does not appear to be a factor explaining the difference between humans and rats.

Gastrectomy causes a decrease in plasma ghrelin levels (10). In agreement with this previous finding, plasma total ghrelin levels were lower in the patients than in normal subjects in the present study, suggesting that additional sources of ghrelin production are not sufficient to maintain plasma ghrelin levels. It is difficult to exclude the possibility that low plasma ghrelin level may have affected the GH response to ghrelin.

The results of Date *et al.* (12) in rats would indirectly suggest that the low dose of ghrelin utilized in these vagotomized patients may in part be a reason for concluding that ghrelin administration did not release GH via the vagal nerve pathway. It is still unclear why the GH response to ghrelin differed between vagotomized humans and rats. However, intravenously administered ghrelin seems to stimulate GH release by its direct action on the hypothalamus/pituitary region rather than via vagal afferent nerve in humans, at least under the present experimental conditions.

In summary, intravenous administration of ghrelin caused marked GH response in patients with vagotomy as well as in normal subjects. Under the present experimental conditions, the involvement of the afferent vagal nerve in ghrelin-induced GH secretion was not confirmed in humans.

Acknowledgements

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Ghrelin mRNA and GH secretagogue receptor mRNA in human GH-producing pituitary adenomas is affected by mutations in the α subunit of G protein

Kyongsong Kim*†, Naoko Sanno*, Keiko Arai†, Koji Takano‡, Junko Yasufuku-Takano‡, Akira Teramoto* and Tamotsu Shibasaki†

*Departments of *Neurosurgery and †Physiology, Nippon Medical School and ‡Department of Nephrology and Endocrinology, University of Tokyo, Tokyo, Japan*

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Summary

OBJECTIVE Ghrelin and its receptor, growth hormone secretagogue (GHS) receptor (GHSR), are expressed in the normal pituitary gland and various types of pituitary adenoma. Somatic mutations in the subunit of Gs α protein (*gsp*), which led to a constitutive activation of adenylyl cyclase, are reported in GH-producing pituitary adenomas. We analysed the relationship between ghrelin mRNA and GHSR mRNA expression levels in *gsp* mutation-positive and -negative GH-producing pituitary adenomas.

PATIENTS Pituitary adenoma tissue was obtained at surgery from 20 patients with acromegaly.

METHODS The expression levels of human ghrelin mRNA and GHSR mRNA were quantified using a competitive RT–PCR method. To detect the *gsp* mutations, amplified Gs α subunit cDNA fragments were sequenced directly using RT–PCR method.

RESULTS There was no significant difference in the expression of ghrelin mRNA between mutation-positive and -negative adenomas. The expression of GHSR mRNA was significantly lower in *gsp* mutation-positive than -negative adenomas. There was a significant negative correlation between the levels of ghrelin mRNA and GHSR mRNA expression in mutation-negative adenomas; no such correlation was found in mutation-positive adenomas.

CONCLUSION These results suggest that GHSR mRNA expression is downregulated by ghrelin in *gsp* mutation-negative GH-producing pituitary adenomas, and that changes in intracellular signalling pathways in *gsp* mutation-positive GH-producing pituitary adenomas affect the expression of G protein-coupled receptors such as GHSR. The absence of negative correlation between ghrelin and GHSR expression might be induced by lowered GHSR expression in *gsp* mutation-positive GH-producing adenomas.

Correspondence: Kyongsong Kim, Department of Neurosurgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo, 113-8602 Japan. Tel.: + 81-3-3822-2131; Fax: + 81-3-5685-0986; E-mail: kyongson@nms.ac.jp

Ghrelin, an endogenous ligand specific for growth hormone secretagogue (GHS) receptor (GHSR) that stimulates the secretion of GH *in vitro* and *in vivo*, has been isolated from rat stomach and characterized as an acylated peptide of 28 amino acids (Kojima *et al.*, 1999). Different levels of ghrelin mRNA and GHSR mRNA are expressed in the normal pituitary gland and in various types of pituitary adenoma (Kim *et al.*, 2001; Korbonits *et al.*, 2001a).

Somatic mutations of the Gs α (*gsp*), localized at amino acid residues 201 and 227 which are critical sites for the intrinsic guanosine triphosphatase activity of the protein, leads to constitutive activation of adenylyl cyclase and has been found in human GH-producing pituitary adenomas (Vallar *et al.*, 1987; Landis *et al.*, 1989). The frequency of *gsp* mutations in GH-producing pituitary adenomas is approximately 40% (Landis *et al.*, 1990). Clinical and biological characteristics of patients bearing *gsp* mutation-positive pituitary GH-producing adenomas are high adenylyl cyclase activity in the GH-producing adenomas, low GH response to GH-releasing hormone (GHRH), and a high paradoxical response of GH to thyrotropin-releasing hormone (TRH) (Landis *et al.*, 1990; Spada *et al.*, 1990; Harris *et al.*, 1992; Adams *et al.*, 1993, 1995; Fagia *et al.*, 1996; Yang *et al.*, 1996). The expression of GHSR in the normal rat pituitary is downregulated by GHS (Kineman *et al.*, 1999). However, there is no significant correlation between the expression of ghrelin mRNA and GHSR mRNA in human GH-producing pituitary adenomas (Kim *et al.*, 2001; Korbonits *et al.*, 2001a). In these reports, the *gsp* mutation status of the adenomas was not addressed; therefore, in the present study, we first examined the adenomas for the presence of *gsp* mutations. We then quantified the expression levels of ghrelin mRNA and GHSR mRNA and analysed their relationship in both *gsp* mutation-positive and -negative GH-producing pituitary adenomas to examine the

influence of mutation-induced abnormal signal transduction on their expression in GH-producing pituitary adenomas.

Materials and methods

Patients

Informed prior consent was obtained from all 20 acromegalic patients (14 women and six men; mean ± SD age, 49·5 ± 2·3 years; range 26–64 years) enrolled in this study. Several days before surgery, blood was drawn and plasma samples were frozen and kept at −20 °C until enzyme immunoassays for GH, TSH, PRL, FSH and LH, and radioimmunoassays for adrenocorticotropin (ACTH) and IGF-I. Pituitary adenoma tissue was obtained during transsphenoidal surgery. All samples were frozen immediately at −80 °C until RNA extraction. Tumours were examined by immunohistochemical staining for ACTH, GH, PRL, FSH-β, LH-β, TSH-β and α-subunit. The sizes of the pituitary tumours were determined by measuring their maximum diameter using magnetic resonance imaging (MRI). The clinical characteristics of the patients and the histological characteristics of the adenomas are summarized in Table 1.

RNA preparation and RT–PCR analysis

Total RNA was extracted from adenoma tissue within 2 months of surgery and first-strand cDNA was synthesized from 5 μg of total RNA by reverse transcription after removing contaminating genomic DNA. To amplify the human ghrelin cDNA fragment (385 bp) we designed a sense primer (g5 = 5′-CCGGATCCCT-GAACACCAGAGAGTCCAG-3′), and an antisense primer (g3 = 5′-CCGAATTCCTCCTGAGCTTGTACAACAG-3′). The PCR method used to amplify ghrelin cDNA consisted of initial denaturation at 95 °C for 4 min, followed by 38 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s (an adequate number of cycles to detect the target cDNA), and a final extension at 72 °C for 4 min. To amplify the human GHSR-Ia cDNA fragment (442 bp) we also designed a sense primer (G5 = 5′-CCGGATC-CCTGGTCATCTTCGTCATCTG-3′) and an antisense primer (G3 = 5′-CCGAATTCAGTACTGGCTGATCTGAGCA-3′). The PCR procedure was as described above except that the annealing temperature was 58 °C. PCR products were electrophoresed through 2% agarose gels, stained with ethidium bromide and the density of bands was analysed using a fluorescent image analyser (FLA-3000; Fuji Photo Film Co. Ltd, Tokyo, Japan). To confirm that the PCR products corresponded to the cDNA fragment of ghrelin or GHSR-Ia, they were sequenced directly as previously described (Kim *et al.*, 2001). The integrity of mRNA from each specimen was verified by RT–PCR for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as previously described (Kim *et al.*, 2001). We designed a sense primer according to the nucleotide sequence 482–503 (5′-ACAACAGCCTCAAGATCATCAG-3′), and an antisense

Table 1 Summary of the clinical characteristics of the patients and their adenomas and the level of ghrelin and GHSR-Ia mRNA expression in GH-producing pituitary adenomas

Patient no.	Tumour size (mm)	IGF-I (μg/l)	GH (μg/l)	*gsp* mutation	ghrelin mRNA ($\times 10^{-2}$ attomol/μg total RNA)	GHSR mRNA ($\times 10^{-2}$ attomol/μg total RNA)
1	9	424	8·3	Arg 201 Cys	28·03	ND
2	16	430	157·6	–	17·35	3·71
3	35	1300	116·3	Arg 201 Cys	15·63	3·23
4	16	910	74·6	–	12·04	0·27
5	8	820	8·7	–	11·96	0·77
6	9	760	19·8	–	10·17	6·55
7	16	1150	29	Arg 201 Cys	6·79	0·48
8	12	1100	29·8	Arg 201 Cys	6·64	1·22
9	21	310	7·5	–	4·25	9·70
10	12	1244	49·9	Gln 227 Leu	4·20	4·65
11	20	750	193	Arg 201 Cys	4·12	1·87
12	12	930	35·6	–	3·61	9·89
13	38	650	629	–	2·83	8·00
14	11	420	11·5	Gln 227 Leu	2·76	1·76
15	30	960	8·7	–	2·71	3·48
16	26	660	129	–	2·51	9·99
17	18	630	51·6	Arg 201 Cys	2·48	ND
18	8	630	6·1	Arg 201 Cys	2·46	4·00
19	11	685	36·5	Arg 201 Cys	1·59	2·05
20	40	950	396	Arg 201 Hys	0·93	4·17

–, undetected *gsp* mutation; ND, not determined.

primer according to the nucleotide sequence 774–793 (5′-GGTCCACCACTGACACGTTG-3′) of the human GAPDH cDNA (GenBank accession no. M33197). The PCR reaction used to amplify the cDNA fragment of GAPDH was performed in the same way as described above, except that the temperature for annealing was changed to 57 °C and the cycles for amplification were changed to 20 cycles. The density of bands was analysed using a fluorescent image analyser. The density of the amplified GAPDH mRNA fragments were compared each other, and the concentrations of first-strand cDNA were fine-tuned.

Quantification of the level of ghrelin mRNA and GHSR-Ia mRNA

To quantify the PCR products corresponding to the cDNA fragment of ghrelin, we synthesized a competitor of ghrelin that was 77 bp shorter than the target cDNA fragment. This competitor was added to the PCR reaction mixture at concentrations of 0·001, 0·01, 0·1, 1 and 10 attomol/μg total RNA. We found that 23, 28, and 32 cycles were suitable for specific amplification of the ghrelin cDNA fragment under the above conditions in the linear range of 0·01, 0·1 and 1 attomol/μg total RNA for both competitor and target cDNA. For quantification of ghrelin mRNA levels in pituitary adenoma tissue, we first used 1 μl of the fine-tuned first cDNA solution and 1 μl of competitor solution at the serial dilutions described above for 28 PCR cycles. To quantify ghrelin mRNA levels, the density of the amplified ghrelin mRNA fragment was compared to that of the competitor, and the concentration of the target cDNA was then estimated from the density most similar to that of the competitor. Next, to quantify the concentration of the target fragment of ghrelin mRNA more exactly, we amplified 5 × twofold serial dilutions of the competitor under the conditions described above, and then quantified the concentration of ghrelin mRNA.

To quantify the PCR products corresponding to the cDNA fragment of GHSR-Ia, we synthesized a competitor of GHSR-Ia that was 96 bp shorter than the target cDNA fragment, and added this competitor to the PCR reaction mixture at concentrations of 0·001, 0·01, 0·1, 1 and 10 attomol/μg total RNA. We found that 23, 28 and 32 cycles were suitable for specific amplification of the GHSR-Ia cDNA fragment under the above conditions in the linear range of 0·01, 0·1 and 1 attomol/μg total RNA for both competitor and target cDNA fragments. For quantification of GHSR-Ia mRNA levels in the pituitary adenoma tissues, PCR reactions were performed using the methods described above; the annealing temperature was 58 °C.

