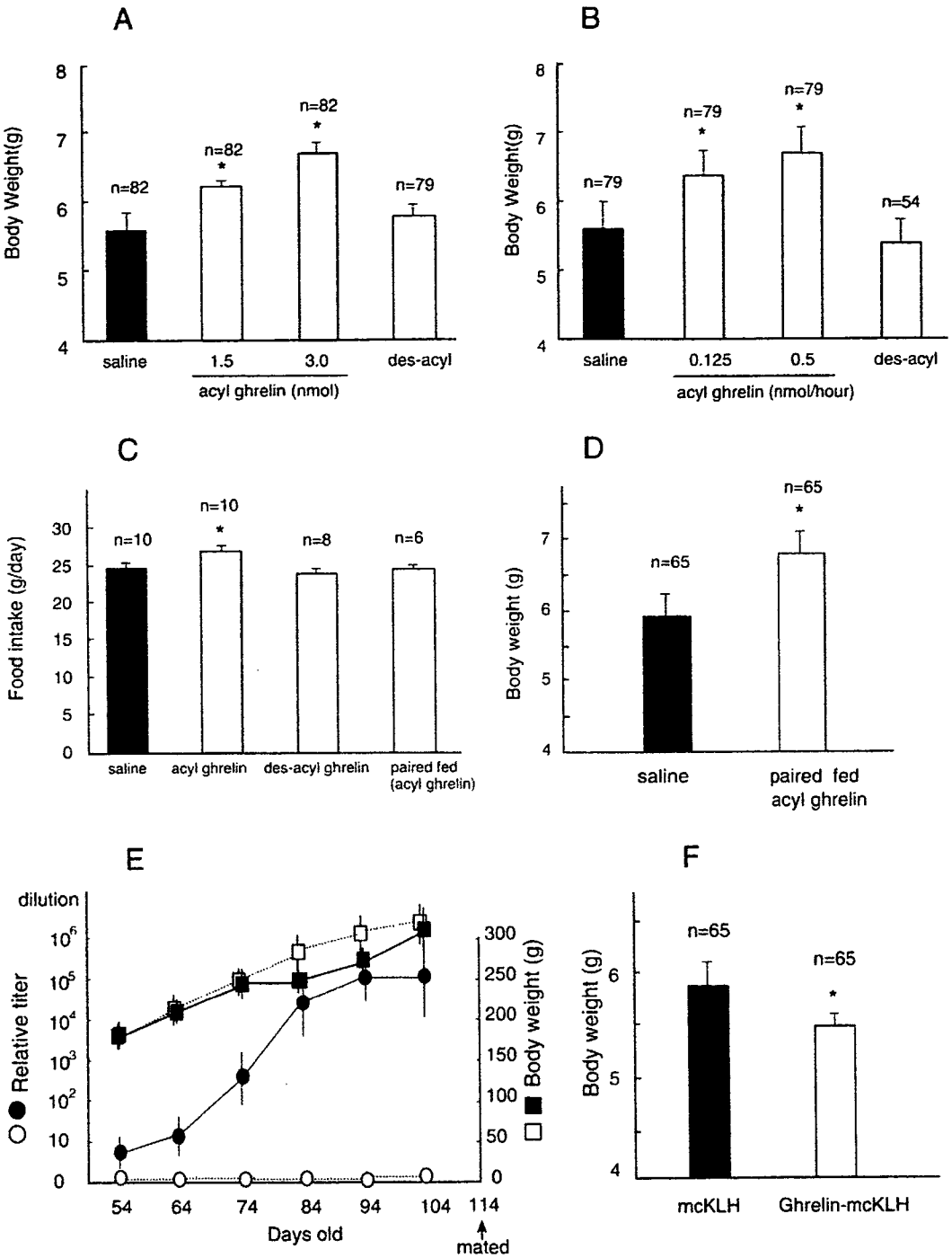

FIG. 3. A and B, Effect on neonatal body weight at birth of daily sc injections of ghrelin (A) or continuous infusion of ghrelin with an osmotic minipump (B) into pregnant rats. Treatment with ghrelin was performed from d 14 until delivery. C, Daily food intake in pregnant rats (A) and in pair-fed pregnant rats treated with acyl ghrelin on the same schedule as A. D, The effect of daily sc injection of 3 nmol acyl ghrelin into pair-fed pregnant rats on neonatal body weight at birth. Acyl ghrelin was injected three times daily from d 14 until delivery. Paired feeding for the saline-treated group also begun on d 15 of pregnancy. E, Comparison of body weight and relative antibody titer between rats immunized against ghrelin-mcKLH complex (●, ■) or carrier protein alone (○, □). The relative titer (●, ○) was expressed as a dilution rate at 50% binding capacity. Each *bar* (A–E) and *symbol* (F) and *vertical line* represent the mean ± SEM. The *upper numbers* represent the total number of newborn rats compared in each group. *Asterisks* indicate significant differences ($P < 0.05$ *vs.* control). F, Comparison of the body weight of newborn rats delivered from rats passive-immunized with the ghrelin-mcKLH complex or with carrier protein alone (mcKLH).

demonstrated previously, ghrelin-positive cells were not evident in the fetal stomach until E19 by immunohistochemistry using an antibody recognizing the N-terminal of acyl ghrelin, suggesting that fetal plasma ghrelin originates from the maternal placenta and/or the maternal blood (3, 22). Indeed, acyl ghrelin levels in fetal plasma increased rapidly

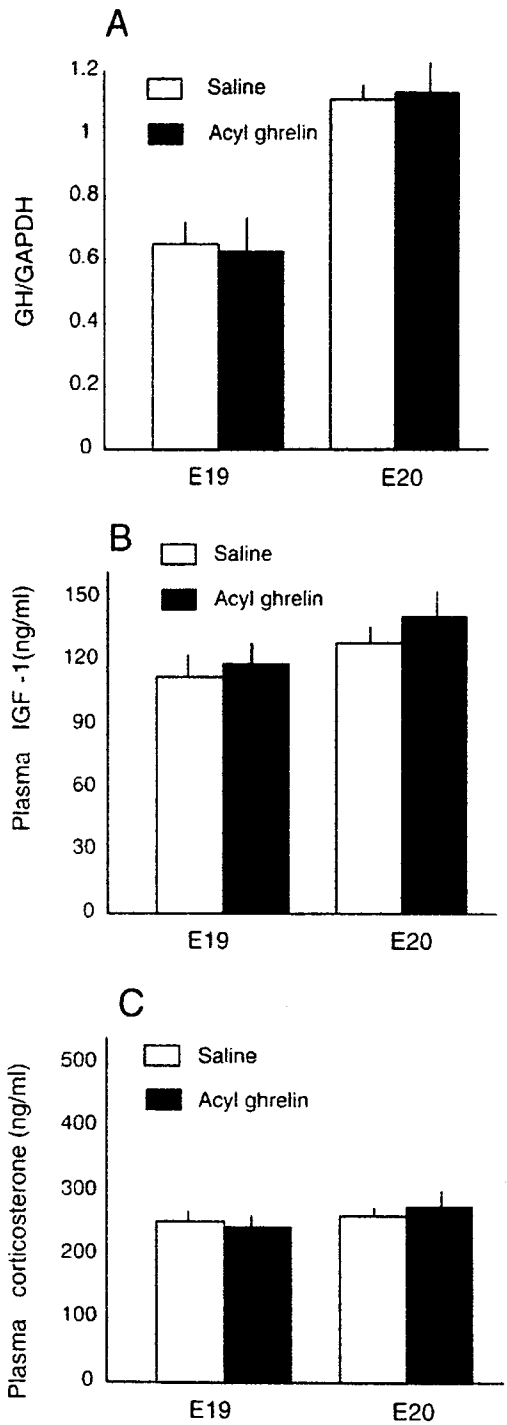

FIG. 4. A, The effect of continuous infusion of acyl ghrelin into pregnant rats on fetal pituitary GH mRNA levels (A) and fetal plasma IGF-I (B) and corticosterone levels (C). Acyl ghrelin was infused at a rate of 0.5 nmol per hour with an osmotic minipump beginning on d 14 and lasting until delivery. The fetal pituitary and plasma were collected at E19 and E20. Each *bar and vertical line* represent the mean ± SEM (n = 6).

