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MicroRNAs in hepatobiliary and pancreatic cancers

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MicroRNAs (miRNAs) are small non-coding RNAs that function as endogenous silencers of numerous target genes. Hundreds of miRNAs have been identified in the human genome. miRNAs are expressed in a tissue-specific manner and play important roles in cell proliferation, apoptosis, and differentiation. Aberrant expression of miRNAs may also contribute to the development and progression of human hepatobiliary and pancreatic cancers. Recent studies have shown that some miRNAs play roles as tumor suppressors or oncogenes in hepatobiliary and pancreatic cancers. *miR-122*, *let-7* family, and *miR-101* are down-regulated in hepatocellular carcinoma (HCC), suggesting that it is a potential tumor suppressor of HCC. *miR-221* and *miR-222* are up-regulated in HCC and may act as oncogenic miRNAs in hepatocarcinogenesis. miRNA expression profiling may be a powerful clinical tool for diagnosis and regulation of miRNA expression could be a novel therapeutic strategy for hepatobiliary and pancreatic cancers. In this review, we summarize current knowledge about the roles of important tumor suppressor microRNAs and oncogenic microRNAs in hepatobiliary and pancreatic cancers.

Keywords: microRNA, oncogene, tumor suppressor, epigenetics, hepatocellular carcinoma, cholangiocarcinoma, pancreatic cancer

THE BIOGENESIS OF microRNAs

MicroRNAs (miRNAs) are ~22 nucleotide (nt) non-coding RNAs that can post-transcriptionally downregulate the expression of various target genes. Currently, ~1400 human miRNAs have been identified in the human genome, and each miRNA potentially controls hundreds of target genes. miRNAs are expressed in a tissue-specific manner and play important roles in cell proliferation, apoptosis, and differentiation. Moreover, recent studies have shown a link between aberrant expression of miRNAs and the development of cancer (Saito et al., 2009a; Calin and Croce, 2007; Cho, 2007).

In animals, miRNA genes are generally transcribed by RNA polymerase II (pol II) to form primary transcripts (pri-miRNAs). Pol II transcribed pri-miRNAs are capped with 7-methylguanosine and polyadenylated. The nuclear RNase III enzyme Drosha and its co-factor Pasha (also known as DGCR8) process pri-miRNAs into ~60 nt precursor miRNAs (pre-miRNAs) which form an imperfect stem–loop structure. Pre-miRNAs are transported into the cytoplasm by the RAN GTP-dependent transporter exportin 5 and are subsequently cleaved by the cytoplasmic RNase III enzyme Dicer into mature miRNAs which are then loaded into the RNA-induced silencing complex (RISC). The miRNA/RISC complex downregulates specific gene products by translational repression via binding to partially complementary sequences in the 3′ untranslated regions (3′-UTRs) of the target mRNAs or by directing mRNA degradation via binding to perfectly complementary sequences.

Identification of miRNA target genes is critical to determining the miRNA functions. There are several databases of human miRNA target predictions using different algorithms such as microRNA.org[1] miRBase[2] PicTar[3] and TargetScan[4] It is generally believed that conserved perfect 6–8 base-pair (bp) matches between the 5′ end of the mature miRNA and the 3′ UTR of the predicted target mRNA (called "seed" matches) are the most reliable way of determining miRNA targets. Experimental confirmation using protein expression and luciferase reporter assays for the miRNA targets is necessary to accurately identify the target genes of miRNAs.

REGULATORY MECHANISMS OF miRNA EXPRESSION

Since miRNAs can have large-scale effects through regulation of a variety of genes during mammalian development and carcinogenesis, an understanding of the regulatory mechanisms controlling miRNA expression, which is quite limited at present, is important. There are several reports of transcription factors binding to the promoter regions of specific miRNA genes and activating the transcription of pri-miRNAs, resulting in increased expression of mature miRNAs. *c-Myc* binds to the regulatory region of the *miR-17-92* cluster and increased expression of *c-Myc* leads to the activation of the miRNAs in the cluster (O'Donnell et al., 2005).

Abbreviations: 5-Aza-CdR, 5-aza-2′-deoxycytidine; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDAC, histone deacetylase; LOH, loss of heterozygosity; miRNA, microRNA; PBA, 4-phenylbutyric acid; PDAC, pancreatic ductal adenocarcinoma.

[1] http://www.microrna.org/microrna/home.do,
[2] http://www.mirbase.org,
[3] http://pictar.mdc-berlin.de,
[4] http://www.targetscan.org.

The location of miRNA genes is also an important factor in the regulation of miRNA expression. Many miRNAs are located in cancer-associated genomic regions that are frequently involved in chromosomal abnormalities, such as loss of heterozygosity (LOH), amplification, and breakpoints (Calin et al., 2004). Chromosomal abnormalities during carcinogenesis could lead to widespread differential expressions of miRNAs in human cancer cells.

Epigenetic alterations such as DNA methylation and histone modification play critical roles in chromatin remodeling and regulation of gene expression in mammalian development and in human diseases. Many miRNAs are expressed in a tissue and tumor specific manner, implying that some miRNAs are subject to epigenetic control. We have recently shown that *miR-127*, which is embedded in a CpG island, is strongly induced by the treatment with the DNA demethylating agent 5-aza-2′-deoxycytidine (5-Aza-CdR) and the histone deacetylase (HDAC) inhibitor 4-phenylbutyric acid (PBA), indicating that some miRNA genes are controlled by epigenetic alterations in their promoter regions and can be activated by chromatin modifying drugs (Saito et al., 2006, 2009b).

MicroRNAs expression profiles can be used to classify the developmental lineages and differentiation stages of tumors. Interestingly, miRNA expression profiles are more accurate for tumor classification than conventional mRNA profiling (Lu et al., 2005). Furthermore, recent studies have demonstrated the association of miRNA expression signatures with prognostic and disease progression factors in human malignancies (Calin and Croce, 2006). These findings indicate that miRNA expression profile might be a powerful clinical tool for the diagnosis and prognosis of malignancies.

IMPORTANT TUMOR SUPPRESSOR AND ONCOGENIC miRNAs IN HCC

Hepatocellular carcinoma (HCC) is the most common type of liver cancer. Most cases of HCC are secondary to either chronic hepatitis or hepatic cirrhosis caused by a viral infection (hepatitis B or C) or alcoholism. HCC accounts for 85–90% of all primary liver cancers and is one of the most lethal cancers, and affects many of the world's population (Farazi and DePinho, 2006). The lethality of liver cancer may cause from its resistance to existing anticancer agents, a lack of biomarkers, and underlying liver disease that limits the use of chemotherapeutic drugs. In addition, the molecular pathogenesis of HCC remains poorly understood.

