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임신중 태반과 췌장에서의 Ghrelin의 발현과 이의 임신중 체중변화와의 관계

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Ghrelin Expression in Placenta and Pancreas during Pregnancy and its Correlation to Maternal Weight Changes

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Objectives: Ghrelin, an orexigenic peptide, is expressed in various central and peripheral human tissues including placenta and pancreas. Ghrelin has been implicated in fetal growth, maternal diet, and energy metabolism during pregnancy. Present study was to examine the expression of ghrelin in human placental tissues of missed abortion and term placenta and to evaluate the pattern of ghrelin expression in mice placenta and pancreas with respect to maternal weight gain during gestation.

Methods: Placental tissue from early abortion and delivery collected in human, ghrelin expression was assessed by immunohistochemistry and real time PCR. Pregnant mice were sacrificed at gestational days 8, 11, 13, 15, and 18 and their placenta and pancreas ghrelin levels were measured by real time PCR.

Results: In human placenta, villous cytotrophoblasts and extravillous trophoblasts showed positive immunostaining of ghrelin. In pregnant mice, term placenta had about three-fold relative ghrelin mRNA levels, while early placenta showed low ghrelin mRNA expression. Ghrelin mRNA expression in pancreas showed a continuously increasing pattern and a linear correlation with maternal weight gain (Pearson correlation coefficient r=0.96, *P*=0.016) during gestation.

Conclusion: Ghrelin expression is increased in placenta and pancreas at a later stage of pregnancy, especially pancreas might be related with the maternal weight gain throughout gestation.

Key words: Ghrelin, Placenta, Pancreas, Pregnancy, Maternal weight

Ghrelin, an endogenous ligand for growth hormone secretagogue receptor, was first purified and identified in rat stomach[1] and is ubiquitously expressed in all the human tissues.[2] It stimulates food intake and induces multiple endocrine and non-endocrine biological actions: neuroendocrine control of lactotropic, corticotropic, gonadotropic axis, metabolic and cardiovascular effects, and regulation of cell proliferation in various tissues and tumors.[3-8]

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Normal pregnancy typically shows a consistent weight gain which is closely related with maternal food intake. Endogenous ghrelin stimulates feeding and release of growth hormone, which is important in development and growth of both fetus and placenta during gestation.[9,10]

In energy balance and nutritional regulation, the pancreas plays a central role as the exocrine cells secreting digestive enzymes through a ductal system into the gastrointestinal system and the endocrine cells secreting the hormones into the bloodstream. In human, a high level of ghrelin was demonstrated in pancreas,[2] and

found to influence insulin secretion and glucose metabolism.[11,12]

Gualillo *et al*. described ghrelin as a placenta-derived hormone [13] and Tanaka *et al*. confirmed that the expression of ghrelin in the decidual and placental tissues during the first trimester.[14] A recent study of the ghrelin and its receptor expression in ovine placenta during pregnancy has shown the expression of ghrelin is highly correlated with the gestational age.[15]

Ghrelin is involved in regulating diet and fetal growth during developmental period; placenta and pancreas play an important role in energy metabolism during pregnancy. Thus, we hypothesize that ghrelin expression in placenta and pancreas as endocrine organs may change according to maternal weight gain during pregnancy.

In this respect, the present study was designed to examine the expression of ghrelin in human placenta. We examined the patterns of ghrelin expression, according to gestational days, in placenta and pancreas from pregnant mice, and then calculated the correlation between ghrelin expression and maternal weight gain.

MATERIALS AND METHODS

Human tissue samples

In human placenta

Term placental tissues were collected from 10 pregnant women immediately after cesarean section delivery (mean gestational age 39 weeks 5 days; 7 cases of obstructive labor and 3 cases of previous cesarean section). First trimester placenta tissues were collected from 10 pregnant women who underwent dilatation and curettage due to missed abortion (mean gestational age of 9 weeks 4 days). Normal non-pregnant human stomach tissues were used as a control.

In animal model

To verify ghrelin expression in pregnant mice as a physiological model, pregnant female ICR mice were purchased from Jung-Ang Lab Animal Inc. (Seoul, Korea). Timed pregnancies (20 days of gestation) were obtained by checking mating plugs. The morning of plug detection was defined as embryonic day 0.5. Five mice were euthanized at selected time points of pregnancy (Day 8, 11, 13, 15, and 18). Pancreas was extracted in circulating state under microscopic area immediately after CO2 inhalation. And then seperated placenta was vigorously washed in phosphate-buffered saline (PBS) buffer solution (pH 7.4) to reduce blood contamination. Collected samples were immediately stored at -80℃ before mRNA extraction. Stomach tissues from non-pregnant mouse mice were used as a control.