within 5 min after administration of acyl ghrelin to the mother, indicating that maternal ghrelin easily transits to the fetal circulation. Although maternal trunk ghrelin levels declined 30 min after injection, fetal trunk ghrelin increased at the time, probably resulting from a longer half-life of ghrelin in fetuses than in adults, and high levels of des-acyl ghrelin might accumulate in the fetal circulation. The existence of GHS-R and an additional GHS-R subtype in fetal tissues, combined with both acyl ghrelin and large quantities of des-acyl ghrelin in the fetal circulation and amniotic fluid, supports the hypothesis that maternal ghrelin plays a critical role in fetal development.

Fetal growth is mainly influenced by the nutrition provided by the mother through the arteria umbilicalis (39, 40). Decreases in the amount of food given to pregnant mothers during the gestational period tend to decrease the size of their neonatal pups in comparison with pups born to mothers fed *ad libitum*. Daily treatment with acyl ghrelin significantly increased daily maternal food intake. The stimulation of fetal growth by maternal ghrelin injection would result from increased nutrition provided by the mother. However, a paired feeding study demonstrated that even when pregnant females treated with acyl ghrelin consumed the same amount of food as saline-treated pregnant females, neonatal body weight was significantly greater in the ghrelin-treated group. This result indicates that maternal ghrelin affected fetal development through a mechanism independent of increased nutrition.

In rats, a rapid increase in fetal body weight occurs during the last quarter of pregnancy. The somatotroph, a GH-secreting cell, appears in the fetal pituitary near E18 (41). Pituitary GH mRNA at E19 and E20 was not altered by ghrelin treatment, indicating that maternal ghrelin-induced fetal development is not due to increased release of fetal GH. The stimulation of maternal GH secretion by daily treatment of ghrelin, leading to the transition of maternal GH to fetal circulation, may stimulate fetal development. Garcia-Aragon and colleagues (42) provided evidence for the wide distribution of GH receptor in the mid-late gestation of rat fetus. The receptor expression markedly increased between E12 and E18; the receptor was present in all major organ systems at E18. Genetically manipulated model mice, Laron dwarfs, with inactivating GH receptor mutations, were shorter in length than normal at birth. Congenitally GH-deficient newborn babies are also much shorter (43, 44). In contrast, the fetuses of GH-deficient dwarf rats were proportionately smaller in size (45). However, we previously reported that continuous infusion of ghrelin to rats stimulated GH secretion for several days, but that the effect decreased after prolonged administration (26). Levels of GH mRNA within the pituitary were also decreased by these treatments (26), probably due to transcriptional down-regulation. In addition, fetal plasma IGF-I levels were not affected by maternal treatment with ghrelin. We found no significant change in fetal circulating levels of corticosterone and prolactin during maternal ghrelin administration. Therefore, the stimulation of fetal development by maternal ghrelin administration is probably not due to the maternal GH and fetal circulating IGF-I and corticosterone levels.

Both [^{3}H]thymidine and BrdU incorporation increased significantly after treatment with acyl ghrelin in a dose-dependent and time-dependent manner. Interestingly, des-acyl ghrelin stimulated proliferation more potently than acyl ghrelin. The GHS-R antagonist [D-Lys^3]-GHRP-6 inhibited