As shown in **Table 1**, some miRNAs are overexpressed in HCCs, indicating that they may have roles as oncogenes accelerating the development of HCC. Some miRNAs are down-regulated in HCCs, suggesting that they may act as tumor suppressors. miRNAs have critical roles in human hepatocarcinogenesis and that aberrant expression of miRNAs may contribute to the initiation and progression of HCC.

miR-122

miR-122 is specifically expressed and highly abundant in the human liver. Recent studies have reported that *miR-122* may modulate and facilitate replication of hepatitis C virus (HCV), suggesting that *miR-122* could be a target for antiviral intervention (Jopling et al., 2005; Li et al., 2011).

Table 1 | Putative tumor suppressor and oncogenic miRNAs in HCC.

miRNA	Target genes	References
PUTATIVE TUMOR SUPPRESSOR miRNAs IN HCC		
let-7	*c-Myc, Bcl-xL, COL1A2*	Ji et al. (2010)
		Lan et al. (2011)
		Shimizu et al. (2010)
miR-101	*Mcl-1, FOS*	Su et al. (2009)
		Li et al. (2009)
miR-122	*Cyclin G1, SRF, Igf1R, Bcl-w, ADAM10, ADAM17*	Gramantieri et al. (2007)
		Lin et al. (2008)
		Bai et al. (2009)
		Fornari et al. (2009)
		Tsai et al. (2009)
		Ma et al. (2010)
PUTATIVE ONCOGENIC miRNAs IN HCC		
miR-221/222	*p27, p57, DDIT4, PTEN, Bmf, TIMP3, PPP2R2A*	le Sage et al. (2007)
		Fornari et al. (2008)
		Garofalo et al. (2009)
		Gramantieri et al. (2009)
		Pineau et al. (2010)
		Wong et al. (2010)

miR-122 can modulate cyclin G1 expression in HCC-derived cell lines and an inverse correlation between *miR-122* and cyclin G1 expression exists in HCCs, indicating that cyclin G1 is a target of *miR-122* (Gramantieri et al., 2007). In patients of HCC, lower *miR-122* levels were associated with a shorter time to recurrence, whereas higher cyclin G1 expression was related to a lower survival, suggesting that *miR-122* might represent an effective molecular target for HCC (Fornari et al., 2009).

miR-122 also modulates Bcl-w expression by directly targeting binding site within the 3′-UTR. The cellular mRNA and protein levels of Bcl-w were repressed by elevated levels of miR-122, which subsequently led to reduction of cell viability and activation of caspase-3, suggesting that Bcl-w is a direct target of miR-122 that functions as an endogenous apoptosis enhancer in HCC cells (Lin et al., 2008). Ma et al. (2010) generated a recombinant adenoviral vector expressing miR-122 (Ad-miR-122). Infection of tumor cells with Ad-miR-122 resulted in inhibition of growth of cancer cells. This antitumor activity was related to the induction of apoptosis and/or cell-cycle arrest in cancer cells. Infection with Ad-miR-122 resulted in decreased expression of Bcl-w and CCNG1 in cancer cells. Induction of *miR-122* via adenoviral vector could be a promising strategy for cancer treatment (Ma et al., 2010).

A distintegrin and metalloprotease family 10 (ADAM10), serum response factor (SRF), and insulin-like growth factor 1 receptor (Igf1R) that promote tumorigenesis were validated as targets of miR-122. ADAM10, SRF, and Igf1R were up-regulated in primary human HCCs compared with the matching liver tissue, suggesting that the loss of multifunctional *miR-122* contributes to the malignant phenotype of HCC cells (Bai et al., 2009).

Tsai et al. (2009) have shown that *miR-122* is significantly down-regulated in HCCs with intrahepatic metastasis and negatively regulates tumorigenesis. Restoration of *miR-122* in metastatic cells significantly reduced *in vitro* migration, invasion,

and anchorage-independent growth as well as *in vivo* tumorigenesis, angiogenesis, and intrahepatic metastasis. They have further shown that one of the *miR-122* targets, a disintegrin, and metalloprotease 17 (ADAM17) is involved in metastasis. Silencing of ADAM17 resulted in a dramatic reduction of *in vitro* migration, invasion, *in vivo* tumorigenesis, angiogenesis, and local invasion in the livers of nude mice, indicating that *miR-122* plays a role in the intrahepatic metastasized HCC by the suppression of angiogenesis via regulation of ADAM17 (Tsai et al., 2009).

These studies demonstrate that *miR-122* is significantly downregulated in HCCs and has multiple functions as a tumor suppressor miRNA during hepatocarcinogenesis. *miR-122* is a promising target for HCC treatment as well as a diagnostic and prognostic marker for the progression of HCC. *miR-122* alone or in combination with anticancer drugs can be a promising therapeutic strategy against HCC. Further studies are necessary to develop miRNA-based therapy for HCC treatment. Since *miR-122* can modulate HCV replication, the status of HCV infection in HCC patients should be detected carefully.

let-7 FAMILY

The *let-7* family plays critical roles in tumorigenesis by functioning as potential tumor suppressor. The expression of *let-7g* was markedly decreased in HCC cells. Proliferation of HCC cell line was significantly inhibited after the transfection of *let-7g*. Concurrently, the mRNA and protein levels of c-Myc were found significantly decreased in HCC cells after transfection of *let-7g*. *let-7g* may act as a tumor suppressor gene that inhibits HCC cell proliferation by downregulating c-Myc (Lan et al., 2011). In addition, the level of *let-7g* was significantly lower in metastatic HCCs compared to metastasis-free HCCs. The low expression level of *let-7g* in tumor was predictive of poor survival in HCC patients. Type I collagen alpha2 (COL1A2) was validated as a direct target of *let-7g*, suggesting that *let-7g* may suppress HCC metastasis through targeting COL1A2 (Ji et al., 2010).

Bcl-xL, an anti-apoptotic member of the Bcl-2 family, is identified as a target of *let-7c* and *let-7g*. Human HCC tissues with low expression of *let-7c* displayed higher expression of Bcl-xL protein than those with high expression of *let-7c*, suggesting that low *let-7* miRNA expression contributes to Bcl-xL overexpression. *let-7* miRNAs negatively regulate Bcl-xL expression and induce apoptosis in human HCCs (Shimizu et al., 2010).

miR-101

Expression level of *miR-101* was significantly decreased in HCC cell lines and HCC tissues compared with their non-tumor counterparts. Ectopic expression of *miR-101* dramatically suppressed the ability of HCC cells to form colonies *in vitro* and to develop tumors in nude mice. In addition, *miR-101* repressed Mcl-1 expression as its target oncogene. These results indicate that *miR-101* may exert its proapoptotic function via targeting Mcl-1 (Su et al., 2009). Li et al. (2009) have reported that *miR-101* was significantly downregulated in HCC tissues compared with the matching non-tumor liver tissues. They also showed that *miR-101* repressed the expression of v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS) oncogene, a key component of the activator protein-1 (AP-1) transcription factor. In *in vitro* invasion and migration assays, enhanced *miR-101* expression inhibited the invasion and migration of cultured HCC cells. These findings suggest that *miR-101*, which is aberrantly expressed in HCC, could repress the expression of the FOS oncogene and may play an important role as a tumor suppressor in HCC (Li et al., 2009).