Detection of Gsα subunit mutations in tumour cDNA

To amplify the specific Gs α subunit cDNA fragment (249 bp) including codons 201 and 227 of the Gs α gene, we designed a sense primer corresponding to nucleotide sequence 562–585 (Gsp5 = 5′-GTGTATCAAGCAGGCTGACTATGTG-3′), and an antisense primer corresponding to nucleotide sequence 793–819 (Gsp3 = 5′-CAGGCGGTTGTTCTGGTT-3′; Harris, 1988). Using these primers, the PCR reaction was performed to amplify first-strand cDNA as described above, except that the temperature for annealing was changed to 60 °C for 40 cycles. The PCR products were sequenced directly using the above method.

Statistical analysis

Data are expressed as the mean ± SEM. Data were subjected to analysis of variance (ANOVA) following Fisher's PLSD test. Correlations were analysed by simple linear regression analysis. Statistical significance was established at the $P < 0·05$ level.

Results

Detection of Gsα subunit mutations in tumour cDNA

A CGT to TGT mutation (Arg to Cys) was detected in eight of 20 adenomas and a CGT to CAT mutation (Arg to His) in one of 20 adenomas at codon 201. A CAG to CTG mutation (Gln to Leu) was detected in two of 20 adenomas at codon 227.

There was no significant difference in tumour size, or serum levels of GH or IGF-I between GH-producing adenomas with and without *gsp* mutations.

Expression of ghrelin mRNA and GHSR-Ia mRNA

RT–PCR detected ghrelin mRNA and GHSR-Ia mRNA in all GH-producing pituitary adenoma samples examined (Fig. 1). The size of all PCR products was as expected. Some PCR products from GH-producing pituitary adenoma samples were directly sequenced; they corresponded to the partial fragment of ghrelin cDNA and GHSR-Ia cDNA.

Quantification of the level of ghrelin mRNA expression

PCR amplification was performed after reverse transcription with the competitor at the concentrations shown in Fig. 2(a). Further PCR amplification was performed with the competitor in twofold serial dilutions at five points (Fig. 2b). As shown in Table 1, the mean level of ghrelin mRNA expression in mutation-positive adenomas was $6·88 \pm 2·45 \times 10^{-2}$ attomol/μg total RNA ($n = 11$); in adenomas without *gsp* mutations it was $7·45 \pm 1·83 \times 10^{-2}$ attomol/μg total RNA ($n = 9$). Although expression tended to be lower in *gsp*-positive adenomas than in mutation-negative adenomas, the difference was not statistically significant ($P = 0·848$; Fig. 3).

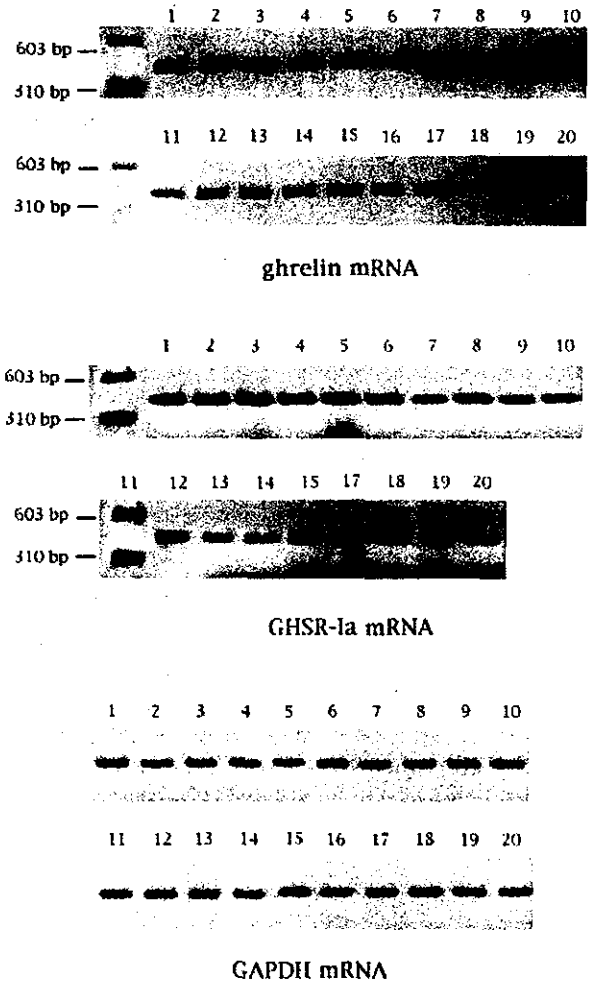

Fig. 1 RT–PCR detected ghrelin mRNA and GHSR mRNA in all GH-producing pituitary adenomas.

Quantification of the level of GHSR-1a mRNA expression

GHSR-Ia mRNA expression was quantified using the same two-step competitive PCR we used for the quantification of ghrelin mRNA. As shown in Table 1, the mean concentration of GHSR-Ia mRNA in mutation-positive adenomas ($2{\cdot}13 \pm 0{\cdot}51 \times 10^{-2}$ attomol/μg total RNA; $n = 9$) was significantly lower than in mutation-negative adenomas ($5{\cdot}82 \pm 1{\cdot}29 \times 10^{-2}$ attomol/μg total RNA; $n = 9$, $P < 0{\cdot}05$; Fig. 4).

Comparison of the expression levels of ghrelin mRNA and GHSR-1a mRNA

There was no significant correlation between the expression levels of ghrelin mRNA and GHSR-Ia mRNA in *gsp* mutation-positive adenomas ($R = 0{\cdot}125$, $P = 0{\cdot}748$; Fig. 5). On the other hand, there was a significant negative correlation between the levels of ghrelin mRNA and GHSR-Ia mRNA expression in *gsp* mutation-negative adenomas ($R = -0{\cdot}675$, $P = 0{\cdot}046$; Fig. 6).

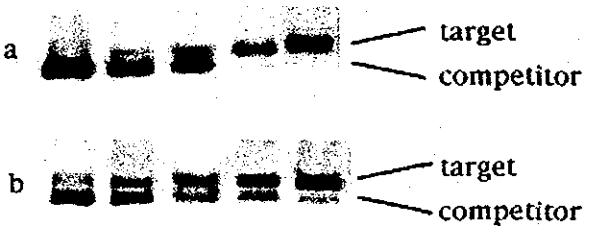

Fig. 2 Competitive RT–PCR analysis of ghrelin mRNA expression in GH-producing pituitary adenoma (G-3 in Fig. 1, upper lane) and of competitor (lower lane). (a) Quantitative competitive RT–PCR. First-strand cDNA and 1 μl of serial dilutions of competitor (0·001, 0·01, 0·1, 1·0 and 10 attomol/μg total RNA) were subjected to 23 PCR cycles. GHRHR PCR products were competed out by the competitor at concentrations ranging between 1 and 0·1 attomol/μg total RNA, allowing the concentration of ghrelin cDNA to be calculated. (b) Further quantitative competitive RT–PCR. The competitor was diluted twofold five times, and 23-cycle PCR amplification was performed. The ghrelin PCR products were competed out by the competitor at concentrations between the second and third dilution and the concentration of ghrelin cDNA was calculated.

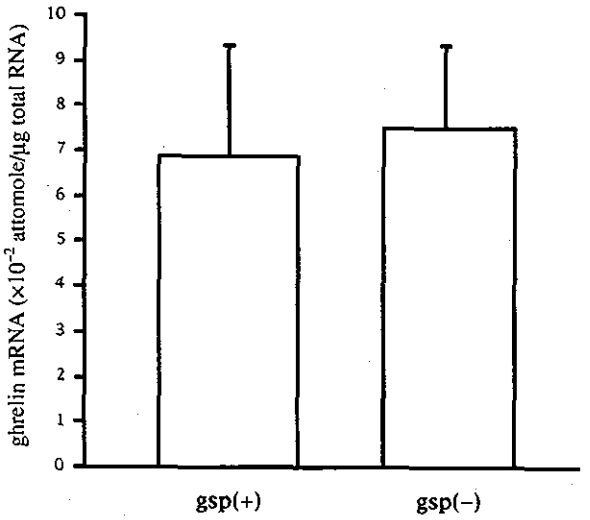

Fig. 3 Mean levels of ghrelin mRNA in GH-producing pituitary adenomas with and without *gsp* mutations. There was no significant difference between the two groups ($P = 0{\cdot}848$).

Discussion

Gsp mutations such as Arg201 to Cys, His, or Ser, and of Gln227 to Arg or Lys, are seen in GH-producing pituitary adenomas

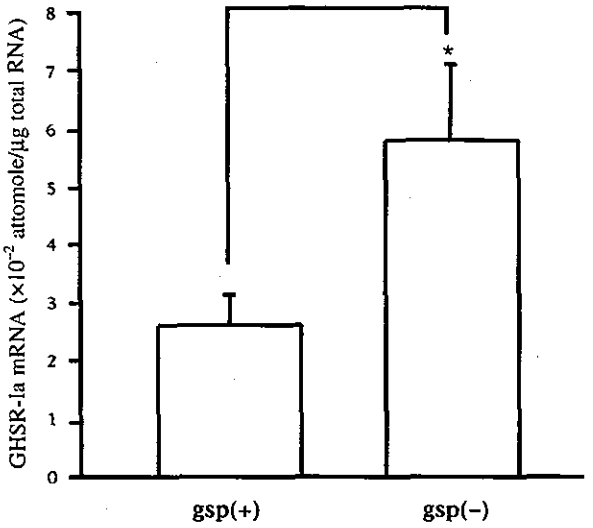

Fig. 4 Mean levels of GHSR-Ia mRNA in GH-producing pituitary adenoma with and without *gsp* mutations. The mean expression level in the *gsp*-positive adenomas was significantly lower than in the *gsp*-negative adenomas ($P < 0{\cdot}05$).

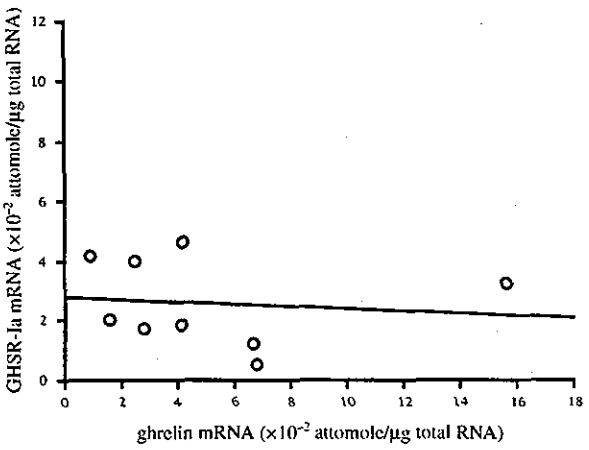

Fig. 5 Simple linear regression analysis of the correlation in the expression of ghrelin mRNA and GHSR-Ia mRNA in GH-producing adenomas with *gsp*-mutation. There was no significant correlation.