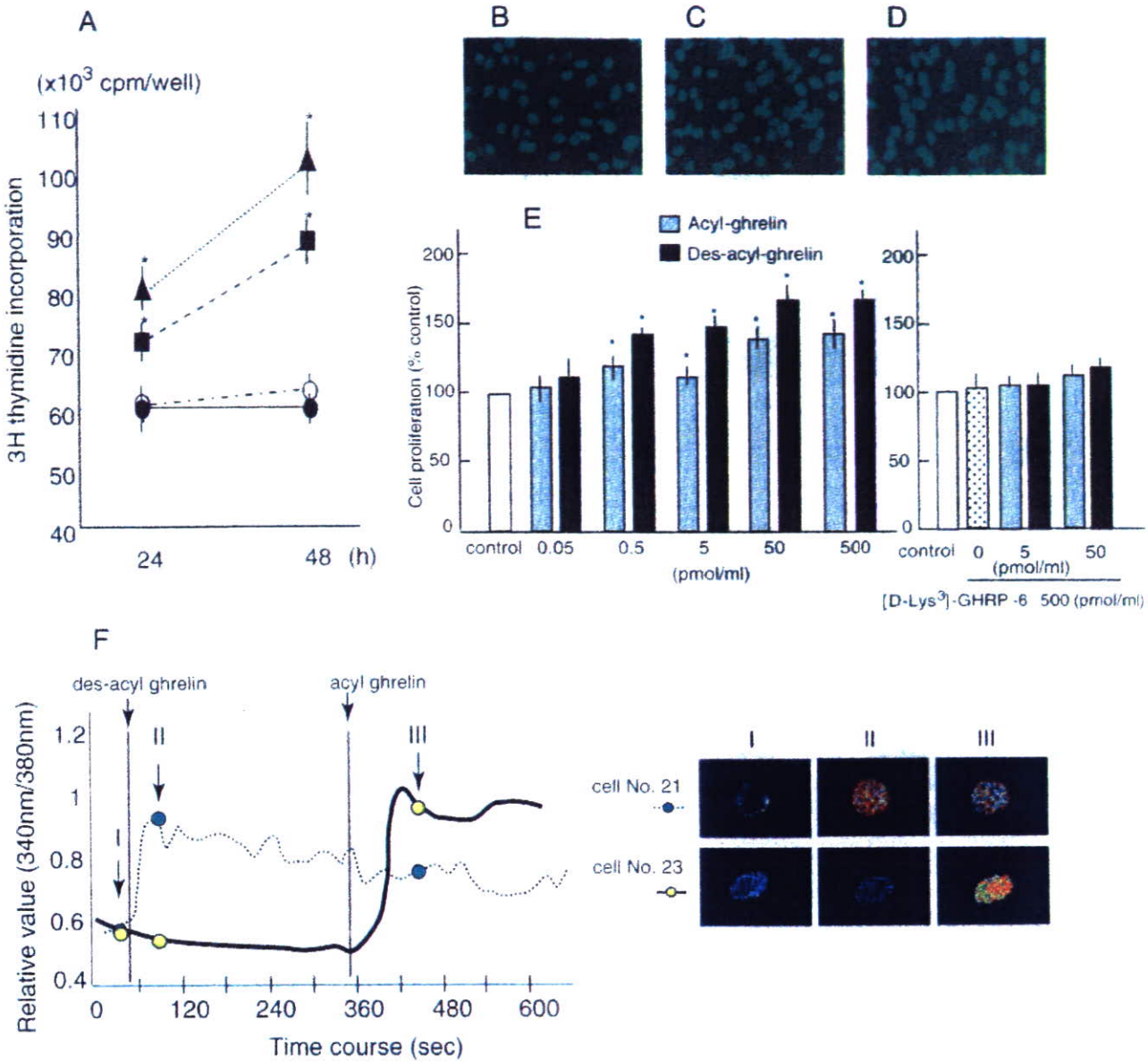

FIG. 5. A, The effect of acyl ghrelin on [^{3}H]thymidine incorporation by cultured fetal (E17) skin cells. One day after initiation of culture, acyl ghrelin (●, 0 pmol; ○, 0.5 pmol; ■, 5 pmol; ▲, 50 pmol/ml) and [^{3}H]thymidine (2 μCi/ml) were added for a 24- or 48-h incubation period. Each *symbol* and *vertical line* represents the mean ± SEM (n = 16 wells). *Asterisks* indicate significant differences ($P < 0.05$ *vs.* ●). B–D, Example of BrdU incorporation (shown in E) into the nucleus of cultured fetal (E17) skin cells incubated for 24 h with 50 pmol/ml acyl ghrelin (C), 50 pmol/ml des-acyl ghrelin (D), or without ghrelin (B). E (*left*), The effect of various doses of acyl and des-acyl ghrelin on BrdU incorporation by cultured fetal skin cells. Fetal (E17) skin cells were cultured for 24 h with or without ghrelin. E (*right*), Effect of [D-Lys3]-GHRP-6, a GHS-R antagonist, on the ghrelin-stimulated proliferation of fetal skin cells. Various doses of acyl or des-acyl ghrelin were added to the culture medium together with 500 pmol [D-Lys3]-GHRP-6. F, Calcium imaging analysis of fetal skin cells. The skin cells were prepared from E17 fetuses. Cell no. 21 responded to des-acyl ghrelin, but not to acyl ghrelin. In contrast, cell no. 23 responded to acyl ghrelin, but not to des-acyl ghrelin. Photographs on the *right* represent the calcium response in cells at points I, II, and III. The *red and yellow color spots* indicate high intracellular Ca^{2+} concentration. The *blue color* shows the basal condition. Des-acyl ghrelin and acyl ghrelin were added to the culture medium at the point indicated by the *arrow*. The *graph on the left* represents the transition of the relative value of Ca^{2+} concentration analyzed by an imaging scanner.

acyl ghrelin- and des-acyl ghrelin-stimulated cell proliferation. These results clearly indicate that both acyl ghrelin and des-acyl ghrelin stimulate proliferation of fetal skin cells. Acyl ghrelin induces neurogenesis in the dorsal motor nucleus (46) and stimulates bone formation (47). During pregnancy, maternal ghrelin is likely transferred to the fetal circulation, and then would prompt fetal growth through stimulation of cell proliferation. Calcium-imaging analysis revealed that two types of cells exist in cultured fetal skin cells: one responds only to des-acyl ghrelin, and the other one responds only to acyl ghrelin. These results strongly suggest that fetal skin cells have different type of receptors: one is a classical receptor for acyl ghrelin, GHS-R 1a, and the other is a novel receptor for des-acyl ghrelin that mediates intracellular calcium mobilization.

In this study, we detected high levels of des-acyl ghrelin

in the fetal circulation and amniotic fluid. These findings suggest that amniotic fluid serves, in part, as an incubation medium to provide des-acyl ghrelin to the fetus. In this way, des-acyl ghrelin may act on fetal development by direct stimulation of proliferation. If this is true, however, the lack of an effect of des-acyl ghrelin treatment on neonatal body weight at birth (Fig. 3) remains to be explained. We speculate that, late in pregnancy, high endogenous quantities of des-acyl ghrelin in the fetal circulation and amniotic fluid saturate the GHS-R 1a subtype des-acyl ghrelin receptors, effectively preventing the exogenous des-acyl ghrelin from exerting an effect. It has been reported that ghrelin knockout mice do not exhibit any changes in development (probably including fetal development) (13). We do not know the reason for the discrepancy of neonatal body weights between mothers passive-immunized against acyl ghrelin and ghrelin knockout mice. Further studies are required to elucidate this discrepancy.

In conclusion, the present study has demonstrated that maternal ghrelin is easily transferred to the fetal circulation, and then prompts fetal growth through stimulation of cell proliferation during the late half of pregnancy. Recent reports that ghrelin directly stimulates bone formation (47) also supports this hypothesis. These findings may have implications for the clinical application of ghrelin for pregnant subjects.

Acknowledgments

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Address all correspondence and requests for reprints to: Noboru Murakami, Ph.D., Department of Veterinary Physiology, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2155, Japan. E-mail: a0d201u@cc.miyazaki-u.ac.jp.

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CHARACTERIZATION OF A NOVEL METALLOPROTEINASE IN DUVERNOY'S GLAND OF *RHABDOPHIS TIGRINUS TIGRINUS*

Koji KOMORI[1], Motomi KONISHI[1], Yuji MARUTA[1], Michihisa TORIBA[2], Atsushi SAKAI[2], Akira MATSUDA[3], Takamitsu HORI[3], Mitsuko NAKATANI[4], Naoto MINAMINO[4] and Toshifumi AKIZAWA[1]

[1]*Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Setsunan University, 45-1 Nagaotogecho, Hirakata, Osaka 573-0101, Japan*

[2]*The Japan Snake Institute, 3318 Yabuzuka Ota, Gunma 379-2301, Japan*

[3]*Department of Biochemistry, Faculty of Pharmaceutical Sciences, Hiroshima International University, 5-1-1 Hirokoshingai, Kure, Hiroshima 737-0112, Japan*

[4]*Department of Pharmacology, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan*

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ABSTRACT — During the characterization of hemorrhagic factor in venom of *Rhabdophis tigrinus tigrinus*, so-called Yamakagashi in Japan, one of the Colubridae family, a novel metalloproteinase with molecular weight of 38 kDa in the Duvernoy's gland of Yamakagashi was identified by gelatin zymography and by monitoring its proteolytic activity using a fluorescence peptide substrate, MOCAc-PLGLA$_2$pr(Dnp)AR-NH$_2$, which was developed for measuring the well-known matrix metalloproteinase (MMP) activity.

After purification by gel filtration HPLC and/or column switch HPLC system consisting of an affinity column, which was immobilized with a synthetic BS-10 peptide (MQKPRCGVPD) originating from propeptide domain of MMP-7 and a reversed-phase column, the N-terminal amino acid sequence of the 38 kDa metalloproteinase was identified as FNTFPGDLK which shared a high homology to *Xenopus* MMP-9.

The 38 kDa metalloproteinase required Zn^{2+} and Ca^{2+} ions for its proteolytic activity. In addition, the proteolytic activity was almost completely inhibited by BS-10, a MMP inhibitor, but not by the serine proteinase inhibitors, cysteine proteinase inhibitors and aspartic proteinase inhibitors.Together these results demonstrated that the 38 kDa proteinase is a novel snake verom metalloproteinase (SVMP) containing HExGHxxGxxH motif which possesses high affinity to the BS-10 peptide, into its molecule, and the enzymatic properties are closed to that of MMPs.

Based on the results obtained in the present study, we concluded that the 38 kDa metalloproteinase is a novel metalloproteinase whose activity may be regulated by the cysteine switch mechanism, and could be classified as one of the matrix metalloproteinases rather than snake venom metalloproteinases.

KEY WORDS: Snake venom metalloproteinase, Matrix metalloproteinase, Yamakagashi, *Rhabdophis tigrinus tigrinus*, Cysteine switch mechanism, Column switch HPLC

INTRODUCTION

Rhabdophis tigrinus tigrinus, so-called Yamakagashi in Japan, is one of the Colubridae that has been recognized as a nonpoisonous snake for a long times. In most cases, continuous bleeding from the gums, old abrasions and needle points, bright-red urine and decrease in fibrinogen contents were observed when bitten by this snake (Mittleman and Goris, 1974; Mori *et al.*, 1983). In a serious case, disseminated intravascular coagulation (DIC), acute renal failure and brain hemorrhage often occurr (Sakai *et al.*, 1990; Akimoto *et al.*,

Correspondence: Toshifumi AKIZAWA (E-mail: akizawa@pharm.setsunan.ac.jp)

1991).