miR-221 AND *miR-222*

miR-221 is overexpressed in human HCC as well as in other malignancies. p27 Was identified as a target of *miR-221* and *miR-222* (le Sage et al., 2007). Fornari et al. (2008) have reported that the cyclin-dependent kinase inhibitor p57 is also a direct target of *miR-221*. Indeed, downregulation of both p27 and p57 occurs in response to *miR-221* transfection into HCC-derived cells and a significant upregulation of both p27 and p57 occurs in response to anti-*miR-221* transfection. They suggest that *miR-221* has an oncogenic function in hepatocarcinogenesis by targeting p27 and p57, hence promoting proliferation by controlling cell-cycle inhibitors (Fornari et al., 2008). *miR-221* also targets Bmf, a proapoptotic BH3-only protein, and inhibits apoptosis of cells. *miR-221* overexpression is associated with a more aggressive phenotype of HCC (Gramantieri et al., 2009). In addition, DNA damage-inducible transcript 4 (DDIT4), a modulator of mTOR pathway, was identified as a target of *miR-221*, indicating an important contribution for *miR-221* in hepatocarcinogenesis and suggest a role for DDIT4 dysregulation in this process (Pineau et al., 2010). Taken together, these findings indicate that *miR-221* simultaneously affects multiple pro-oncogenic pathways in hepatocarcinogenesis.

Garofalo et al. (2009) have reported that *miR-221* and *miR-222* are overexpressed in HCC cells, as compared with normal liver cells. They also show that *miR-221* and *miR-222* induce TRAIL resistance and enhance cellular migration through the activation of the AKT pathway and metallopeptidases by targeting PTEN and TIMP3 tumor suppressors and that the MET oncogene is involved in *miR-221* and *miR-222* activation through the c-Jun transcription factor (Garofalo et al., 2009). The protein phosphatase 2A subunit B (PPP2R2A) was identified as a target of *miR-222*. *miR-222* overexpression is common in HCC and could confer metastatic potentials in HCC cells, possibly through activating AKT signaling (Wong et al., 2010).

These studies strongly suggest that *miR-221* and *miR-222* are oncogenic miRNAs that play critical roles in the initiation and progression of human HCC. The use of synthetic inhibitors of *miR-221* and *miR-222* may be a promising approach to HCC treatment.

ABERRANT EXPRESSION OF miRNAs IN CHOLANGIOCARCINOMA

Meng et al. (2006) have reported that *miR-21*, *miR-141*, and *miR-200b* were highly overexpressed in malignant cholangiocytes. They show that *miR-21* modulates gemcitabine-induced apoptosis by phosphatase and tensin homolog deleted on chromosome 10 (PTEN)-dependent activation of PI 3-kinase signaling. This study suggests that alterations in miRNA expression contribute to tumor growth and response to chemotherapy (Meng et al., 2006).

They also provide evidence that overexpression of IL-6 cause upregulation of *let-7a* and reduction of *miR-370* in malignant human cholangiocytes. *let-7a* Contributes to the constitutively increased phosphorylation of STAT-3 by a mechanism

Table 2 | Putative tumor suppressor and oncogenic miRNAs in pancreatic cancer.

miRNA	Target genes	References
PUTATIVE TUMOR SUPPRESSOR miRNAs IN PANCREATIC CANCER		
let-7	*K-ras*	Torrisani et al. (2009)
miR-34a	*CDK6*	Lodygin et al. (2008)
		Chang et al. (2007)
PUTATIVE ONCOGENIC miRNA IN PANCREATIC CANCER		
miR-155	*TP53INP1*	Gironella et al. (2007)

involving the neurofibromatosis 2 (NF2) gene. The oncogene mitogen-activated protein kinase kinase kinase 8 (MAP3K8) was identified as a target of *miR-370*. Thus, *let-7a* modulates IL-6-dependent STAT-3 survival signaling in malignant human cholangiocytes. In addition, IL-6 may contribute to tumor growth by modulation of expression of selected miRNAs, such as *miR-370*. Aberrantly expressed miRNA or their targets will provide mechanistic insight and therapeutic targets for cholangiocarcinoma (Meng et al., 2007, 2008).

ABERRANT EXPRESSION OF miRNAs IN PANCREATIC CANCER

Pancreatic ductal adenocarcinoma (PDAC) is still the fourth leading cause of cancer-related deaths in Western countries with increasing incidence. Neither effective prognostic markers nor therapies exist for PDAC.

As shown in **Table 2**, *let-7* miRNA expression is remarkably reduced in PDAC samples, as compared with adjacent tissue. Restoring *let-7* levels in cancer-derived cell lines strongly inhibits cell proliferation, K-ras expression, and mitogen-activated protein kinase activation (Torrisani et al., 2009). Chang et al. (2007) have demonstrated that *miR-34a* is commonly deleted in human cancers and frequently absent in pancreatic cancer cells. *miR-34a* is directly transactivated by the tumor suppressor p53. Expression of *miR-34a* causes dramatic reprogramming of gene expression and promotes apoptosis (Chang et al., 2007). *miR-34a* expression is silenced in several types of cancer including pancreatic cancer due to aberrant CpG methylation of its promoter. Re-expression of *miR-34a* in pancreas carcinoma cell line induced senescence and cell-cycle arrest at least in part by targeting CDK6. These results show that *miR-34a* represents a tumor suppressor gene which is inactivated by CpG methylation in pancreatic cancer (Lodygin et al., 2008).

Gironella et al. (2007) have shown that oncogenic miRNA *miR-155* is overexpressed in PDAC cells and repress tumor protein 53-induced nuclear protein-1 (TP53INP1) as its target. TP53INP1 expression is dramatically reduced in PDAC and this decrease occurs early during pancreatic cancer development (Gironella et al., 2007; **Table 2**).

Besides *miR-155*, several miRNAs including *miR-21*, *miR-221*, and *miR-222* have been reported to be up-regulated in pancreatic cancer, suggesting that they are potential oncogenic miRNAs (Lee et al., 2007).

PERSPECTIVES AND CONCLUSION

The distinct connection between aberrant expressions of miRNAs and the development of hepatobiliary and pancreatic cancers indicates that miRNAs can be promising therapeutic targets. A recent study have shown that chemically engineered oligonucleotides, termed locked-nucleic-acid (LNA)-modified miRNAs, were found to be specific inhibitors of endogenous miRNAs in non-human primates (Elmen et al., 2008). They could be used to silence oncogenic miRNAs, such as *miR-221* and *miR-222* as a strategy for HCC treatment. Regulation of miRNA expression by epigenetic therapy using DNA methylation inhibitors and histone deacetylase (HDAC) inhibitors also has clinical promise for the treatment of human malignancies. In addition, miRNA expression profiling may be a powerful clinical tool for diagnosis of hepatobiliary and pancreatic cancers.