(Landis *et al.*, 1990; Yang *et al.*, 1996). The mutated G protein inhibits GTPase activity and keeps the adenylyl cyclase system in a continuous turned-on state, mimicking the stimulatory effect of GHRH on the cell membrane signal transduction system (Landis *et al.*, 1989). We detected a CGT to TGT mutation (Arg to Cys) in eight and a CGT to CAT mutation (Arg to His) in one of 20 adenomas examined at a codon 201; a CAG to CTG mutation (Gln to Leu) was detected at codon 227 in two of 20

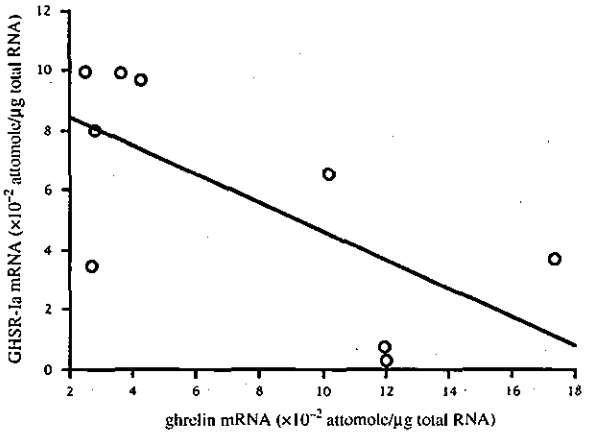

Fig. 6 Simple linear regression analysis of the correlation in the expression of ghrelin mRNA and GHSR-Ia mRNA in GH-producing adenomas without *gsp*-mutation. There was a significant negative correlation ($R = 0{\cdot}675$, $P = 0{\cdot}046$).

adenomas. There was no significant difference with respect to age, tumour size, or serum levels of GH or IGF-I in our acromegalic patients with or without *gsp* mutations; confirming the findings of others (Landis *et al.*, 1990; Spada *et al.*, 1990; Adams *et al.*, 1993; Faglia *et al.*, 1996). The mean levels of ghrelin mRNA expression did not differ between *gsp*-positive and -negative adenomas. It has been reported that ghrelin mRNA expression between *gsp* mutation-positive and -negative GH-producing adenomas was not significantly different, although the level of ghrelin mRNA expression of *gsp* mutation-positive adenomas tended to lower than that of *gsp* mutation-negative adenomas (Korbonits *et al.*, 2001b). This suggests that *gsp* mutations, which led to the activation of adenylyl cyclase, do not affect the levels of ghrelin mRNA expression in the adenoma cells.

GHSR belongs to the G protein-coupled receptor family and consists of seven transmembrane domains (Howard *et al.*, 1996). It mediates the effects of GHS through the activation of the inositol phospholipid-calcium protein kinase C (IP3-PKC) transduction pathway, whereas GH-releasing hormone receptor (GHRHR) activates cAMP-dependent mechanisms (Mayo, 1992; Hanew *et al.*, 1998). Two subtypes of human GHSR have been cloned (Howard *et al.*, 1996). GHSR-Ia binds and responds to GHS, whereas GHSR-Ib, 77 amino acids shorter than GHSR-Ia, is biologically inactive (Howard *et al.*, 1996). Both types of receptor are expressed in GH-producing pituitary adenomas (Kim *et al.*, 2001; Korbonits *et al.*, 2001a). In the present study, we quantified the levels of GHSR-Ia mRNA expression and found that they were significantly lower in *gsp*-positive adenomas than in *gsp*-negative adenomas. It was reported that the expression of GHSR-Ia in *gsp* mutation-positive adenomas tended to be lower than that of *gsp* mutation-negative adenomas

(Korbonits *et al.*, 2001b). The authors suggested that the lack of statistically significance might be due to the small sample size. These result seems to be incompatible with the report by Kineman *et al.* (1999) that pituitary GHSR is upregulated in normal rats after 4-h infusion of GHRH as in *gsp* mutation-positive adenomas, the signal transduction system is in a continuous turned-on state of GHRH action. They also found that this GHRH-induced upregulation of GHSR expression was not affected by somatostatin antiserum, and that the upregulation occurred when GHRH was infused into spontaneous dwarf rats that lack GH. Their results suggest that GHRH-induced up-regulation of GHSR synthesis is independent of somatostatin or circulating GH. Furthermore, GHRH had no significant effect on pituitary GHSR mRNA levels *in vitro* (Kineman *et al.*, 1999). Therefore, we suggest that factors besides GHRH also play a role in regulating the levels of GHSR mRNA in GH-producing pituitary adenomas.

The GHSR promoter does not contain any putative cAMP response element(s) for binding the transcription factor cAMP response element-binding protein which could transduce the signalling cascade induced by GHRH to the GHSR promoter (Petersenn *et al.*, 2001). In addition, the activity of the GHSR promoter in GH4 rat pituitary cells, which lack endogenous GHRH receptor, is not influenced by forskolin, an activator of protein kinase A (Petersenn *et al.*, 2001). This suggests that factors other than cAMP response element-binding protein are required for GHRH to affect GHSR mRNA. We found a significant negative correlation between the expression of ghrelin mRNA and of GHSR-1a mRNA in human GH-producing pituitary adenomas without *gsp* mutations. No such correlation was detected in *gsp* mutation-positive adenomas. These observations suggest that GHSR in *gsp* mutation-negative adenomas is downregulated through autocrine/paracrine mechanisms by ghrelin synthesized in adenoma cells. Our hypothesis is supported by the finding that GHSR mRNA in the normal rat pituitary was decreased by a 4-h infusion of L-692 585, a GHS (Kineman *et al.*, 1999). Further analyses of large numbers of GH-producing adenomas will be required to confirm this issue.

On the other hand, in *gsp*-positive adenomas with constitutive activation of adenylyl cyclase, the expression of GHSR-1a was suppressed (Vallar *et al.*, 1987). The elevated activity of adenylyl cyclase induced by *gsp* mutations is not further stimulated by GHRH, as *in vivo* and *in vitro* GH responses to GHRH are abolished in mutation-positive GH-producing adenomas (Landis *et al.*, 1990; Spada *et al.*, 1990; Adams *et al.*, 1993, 1995; Yang *et al.*, 1996). GHS stimulates cAMP production and the IP3-PKC pathway in *gsp*-positive adenomas; only the IP3-PKC pathway is stimulated by GHS in *gsp*-negative adenomas (Adams *et al.*, 1996). Therefore, the function of GHSR may be modified by intracellular changes such as increased adenylyl cyclase activity induced by *gsp* mutations.

In summary, there was no significant difference in the expression of ghrelin mRNA between *gsp* mutation-positive and -negative GH-producing pituitary adenomas. The expression of GHSR mRNA was significantly lower in *gsp* mutation-positive than -negative adenomas. There was a significant negative correlation between the expression of ghrelin mRNA and GHSR mRNA in the *gsp* mutation-negative adenomas and no such correlation was present in *gsp* mutation-positive adenomas. These results suggest that GHSR mRNA expression is downregulated by ghrelin in *gsp* mutation-negative GH-producing pituitary adenomas, and that changes in intracellular signalling pathways in *gsp*-positive GH-producing pituitary adenomas induce inhibitory effects on the expression of G protein-coupled receptors such as GHSR.

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Plasma Levels of Intact and Degraded Ghrelin and Their Responses to Glucose Infusion in Anorexia Nervosa

MARI HOTTA, RINA OHWADA, HIDEKI KATAKAMI, TAMOTSU SHIBASAKI, NAOMI HIZUKA, AND KAZUE TAKANO

Health Services Center (M.H.), National Graduate Institute for Policy Studies, 162-8677 Tokyo, Japan; Department of Medicine (R.O., N.H., K.T.), Institute of Clinical Endocrinology, Tokyo Women's Medical University, School of Medicine, 162-8666 Tokyo, Japan; Department of Medicine (H.K.), Miyazaki University, School of Medicine, 889-1692 Miyazaki, Japan; and Department of Physiology (T.S.), Nippon Medical School, 113-8602 Tokyo, Japan

Octanoylated ghrelin (1–28) (intact ghrelin) is rapidly and easily degraded to desoctanoyl forms or smaller fragments (degraded ghrelin). Plasma levels of intact and degraded ghrelin were examined in 30 patients with anorexia nervosa (AN) (body mass index, 8.81–22.4 kg/m^2) and 16 age-matched healthy women using several assay methods. Plasma levels of ghrelin measured using immunocomplex transfer-enzyme immunoassay, which specifically detects intact ghrelin, were lower in AN than controls. Plasma ghrelin levels in AN measured using the active ghrelin ELISA kit, which is advertised as specifically detecting intact ghrelin, did not differ significantly from controls. Plasma levels of desoctanoyl ghrelin using the desacyl-ghrelin ELISA kit, N-terminus ghrelin using the ghrelin active RIA kit, and C-terminus ghrelin using the ghrelin total RIA kit were significantly higher in AN than controls, and displayed significant negative correlations with body mass index. Plasma levels of ghrelin determined using immunocomplex transfer-enzyme immunoassay or active ghrelin ELISA during iv glucose infusion were suppressed in both AN and controls, whereas plasma levels of degraded ghrelin levels were not significantly decreased in AN. Plasma levels of intact ghrelin are therefore not higher in AN than controls, whereas degraded forms of ghrelin are elevated in AN. Rapid suppression of plasma intact ghrelin, but not degraded ghrelin, occurs in AN in response to glucose infusion. The profiles of intact and degraded forms of ghrelin in plasma of AN patients differ from those of healthy women. (*J Clin Endocrinol Metab* 89: 5707–5712, 2004)

GHRELIN, AN ENDOGENOUS ligand for GH secretagogue receptor, exerts potent stimulatory effects on food intake and GH secretion (1). Ghrelin also decreases fat utilization, causing increased energy storage. Comprising 28 amino acid residues with an *n*-octanoyl ester at Ser^3 (intact ghrelin) that is necessary for bioactivity (1), intact ghrelin is quite unstable and rapidly degraded to inactive desoctanoyl form or smaller fragments (degraded ghrelin) (2).

Glucose load and food intake lead to rapid falls in plasma concentrations of ghrelin in normal or obese subjects, indicating that plasma ghrelin levels reflect the acute feeding state and short-term energy balance (3, 4). Plasma ghrelin levels in patients with anorexia nervosa (AN) with poor nutritional status and elevated plasma GH levels are reportedly significantly higher than those in healthy women. Such data support the hypothesis that elevated ghrelin secretion reflects energy demand and stimulates GH secretion in AN. However, antibodies for measuring plasma ghrelin levels in previous studies have been against C-terminus ghrelin (13–28) (3, 5, 6), ghrelin (1–11) (7) or full-length ghrelin with or without octanoylation at Ser^3 (8–11). Concentrations of intact ghrelin actually represent less than 10% of total circulating ghrelin levels, which include acylated and desacylated ghrelin immunoreactivities (12). Plasma levels of total ghrelin, but not intact ghrelin, are significantly correlated with renal function (12). Chronic dehydration and reduced renal flow due to decreased food intake or vomiting induce decreased renal clearance of creatinine and other metabolites in AN. Plasma levels of intact and degraded ghrelin seem likely to be profoundly affected by reduced renal function in AN.

To determine differences in plasma levels of intact and degraded ghrelin between AN and healthy women, plasma levels of intact, desoctanoyl, N-terminus, and C-terminus ghrelin were investigated using a novel immunocomplex transfer-enzyme immunoassay (ICT-EIA), and four other commercially available ELISA and RIA kits. Response of plasma ghrelin levels to acute iv infusion of glucose was also investigated to clarify how the suppressive action of acute elevation of plasma glucose levels on ghrelin secretion is modified in AN.

Subjects and Methods

Subjects

Subjects in the present study comprised 30 Japanese female amenorrheic AN patients with mean age of 24 ± 3 yr (mean ± SD) (range 17–33.2 yr) and mean body mass index (BMI) of 15.54 ± 2.62 kg/m^2 (range 8.81–22.42 kg/m^2). Patients met the Diagnostic and Statistical Manual IV criteria for AN (13), in addition to the criteria put forth by the Survey Committee for Eating Disorders of the Japanese Ministry of Health, Labor, and Welfare (14). Of the 30 patients, 21 had restricting AN, whereas the remaining nine had binge-eating/purging AN. Control samples were obtained from 16 healthy, age-matched women (mean age 25.6 ± 2.1 yr; range 23–30 yr) with mean BMI 20.28 ± 1.93 kg/m^2 (range 18.36–25.39 kg/m^2). The effects of glucose infusion on plasma ghrelin

Abbreviations: AN, Anorexia nervosa; BMI, body mass index; ICT-EIA, immunocomplex transfer-enzyme immunoassay.

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levels were investigated in six healthy controls (age 23–29 yr; BMI, 18.4–21.5 kg/m^2) and six AN patients (age 21–33 yr; BMI 10.2–14.0 kg/m^2) from among the above subjects. Two AN patients were identified as *Helicobacter pylori* positive.