Morita *et al.* have reported that mammalian coagulation factor was activated by 170 kDa protein in the Duvernoy's gland as a venomous coagulant and that activators of coagulation factor appeared to be single-chain enzymes with molecular weights ranging between 50 and 90 kDa. These proteins activated prothrombin, but were different from factor X (Morita *et al.*, 1988).

It is believed that snake venom metalloproteinases (SVMPs) play an impotant role in the bleeding. To date over a hundreds SVMPs have been reported, and classified into the following four classes according to the domain structure/molecular size: P-I, composed of only metalloproteinase domain/20-30 kDa; P-II, containing metalloproteinase and disintegrin domains/30-50 kDa; P-III, consisting of metalloproteinase, disintegrin-like and Cys-rich/50-80 kDa; and P-IV, containing an additional C-type lectin sequence at the C-terminal of P-III/80-100 kDa (Hite *et al.*, 1994; Kini and Evans, 1992). All the metalloproteinases have zinc-binding sites (HExGHxxGxxH), and cysteine residue in prodomain chelates to zinc in the latent form. They are most likely stored as inactive zymogens in order to prevent autodigestion. Moreover, the active form of these metalloproteinases has a shortage of prodomain from precursor protein. This activation mechanism has been suggested to be similar to the cysteine switch mechanism as proposed for matrix metalloproteinases (MMPs) (Grams *et al.*, 1993). Although there are a large number of studies dealing with snake venoms, no report of the metalloproteinase has been found from the Duvernoy's gland of *Rabdophis tigrinus tigrinus*.

During the determination of the hemorrhagic factor, a novel metalloproteinase was found in the Duvernoy's gland of Yamakagashi. In the present study, characterization of this new MMP was undertaken.

MATERIALS AND METHODS

Materials

Gelatin and Triton X-100 were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Molecular weight marker low range for electrophoresis was obtained from BIO-Rad Laboratories (Calfornia, USA). All electrophoresis media were from nacalai tesque, inc. (Kyoto, Japan). Tris was from ICN. Biomedical inc. (OH, USA). Sodium dodecyl sulfate (SDS), calcium chloride and zinc chloride were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA) was purchased from Kishida Chemicals (Osaka, Japan). 1,10-phenantholine was from nacalai tesque, inc. (Kyoto, Japan). *O,O'*-Bis(2-aminoethyl) ethyleneglycol-*N,N,N',N'*-tetraacetic acid (EGTA), phosphoramidon, luepeptin, phenylmethylsulfonyl fluoride (PMSF), antipain, E-64, and pepstatin A were purchased from Calbiochem Merck KGaA (Darmstadt, Germany). All other reagents were analytical grade from commercial sources. Fluorescence substrates, 3163-v: (7-methoxycoumarin-4-yl)acetyl-Pro-Leu- Gly-Leu-{N_3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl}-Ala-Arg-NH_2, 3167-v: (7-methoxycoumarin-4-yl) acetyl-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys-{N_3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl}-NH_2, and 3168-v: (7-methoxycoumarin-4-yl)acetyl-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys-{N_3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl}-NH_2 were purchased from Peptide Institute Inc. (Osaka Japan).

Venom

The venom was collected from the Duvernoy's gland of *Rhabdophis tigrinus tigrinus* at the Japan Snake Institute (Gunma, Japan). This gland was excised and minced, and the venom was extracted by water. The insoluble material was removed by centrifugation, and the supernatant was then lyophilized. The lyophilized venom was stored at –40°C before use, and dissolved in distilled water in use (Sakai *et al.*, 1983).

Preparation of recombinant MT1-MMP

Recombinant human soluble MT1-MMP was prepared according to the method described previously (Itoh *et al.*, 1996; Oku *et al.*, 2003). In brief, the cDNAs for procatalytic domains of human MT1-MMP were prepared by polymerase chain reactions using sets of primers (5' primer, GGCGGATCCATGCTCGCCTCCCTCGGCTCG, 3' primer, GCCGTCGACGTTCCCGTCACAGATGTTGGG) based on the reported sequences, and the template (poly (A)$^+$ RNA isolated from a human rectal carcinoma cell line pME18S-MTMMP having a 3.5 Kb cDNA fragment of MT1-MMP). The resulting PCR fragments were inserted into the bacterial expression plasmid pTH-72, having a tandem repeat of the T7 promoter and a hexahistidine-Tag encoding sequence. The expression, purification, and refolding of the human recombinant MMPs were performed by the following procedures: Human recombinant proMMPs were produced in *E. coli* strain BL21 (DE3) transfected with the corre-

sponding expression plasmids derived from pTH-72, solubilized in 8 M urea/10 mM Tris-HCl/100 mM Na-phosphate (pH 8.0)/100 mM β-mercaptoethanol, purified with Ni-NTA resin (QIAGEN Inc., U.S.A), and refolded reducing the urea concentration.

Microplate assay

Inhibitory effects of various inhibitors were measured by a microplate reader assay method with fluorescence substrates described previously (Oku *et al.*, 2003). Each 20 μl of inhibitor solution, assay buffer (50 mM Tris, pH 8.0, 5 mM $CaCl_2$, 200 mM NaCl, and 50 μM $ZnCl_2$, 0.1% Brij), distilled water and proteinase solution were mixed and pre-incubated in each well of 96 well microplates at 37°C for 15 min. Then 120 μl of fluorogenic substrate peptide (2.5 μM as final concentration) was added to initiate the proteolytic reaction. The fluorescence intensity transcribed resonance fluorescence unit (RFU) was measured at 320 nm for excitation and 390 nm for emission every 15 min for 2 hr by using an fmax fluorescence microplate reader (Molecular Devices, USA). The inhibitory effects were calculated based on the reaction data at 2hr. For measuring the proteolytic activities in each elution from HPLC, 20 μl of distilled water was added instead of the test sample solution. For the measurement of proteolytic activity of enzymes, 20 μl of distilled water was added instead of inhibitor solution. The concentration of enzymes (100 – 200 RFU at 2 hr) was determined by measuring the proteolytic activity of various dilution enzymes using 3163-v.

Purification of the 38 kDa metalloproteinase by gel-filtration HPLC

A gel-filtration HPLC was carried out with TSK-gel G-3000sw column (Tosoh, Tokyo, Japan; 600 × 7.5 mm i.d.) using 200 mM Tris-HCl (pH 6.7) buffer below 4°C. The flow rate was set at 0.6 ml/min, and chromatogram monitored at UV 220 nm with CD detector CD-1595 (Jasco, Japan).

Preparation of synthetic peptides

BS-10 (MQKPRCGVPD) was synthesized by the Fmoc method with 433A peptide synthesizer (Applied Biosystems, CA., U.S.A.). After deprotection according to the manufacturer's protocol, peptide was purified by a reversed-phase HPLC column (Shiseido co., Ltd.,Tokyo, Japan; Capcell Pak C18, 250 × 10 or 15 mm i.d.) with a linear gradient elution system from 0.1% TFA to 50% CH_3CN containing 0.1% TFA for 30 min. The peptide purity was confirmed by an analytical HPLC system via reversed-phase HPLC using a Capcell Pak C18 (Shiseido co., Ltd.,Tokyo, Japan; Capcell Pak C18, 250 × 4.6 mm i.d.) with a linear gradient elution system from 0.1% TFA to 70% CH_3CN containing 0.1% TFA for 30 min monitoring with a photodiode array detector.