Since the link between miRNAs and hepatobiliary and pancreatic cancers has only just begun to be understood and the number of miRNA genes identified is increasing, there could be a large number of therapeutic targets for HCCs, cholangiocarcinomas, and pancreatic cancers. Further studies are necessary to investigate whether miRNA-mediated therapy can be an effective approach for the chemoprevention of hepatobiliary and pancreatic cancers.

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Review

Roles of oxidative stress in stomach disorders

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The stomach is a sensitive digestive organ that is susceptible and exposed to exogenous pathogens from the diet. In response to such pathogens, the stomach induces oxidative stress, which might be related to the development of gastric organic disorders such as gastritis, gastric ulcers, and gastric cancer, as well as functional disorders such as functional dyspepsia. In particular, the bacterium *Helicobacter pylori* plays a major role in eliciting and confronting oxidative stress in the stomach. The present paper summarizes the pathogenesis of oxidative stress in the stomach during the development of various stomach diseases.

Key Words*:** **gastric mucosa, oxidative stress, *Helicobacter pylori

Oxidative Stress in the Process of Gastric Mucosal Injury

Physiological responses to stressors include increased activity of the hypothalamic-pituitary-adrenal axis as well as changes in gastrointestinal tissue. According to Selye's formulation of the general adaptation syndrome, an increase in adrenocortical activity is related to an increase in the incidence of gastric ulceration. The main candidate for the cause of stress ulcers is oxidative stress. There is some evidence that psychological stress, in addition to physical stress such as surgical intervention and microbial infection including *Helicobacter pylori* (*H. pylori*),[1] leads to oxidative stress in the stomach. Oxidative stress, which is a state of elevated levels of reactive oxygen species (ROS), causes a variety of conditions that stimulate either additional ROS production or a decline in antioxidant defenses. Oxidative stress is not only involved in the pathogenesis of gastric inflammation, ulcerogenesis, and carcinogenesis in *H. pylori* infection, but also in that of lifestyle-related diseases including atherosclerosis, hypertension, diabetes mellitus, ischemic heart diseases, and malignancies.[2] Several phenotypes of gastrointestinal diseases, such as peptic ulcer disease and gastroparesis, are known to be related to antioxidant property dysfunction.

Ethanol. The effects of ethanol on gastric mucosa are complicated and multifaceted. They may be associated with a disturbance in the balance between gastric mucosal protective and aggressive factors. Gastric mucosa is exposed to gastric acid, pepsin, and stimulants among others, while gastroprotective factors maintain the integrity of the gastric mucous layer, microcirculatory system, HCO_3^-, prostaglandins (PGs), epidermal growth factor synthesis, and epithelial cell restitution. Ethanol injures the vascular endothelial cells of the gastric mucosa and induces microcirculatory disturbance and hypoxia, linking to the overproduction of oxygen radicals.

Pan *et al.*[3] report the role of mitochondrial energy charge in the pathogenesis of ethanol-induced gastric mucosal injury. The gastric mucosal lesion index is correlated with the thiobarbituric acid (TBA)-reactive substance (TBARS) content in gastric mucosa. As the concentration of ethanol increases and the exposure time to ethanol is extended, the TBARS content in gastric mucosa and the extent of gastric mucosal damage increase. The ultrastructural pathological changes in mitochondria are positively related to ethanol concentration and exposure time. The expressions of mitochondrial DNA ATPase subunits 6 and 8 mRNA decline with increasing TBARS content in gastric mucosa produced as a result of ethanol gavage. As mentioned above, ethanol-induced gastric mucosal injury is related to oxidative stress, which disturbs the energy metabolism of mitochondria and plays a critical role in the pathogenesis of ethanol-induced gastric mucosal injury.

Ischemia/reperfusion injury. Ischemia/reperfusion damages the gastric mucosa by inducing oxidative stress. Specifically, ROS such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) induce inflammatory responses and tissue damage by fragmenting cellular DNA. In the gut, ROS can also be generated by non-steroidal anti-inflammatory drugs (NSAIDs), cold stress, ethanol, and *H. pylori* infection. NADPH oxidase found in phagocytic cells, vascular smooth muscle cells, endothelial cells, fibroblasts, and adipocytes convert oxygen into superoxide anions. Nakagiri *et al.*[4] recently reported that NADPH oxidase activity is elevated in ischemia and ischemia/reperfusion and is involved in the resultant gastric mucosal damage. This increased NADPH oxidase activity may also upregulate cyclooxygenase-2.

Peskar *et al.*[5] report that during ischemia/reperfusion, inhibitors of the cyclooxygenase and lipoxygenase pathways increase gastric mucosal damage in a dose-dependent manner. The synergism observed as a result of the combination of cyclooxygenase and lipoxygenase inhibitors suggests that both pathways are important in gastric mucosal defense during ischemia/reperfusion. PGE_2 theoretically antagonizes the effects of cyclooxygenase and lipoxygenase inhibitors. Similarly, lipoxin A_4, a lipoxygenase-derived product of arachidonate metabolism, also antagonizes the effects of cyclooxygenase and lipoxygenase inhibitors; moreover, it could replace PGE_2 for the prevention of gastric mucosal damage caused by cyclooxygenase inhibitors during ischemia/reperfusion.

Portal hypertensive gastropathy. Portal hypertensive gastropathy (PHG) is a common complication of liver cirrhosis and is associated with impaired gastric mucosal healing. PHG may be related to increased ROS and lipid peroxide (LPO) production. Kinjo *et al.*[4] report increased levels of LPO and nitrotyrosine, an indicator of nitration of tyrosine residues due to peroxynitrite, in the gastric mucosa of portal hypertensive rats; in addition, they report impaired ERK1/2 phosphorylation related to increased nitration by peroxynitrite. The gastroprotective, anti-inflammatory agent rebamipide prevents free radical production by scavenging

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hydroxyl radicals.[5,6] Rebamipide decreases LPO and nitrotyrosine levels, normalizes ERK1/2 phosphorylation, and improves the ulcer index; this suggests that defects in the mitogen-activated protein kinase (MAPK) pathway are involved in the increased susceptibility to gastric mucosal injury observed in portal hypertensive gastropathy, indicating a potential role of rebamipide for treatment.