The study was performed with patients' informed consent with an approval by Institutional Review Board and Institutional Animal Care and Use Committee of CHA Gangnam Medical Center, CHA University (Seoul, Korea).

Immunohistochemistry

The placental tissue was embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), immediately frozen in liquid nitrogen-cooled isopentane and stored at -80℃. Sections of 3-4 μm thick were processed by standard histologic techniques on glass slides coated with Vectabond® (Vector Laboratories, Burlingame, CA, USA). Briefly the sections were air-dried, fixed with acetone, and incubated within 0.6% H2O2 in methanol for 30 min to block endogenous peroxidase activity, followed by equilibration in 10 mM PBS buffer and incubation for 30 min in 10% normal blocking serum (Vector Laboratories, Burlingame, CA, USA). Then the sections were incubated overnight at 4℃ with anti-ghrelin antibodies (Santa Cruz Biotechnology, CA, USA, dilution 1:200). After washing the slides with PBS buffer three times for 5min each time, incubation was performed according to the standard avidin-biotin method with the Vectastatin ABC kit® (Vector Laboratories, Burlingame, CA, USA). Color development was achieved with the Vector NovaRED substrate kit® (Vector Laboratories, Burlingame, CA, USA) and the sections were counterstained with hemotoxylin. Positive and negative control sections were processed with orwithout the primary antibody incubation.

Quantification of ghrelin in placenta and pancreas

Triplicate quantitative real-time PCR were performed in 384-well plates; each 20 μL reaction consisted of 10 μL of SYBR Green Master Mix (Qiagen, UK), 0.8 μL of 10 pM forward and reverse primers of ghrelin and GAPDH. The amplification profile was a 10-min incubation at 95℃ and a 2-min incubation at 50℃ followed by 40 cycles of 95℃ for 30s, annealing at 60℃ for 30s, and extension at 72℃ for 30s. The amplification was completed with an additional step at 72℃ for 10 min. To normalize the results for differences in RNA sampling, the second half of the RT reactions were used to amplify the mouse GAPDH as a control. The sequences of the primers were designed as follows: 5'-AACA-CCAGAGAGTCCAGCA-3' (sense) and 5'-CAACATCAAAGG-GGGCGTT-3' (antisense) for ghrelin, 5'-ACCCTTCGAGACC TGTTTGA-3' (sense) and 5'-GTGACCAGATGATACAGAGG-3' (antisense) for GAPDH. Each of the 384-well plates included serial dilutions (1, 1/2 and 1/4, 1/8, 1/16) of cDNA, which were used to generate relative standard curves for ghrelin and GAPDH. The amplification products of ghrelin (as a 380 bp product spanning through the first and fourth exons) was confirmed by electro-phoresis with DNA ladder markers. The real-time PCR analysis was performed on an Applied Biosystems Prism 7900 Sequence Detection System.

RESULTS

Expression of ghrelin in human placenta

In immunohistochemical staining, ghrelin was shown positive in villous cytotrophoblasts and extravillous trophoblasts as red cytoplasmic staining at low power (Fig. 1 A, B). The ghrelin mRNA expression level in term placenta was approximately three times higher than the control. We counted that the average level of ghrelin expression in the stomach was defined as 1. While the ghrelin mRNA expression level in chorion from missed abortion was significantly lower than the control (Fig. 1C).

Expression of ghrelin mRNA of placenta and pancreas with maternal weight changes during pregnancy in mouse

In mouse placenta, the ghrelin mRNA expression levels were higher compared with the control at gestational days 8, 15, and 18. However, mid-gestational days (day 11 and 13) showed lower expression levels than the control as illustrated in Fig. 2.

In the maternal pancreas, ghrelin mRNA expression level was consistently higher than the control throughout gestation. Gestational day 15 showed the highest ghrelin expression up to six-fold. Ghrelin in the pancreas also had a significant linear correlation with maternal weight gain (Pearson correlation coefficient r=0.96 with P=0.016) (Fig. 2). Relative maternal weights at gestational day 11 from before gestation, 15 from day 11, and 18 from day 15 were 0.56, 1.3, and 1.27 respectively.

DISCUSSION

Maternal weight gain is known to be affected by numerous hormonal and molecular components such as growth hormones, obesity related hormones, insulin, and cytokines. Among them, ghrelin with an orexigenic effect was examined for possible correlation between its expression with maternal weight change during gestation.[16] Ghrelin is produced in enteroendocrine cells as well as in pancreatic endocrine cells.