Column switch HPLC system

The column switching HPLC consisted of an affinity column (BS-10 column) for the first separation and a reversed-phase column for the second separation (Fig. 1). For the first separation, an affinity HPLC column was prepared according to the following steps: TSK-GEL Tresyl-5PW packed with $CH_2OSO_2CH_2CF_3$ group conjugating resin (Tosoh; 40 × 6.0 mm i.d.) was washed with water for 10 min, and then equipped with 0.5 M phosphate buffer (pH 8.0) for another 10 min. For immobilization of BS-10, 10 ml of BS-10 solution (1.87 g/ml) in 0.5 M phosphate buffer (pH 8.0) was passed into the column and recycled for 24 hr, and then non-immobilization resin was blocked with 0.2 M Tris-HCL (pH 8.0) for 1 hr. During all steps, the flow rate was set at 0.5 ml/min. The amount of immobilized BS-10 was 8.98 μmole.

In the first separation, the flow rate was set at 1.0 ml/min, and the elution was monitored by UV absorption at 280 nm (Detector A). The BS-10 column (Affinity column) was equipped with 10 mM Tris-HCl (pH 8.0) containing 400 μM $ZnCl_2$ (Binding buffer) for 15 min before injection. Various test samples were loaded into the BS-10 column, and then the column was washed with the binding buffer for 8 min. In this step, the peptide or protein was bound to BS-10 column (Fig. 1b, left). Then the elution solvent changed to 10 mM Tris-HCl (pH 8.0) containing 3 mM EDTA instead of $ZnCl_2$ (Elution buffer) using solvent selection unit by manual mode. At the same time, the column selection unit was switched by manual manner, and the elution from BS-10 column was loaded into a second column with on-line. During another 10 min, the elution of the first column was loaded to the second column (Fig. 1b, center). The second separation was carried out using a reversed-phase HPLC column (Kanto Chemical, Tokyo, Japan; Migthysil RP-18, 75 × 4.6 mm i.d.). After that, the elution of the second column was changed to 0.1% TFA, and kept for another 12 min (Fig. 1b right). In this step, the elution buffer was washed out from the second column. A linear gradient system from 0.1% TFA to 50 % CH_3CN containing 0.1% TFA for 30 min was then started 12 min after switching the column. The flow rate of the second col-

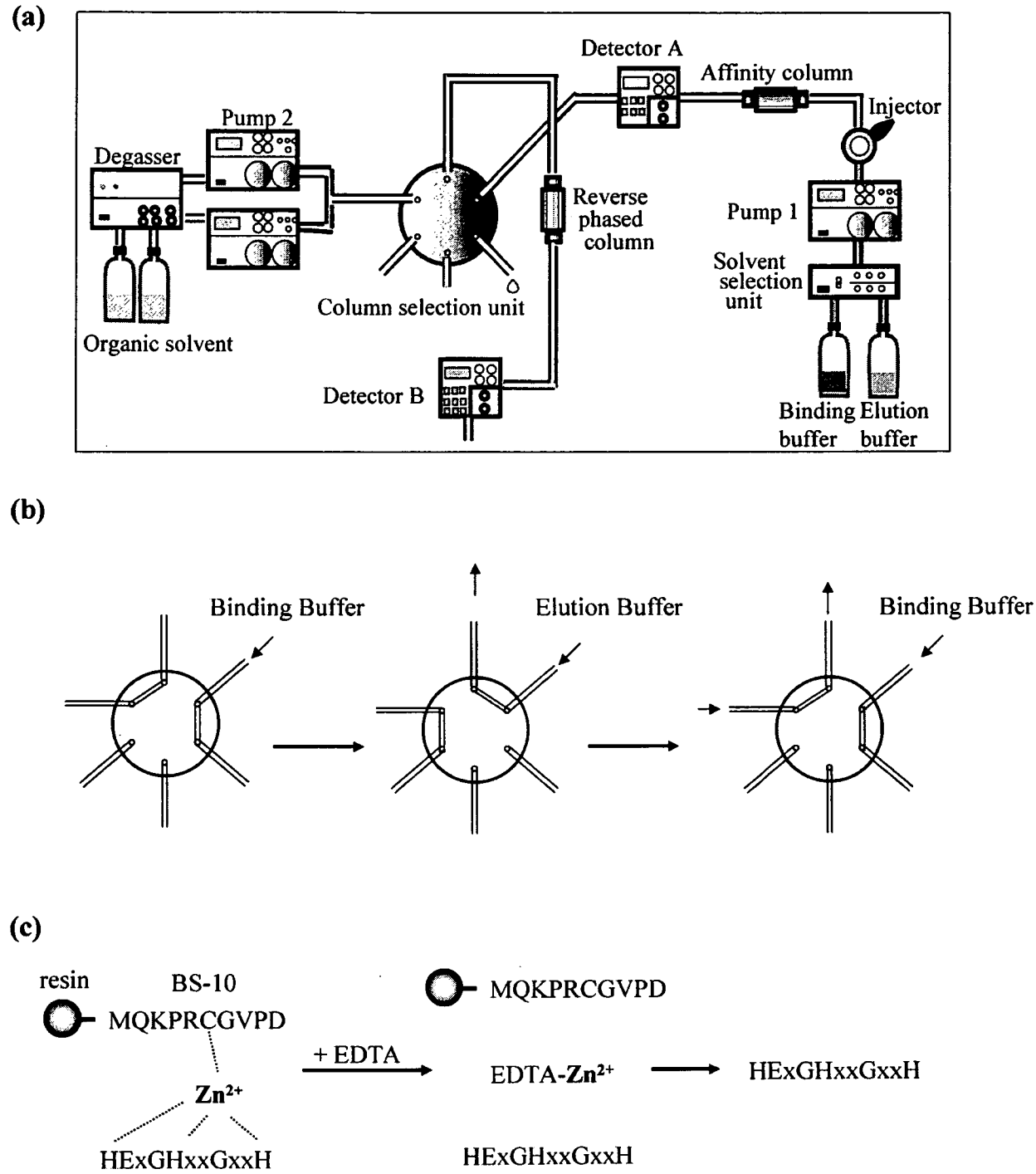

Fig. 1. (a) Apparatus of column switch HPLC system, (b) Connection of column selection unit, (c) Principle of affinity column for purification of 38 kDa metalloproteinase.

umn was set at 1.0 ml/min. The chromatogram was monitored at UV absorption at 220 nm (Detector B).

Gel electrophoresis and Zymography

SDS-polyacrylamide gel electrophoresis (PAGE) was done in a Tris-glycine buffer system as described using a 3% stacking gel and 12.5% separation gel. Zymography was performed as previously described (Miyazaki *et al.*, 1990; Chin *et al.*, 1985). Briefly, samples were mixed with SDS sample buffer in the absence of a reducing agent, incubated for 20 min at 37°C and separated on a 12.5% polyacrylamide gel containing 1 mg/ml of casein or gelatin. After electrophoresis, gels were soaked in 2.5% Triton X-100 for 1 h then digested by incubating the gel in 50 mM Tris-HCl (pH 8.0) containing 200 mM NaCl, 5 mM $CaCl_2$ and 50 μM $ZnCl_2$ at 37°C for overnight. The gels were stained with 0.1% Coomassie Brilliant Blue G250, and the location of proteolytic activity was detected as clear bands in a background of uniform staining.

Analysis by reversed-phase column

Crude venom (500 μg) was loaded into a reversed-phase column (Waters; Puresil C18 150 × 4.6 mm i.d.) monitoring with photodiode array detector. The flow rate was set at 1.0 ml/min. Each 200 μl of elution was collected into a 96 well microplate twice under linear gradient elution for 30 min from H_2O to 70% CH_3CN containing 0.1% TFA. A 20 μl of each fraction was used for the measurement of the proteolytic activity by microplate reader assay.