NSAIDs and aspirin. In addition to inhibiting cyclooxygenase and decreasing prostaglandin production, NSAIDs induce mucosal damage *via* ROS produced by recruited leukocytes. ROS-mediated mitochondrial damage as well as lipid, protein, and DNA oxidation lead to apoptosis and mucosal injury. Proton pump inhibitor (PPI) therapy is thought to primarily protect gastric mucosa by inhibiting gastric acid secretion. Nevertheless, Maity *et al.*[7] recently demonstrated that a PPI, lansoprazole, also inhibits NSAID-induced gastropathy by inhibiting mitochondrial and Fas-mediated apoptosis pathways. Lansoprazole's anti-apoptotic activity appears to be mediated by preventing NSAID-induced reductions in anti-apoptotic genes (e.g., Bcl and Bcl-2) while inhibiting increases in Fas and Fas ligand as well as pro-apoptotic genes (e.g., Bax and Bak).

On the other hand, aspirin increases the permeability of cultured gastric epithelial cell monolayers. The disruption in barrier integrity is mediated by p38 MAPK and involves the downregulation of claudin-7, a protein component of tight junctions.[8] This differs from the effects of other NSAIDs (i.e., non-aspirin NSAIDs), which increase epithelial permeability coupled to cyclooxygenase-1 inhibition; this increase can be restored by PGE_2 administration.

Heat shock proteins. Heat shock proteins, especially HSP70, provide cellular protection against stressor-induced tissue damage by refolding or degrading denatured proteins produced as a result of these stressors. Otaka *et al.*[9] recently used affinity chromatography to identify cytoskeletal myosin and actin as the first molecules bound by HSP70 after gastric mucosal injury in rats. Transcriptional upregulation of HSPs occurs via the binding of the transcription factor, heat shock factor 1 (HSF1), to heat shock element, which is located upstream of the HSP genes. In HSF1 null and HSP70-expressing transgenic mice, HSPs protect against irritant (e.g., ethanol or NSAIDs)-induced gastric lesions; moreover, geranylgeranylacetone (GGA), a gastroprotective agent, induces HSPs. Furthermore, HSP70 protects the gastric mucosa by inhibiting apoptosis, proinflammatory cytokines, and cell adhesion molecules involved in leukocyte infiltration. After induction by GGA, HSPs exhibit protective effects in mouse models of inflammatory bowel disease as well as in NSAID-induced lesions of the small intestine. Therefore, HSP inducers such as GGA may have therapeutic benefits in numerous diseases.

Enlarged Fold Gastritis and Oxidative Stress

H. pylori eradication therapy increases Runt domain transcription factor 3 (RUNX3) expression in glandular epithelial cells in enlarged-fold gastritis. Recently, we reported that RUNX3 is expressed in gastric epithelial cells and that *H. pylori* eradication significantly increases RUNX3 expression in the glandular epithelium of the corpus; however, no changes were observed in the antrum.[10] The mucosal chemiluminescence value, a marker of oxidative stress, is 4-fold higher in the corpus than in the antrum. *H. pylori* eradication significantly decreases the mucosal chemiluminescence values in both portions of the stomach to nearly undetectable levels. We conclude that the glandular epithelium is exposed to high levels of carcinogenic oxidative stress and expresses low levels of the tumor-suppressing molecule, RUNX3; however, RUNX3 expression was restored after eradication, suggesting a high risk of carcinogenesis associated with *H. pylori*-induced enlarged-fold gastritis of the corpus.[10]

Oxidative Stress during *H. pylori* Infection

Antioxidant ability of *H. pylori* to establish chronic infection. *H. pylori* infection induces a strong inflammatory host response, leading to the generation of a number of ROS and reactive nitrogen species (RNS), which are mediated by neutrophils and macrophages.[11] The generation of ROS and/or RNS is an important host immune response against persistent pathogens. Therefore, *H. pylori* must combat oxidative stress generated by the host immune response using an antioxidant protein in order to establish long-term colonization.[12,13] The mechanisms for ROS detoxification are of particular interest in understanding the *H. pylori*-associated pathogenesis. It is well known that *H. pylori* has a variety of enzymes acting as antioxidant systems to combat the toxic effects of ROS including catalase (KatA), iron-cofactored superoxide dismutase (SodB), and alkyl hydroperoxide reductase (AhpC).[13,14]

Superoxide dismutase (SOD) catalyzes the conversion of superoxide anions to hydrogen peroxide, which is degraded to oxygen and water by catalase.[13,15] SOD is a metalloenzyme; 3 structurally different forms have been identified depending on the metal cofactor. In general, organisms encode different sets of SOD enzymes. For example, *Escherichia coli* has 3 SODs: Fe-SOD (SodB) and Mn-SOD (SodA) in the cytoplasm and Cu/Zn-SOD in the periplasm.[16] On the other hand, *H. pylori* produces only a single SodB encoded by the *sodB* gene.[17,18] It was recently reported that *sodB* deletion in *H. pylori* causes the bacterium to lose its capacity for gastric mucosal colonization in mice.[19] This indicates that SodB is an important determinant of the host colonization capability of *H. pylori*. The regulation of *sodB* mRNA expression is also important for ROS detoxification. The mRNA expression of *sodB* in *H. pylori* is directly regulated by ferric uptake regulator (Fur) protein.[20] Fur functions as a global transcriptional regulator in *H. pylori*.[21–24] It is reported that Fur binds to ferrous iron (Fe^{2+}) and that the genes for iron uptake are suppressed by the iron-binding form of Fur.[25,26] On the other hand, *sodB* expression is suppressed by the iron-free form of Fur (apo-Fur).[20] Apo-Fur binds to a specific consensus sequence called Fur-Box located on the *sodB* promoter, blocking the binding of RNA polymerase.[20,27,28] It was recently reported that nucleic-acid mutations in Fur-Box and/or amino-acid mutations in Fur decrease the affinity of apo-Fur for Fur-Box in *H. pylori*, halting the suppression of *sodB* mRNA expression.[29–31] In particular, stopping the suppression of *sodB* mRNA expression in *H. pylori* by amino-acid mutations in Fur (i.e., C78Y and P114S) determines the development of metronidazole (Mtz) resistance.[31] This is because, when Mtz enters cells, its antimicrobial toxicity is dependent on the reduction of its nitro group to nitro anion radicals and the generation of superoxide.[32,33]

The SodB of *H. pylori* shares 53% sequence identity with the corresponding protein from *E. coli*. Interestingly, *H. pylori* SodB is significantly different from other Fe-SODs; its most distinguishing characteristic is its extended C-terminal tail,[34] although the role of this tail remains unclear. The structure of SodB has been clarified; it is now known to exist as a dimer composed of 2 identical subunits.[34] Furthermore, it is identified as having 4 ferrous ion (Fe^{2+})-coordinating residues (i.e., an iron-binding domain; His 26, His 73, His 160, and Asp 156) (Fig. 1). In fact, SodB needs to recruit ferrous ion (Fe^{2+}) to express its activity.[15,34] It is expected that SodB activity might be suppressed by preventing the uptake of iron ion (Fe^{2+} and/or Fe^{3+}).