The study protocol was approved by the institutional review board of Tokyo Women's Medical University. All patients and controls provided written informed consent to participate in this study.

Methods

At 0800 h after overnight fasting longer than 12 h, blood was taken from subjects and transferred into tubes with 1 mg/ml EDTA-2Na and 500 U/ml aprotinin. Blood samples were immediately centrifuged at 4 C. Plasma samples were then acidified with 1 normal HCl and stored at −80 C until assay.

Assay methods and antibodies used in the present study are shown in Fig. 1. Describing briefly ICT-EIA, this assay is designed to measure intact human ghrelin based on the principle of a two-site sandwich ELISA. ICT-EIA uses two different polyclonal antibodies raised against Cys^{12} ghrelin (1–11) and Cys^{29} ghrelin (1–28). In ICT-EIA, an immunocomplex comprising ghrelin, antibody raised against Cys^{29} ghrelin (1–28) bound to 2, 4-dinitrophenol and biotin, and another antibody raised against Cys^{12} ghrelin (1–11) bound to β-D-galactosidase is attached to the first solid phase via anti-2, 4-dinitrophenol Ig, and immunocomplexes are then transferred to the second solid phase coated with streptavidin to reduce nonspecific binding. ICT-EIA specifically detects intact human ghrelin (15) but does not detect shorter fragments such as ghrelin (1–14), ghrelin (1–18), ghrelin (13–28), or desoctanoyl ghrelin at all. No significant cross-reactivities with murine ghrelin, motilin, or other brain-gut peptides are seen. Minimum detectable concentration of ghrelin is 1 pg/ml (0.34 pmol/liter), and 10 μl of plasma sample are used for measuring without any extraction procedure (15). The active ghrelin ELISA kit (Mitsubishi Kagaku Iatron, Tokyo, Japan) uses a combination of two monoclonal antibodies raised against ghrelin (1–10) and ghrelin (13–28) and is reported to specifically detect intact human or murine ghrelin (16). Plasma levels of desoctanoyl ghrelin were measured using the desoacyl-ghrelin ELISA kit (Mitsubishi Kagaku Iatron), which uses a combination of two antibodies raised against desoctanoyl ghrelin (1–10) and ghrelin (13–28) (16). Plasma levels of N-terminus and C-terminus ghrelin were measured using the ghrelin active RIA and the ghrelin total RIA kits (Linco Research, St. Charles, MO), respectively. The antibody used in the ghrelin active RIA kit recognizes intact ghrelin and octanoyl ghrelin (1–10) but not desoctanoyl ghrelin, whereas the antibody used in the ghrelin total RIA kit recognizes intact and desoctanoyl ghrelin and ghrelin (14–28). Immunoradiometric assays were used to measure levels of plasma GH (Eiken Chemical Co., Tokyo, Japan) and serum IGF-I (Daiichi Pharmaceutical Co., Tokyo, Japan). Plasma insulin measurements were performed using an ELISA kit (Eiken).

To investigate the effect of glucose infusion on plasma ghrelin levels, subjects were administered iv 500 ml of either 10% glucose solution (50 g glucose, 200 cal) or saline for 2 h from 0800 h. Blood was taken before and at 1 and 2 h after infusion. The six healthy women ate meals totaling 1500–2300 cal/d before testing. The six AN patients who participated in this study had an oral intake of food of no more than an estimated 800 cal/d and consequently required hospitalization and glucose infusion therapy to prevent further deterioration of nutritional state. These patients had been receiving 500 ml infusion for a minimum of 1 wk before testing, and no medication had been initiated. Both healthy controls and AN patients had slept for a minimum of 5 h and had not performed any exercise the day before the testing.

Statistics

Data are expressed as mean ± SEM. Comparisons between groups were performed using unpaired *t* tests. Changes in plasma ghrelin levels after fasting and glucose infusion were compared using paired *t* tests. Correlations between plasma ghrelin levels and other hormones were analyzed using Spearman's ranked correlations.

Results

Plasma levels of ghrelin in controls and AN patients are shown in Table 1. Mean plasma level of ghrelin measured using ICT-EIA was significantly lower in AN patients than controls [49.2 ± 2.9 (mean ± SEM) *vs.* 65.0 ± 4.9 pmol/liter, $P = 0.043$; range 25.0–83.9 *vs.* 35.3–111.0 pmol/liter]. Mean plasma level of ghrelin as determined by active ghrelin ELISA tended to be slightly higher in AN patients than controls (34.7 ± 3.2 *vs.* 29.9 ± 3.1 pmol/liter), but no significant difference was identified ($P = 0.267$). Mean plasma level of desoctanoyl ghrelin using the desacyl-ghrelin ELISA kit was significantly higher in AN patients than controls (223.5 ± 37.3 *vs.* 94.1 ± 7.5 pmol/liter, $P < 0.0001$). The ratio

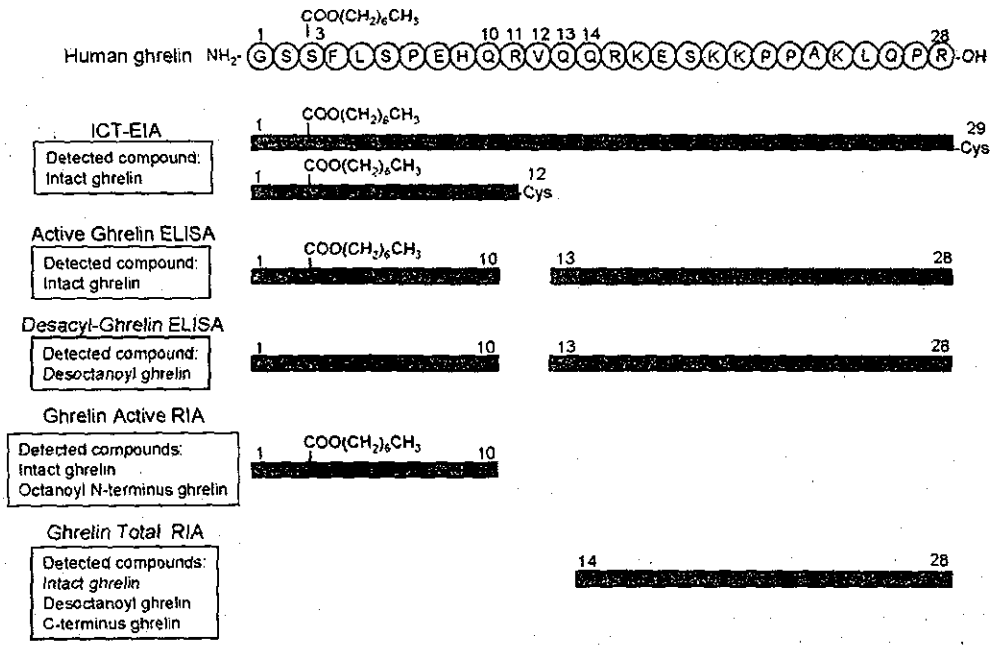

FIG. 1. Schema of intact ghrelin and its fragments against which the antibodies for the five assays are raised.

of values from desacyl-ghrelin ELISA to those from active ghrelin ELISA was significantly higher in AN patients than controls (6.14 ± 0.44 *vs.* 3.34 ± 0.24, $P < 0.0001$). Plasma levels of N-terminus ghrelin measured using the ghrelin active RIA kit and C-terminus ghrelin measured using the ghrelin total RIA kit were also significantly higher in AN patients than controls ($P = 0.048$ and $P < 0.0001$, respectively).

Correlations between plasma levels of ghrelin using ICT-EIA and the active ghrelin ELISA kit, both of which are considered to specifically detect intact ghrelin, are shown in Fig. 2. Plasma levels of ghrelin as measured using ICT-EIA were significantly and highly correlated with values from active ghrelin ELISA in both controls ($r = 0.876$, $P = 0.001$) and AN patients ($r = 0.796$, $P < 0.0001$). The ratio of values from active ghrelin ELISA to those from ICT-EIA was significantly higher in AN patients than controls (0.70 ± 0.04 *vs.* 0.47 ± 0.02, $P < 0.0001$). In controls, plasma levels of ghrelin measured using both ICT-EIA and the active ghrelin ELISA kit were significantly correlated with plasma levels determined using the ghrelin active RIA kit, which detects N-terminus ghrelin including intact ghrelin in addition to octanoyl ghrelin (1–10) ($r = 0.792$, $P = 0.002$; and $r = 720$, $P = 0.005$, respectively). No significant correlations were found between plasma levels of intact ghrelin measured using ICT-EIA or the active ghrelin ELISA kit and other degraded ghrelin levels in controls or AN patients (data not shown).

Relationships between BMI and plasma levels of ghrelin in controls and AN patients are presented in Figs. 3 and 4. No significant correlations were observed between BMI and plasma levels of ghrelin using ICT-EIA or the active ghrelin ELISA kit (Fig. 3). BMI was significantly correlated with plasma levels measured using the ghrelin active RIA kit ($r = -0.391$, $P = 0.009$), the ghrelin total RIA kit ($r = -0.641$, $P < 0.0001$), the desacyl-ghrelin ELISA kit ($r = -0.693$, $P < 0.0001$), and the ratio of values from desacyl-ghrelin ELISA to those from active ghrelin ELISA ($r = -0.737$, $P < 0.0001$) (Fig. 4).

Mean values of plasma GH were 2.99 ± 1.03 μg/liter in controls, and 13.96 ± 4.44 μg/liter in AN patients. Mean levels of serum IGF-I were 285.9 ± 16.5 μg/liter in controls and 103.1 ± 20.3 μg/liter in AN patients. Plasma levels of intact ghrelin measured using ICT-EIA or the active ghrelin ELISA kit in AN patients displayed no significant correlations with plasma GH or serum IGF-I levels.

Glucose infusion in six controls and six AN patients resulted in increased plasma glucose levels (controls: from 92.3 ± 2.3 to 182.0 ± 15.1 mg/dl; AN: from 68.7 ± 6.5 to 227.0 ± 29.7 mg/dl), and plasma ghrelin levels as determined by ICT-EIA promptly decreased in both groups (controls: 58.8 ± 3.3% *vs.* AN: 63.2 ± 9.8% of the basal levels, $P = 0.206$), whereas plasma levels of ghrelin and glucose during saline infusion were not significantly changed in either group (Fig. 5). Changes in plasma ghrelin level displayed no correlation with plasma insulin levels ($r = -0.121$, $P = 0.556$). After glucose infusion in controls, plasma levels of ghrelin as determined using the active ghrelin ELISA kit, desoctanoyl ghrelin using the desacyl-ghrelin ELISA kit, and N-terminus ghrelin using the ghrelin active RIA kit were all significantly decreased, whereas plasma levels of C-terminus ghrelin measured using the ghrelin total RIA kit showed a nonsignificant tendency to decrease, compared with levels after saline infusion. In AN patients, plasma levels of ghrelin measured by the active ghrelin ELISA kit decreased significantly after glucose infusion. Conversely, plasma levels of degraded ghrelin such as desoctanoyl ghrelin using the desacyl-ghrelin ELISA kit, N-terminus ghrelin using the ghrelin active RIA kit, and C-terminus ghrelin using the ghrelin total RIA kit displayed no significant changes after glucose infusion in AN patients.

TABLE 1. Plasma levels of ghrelin in controls and AN

	Controls	AN
n	16	30
ICT-EIA (pmol/liter)	65.0 ± 4.9	49.2 ± 2.9[a]
Active ghrelin ELISA (pmol/liter)	29.9 ± 3.1	34.7 ± 3.2
Desacyl-ghrelin ELISA (pmol/liter)	94.1 ± 7.5	223.5 ± 37.3[a]
Ratio of desacyl to active ghrelin ELISA	3.34 ± 0.24	6.14 ± 0.44[a]
Ghrelin active RIA (pmol/liter)	104.1 ± 9.5	136.7 ± 12.9[a]
Ghrelin total RIA (nmol/liter)	1.85 ± 0.13	2.87 ± 0.25[a]
Ratio of ghrelin total to active RIA	19.9 ± 2.1	21.9 ± 1.2

Data are expressed as mean ± SEM.
[a] $P < 0.05$ compared to values of controls.