N-terminal sequence analysis

For the N-terminal amino acid sequence, 50 μl of crude venom (10 mg/ml) was loaded into a reversed phase column (see **Analysis by reversed-phase column**) twice, and the active fractions were collected into micro tube. The combined fraction obtained was concentrated to approximately 30 μl by Speed vac concentrator (Savant, U.S.A). A 13 μl of concentrated sample was treated with the same volume of SDS, and then 20 μl was applied to SDS-PAGE (see **Gel electrophoresis and Zymography**). After transfer, PVDF membrane was stained by 0.1% Coomassie Brilliant Blue G250. The band of the 38 kDa metalloproteinase was cut off and destained twice by small volume of methanol. The amino-terminal of the purified 38 kDa metalloproteinase on PVDF membrane was determined by Edman degradation with a Procise-cLC sequencer (PE Biosystems, Branchburg, N.J.) operated according to the manufacturer's protocol.

RESULT

Purification of the 38 kDa metalloproteinase

In preliminary experiments, we found the crude venom of *Rhabdophis tigrinus tigrinus* contained many proteins with molecular weights of 30 to 90 kDa. Among them, only 38 kDa protein possessed proteolytic activity in gelatin and casein zymography. In addition, the proteolytic activity was inhibited by EDTA but not PMSF, a serine protease inhibitor, suggesting that a 38 kDa protein may be a metalloproteinase such as SVMP or MMP.

To characterize the 38 kDa protein, the crude venom from Yamakagashi was purified by column switch HPLC system (Fig. 1). The first separation in this system was perfomed based on cysteine switch mechanism using a BS-10 peptide affinity column. BS-10 peptide (MQKPRCGVPD) consisting of cysteine switch with essential zinc atom was chemically synthesized. The molecule containing the zinc-binding motif (HExGHxxGxxH) was retained by the column and eluted by EDTA solution. Our preliminary results indicate that the BS-10 column possesses high affinity to the catalytic domain of MMP or SVMP.

The partially purified enzyme from the snake venom was prepared by gel filtration HPLC (Fig. 2a). As shown in Fig. 2c, fraction A contained several proteins. However, only a single protein band with molecular weight of 38 kDa was identified by gelatin zymography (Fig. 2c). The enzyme in fraction A was further purified by a column switch HPLC. The proteolytic activity was identified in the fraction eluted at 29 min by using 3163-v as a substrate (Fig. 2b). The active fractions at 29 min were combined as fraction α. SDS-PAGE analysis of fraction α revealed that the 38 kDa protein was purified as a single band by a column switch HPLC (Fig. 2d). These result suggest that the 38 kDa metalloproteinase binds to BS-10 through a cysteine switch mechanism and possesse a homolugious amino acid sequence to MMPs and SVMPs

Analysis of N-terminal amino acid sequence

To analyze the N-terminal amino acid sequence of the 38 kDa protein, crude venom was purified by a reversed-phase column chromatography twice. The active fractions were separated by SDS-PAGE, and then transferred onto PVDF membrane. The 38 kDa protein was cut off and analyzed by automatic Edoman sequencer. N-terminal amino acid sequence of the 38 kDa protein was determined as FNTFPGDLK. This sequence possessed a high homology to the active site

N-terminal of *Xenopus* MMP-9 (Table 1). These results suggested that the 38 kDa might be a novel SVMP.

Enzymatic property of the 38 kDa metalloproteinase

Since the N-terminal amino acid of the 38 kDa metalloproteinase shared a high homogogy to *Xenopus* MMP-9, we next examined the substrate specificity of

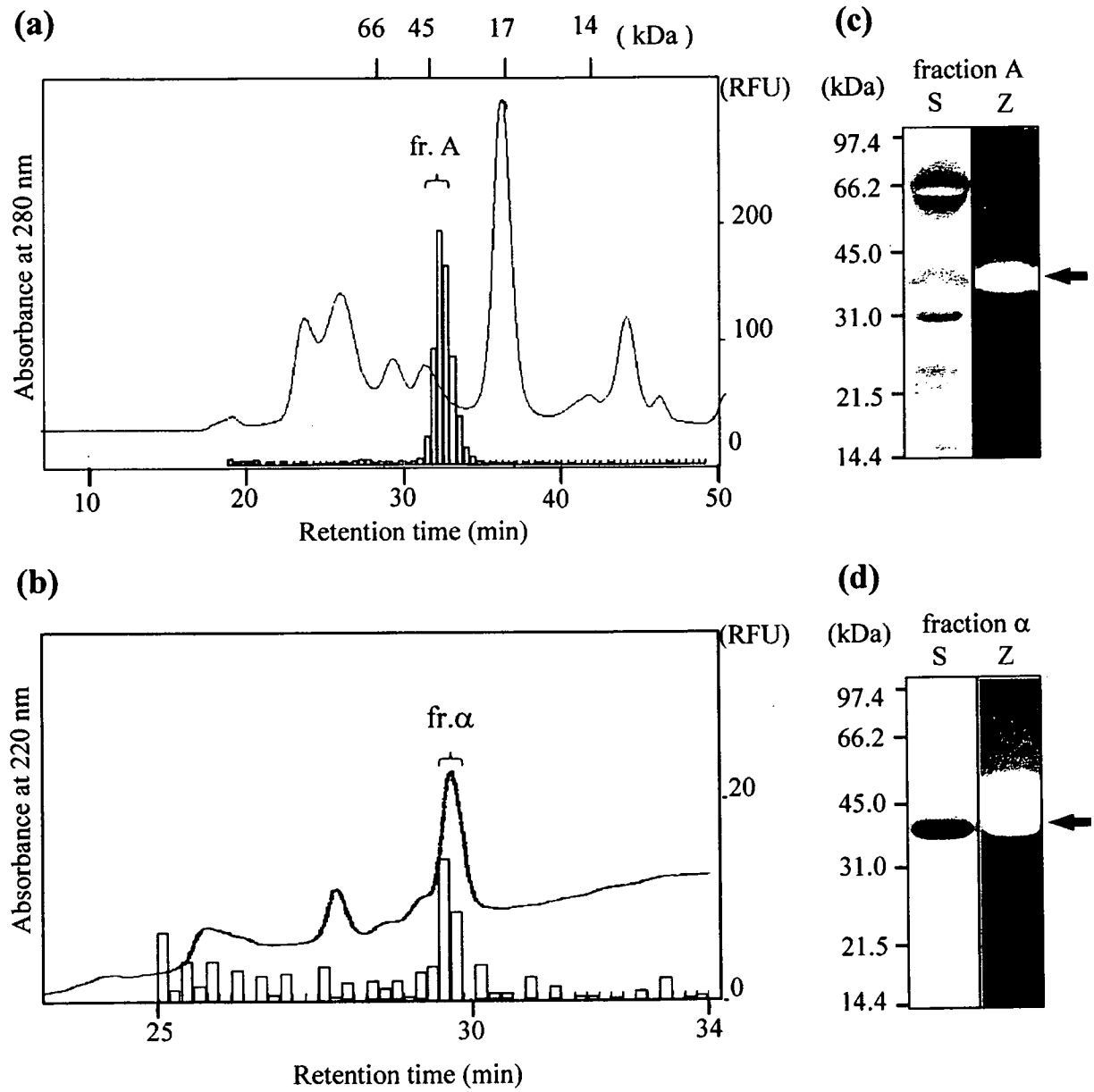

Fig. 2. Separation of 38 kDa metalloproteinase. The 38 kDa metalloproteinase in the venom from *Rhabdophis t. tigrinus* was separated by gel-filtration HPLC (a) and the active three fractions combined as "Fraction A". The 38 kDa metalloproteinase in Fraction A was next separated by column switch HPLC (b) and the active two fractions combined as "Fraction α". The proteolytic activity □ () was monitored by using 3163-v a fluorescence substrate. The 38 kDa metalloproteinase in Fraction A (c) and Fraction α (d) was then analyzed by SDS-PAGE with silver staining (lane S) and gelatin zymography (lane Z).

the 38 kDa metalloproteinase in crude venom, and compared it to those of r-MT1-MMP. As shown in Fig. 3, the enzyme in crude venom cleaved 3163-v strongly, 3167-v at medium and 3168-v very weakly. This proteolytic pattern was the same as that of r-MT1-MMP, the well-known metalloproteinase. r-MT1-MMP specifically cleaved the 3163-v between Gly and Leu residues with high sensitivity. In contrast, the MMP enzyme in crude venom of *Agkistrodon contortrix contortrix* showed a proteolytic activity restricted to 3168-v (data not shown).