Generation of oxidative stress as a virulence factor in *H. pylori*-infected hosts. ROS released from activated neutrophils are also potential virulence factors involved in *H. pylori*-infected host cells. In *H. pylori*-infected host cells, hypochlorous anions (OCl^-) are generated from H_2O_2 in the presence of Cl^-. The hypochlorous anions subsequently react with ammonia (NH_3), which is derived from urea by urease produced by *H. pylori*,

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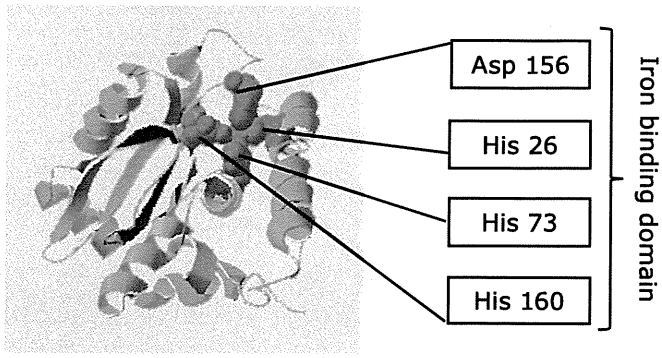

Fig. 1. Homology modeling of *Helicobacter pylori* SodB. Four amino acids (His 26, His 73, His 160, and Asp 156) of SodB are ferrous ion (Fe^{2+})-coordinating residues (i.e., an iron-binding domain).

ultimately yielding monochloramine (NH_2Cl). NH_2Cl induces mucosal cytotoxicity due to its lipophilic properties and freely penetrates biological membranes to oxidize intracellular components.[35–37]

In addition, it is well known that *H. pylori* produces a γ-glutamyltranspeptidase (EC 2.3.2.2; GGT) in the periplasm.[38] *H. pylori* GGT catalyzes the transpeptidation and hydrolysis of the γ-glutamyl groups of glutamine and glutathione. Interestingly, these findings indicate that *H. pylori* GGT performs 2 functions. First, GGT functions in the physiological functioning of *H. pylori*. *H. pylori* is unable to take up extracellular glutamine and glutathione directly; GGT hydrolyzes these substances to glutamate. The glutamate is then transported into *H. pylori* cells via a Na^+-dependent reaction and is mainly incorporated into the TCA cycle.[39] Second, GGT acts as a virulence factor by disrupting the antioxidant ability of host cells. Although glutathione has antioxidant potential in host cells, *H. pylori* GGT reduces extracellular glutathione levels. In fact, *H. pylori* GGT reduces the ROS resistance of the host cells and induces apoptosis or necrosis.[38,40]

Excess ROS are produced in *H. pylori*-colonized human stomachs; this induces oxidative stress to both the gastric mucosa and *H. pylori*. Because *H. pylori* has a deft capability of detoxifying ROS using a variety of enzymes to establish long-term colonization,[12,13] excess ROS leads solely to host cell damage.

Oxidative Stress in the Progression of Gastric Motility Disorders

Gastric motility disorders can occur in many clinical settings with a wide variety in the severity of symptoms with or without gastric mucosal injuries. Gastric motility disorders are attributable to either damage within the smooth muscle itself or dysfunctions within the neuromuscular components including the enteric nerves and interstitial cells of Cajal (ICC), which regulate smooth muscle function. How oxidative stress is involved in these dysfunctions is discussed in the following situations.

Gastrointestinal Complications in Sepsis

Oxygen radicals are implicated as relevant mediators in sepsis and septic shock in animals including humans.[41,42] Sepsis is a systemic response caused by bacterial endotoxins such as lipopolysaccharide, which induce the release of ROS and the generation of numerous pro-inflammatory factors and nitric oxide. During sepsis, the most frequent complications within the gastrointestinal tract are gastrointestinal motility disturbances and mucosal barrier dysfunction. Experimental administration of LPS delays gastric emptying and upregulates inducible nitric oxide synthase in order to downregulate neuronal nitric oxide synthase (nNOS) and synthesize PGs.[43] It is also reported that SOD reverses the endotoxin-induced delay in gastric emptying and diminishes the presence of nitrotyrosine, 4-hydroxy-2-nonenal in gastric mucosa, and inducible nitric oxide synthase-positive residential macrophages in the external musculature; these suggest the involvement of oxidative and nitrosative stresses in the pathogenesis of lipopolysaccharide-induced gastrointestinal dysmotility.[44]

Gastrointestinal Complications in Ischemia/Reperfusion Injury

The gastrointestinal tract is one of the most susceptible organ systems to ischemia. Previous investigations demonstrate that ischemia/reperfusion is a major contributor to gastric mucosal injury caused by stresses such as burn stress or hemorrhagic shock, NSAIDs, and *H. pylori* infection. In addition to mucosal injuries, delayed gastric emptying is also reported after gastric ischemia/reperfusion associated with disruption of the ICC network and nNOS-positive neurons.[45] ICC play critical roles in gastrointestinal motility in that they are the source of the electrical slow waves underlying the phasic contractions of the gastric musculature and mediate excitatory and inhibitory inputs to the musculature from the enteric motor neurons. Neuronal NOS generates neuronally derived NO, which is the major inhibitory neurotransmitter in the gastrointestinal tract. ICC and nNOS-positive neurons are both important factors for gastric emptying. Oxidative stress produced by the xanthine–xanthine oxidase system after ischemia/reperfusion may play a major role in these events, although the precise mechanism is unclear.

Diabetes Mellitus

Oxidative stress is a strong pathogenic co-factor involved in the development of complications of diabetes. Increased glucose levels in diabetes react non-enzymatically with proteins and become advanced glycation end products (AGEs); AGEs activate endothelial NADPH oxidase and increase endothelial ROS,[46] which occurs in animal models of diabetes[47] and diabetes patients.[48] Gastric neuromuscular dysfunction occurs in up to 30–50% of patients after 10 years of type 1 or 2 diabetes associated with histological changes including the loss of nNOS and ICC in both humans and animal models. It is reported that increased oxidative stress is attributable to the loss of upregulation of heme oxygenase-1; this results in the loss of ICC, decreased nNOS expression, and delayed gastric emptying in non-obese diabetic mice. These changes can be reversed by heme oxygenase-1 induction, demonstrating an important role of oxidative stress in the development of diabetic gastroparesis.[49]

Aging and Oxidative Stress

Gastrointestinal function declines with aging, including delayed gastric emptying, decreased peristalsis, and slowed colonic transit; these impair quality of life and increase morbidity and mortality. Notable changes in gut neuromuscular function that accompany advanced age are reported in human and animal models.[50,51] Cowen *et al.*[52] report a 50% reduction in ileal myenteric neurons in 24-month-old Sprague-Dawley rats fed *ad libitum*; this was prevented by caloric restriction, which reduces oxidative stress.[53] In the study using progeric mice deficient in the anti-aging peptide Klotho, progeric mice exhibited a gastric phenotype resembling that of human aging involving profound ICC loss with reduced slow wave amplitude and nitrergic inhibitory junction potentials. Klotho protects ICC by preserving their precursors, limiting oxidative stress, and maintaining nutritional status and normal levels of trophic factors important for ICC differentiation.[54] Increased oxidative stress in combination with a decrease

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in circulating and tissue factors that regulate ICC differentiation and survival contribute to the profound depletion of mature ICC and impair gastric function.