The expression pattern of ghrelin, a placental hormone, during pregnancy was first described in rats.[1] Recently, some authors reported distribution of ghrelin and its receptor in pancreas.[17] The expression of ghrelin was confirmed in a new cell type in fetal pancreas islet and proposed that these be a common precursor of insulin and ghrelin producing cells.[18,19] However, plasma ghrelin concentration was not correlated with the production of placental ghrelin during pregnancy.[20] The pathophysiologic role of ghrelin during pregnancy remains unclear.

In this study, the mice placenta showed detectable expression of ghrelin at early pregnancy days, while the human placenta from missed abortion did not show ghrelin expression. We suspect this

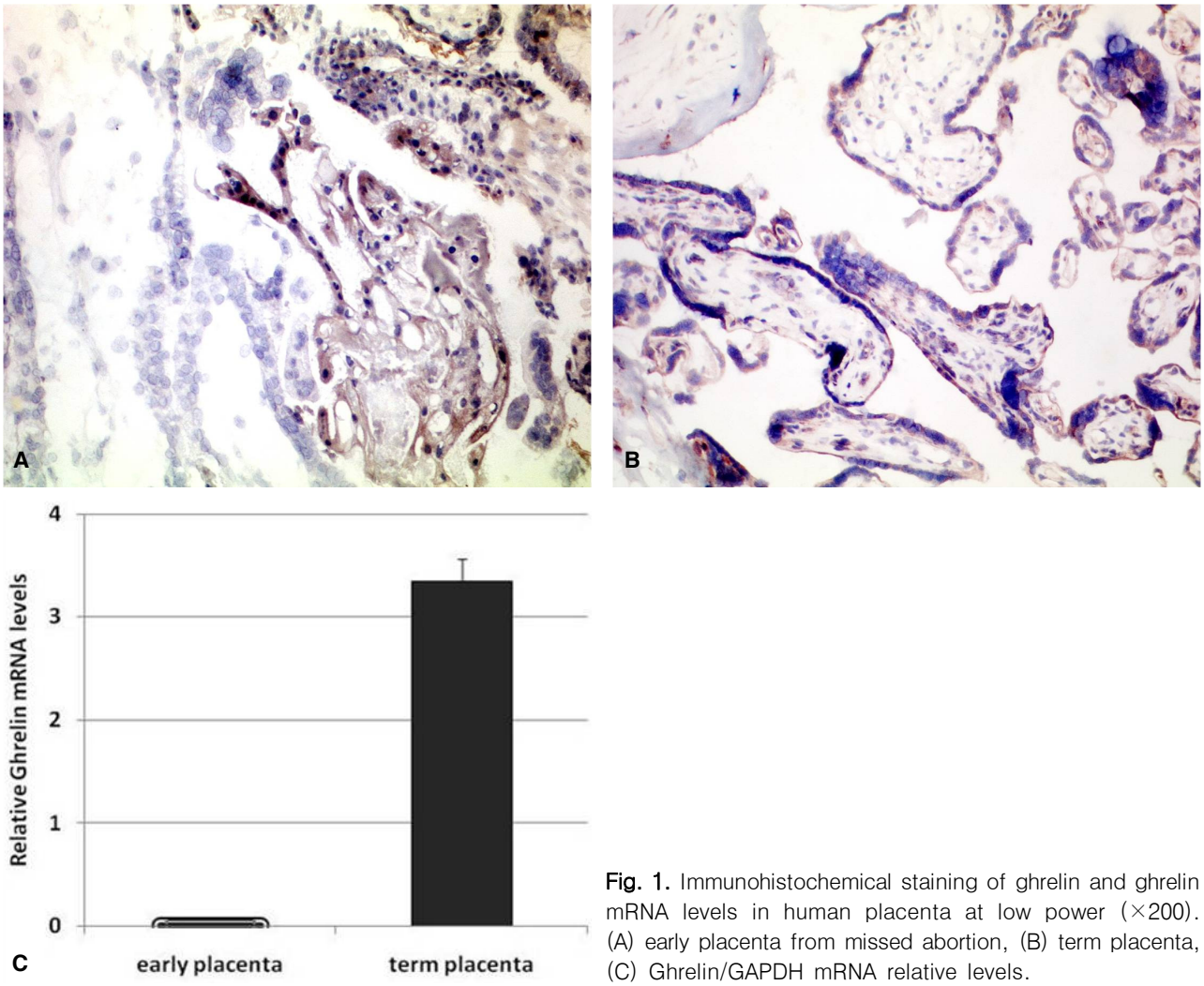

Fig. 1. Immunohistochemical staining of ghrelin and ghrelin mRNA levels in human placenta at low power (×200). (A) early placenta from missed abortion, (B) term placenta, (C) Ghrelin/GAPDH mRNA relative levels.