The pH dependency of the 38 kDa metalloproteinase using 3163-v as a substrate was shown in Fig. 4. The 38 kDa enzyme in both crude venom and fraction A showed a maximum activity at pH ranging 7.0 ~ 7.5. The pH dependency is very similar to that of r-MT1-MMP (Fig. 4). The results of substrate specificity and pH dependency noted for this 38 kDa enzyme has amazing similarity to MT1-MMP.

Next, the requirement of essential cation ions for proteolytic activities of the metalloproteinase was examined. The patterns of cation ion requirement were very similar to each other. In the case of *Rhabdophis. t. tigrinus* and r-MT1-MMP, Ca^{2+} and Zn^{2+} ions were essential for expressing the proteolytic activity. The proteolytic activities of the enzyme in both crude venom, fraction A and r-MT1-MMP were decreased to 79, 80 and 43%, respectively, in the absence of Zn^{2+} ion. In addition, the proteolytic activities were almost lost in the absence of Ca^{2+} ion. These results revealed that the Ca^{2+} ion is required for the metalloproteinase to express full proteolytic activity (Fig. 5).

Effects of various inhibitors on the 38 kDa metalloproteinase

The effects of various protease inhibitors on the proteolytic activity of the 38 kDa metalloproteinase in both crude venom and fraction A were further examined (Table 2). The inhibitory effects on the crude venom were very similar to fraction A. The proteolytic activities in fraction A were inhibited by a series of metalloproteinase inhibitors such as EDTA, EGTA and 1,10-phenantholine, but not inhibited by PMSF, phosphoramidon and luepeptin, a series of serine protease inhibitors. Cysteine protease inhibitors such as antipain, E-64, and aspratic protease inhibitor pepstatin A showed a weak inhibitory effect on the enzyme. BS-10 had a high inhibitory effect on the metalloproteinase in fraction A as well as r-MT1-MMP. Interestingly, the responsibility of the inhibitory effects on the 38 kDa metalloproteinase in fraction A to various inhibitors was almost the same as that of r-MT1-MMP.

These results suggested that the properties and structure of the 38 kDa metalloproteinase are more similar to the MMP family than SVMP.

DISCUSSION

In the present study, we demonstrated for the first time that a novel metalloproteinase with molecular

Table 1. Comparison of N-terminal of catalytic domain and active site of 38 kDa metalloproteinase and various MMPs.

	N-ter of catalytic domain	Active site
38 kDa proteinase	FNTFPGDLK*	HExGHxxGxxHx
Human MMP-7	SLFPNSPK	HELGHSLGMGHS
Human MT1-MMP	YAIQGLK	HELGHALGLEHS
Chicken MMP-9	FLTFEGDLK	HEFGHSLGLDHS
Xenopus MMP-9	FNTFEGDLK	HEFGHALGLDHS
Fibrolase (P-I)	EQRFPQRYV	HEIGHNLGMNHD
Bilitoxin-1 (P-II)	ERYNPYKYI	HEIGHNLGMGHD
Jararhagin (P-III)	EQQRYDPYK	HEMGHNLGIHHD
RVVh (P-IV)	LVSTSAQFN	HELSHNLGMYHD
Mouse ADAM-17	RADPDPMKN	HELGHNFGAEHD

Human MMP-7 and MT1-MMP; Massova *et al.*, 1998.
Chicken MMP-9; Hahn-Dantona *et al.*, 2000, *Xenopus* MMP-9; Carinato *et al.*, 2000,
Fibrolase; Randolph *et al.*, 1992, Bilitoxin-1; Nikai *et al.*, 2000, Jararhagin; Paine *et al.*, 1992,
RVVh; Takeya *et al.*, 1992, Mouse ADAM-17; Black *et al.*, 1997.
* N-terminal of 38 kDa proteinase.

mass of 38 kDa exist in Duvernoy's gland of *Rhabdophis tigrinus tigrinus*. The snakes belonging to the Colubridae are classified as non-poisonous snakes such as *Cyclophiops herminae, Elaphe climacophora, Elaphe quadrivirgata* and *Amphiesma vibakari*, and poisonous snakes such as *Rhabdophis t. tigrinus, Molpolon monspessulanus, Spalerosophis diadema clifordi* and *Thamnophis sirtalis parietaris*, whose venoms have LD_{50} of 0.265 (i.v.), 6.5 (i.v.), 2.75 (i.v.) and 33.3 (i.p) mg/kg to mice, respectively (Sakai *et al.*, 1983; Rosenberg *et al.*, 1985). Although the lethal activity of *Rhabdophis t. tigrinus* is the most potent among poisonous Colubridae snakes, there are few reports of a fatal case by biting with this snake. However, once the venom was injected into an animal body, serious symptons were caused (Sakai *et al.*, 1984). Thus the antivenom was prepared for cure in Japan.

The hemorrhagic and nonhemorrhagic metalloproteinases in the snake venom have been reviewed by Takeya *et al.* (1993). Hemorrhage is a common mani-

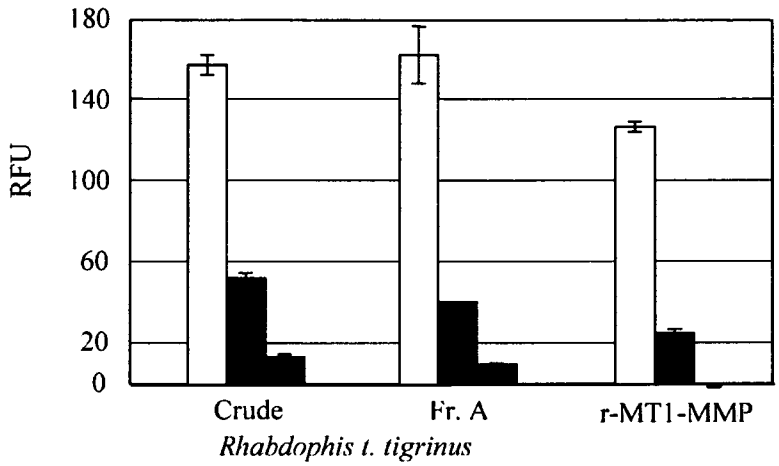

Fig. 3. Substrate specificity. The fluorescence substrates 3163-v □ (), 3167-■ () and 3168-v (■), were used to determine the proteolytic activity of 38 kDa metalloproteinase from *Rhabdophis t. tigrinus*, r-MT1-MMP.