Conclusion

In conclusion, oxidative stress is one of the major contributors to the development of stomach diseases. Recent therapeutic options such as gastroprotective agents including antioxidant properties (e.g., rebamipide) can modulate the level of oxidative stress to enhance anti-inflammatory or antioxidant capacity. The stomach is an organ in direct contact with external pathogens; by presenting a strong acid environment, it has a special biological defense mechanism that eliminates such pathogens. However, *H. pylori* manages to live in the stomach by breaking through this defensive line. In response to the colonization of this bacterium, gastric mucosa can be exposed to severe oxidative stress with considerable levels of inflammatory cell accumulation, which might be related to the development of gastric mucosal as well as neuromuscular disorders.

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Abbreviations

AGEs	advanced glycation end products
GGA	geranylgeranylacetone
GGT	γ-glutamyltranspeptidase
HSF	heat shock factor
ICC	interstitial cells of Cajal
LPO	lipid peroxide
MAPK	mitogen-activated protein kinase
Mtz	metronidazole
nNOS	neuronal nitric oxide synthase
NSAIDs	non-steroidal anti-inflammatory drugs
PGs	prostaglandins
PHG	portal hypertensive gastropathy
PPI	proton pump inhibitor
RNS	reactive nitrogen species
ROS	reactive oxygen species
RUNX3	Runt domain transcription factor 3
SOD	superoxide dismutase
TBARS	thiobarbituric acid-reactive substance

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ORIGINAL ARTICLE

Enhanced Gastric Ghrelin Production and Secretion in Rats with Gastric Outlet Obstruction

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Abstract
Background and Aim Ghrelin has distinct effects on gastrointestinal motility through the vagus nerve and gastric excitatory neural plexus. The objectives of this study were to investigate the dynamics of ghrelin and expression of neuromuscular markers in a newly established surgically manipulated rat model of gastric outlet obstruction (GOO), akin to the pyloric stricture associated with duodenal ulcer, advanced gastric cancer, and other conditions, in the clinical setting.
Material and Methods The rats were divided into two groups, a control group (sham operation) and the GOO group (proximal duodenal stricture). The animals were sacrificed 2 weeks after the operation. Plasma and gastric ghrelin were measured by radioimmunoassay. mRNA expression in the stomach of neural choline acetyltransferase (ChAT), c-kit, and membrane-bound stem cell factor (SCF) were analyzed by quantitative RT-PCR. In addition, gastric mRNA expression of the aforementioned were also evaluated 60 min after intraperitoneal administration of a synthetic GHS-R1a antagonist ([D-Lys3] GHRP-6 6.0 mg/kg).
Results Mechanical GOO induced increases of fasting plasma ghrelin levels and hyperplasia of the gastric muscle layers, with enhanced expression of the gastric neuromuscular markers. Administration of [D-Lys3] GHRP-6 normalized the enhanced expression of c-kit and SCF.
Conclusion GOO stimulates ghrelin dynamics and then enhances the mechanistic expression of gastric cellular communication network molecules between nerves and smooth muscle cells.

Keywords Ghrelin · Gastric emptying · Motility · Gastric outlet obstruction

Introduction

Ghrelin, a 28-amino-acid motilin-related peptide, was first purified from the rat stomach as a natural ligand for the growth hormone secretagogue receptor (GHSR) [1]. It has also been shown to stimulate food intake, induce body weight gain, and enhance gastric motility. Recent animal studies have shown that ghrelin has distinct effects on gastrointestinal motility, which may be mediated through the GHSR expressed on the vagus nerve and enteric nerve endings [2, 3]. Ghrelin has been reported to enhance gastric motility and accelerate gastric emptying in rats and mice and to stimulate small intestinal transit [2, 4]. Fujino et al. reported that ghrelin induces accelerated motor activity of the gastrointestinal tract via ghrelin receptors expressed on vagal afferent nerve terminals and activated neuropeptide Y neurons in the brain [5].

Chasen et al. reported that abnormal electrogastrography diagnosis and increased levels of plasma ghrelin were

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found in most patients with advanced cancer [6]. We have previously shown increased fasting plasma levels of ghrelin in patients with functional dyspepsia (FD), especially those with dysmotility-like FD, possibly originating from gastric motility disorders, including delayed gastric emptying [7]. Although the precise molecular mechanisms are not yet clear, such clinical manifestations suggest that ghrelin production might be increased by impaired gastric motility.

The objectives of this study were to investigate the dynamics of ghrelin and expression of neuromuscular markers in a newly established surgically manipulated rat model of gastric outlet obstruction (GOO), akin to the pyloric stricture associated with duodenal ulcer, advanced gastric cancer, and other clinical conditions.

Methods

Animal Procedures

This study was conducted with the approval of Keio University Animal Research Committee (no. 056188). Seven-week-old male specific-pathogen-free (SPF) Sprague–Dawley (SD) rats (Sankyo Laboratory Service, Tokyo, Japan) were used for the study after acclimatization for 7 days in an animal room at a controlled temperature (24 ± 2°C). The rats were fed standard chow and deprived of food for 24 h before the operation. After induction of anesthesia by intraperitoneal injection of 50 mg/kg sodium pentobarbital, the hair was shaved off the upper abdomen of the animals. The abdomen was opened via a 25-mm-long median incision, and the stomach was exposed. The proximal duodenum was then carefully covered with an 18-Fr Nelaton catheter (diameter, 4.0 mm; Nippon Sherwood, Tokyo, Japan) and sutured with a 5–0 nylon thread (Fig. 1). The width of the catheter was 2.0 mm. This surgical duodenal stricture induced incomplete gastric outlet obstruction with gastric retention; this animal group was named the GOO group. After the operation, the animals were deprived of food for 24 h, but allowed free access to water. A sham operation, involving only abdominal incision, was also undertaken on the same number of rats of the control group.

Because weight loss is known to increase circulating levels of ghrelin, we designed a pair-feeding method to evaluate the dynamics of ghrelin without the confounding effect of body weight changes. The results of our preliminary experiments revealed that food intake in the GOO group was lower than that in the control group. We measured daily food intake in the GOO group, and the control group of rats received the same amount of food on the following day. After 2 weeks, after food deprivation for 24 h, the rats were sacrificed under ether anesthesia and the gastric wet weight and intraluminal pH were measured. The thicknesses of the gastric antral mucosal layer and muscular layer were measured by light-microscopic examination of hematoxylin–eosin-stained sections. The average thicknesses 1, 1.5, and 2.0 mm from the pylorus were measured.