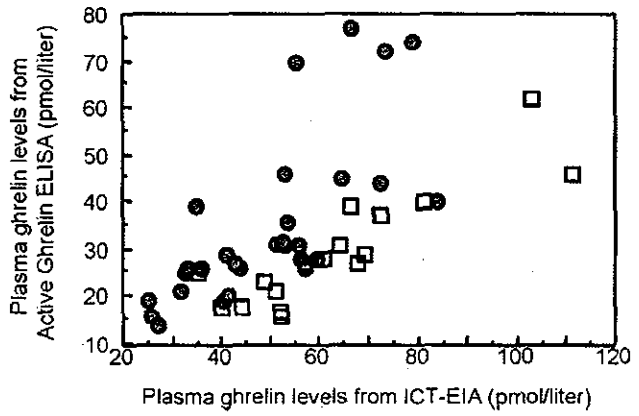

FIG. 2. Relationship between plasma ghrelin levels from ICT-EIA and the active ghrelin ELISA kit. Plasma ghrelin levels from ICT-EIA were significantly and highly correlated with values from the active ghrelin ELISA kit in healthy women ($r = 0.876$, $P = 0.0007$), AN patients ($r = 0.796$, $P < 0.0001$), and all subjects ($r = 0.749$, $P < 0.0001$). *Open squares* and *closed circles* represent healthy women and AN, respectively.

Discussion

The present results demonstrated that mean plasma levels of ghrelin in AN patients obtained using ICT-EIA or the active ghrelin ELISA kit, which is reported to specifically recognize intact ghrelin, vary from lower than to similar to levels in healthy women, and degraded forms of ghrelin such as desoctanoyl ghrelin, octanoyl N-terminus ghrelin, and C-terminus ghrelin are elevated in AN patients. These findings for degraded ghrelin are consistent with previous reports showing that plasma levels of ghrelin are higher in AN patients than in healthy women (3, 5–11), in which the antibodies used detect C-terminus ghrelin (13–28) (3, 5, 6) or full-length ghrelin including intact and desoctanoyl ghrelin

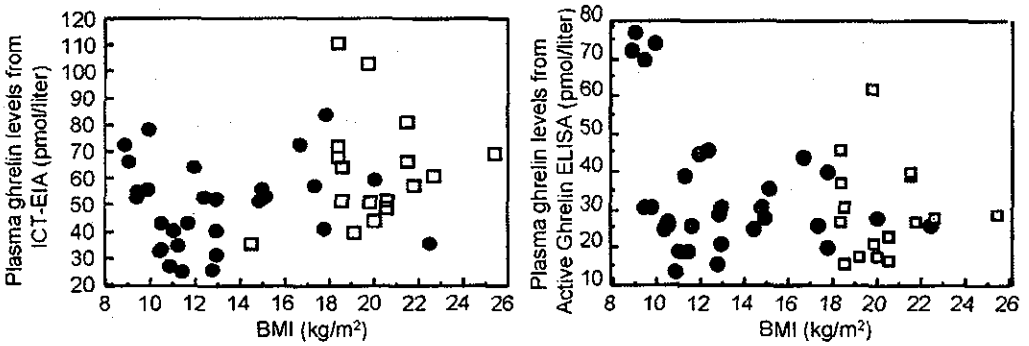

FIG. 3. Relationship between BMI and plasma ghrelin levels from ICT-EIA or the active ghrelin ELISA kit. No significant correlation existed between BMI and plasma ghrelin levels measured using ICT-EIA (*left panel*) or the active ghrelin ELISA kit (*right panel*). *Open squares* and *closed circles* represent healthy women and AN, respectively.

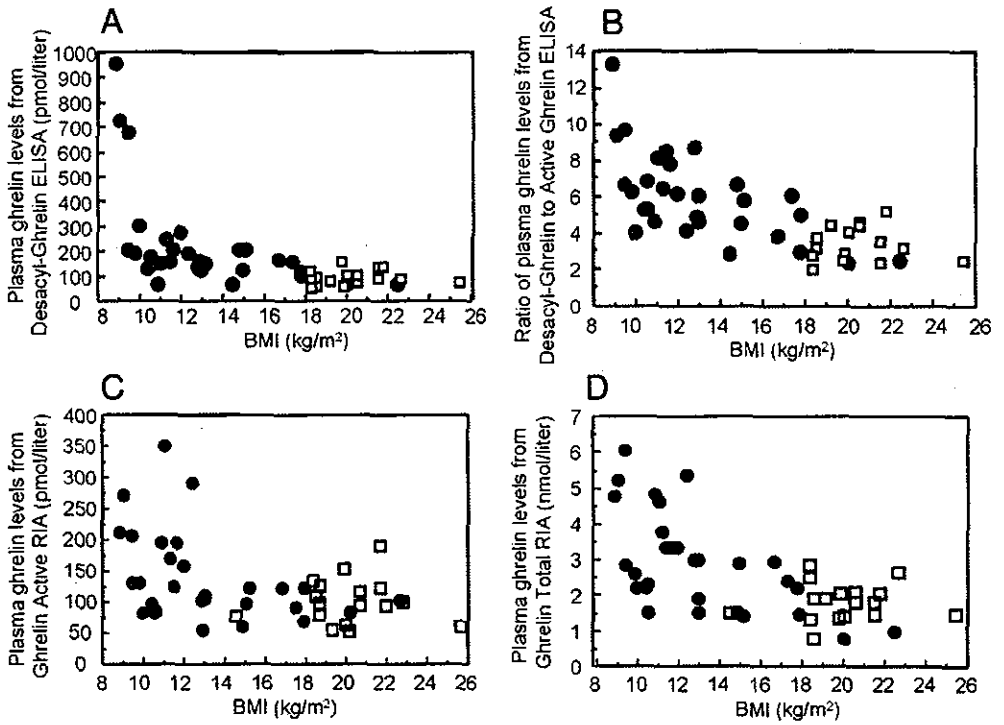

FIG. 4. Relationship between BMI and plasma ghrelin levels from the desacyl-ghrelin ELISA, the ghrelin active RIA, or the ghrelin total RIA kits. BMI was significantly correlated with plasma ghrelin levels from the desacyl-ghrelin ELISA kit ($r = -0.693$, $P < 0.0001$) (A), ghrelin active RIA kit ($r = -0.391$, $P = 0.0087$) (C), and ghrelin total RIA kit ($r = -0.641$, $P < 0.0001$) (D), and the ratio of values from desacyl-ghrelin ELISA to those from active ghrelin ELISA ($r = -0.737$, $P < 0.0001$) (B). *Open squares* and *closed circles* represent healthy women and AN, respectively.

(8–11). Evaluation of plasma ghrelin levels thus depends on the specificity of the ghrelin antibody. Of note is the fact that plasma levels measured by the desacyl-ghrelin ELISA kit and the ratio of values from desacyl-ghrelin ELISA to those from active ghrelin ELISA were much higher in AN patients than controls. These results indicate that the profiles of intact and degraded forms of ghrelin in plasma of AN patients differ from those of healthy women.

The present results also show that no correlation exists between BMI and ghrelin as measured by ICT-EIA or the active ghrelin ELISA kit in AN patients and controls. In contrast, plasma levels measured using the desacyl-ghrelin ELISA, ghrelin active RIA, and ghrelin total RIA kits, and the ratio of values from desacyl-ghrelin ELISA to those from active ghrelin ELISA all displayed negative correlations with BMI in AN patients and controls. The kidney represents an important site for the clearance and/or degradation of ghrelin (12). In patients with end-stage renal disease, plasma levels of C-terminus ghrelin are significantly correlated with serum creatinine levels (12). Elevated plasma levels of C-terminus ghrelin have recently been demonstrated in lung cancer cachexia (17), cardiac cachexia (18), and hepatic cachexia (19) with deterioration of the clinical status as determined by signs such as ascites or reduced renal clearance. AN is also usually complicated by dehydration, reduced glomerular filtration rate, and decreased creatinine clearance (20). Elevation of plasma levels of degraded ghrelin such as desoctanoyl, octanoyl N-terminus, and C-terminus ghrelin in

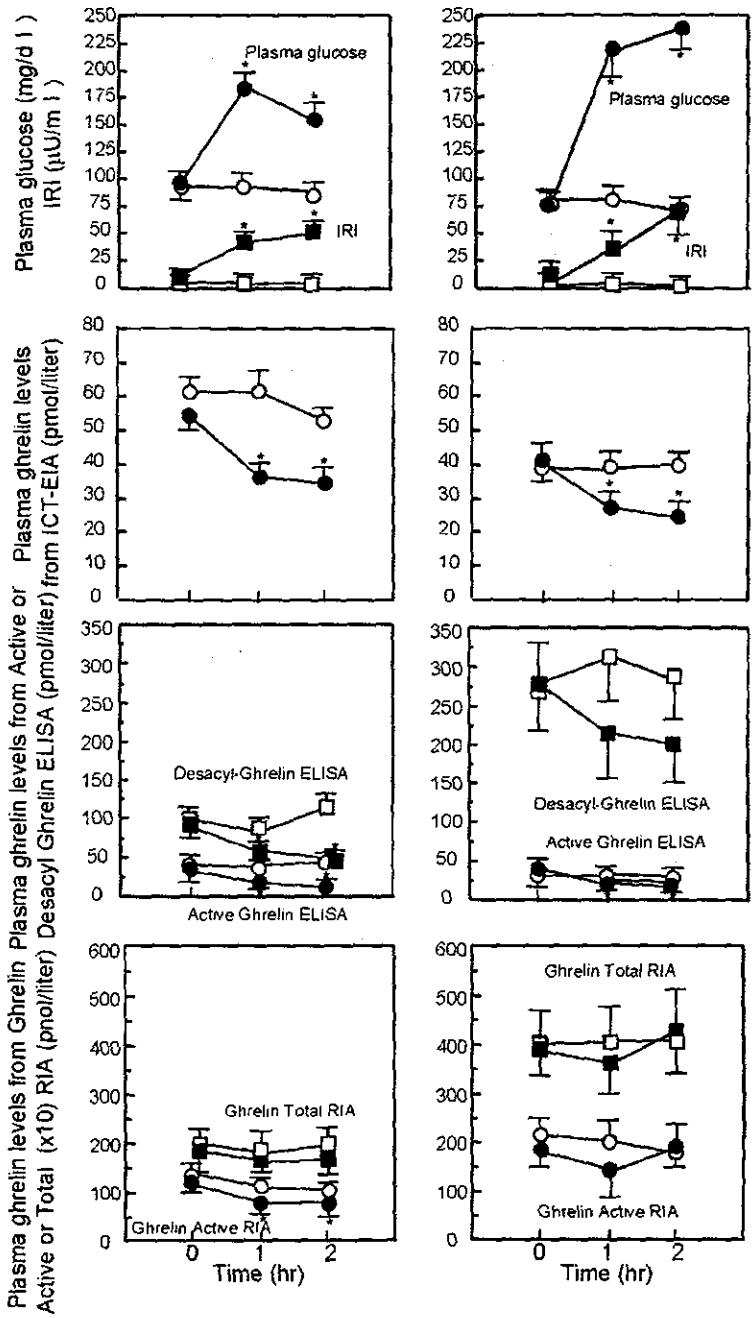

FIG. 5. Effects of iv glucose (●, ■) or saline (○, □) on plasma ghrelin, glucose, and insulin levels in six healthy women (*left panel*) and six AN patients (*right panel*). Plasma ghrelin levels measured using ICT-EIA during glucose infusion were suppressed to 58.8 ± 3.3% of basal level in healthy women and 63.2 ± 9.8% of basal level in AN patients, whereas plasma levels of ghrelin and glucose during saline infusion did not change significantly in either group. In controls, plasma ghrelin levels (except for C-terminus ghrelin as measured by the ghrelin total RIA kit) were decreased during glucose infusion. In AN patients, plasma ghrelin levels as determined by the active ghrelin ELISA kit during iv glucose infusion were suppressed as in controls, whereas plasma levels of desoctanoyl, N-terminus, and C-terminus ghrelin showed no significant decreases following increased plasma glucose levels. For conversion from metric units in the figure to SI units, divide by 18 to get millimoles per liter for glucose, and multiply by 7.241 to get picomoles per liter for insulin. Data are expressed as mean ± SEM. *, $P < 0.05$, compared with values on saline infusion.