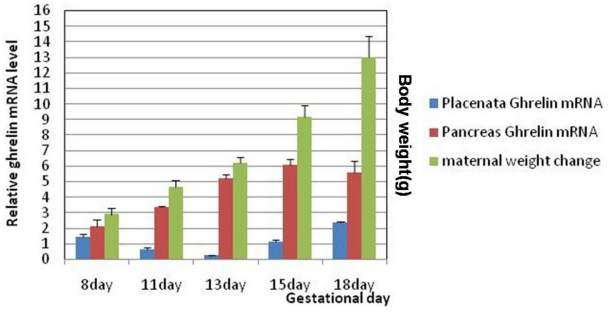

Fig. 2. Ghrelin mRNA levels and maternal weight changes in mice during pregnancy.

disagreement might originate from the samples under different time to sample collection with between alive and dead fetus.[14] At later gestational days, placental ghrelin levels in both human and mouse were the highest throughout gestation. This result is consistent with Shibata *et al*.'s finding of high ghrelin expression in late gestational days.[13,21] During pregnancy, placenta undergoes growth and enlargement and weights about one sixth of the total fetal weight at term. Placenta is also highly vascularized organ with rich blood supply, which might affect the ghrelin levels at late gestation.

Other groups have shown that the changes in the ghrelin peptide concentration in the stomach are not significantly different between pregnant and non-pregnant rats.[21,22] The ghrelin expression level in the pancreas was lower than the stomach in non-pregnant

state;[17,23] however, our study shows the ghrelin level in the pancreas during pregnancy is higher than the stomach with an increasing pattern during gestation.

Pregnant mice gained much more weight at late gestational days than at early gestational days. The ghrelin levels in both placenta and pancreas were higher at late gestational days than early gestation. Weight gain is suspected as a result of increased ghrelin secretion from the placenta and the maternal pancreas. However, the mechanisms how ghrelin might affect the maternal weight gain need to be further investigated with a careful consideration of possible interaction with other hormonal and molecular components.

In conclusion, our results show different patterns of ghrelin expression between placenta and pancreas with increasing maternal weight according to gestational days. We demonstrated that high level of ghrelin expression at late pregnancy is correlated with maternal weight gain in mice. Similarly in human, the expression of ghrelin was significantly increased in placenta at late gestation, suggesting a role of ghrelin in weight gain at late gestation.

However, the correlation between the ghrelin expression in organs and the level of circulating ghrelin in blood during pregnancy still needs to be elucidated. For a better understanding of pathophysiology of ghrelin to provide a further clue for the clinical management of ghrelin in a pregnant milieu, the expression of ghrelin receptors in placenta should be investigated.

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「국문초록」

목적: Ghrelin은 태반과 췌장을 비롯한 다양한 인간의 조직에서 분비되는 식욕과 관련된 단백질로서, 이는 태아의 성장과 임신중 식욕, 임신기간 동안의 에너지대사에 관여한다. 이 연구는 인간에서 계류 유산과 분만 후 태반에서의 Ghrelin의 발현을 살펴보고, 임신 쥐에서의 태반과 췌장의 ghrelin의 발현과 임신중의 쥐의 체중변화에 따른 이들 발현의 정도를 살펴보기로 하였다.

연구방법: 인간의 태반조직에서 면역염색과 real time PCR을 통해 Ghrelin의 발현을 보았고, 임신 쥐에서는 임신 8, 11, 13, 15, 18일에 임신 쥐의 체중을 측정하고 각각의 시기에 태반과 췌장의 ghrelin의 발현을 real time PCR을 통해 살펴보았다.

결과: 인간의 태반에서 ghrelin은 영양융모세포에서는 발현되었다. 임신쥐의 태반에서 ghrelin의 발현은 임신 말기가 임신 초기에 비해 약 세배 정도의 높은 발현을 보였다. 또한 임신쥐의 체중과 관련하여 임신이 진행됨에 따라 증가하는 체중과 ghrelin의 발현 정도는 양의 선형의 관계를 보였다 (Pearson correlation coefficient r=0.96, *P*=0.016).

결론: 임신중의 태반과 췌장에서 ghrelin의 발현은 임신 말기에 증가하고, 임신 중 체중증가와 관련이 있을 것이다.

중심 단어: ghrelin, 태반, 췌장, 임신, 임신중 체중