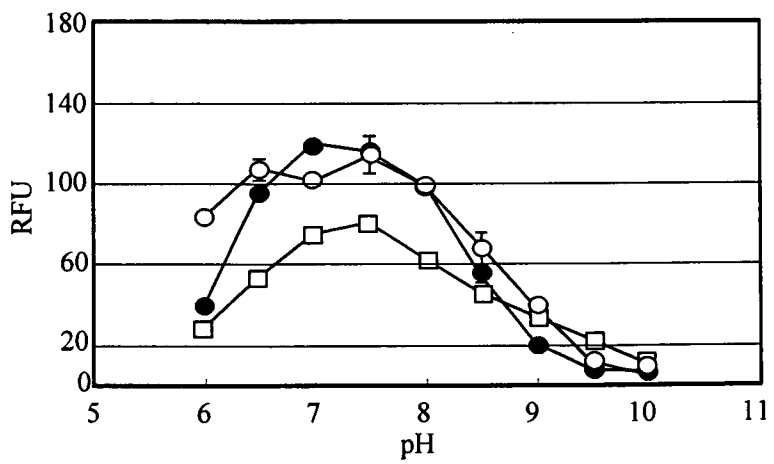

Fig. 4. pH dependency of r-MT1-MMP (□) and 38 kDa metalloprteinase in crude venom (●) and partially purified fraction A of *Rhabdophis t. tigrinus*. (○).

festation in the victim following the bite of crotalid and viperid snakes (Ohsaka, 1979). Various components, such as hemorrhagic factors and metalloproteinases (Tu, 1991), in the snake venoms would cause localized hemorrhage by direct actions on the blood vessel walls. In our preliminary experiments, when crude venom was intravenously injected, mice were dead with disseminated intravascular coagulation syndrome and hemorrhage in the tissues having rich capillary blood vessels such as the lungs and brain. This lethal effect was thought to be due to the hemorrhagic factor (Ogawa and Sawai, 1986).

The 38 kDa metalloproteinase in the Yamakagashi's venom could be separated from the components showing lethal activity and prothrombin activating activity which were eluted in the high molecular fraction (Morita *et al.*, 1988). These results suggest that the 38 kDa metalloproteinase is a nonhemorrhagic metalloproteinase and may act as a cofactor which can enhance lethal activity. In our screening test, the crude venom from 36 snakes belonging to *Trimeresurus, Agkistrodon, Crotalus* and *Naja* had high proteolytic activity to cleave 3168-v. However, the crude venom of *Rhabdophis. t. tigrinus* showed high specificity to cleave 3163-v, and the substrate specificity is very similar to the well-known MMPs (Fig. 3). Most of the MMPs are initially expressed as inactive zymogens, and activate subsequently by removing the N-terminal

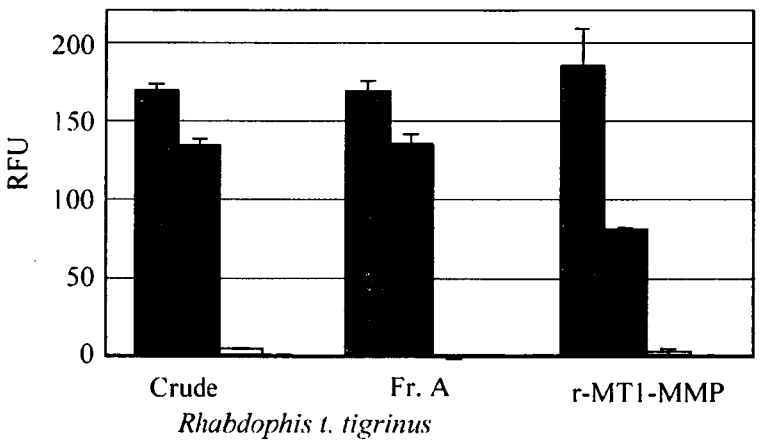

Fig. 5. Metal requirement. The proteolytic activity of 38 kDa metalloproteinase was determined in the presence of Zn^{2+} and Ca^{2+} ■), Ca^{2+} (■), Zn^{2+} (□), and in absence of both Zn^{2+} and Ca^{2+} (□).

Table 2. Effect of various inhibitors on 38 kDa metalloproteinase.

Inhibitors	Concentration (mM)	Inhibition (%)		
		R. t. tigrinus		r-MT1-MMP
		Fr. A	Crude	
EDTA	5.0	100	100	98.6
EGTA	5.0	97.3	90.1	97.0
1,10-phenanthloline	0.5	98.5	100	100
PMSF	0.5	< 5.0	< 5.0	9.3
Phosphoramidon	0.5	7.7	6.5	5.4
Leupeptin	0.5	5.8	7.1	< 5.0
Antipain	0.5	13.0	19.7	21.9
E-64	0.5	8.5	29.6	13.3
Pepstatin A	0.5	39.4	31.8	28.1
BS-10	0.5	98.8	95.1	91.8

prodomain, which has a cysteine residue masking the zinc-binding site in catalytic domain (Van wart and Birkedal-Hansen, 1990). It is well known that zinc metalloproteinases such as MMPs and SVMPs possessing HExGHxxGxxHS motif and HExGHxxGxxHD motif, respectively, in their molecule (Hooper, 1994), are able to bind to BS-10 through a cysteine switch mechanism (Fig. 1). Indeed, the BS-10 column possessed a high affinity to HExGHxxGxxH motif through the cysteine switch mechanism with essential zinc ion and was available for purifying zinc metalloproteinases. As expected, the 38 kDa metalloproteinase could be purified by the column switch HPLC system using the BS-10 conjugating affinity column with higher recovery (Fig. 2). These results demonstrate that the 38 kDa metalloproteinase possesses HExGHxxGxxH sequence in its molecule which has been shown to be conserved in many reprolysins and MMPs.

The N-terminal amino acid sequence of the 38 kDa metalloproteinase shared a high homology to that of *Xenopus* MMP-9 in comparison with those of the human MMP, ADAM and SVMP (Table 1). The predicted molecular mass of MMP-9 identified from humans (Murphy and Cockett, 1995), chickens (Hahn-Dantona *et al.*, 2000) and *Xenopus* (Carinato *et al.*, 2000) was 92.kDa, 75 kDa and 75 kDa, respectively, and has been shown to consist of signal peptide domain, propeptide domain, catalytic domain, fibronectin type-II -like domain, hinge and hemopexin-like domain in its molecule. Judging from the molecular size, the 38 kDa metalloproteinase seemed to consist of catalytic domain only, and might be produced from latent pro-form and activated by a similar machanism as a proposition for many MMPs. The hemopexin-like domain of MMPs is known to play an important role in the recognition of substrate in tissue. If the 38 kDa metalloproteinase resulted from its precursor protein consisting of propeptide domain, catalytic domain, hinge and hemopexin-like domain similar to the structure of MMP-9 after removing the hemopexin-like domain by autodigestion during the preparation, a corresponding protein with larger molecular mass than 38 kDa should be detected in the fresh Yamakagashi venom. However, no protein with larger or smaller molecular mass than 38 kDa was detected to express proteolytic activity in the fresh Yamakagashi venom. In addition to this observation, the properties of the purified 38 kDa metalloproteinase were almost the same as that in crude venom of *Rhabdophis. t. tigrinus*, suggesting that the 38 kDa metallproteinase exists from the first.

The biochemical characteristics of the 38 kDa metalloproteinase obtained by gelatin zymography, pH dependency, metal requirements and responsibility to EDTA and EGTA were strongly similar to those of well-known MMPs. In addition, the substrate specificity and responsibility to various proteinase inhibitors of this 38 kDa metalloproteinase are also similar to the well-known MMPs

Based on the results obtained in the present study, we concluded that the 38 kDa metalloproteinase was a novel metalloproteinase whose proteolytic activity may be regulated by a cysteine switch mechanism and could be classified as one of the matrix metalloproteinases rather than snake venom metalloproteinases.

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