AN patients may therefore result from decreased clearance related to the decreased BMI.

One question raised by the present study is why plasma levels of intact ghrelin were not increased in AN patients? Plasma ghrelin levels reportedly increase in asymptomatic subjects after cure of *H. pylori* infection (21). One recent study reported that gastric banding procedure strongly suppresses plasma ghrelin levels (including intact and desoctanoyl ghrelin) despite a massive and permanent reduction in body weight (22) as gastric banding reduces plasma levels of motilin. These results suggest that injury of the gastric mucosa or impaired gastric peristalsis could induce decreased ghrelin secretion. Chronic malnutrition induces both functional and organic changes in the stomach (23, 24), and atrophy of the gastric mucosa and alteration of peristalsis are observed in AN (25, 26) and would thus seem likely to reduce ghrelin secretion. Ghrelin secretion might be stimulated by overnight fasting as potently in AN patients as in healthy women, but ghrelin production would depend on the viability of ghrelin-secreting cells in the stomach in AN patients.

Intravenous infusion of 50 g glucose or oral administration of 75 g glucose suppresses secretion of C-terminus ghrelin in healthy subjects (5, 27). However, the effects of oral administration of a meal or 75 g glucose on the plasma levels of

ghrelin remain contentious in AN patients (7, 11). Gastric excretion time is delayed in AN (23–26), and changes in plasma glucose levels and insulin secretion are extremely variable after oral glucose tolerance testing or food eating in AN (28). We therefore selected the glucose infusion test to investigate the effects of hyperglycemia on plasma ghrelin levels. The suppressive effect of glucose infusion on plasma levels of intact ghrelin as determined by ICT-EIA or the active ghrelin ELISA kit was almost identical in AN patients and controls. These findings suggest that rapid suppression of plasma intact ghrelin levels in response to glucose infusion is preserved in AN. Although plasma ghrelin levels measured using antibody against ghrelin (1–11) are reportedly suppressed to about 50% of basal levels in AN during oral administration of 75 g glucose (7), the present study did not find any significant suppression of plasma levels as determined by the desacyl-ghrelin ELISA or the ghrelin active RIA kits after glucose infusion. These results may suggest that acute elevation of plasma glucose inhibits secretion of intact ghrelin from the stomach and that the substantial increase in fragments of degraded ghrelin in plasma partly due to renal dysfunction would mask the response of ghrelin as measured using kits based on antibodies for desoctanoyl ghrelin or N-terminus ghrelin in AN patients for the present study.

In conclusion, the profiles of intact and degraded forms of ghrelin in plasma differ between AN patients and healthy women. The suppressive effect of glucose infusion on intact ghrelin secretion is preserved in AN patients.

Acknowledgments

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Address all correspondence and requests for reprints to: Mari Hotta, M.D., Health Services Center, National Graduate Institute for Policy Studies, 2-2 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8677, Japan. E-mail: marihs@grips.ac.jp.

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P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• CLINICAL RESEARCH •

Impact of endoscopically minimal involvement on IL-8 mRNA expression in esophageal mucosa of patients with non-erosive reflux disease

Yusei Kanazawa, Hajime Isomoto, Chun Yang Wen, Ai-Ping Wang, Vladimir A Saenko, Akira Ohtsuru, Fuminao Takeshima, Katsuhisa Omagari, Yohei Mizuta, Ikuo Murata, Shunichi Yamashita, Shigeru Kohno

Yusei Kanazawa, Hajime Isomoto, Ai-Ping Wang, Fuminao Takeshima, Katsuhisa Omagari, Yohei Mizuta, Shigeru Kohno, Second Department of Internal Medicine, Atomic Bomb Disease Institute, Nagasaki University School of Medicine, Nagasaki, Japan
Chun Yang Wen, Department of Molecular Pathology, Atomic Bomb Disease Institute, Nagasaki University School of Medicine, Nagasaki, Japan
Vladimir A Saenko, Akira Ohtsuru, Shunichi Yamashita, Department of Molecular Medicine, Atomic Bomb Disease Institute, Nagasaki University School of Medicine, Nagasaki, Japan
Ikuo Murata, Department of Pharmacotherapeutics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan
Correspondence to: Hajime Isomoto, M.D., Second Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. hajimei2002@yahoo.co.jp
Telephone: +81-95-849-7567 **Fax:** +81-95-849-7568
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Abstract

AIM: Little has been known about the pathogenesis of non-erosive reflux disease (NERD). Recent studies have implicated interleukin 8 (IL-8) in the development and progression of gastroesophgeal reflux disease (GERD). The purpose of this study was to determine IL-8 RNA expression levels in NERD patients with or without subtle mucosal changes.

METHODS: We studied 26 patients with NERD and 13 asymptomatic controls. Biopsy sample was taken from the esophagus 3 cm above the gastroesophageal junction and snap frozen for measurement of IL-8 mRNA levels by real-time quantitative polymerase chain reaction (PCR). We also examined mRNA expression of IL-8 receptors, CXCR-1 and -2 by reverse transcriptase PCR. The patients were endoscopically classified into grade M (mucosal color changes without visible mucosal break) and N (neither minimal involvement nor mucosal break) of the modified Los Angeles classification.

RESULTS: The relative IL-8 mRNA expression levels were significantly higher in esophageal mucosa of NERD patients than those of the controls. There was a significant difference in IL-8 mRNA levels between grade M and N. The CXCR-1 and -2 mRNAs were constitutively expressed in esophageal mucosa.

CONCLUSION: Our results suggest that high IL-8 levels in esophageal mucosa may be involved in the pathogenesis of NERD through interaction with its receptors. NERD seems to be composed of a heterogeneous population in terms of not only endoscopically minimal involvement but also immune and inflammatory processes.

Kanazawa Y, Isomoto H, Wen CY, Wang AP, Saenko VA, Ohtsuru A, Takeshima F, Omagari K, Mizuta Y, Murata I, Yamashita S, Kohno S. Impact of endoscopically minimal involvement on IL-8 mRNA expression in esophageal mucosa of patients with non-erosive reflux disease. *World J Gastroenterol* 2003; 9(12): 2801-2804
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INTRODUCTION

Gastroesophageal reflux disease (GERD) is one of the most common chronic disorders in modern humans. In the United States, 44 % of the adult populations reported experiencing heartburn at least once a month, 14 % on a weekly basis, and 7 % daily[1]. Esophageal erosions are the characteristic lesions of GERD seen on endoscopy[2]. A small number of GERD patients develop stricture, Barrett's esophagus and adenocarcinoma of the esophagus. In fact, the majority of GERD patients have endoscopically normal-appearing esophageal mucosa; this group is termed non-erosive reflux disease (NERD) or endoscopy-negative reflux disease[2,3].

The Los Angeles (LA) classification is widely used for endoscopic assessment of GERD[4]. The slightest degree of esophagitis, i.e., grade A, is defined as one or more mucosal breaks confined to the mucosal folds, each no longer than 5 mm. Accordingly, this classification scheme ignores subtle mucosal damage in the absence of mucosal breaks. In this regard, Hoshihara *et al*.[5,6] have proposed a modified LA system, in which grade O was subdivided into M and N, based on the concept of mucosal color changes. Thus, NERD patients can be classified into two subgroups (grade M and N) based on minimal esophageal involvement during endoscopy.

Recently, several studies have shown that mucosal immune and inflammatory responses, characterized by specific cytokine and chemokine profiles, may determine the diversity of esophageal phenotypes of GERD[7-9]. Of note, Fitzgerald *et al*.[7] reported significantly higher expression levels of interleukin 8 (IL-8) messenger ribonucleic acid (mRNA) in patients with reflux esophagitis (RE), assessed by competitive reverse transcriptase polymerase chain reaction (RT-PCR), compared with subjects with non-inflamed or Barrett' s esophagus. Studies from our laboratories have also demonstrated high IL-8 protein levels in esophageal biopsy samples of patients with erosive esophagitis by enzyme linked immunosorbent assay (ELISA)[8]. Furthermore, we also showed significantly high mucosal IL-8 production, which paralleled the endoscopic severity of RE[8]. However, little has been known about the role of IL-8 in NERD.

The aim of the present study was to assess esophageal expression levels of IL-8 mRNA in NERD patients by quantitative real-time PCR procedure, with special reference to the difference between grade M and N subgroups of the modified LA scheme.

MATERIALS AND METHODS

Subjects and samples

We studied 26 patients with NERD and endoscopically confirmed normal-appearing esophageal mucosa who visited

the Outpatient Department between August 2002 and July 2003. They included 19 men and 7 women, aged between 28 and 80 years (mean, 62.0 years). The diagnosis of GERD was made with more than 6 points in the questionnaire for the diagnosis of reflux disease (QUEST) described by Carlsson *et al*[10]. None of these patients had been treated with non-steroidal anti-inflammatory drugs, proton pump inhibitors, histamine H_2-receptor antagonists, anti-cholinergic agents or antibiotics within 4 weeks prior to the present study. Furthermore, patients with severe concomitant diseases, prior esophageal or gastric surgery, peptic ulcer diseases and comorbid conditions that might interfere with esophageal or gastric motility including diabetes mellitus, systemic sclerosis and neurological disorders were excluded. As a control group, we recruited 13 asymptomatic subjects with no hiatal hernia or any lesions in the esophagus, stomach and duodenum at endoscopy for a health check-up.

In each case, a biopsy specimen was obtained from the esophageal mucosa, 3 cm above the gastroesophageal junction[8], snap-frozen in an ethanol-dry ice mixture for quantitative analysis of IL-8 mRNA expression and stored at -80 ℃ until use.

Endoscopic assessment of NERD

NERD was endoscopically classified into grade M and N in accordance with the modified Los Angeles (LA) classification system proposed by Hoshihara *et al*[5,6]. The criteria were: grade M represents minimal changes (irregular redness or whiteness) without any mucosal breaks and grade N represents esophageal mucosa with neither the minimal changes nor mucosal injury. In addition, we also evaluated the presence of hiatal hernia by endoscopy[11].

Real-time quantitative PCR

Total RNA from the biopsy samples was extracted using a commercial kit according to the instructions provided by the supplier (Isogen, Nippon Gene Co., Toyama, Japan). One μg of total RNA was reversely transcribed into complementary DNA (cDNA) in a volume of 25 μl with MuLV reverse transcriptase and random hexamers (both from PE Applied Biosystems, Warrington, UK).

Real-time PCR measurement of IL-8 cDNA was performed in the ABI PRISM 7700 sequence detector (PE Applied Biosystems) with TaqMan assay. The primers and probe sequences for IL-8 were synthesized (PE Applied Biosystems) as described previously[12]: IL-8 forward primer, 5'-CTCTTGGCAGCCTTCCTGATT-3', reverse primer, 5'-TATGCACTGACATCTAAGTTCTTTAGCA-3' and probe, 5'-CTTGGCAAAACTGCACCTTCACACAGA-3', labeled with the reporter dye 6-carboxyfluorescein at the 5' end and quencher dye 6-carboxytetramethylrhodamine at the 3' end. PCR was performed in a total volume of 50 μl of each amplification mixture containing 1 μl of each RT product, 25 μl of 2×Universal Master Mix (PE Applied Biosystems), 200 nM IL-8 forward and reverse primers, 100 nM fluorogenic probe. Thermal cycling was initiated with at 50 ℃ for 2 min, followed by a first denaturation step at 95 ℃ for 10 min, and followed by 50 cycles of at 95 ℃ for 15 s and at 60 ℃ for 1 min.

The *tubulin alpha 3* gene cDNA (internal control) was quantified in the same machinery using SYBR Green PCR Core reagents kit (PE Applied Biosystems). The primers used were: forward, 5'-AGATCATTGACCTCGTGTTGGA-3' and reverse, 5'-ACCAGTTCCCCCACCAAAG-3', which correspond to nucleotides 437-458 and 537-519, respectively (*TUBA3*, GenBank accession number 17986282). PCR was performed in a total volume of 25 μl of each amplification mixture containing 1 μl of each RT product, 3 μl of 25 mM $MgCl_2$, 2.5 μl of 10×SYBR Green buffer, 2 μl of dNTP Mix (5 mM adenosine, deoxycytosine and deoxyguanosine triphosphate and 2.5 mM deoxyuridine triphosphate), 0.625 U AmpliTaq Gold polymerase, 0.125 U AmpErase and 100 nM tubulin alpha 3 forward and reverse primers. Thermal cycling was initiated at 50 ℃ for 2 min, followed by a first denaturation step at 95 ℃ for 10 min, and continued with 40 cycles of at 95 ℃ for 15 s and at 59 ℃ for 1 min.

Each assay included a standard curve, a no-template control and cDNA samples in triplicate. The standard curve was generated by serial 5-fold dilutions of pooled cDNA obtained from gastric tissues that were found to contain high levels of mRNAs of both genes. Contents of the tubulin alpha 3 and IL-8 cDNAs were expressed in arbitrary units calculated according to the standard curve. The relative expression level of IL-8 was expressed as the ratio of IL-8/tubulin alpha 3 in arbitrary units[13].

RT-PCR

Based on the technique described previously[14] with slight modification, the target sequence of CXCR-1 mRNA was amplified through 35 cycles, each consisting of denaturation at 94 ℃ for 30 sec, annealing at 53 ℃ for 30 sec and extension at 72 ℃ for 30 min, followed by a final extension at 72 ℃ for 5 min with specific primers (forward, 5'-CAGATCCACAGA-TGTGGGAT-3' and reverse, 5'-TCCAGCCATTCACCTTG-GAG-3') using an RT-PCR kit (Takara Shuzo Co., Otsu, Japan). Similarly, CXCR-2 mRNA expression was detected under the following conditions: amplification through 35 cycles, each consisting of denaturation at 94 ℃ for30 sec, annealing at 60 ℃ for 30 sec and extension at 72 ℃ for 30 min, followed by a final extension at 72 ℃ for 5 min with specific primers (forward, 5'-AGCTGCTCTTCTGGAGGTGT-3' and reverse, 5'-TTAGAGAGTAGTGGAAGTGTGC-3')[14]. A 10-μl aliquot of each PCR product was analyzed by electrophoresis on 2 % agarose gel containing ethidium bromide, and the bands were examined under ultraviolet light for the presence of amplified DNA. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene transcript was also amplified as described previously[15], and used as an internal control of the processed RNA for each preparation.

Detection of Helicobacter pylori infection

H pylori status was assessed by serology (anti-*H pylori* Immunoglobulin G antibody, HEL-p TEST, Amrad Co., Melbourne, Australia), rapid urease test (Helicocheck, Otsuka Pharmaceutical Co., Tokushima, Japan) and histopathology (hematoxylin-eosin and Giemsa staining) using additional biopsy specimens obtained during endoscopy from the antrum within 2 cm of the pyloric ring and the corpus along the greater curvature. Patients were considered positive for *H pylori* infection when at least two of these examinations yielded positive results. On the other hand, patients were defined as *H pylori*-negative if all the test results were negative[16].

All the samples were obtained with written informed consent of the patients prior to their inclusion in this study, in accordance with the Helsinki Declaration.

Statistical analysis

Statistical analyses were performed using Fisher's exact, χ^2, Student's *t*, Mann-Whitney U, and Kruskal-Wallis tests, whenever appropriate. A *P* value of less than 0.05 was accepted as statistically significant. Data are expressed as mean ± standard deviation (SD).

RESULTS

Patient demographics

According to the modified LA system, 14 patients were classified as grade M and 12 as grade N. There were no

significant differences in age, gender, current tobacco use, alcohol intake, body mass index, the presence of hiatus hernia and *H pylori* status among the patients with grade M and N and the controls (Table 1). None had such complications as stricture, bleeding and columnar-lined esophagus. The overall incidence of *H pylori* infection in our series was 51.3 %.

Table 1 Baseline characterisitics of the enrolled subjects

	Control group *n*=13	Nonerosive reflux disease group	
		Grade M[a] *n*=14	Grade N[a] *n*=12
Mean age, yr, (range)	61.6(39-80)	58.7(28-75)	62.5 (33-75)
Male/female	8/5	11/3	8/4
Smoker	53.8 %(7/13)	28.6 %(4/14)	33.3 %(4/12)
Alcohol drinker	46.2 %(6/13)	57.1 %(8/14)	33.3 %(4/12)
Hiatal hernia	0 %(0/13)	35.7 %(5/14)	50.0 %(6/12)
H pylori infection	53.8 %(7/13)	57.1 %(8/14)	41.7 %(5/12)

[a]According to the modified Los Angeles system.

Relative expression levels of IL-8

We confirmed that both RT-PCR procedures for IL-8 and tubulin alpha 3 yielded 87- and 101-base pair (bp) specific bands, respectively (data not shown). As a whole, NERD patients had significantly higher expression levels of IL-8 than the controls (Figure 1, $P<0.05$). The expression levels of IL-8 in esophageal mucosa of grade M patients with NERD were significantly higher than those of grade N patients ($P<0.05$, Figure 2). In addition, the expression levels of IL-8 were higher in grade M than control group ($P<0.01$, Figure 2), but not significantly different between NERD-grade N and control group.

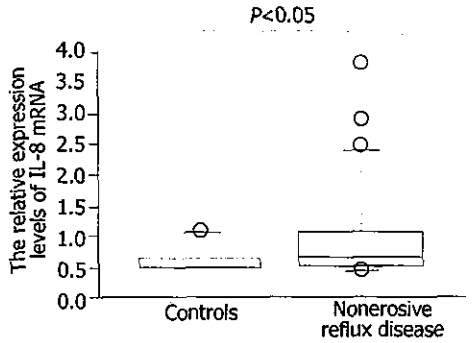

Figure 1 Relative interleukin 8 (IL-8) mRNA expression levels assessed by real-time quantitative polymerase chain reaction in patients with non-erosive reflux disease (NERD) and asymptomatic controls.

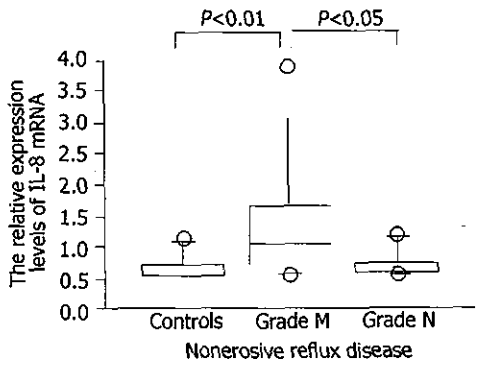

Figure 2 The expression levels of IL-8 assessed by real-time quantitative polymerase chain reaction in patients with non-erosive reflux disease (NERD) of grade M and N and asymptomatic controls.

Expression of CXCR-1 and CXCR-2 genes in esophageal mucosa

We identified the *CXCR-1* and *-2* gene-specific products as 257- and 1 154-bp bands, respectively, by RT-PCR (Figure 3). These mRNAs were constitutively expressed in each subject examined, irrespective of the presence of GERD-related symptoms or endoscopic grading. We confirmed that RT-PCR procedures for *G3PDH* housekeeping gene expression yielded 983 bp specific bands (data not shown).

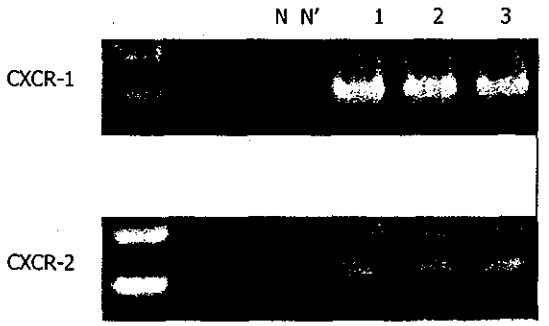

Figure 3 CXCR-1 and -2 mRNA transcripts were detected as 257 and 1 154 base pair (bp) bands with reverse transcription-polymerase chain reaction, respectively. Lane N; negative control, lane N'; RT (-) negative control, lane 1; asymptomatic normal control, lane 2; patient with NERD grade M, lane 3; patient with NERD grade N.

DISCUSSION

Several lines of evidence indicated that a prototype of CXC chemokine, IL-8, played a crucial role in the development and progression of erosive esophagitis[7,8]. In the present study, we focused on the implication of this potent inflammatory mediator in NERD. We found significantly higher expression levels of IL-8 mRNA in esophageal mucosa of patients with NERD than those in asymptomatic controls, suggesting that IL-8 is implicated in the pathogenesis of this incipient form of GERD.

The striking finding of this study was that NERD patients classified as grade M subgroup based on the modified LA system had significantly higher expression levels of IL-8 mRNA compared to those of grade N. IL-8 mediated the recruitment of neutrophils into sites of inflammation[17]. In addition, this potent chemoattractant acted on neutrophils to respiratory burst and release of a variety of reactive oxygen species (ROS), leading to tissue damage[18]. In our previous work employing ELISA, we found a significant association between the presence of intraepithelial neutrophils and increased IL-8 levels in esophageal mucosa of patients with RE[13]. Although we did not perform histopathological evaluation in the current study, elevated mucosal IL-8 expression may be involved even in such subtle mucosal changes as seen in the grade M subcategory, probably through its action on neutrophils, thus triggering chemotaxis and generating harmful ROS. This result also highlighted the possibility that NERD patients could encompass diverse subpopulations in terms of immune and inflammatory reactions. Further studies on the implication of other members of chemokines and proinflammatory cytokines can shed light on our understanding of the mechanisms underlying this poorly studied disorder.

In the present study, we demonstrated constitutive mRNA expression of CXCR-1 and -2 in esophageal biopsy samples by RT-PCR procedure. To date, CXCR-1 and -2 are two distinct receptors for IL-8[19]. It is suggested that the increased IL-8 may facilitate trafficking of neutrophils into the mucosa

affected by GERD process through the interaction with these receptors. Again, recent data from our laboratories showed a significant correlation between IL-8 protein levels and basal layer hyperplasia as well as papillary elongation in patients with RE[13]. IL-8 also exerted mitogenic actions directly or by binding to its receptors on epithelial cells[20,21]. Taken together, it is possible that IL-8, together with other cytokines as well as growth factors[14,22], could contribute to epithelial cell proliferation even in NERD, and could be eventually linked to carcinogenesis. Again, unlike CXCR-1, CXCR-2 is not specific for IL-8 and can bind to other chemokines such as growth related oncogene α, but it has 2- to 5-fold higher affinity for IL-8 than CXCR-1[19]. Further studies on the distribution of diverse IL-8 receptors and the receptor-mediated signaling pathway may help to elucidate the pathogenesis of GERD via IL-8 action.

In conclusion, our study demonstrated significantly enhanced expression of IL-8 mRNA level in NERD by real-time PCR technology. The interaction of IL-8 with CXCR-1 and -2 is likely to be involved in the pathogenesis of NERD. We also showed a significant difference in IL-8 mRNA levels between grade M and N subgroups of the modified LA classification, indicating the heterogeneity of NERD patients both immunologically and endoscopically.

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Insulin-Like Growth Factor-I Enhances Transforming Growth Factor-β-Induced Extracellular Matrix Protein Production Through the P38/Activating Transcription Factor-2 Signaling Pathway in Keloid Fibroblasts

Takehiro Daian, Akira Ohtsuru,* Tatiana Rogounovitch,* Hiroshi Ishihara, Akiyoshi Hirano, Yuri Akiyama-Uchida,* Vladimir Saenko,* Tohru Fujii, and Shunichi Yamashita*

Department of Plastic and Reconstructive Surgery, *Department of Molecular Medicine, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4, Sakamoto, Nagasaki 852-8523, Japan

Keloids are benign dermal tumors, characterized by invasive growth of fibroblasts and concomitant increased biosynthesis of extracellular matrix components, with unclear etiology. We previously demonstrated that keloid fibroblasts overexpress insulin-like growth factor-I receptor. In investigating the role of insulin-like growth factor-I receptor overexpression, insulin-like growth factor-I and transforming growth factor-β interaction was examined in relation to extracellular matrix protein production in cultured human and mouse fibroblasts. Western blotting revealed that collagen type I was expressed in keloid and normal fibroblasts, and its expression was increased by transforming growth factor-β stimulation more significantly in keloid rather than in normal fibroblasts. Insulin-like growth factor-I and transforming growth factor-β1 costimulation markedly increased extracellular matrix proteins (collagen type I, fibronectin, and plasminogen activator inhibitor-1) compared with cultures with transforming growth factor-β1 alone. Insulin-like growth factor-I treatment alone had no stimulatory effect. Real-time reverse transcription–polymerase chain reaction confirmed parallel collagen type I messenger RNA level changes. Luciferase assays were conducted to investigate intracellular signaling pathways in this synergistic stimulation using a mouse fibroblast cell line. Transforming growth factor-β1 (1 or 10 ng per ml) increased the specific signaling activity approximately 10-fold, whereas the increase with insulin-like growth factor-I (100 ng per ml) was less than 2-fold compared with basal activity; however, the combination of transforming growth factor-β1 and insulin-like growth factor-I resulted in an approximately 25-fold increase. Insulin-like growth factor-I markedly enhanced transforming growth factor-β-induced phosphorylation of p38 mitogen-activated protein kinase and activating transcription factor-2. Luciferase assay showed that this synergistic effect was attenuated by the p38 mitogen-activated protein kinase specific inhibitor SB203580 or phosphatidylinositol 3-kinase inhibitor wortmannin, but not by the mitogen-activated protein kinase/extracellular-signal-regulated protein kinase kinase inhibitor PD98059. These results indicate that insulin-like growth factor-I enhances transforming growth factor-β-induced keloid formation through transforming growth factor-β postreceptor signal cross-talk, mainly via the p38 mitogen-activated protein kinase/activating transcription factor-2 pathway. ***Key words: fibrosis/mitogen-activated protein kinase/plasminogen activator inhibitor-1/collagen type I/fibronectin.*** ***J Invest Dermatol 120:956–962, 2003***

Keloids are classified as benign dermal tumors, characterized by the proliferation of dermal fibroblasts, overproduction of extracellular matrix components (ECM), invasiveness beyond the original boundary of the insult, and recurrence. Transforming growth factor (TGF)- β1 has been implicated in several fibrotic disorders, including glomerulonephritis, liver cirrhosis, and lung fibrosis (Border and Noble, 1994). In dermal fibroblasts, TGF-β1 is known to play a crucial part in wound healing processes. TGF-β1 has also been shown to markedly enhance the expression of ECM proteins by cultured fibroblasts, and it has therefore been postulated that TGF-β1 plays a significant part in the development of keloids (Babu *et al*, 1992; Younai *et al*, 1994; Tuan and Nichter, 1998; Chin *et al*, 2001). Furthermore, TGF-β1 is involved in the anti-apoptotic property of keloid fibroblasts (Chodon *et al*, 2000). TGF-β1 mediates signaling through two transmembrane serine/threonine kinase receptors, type I and type II TGF-β receptors. The constitutively active type II receptor recruits the type I receptor upon ligand binding and then phosphorylates its serine and threonine residues in a glycine serine rich domain. Once phosphorylated, the type I receptor activates downstream targets.

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Reprint requests to: Akira Ohtsuru, MD, Department of Molecular Medicine, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4, Sakamoto, Nagasaki 852-8523, Japan. Email: ohtsuru@net.nagasaki-u.ac.jp

Abbreviations: IGF-I, insulin-like growth factor-I; ECM, extracellular matrix; PAI-1, Plasminogen activator inhibitor-1; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular-signal-regulated protein kinase kinase; ATF-2, activating transcription factor-2; PI3K, phosphatidylinositol 3-kinases; JNK, Jun N-terminal kinase.

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Recent evidence indicates that TGF-β1 transduces signals through two different pathways, Smad and mitogen-activated protein kinase (MAPK), including p38, Jun N-terminal kinase (JNK), and extracellular signal regulated kinase (ERK) (Zhou *et al*, 1998; Hanafusa *et al*, 1999; Sato *et al*, 2002).

Insulin-like growth factor-I receptor (IGF-IR) is composed of two extracellular α-subunits and two transmembrane β-subunits that possess intrinsic tyrosine kinase activity (Ullrich and Schlessinger, 1990). Activated IGF-IR phosphorylates various substrates on tyrosine residues, including those of the Ras-Raf-MAPK pathway (Rozakis-Adcock *et al*, 1993; Skolnik *et al*, 1993) and the phosphatidylinositol 3-kinase (PI3K) pathway (Backer *et al*, 1992). In previous studies, we found that keloid fibroblasts overexpressed IGF-IR, IGF-I mediated invasiveness of keloid fibroblasts (Yoshimoto *et al*, 1999; Ohtsuru *et al*, 2000), and keloid fibroblasts are resistant to apoptosis due to IGF-I signaling (Ishihara *et al*, 2000). Although IGF-I/IGF-IR signaling is involved in keloid formation, it is not clear whether IGF-I can modulate TGF-β-mediated effect. In this study we examined how IGF-I affects TGF-β-induced ECM production, focusing on the postreceptor signaling pathway of TGF-β action.

MATERIALS AND METHODS

Materials Keloid samples were obtained from three different Japanese patients after surgical excision and the diagnosis confirmed by routine pathologic examination. In all cases, keloids had developed at sites of unsuspected injury (trivial wounds and acne) and had not been subjected to any kind of treatment. Three normal skin samples were obtained from three Japanese volunteers. Informed consent was obtained from each individual and the study approved by the ethical committee of Nagasaki University (no. 13020615). Patient profiles are listed in **Table I**.

Cell cultures Primary cultures of dermal fibroblasts were established as previously described (Ishihara *et al*, 2000). Explants were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Burlington, Ontario, Canada), 100 U per ml penicillin, and 100 μg streptomycin per ml at 37°C in a humidified incubator with 5% CO_2. Fibroblasts were treated with TGF-β1 (Invitrogen), IGF-I (Sigma, St Louis, MO) or both, at passages 3–8. A hepatoma cell line, HepG2, was cultured as previously described (Akiyama-Uchida *et al*, 2002).

Western blot analysis As the ECM proteins such as collagen type I, fibronectin, and plasminogen activator inhibitor (PAI)-1 are produced and then secreted into extracellular space, we used culture media but not cell lysates as loading samples after the measurement of protein concentration using Bio-Rad assay (BIO-RAD, Richmond, CA). After stimulation for 12 h, culture media was treated with sodium dodecyl sulfate lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue). The proteins were resolved in 7.5% sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes (Hybond-P; Amersham, Arlington Height, IL). The membranes were preincubated with a blocking buffer for 1 h at room temperature. They were then incubated with the primary antibodies to collagen type I (Rockland Immunochemicals, Gilbertsville, PA), fibronectin (Calbiochem, San Diego, CA), PAI-1 (American Diagnostics Inc, Greenwich, CT), phospho-activating transcription factor (phospho-ATF)-2 and control-ATF-2 (Cell Signaling Technology, Beverly, MA), p38 MAPK (New England Biolabs, Hercules, CA), and JNK antibody (Santa Cruz Biotechnology, Santa Cruz, CA). This was followed by incubation with the secondary antibody, a horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology). Proteins were visualized with a chemiluminescent detection system as proscribed by the manufacturer (ECL; Amersham).

Table I. Characteristics of individuals included in the study

	(age, years)	(sex)	(biopsy site)	(duration)*
Keloid				
K-1	77	female	Chest	10 years
K-2	36	female	Shoulder	3 years
K-3	64	male	Chest	45 years
Normal				
N-1	6	male	Foot**	
N-2	43	female	Back	
N-3	17	male	Thigh	

Controls are not site matched.
*Keloid persistence befor the surgery
**Surplus skin of graft for syndactly was used in this subject

Expression of collagen type I mRNA Total RNA was extracted from each sample stimulated in various ways for 6 h. This time point was determined as optimal by the time course study by Luciferase assay (data not shown). One microgram of total RNA reverse transcribed for 1 h at 41°C using random hexamers in a 25 μl reaction. Then 1 μl was used as a template for real-time polymerase chain reaction (PCR), which was carried out in an ABI PRISM® 7700 Sequence Detection System (PE Applied Biosystems, Warrington, U.K.) using the SYBR Green kit (PE Applied Biosystems) according to the manufacturer's guidelines. For each sample, the relative mRNA level of collagen type I was expressed in arbitrary units after normalization against α-tubulin. Standard curves for each real-time PCR run were generated from serial dilutions of a reverse transcription reaction with high content of both collagen I and tubulin mRNA. For each type of real-time PCR assay, 40 biphasic cycles of denaturation at 95°C for 15 s followed by annealing/extension at 60°C for 1 min were performed. Samples were analyzed in triplicates. The primer sequences were as follows: collagen type I (COL1A1) sense ACGCACGGCCAAGAGGAA and anti-sense CGTTGTCGCAGACGCAGATC; α-tubulin sense AGATCATTGACCTCGTGTTGGA and anti-sense ACCAGTTCCCCCACCAAAG; their concentration in the reaction mixture was 200 nM.

Luciferase assay Plasmid p3TP-Lux contains the luciferase reporter gene, under the control of a portion of the PAI-1 promoter region, and three consecutive phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) response elements. Human FAST-1 (human forkhead activin signal transducer-1) (hFAST-1) possesses the ability to bind human Smad2 and activates an activin response element (ARE). Thus the ARE-Lux fusion luciferase vector was used for transcriptional activity determination with FAST-1, to detect Smad signals (Akiyama-Uchida *et al*, 2002). pRL-CMV *Renilla* luciferase was cotransfected as a control reporter vector. Cultured normal and keloid fibroblasts and HepG2 cells at 70% confluence were transiently transfected using the Lipofectamine procedure. The media was changed to serum-free Dulbecco's modified Eagle's medium, and the culture continued for 24 h at 37°C. Cells were incubated for a further 12 h with IGF-I or TGF-β1, or a combination of the two. Cell extracts were prepared and subjected to dual-luciferase assay (Promega, Madison, WI) as proscribed by the manufacturer. To investigate the involvement of p38 and the ERK pathway in the induction of 3TP-Lux, we used the specific inhibitor of p38, SB203580 (5 or 10 μM) (Calbiochem), the MEK1 inhibitor, PD98059 (5 or 10 μM) (New England Biolabs. Inc., Beverly, MA) and the PI3K inhibitor, wortmannin (10 or 100 nM) (Wako Chemical, Osaka, Japan).

RESULTS

Expression of ECM proteins on dermal fibroblasts **Figure 1(*A*,*B*)** shows representative western blots for collagen type I expression in primary cultures of normal and keloid human fibroblasts. Basal expression of collagen type I is observed in keloid and normal fibroblasts. TGF-β1 treatment enhanced its expression in both fibroblast cultures (**Fig 1*A***). IGF-I caused some changes in levels of collagen type I and fibronectin expression, and treatment with TGF-β1 and IGF-I in combination markedly upregulated the expression of both proteins compared with either IGF-I or TGF-β1 treatment alone (**Fig 1*B*,*C***). We then examined the expression of PAI-1 as a marker of fibrosis through TGF-β1 signaling. As shown in **Fig 1(*D*)**, treatment of keloid fibroblasts with TGF-β1 and IGF-I in combination also markedly upregulated PAI-1 expression compared with control, IGF-I, or TGF-β1 treatment alone. Similar results were obtained in normal fibroblasts, but the combination effects were not as strong.

Expression of collagen type I mRNA **Figure 2** shows the expression of collagen type I mRNA in primary cultures of normal and keloid human fibroblasts estimated by real-time