Evaluation of Gastric Emptying

Our surgical procedure is the first reported method for establishing an animal model of GOO. We compared the gastric emptying rate in this model with that in another group of 7-week-old SPF male SD rats. The animals were divided into a control group and GOO group as previously described. Two weeks after the operation, the rats were deprived of food, but allowed free access to water for 24 h before the start of the subsequent experiment. The gastric emptying rate was measured by the phenol red (PR) method reported by Ohnishi et al. [8]. One milliliter of PR (100 μg/ml) was administered orally to the rats, and the rats were sacrificed by cervical dislocation either immediately (Control group, $n = 4$) or 15 min (Control group, $n = 4$; GOO group, $n = 6$) after administration of the PR solution. The standard stomachs (0 min) and test stomachs (15 min) were exposed and ligated at the cardia and pylorus. Each stomach was removed and placed in 10 ml 0.1 M Na_2HPO_4 solution, and the contents of the stomach were washed out. The absorbance of the supernatant was measured at 570 nm with a spectrometer (Shimadzu, Kyoto, Japan). The gastric emptying rate for each rat was calculated as described previously [8].

Measurement of the Ghrelin Dynamics

The rats were divided into two groups, the control group ($n = 14$) and the GOO group ($n = 14$). After 2 weeks, following food deprivation for 24 h, the rats were sacrificed under ether anesthesia. The abdomen and chest were opened via a median incision. Blood was collected from the heart and centrifuged at 3,000 rpm for 10 min to obtain plasma and serum samples for assay. After collection of the blood, the stomachs of the animals were excised and the intraluminal pH was examined by use of a pH meter (Horiba Techno Service, Kyoto, Japan). The stomachs were then cut along the greater curvature and rinsed with isotonic saline. The two radioimmunoassays (RIAs) for measurement of the gastric and plasma ghrelin levels were performed as described previously [9, 10]. Two polyclonal rabbit antibodies were raised against the N-terminal [1–11] (Gly1-Lys11) and C-terminal [13–28] (Gln13-Arg28) fragments of rat ghrelin. [Cys12]-rat ghrelin [1–11] (4 mg) and [Cys0]-rat ghrelin [13–28] (10 mg) were separately conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (mcKLH, Pierce, Rockford, IL, USA; 6 mg) in conjugation buffer (Pierce). Each conjugate was

emulsified with an equal volume of Freund's complete adjuvant. Two corresponding batches of antiserum were obtained by immunization of New Zealand white rabbits by subcutaneous injection. Using these antibodies, two types of RIAs to measure the plasma and gastric ghrelin levels were performed as described previously [11].

Immunohistochemistry for Ghrelin

Stomach samples were fixed in 10% formaldehyde neutral buffer solution for 24 h, then embedded in paraffin. Tissue sections were deparaffinized and hydrated, and endogenous peroxidase was quenched by treatment with 0.3% hydrogen peroxide for 20 min. Nonspecific binding was blocked by use of a blocking reagent (BlockAce; Dainippon Pharmaceuticals, Osaka, Japan). After washing with TBS-T, the tissue slices were incubated for 60 min at 4°C with anti-ghrelin antiserum (final dilution, 1:10,000). Then, after washing again with TBS-T, the slides were incubated with EnVision + Peroxidase rabbit (DAKO Japan, Kyoto, Japan) for 30 min at room temperature, and then visualized after color development with 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution for 1 min. The sections were then counterstained with hematoxylin. The stained sections were examined under high-power magnification (objective lens ×40) by light microscopy equipped with a 3CCD digital camera (C7780; Hamamatsu Photonics, Hamamatsu, Japan). The nuclei were counted using Image-J software (National Institutes of Health, Bethesda, Maryland, USA). The density of the ghrelin-immunoreactive cells was computed by use of the equation: Dghrelin = (Ng/Nt) × 100 (%), where Ng and Nt represent the number of ghrelin-immunoreactive cells and the total cell number, respectively, in the three mucosal regions of the stomach.

Preparation of Total RNA and Quantitative RT-PCR Analysis

Total mRNA was extracted from the stomach tissue by use of the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and DNase treatment was performed with an RNase-free DNase set (Qiagen). RNA was converted into cDNA by use of the PrimeScript RT reagent kit (Takara, Ohtsu, Japan). Cyber green quantitative real-time RT-PCR was performed to detect the mRNA using the Thermal Cycler Dice Real Time System (Takara Bio, Otsu, Japan). The primers used to amplify the target mRNA were: c-kit mRNA 5′-ATC CAG CCC CAC ACC CTG TT-3′, and 5′-TGT AGG CAA GAA CCA TCA CAA TGA-3′, SCF (membrane-bound isoform) mRNA: 5′-TGA GAA AGG GAA AGC CGC-3′, and 5′-TAA GGC TCC AAA AGC AAA GC-3′, choline acetyltransferase (ChAT) mRNA: 5′-CAA CCA TCT TCT GGC ACT GA-3′, and 5′-TAG CAG GCT CCA TAG CCA TT-3′, glyceraldehyde-3-phosphate (GAPDH) mRNA: 5′-GGC ACA GTC AAG GCT GAG AAT G-3′, and 5′-ATG GTG GTG AAG ACG CCA GTA-3′. The target mRNA expression levels were normalized to the GAPDH mRNA expression levels.

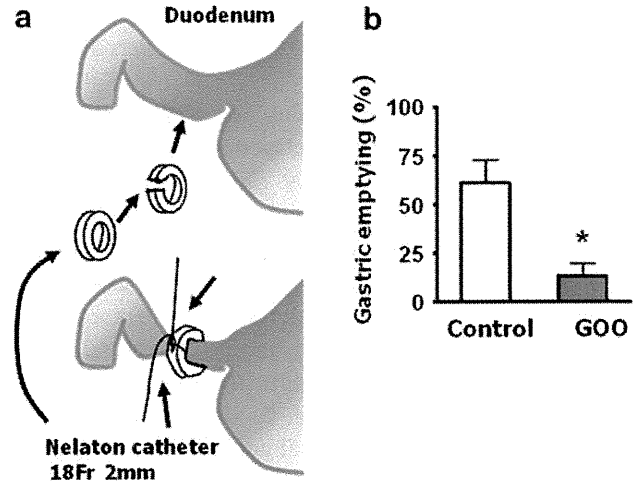

Fig. 1 **a** Surgical manipulation for induction of duodenal stricture. The proximal duodenum was covered with a small piece of an 18Fr-Nelaton catheter. **b** Liquid gastric emptying rates of control rats ($n = 4$, *open bar*) and GOO rats ($n = 6$, *filled bar*) 2 weeks after the operation (mean ± S.E.M. $^*P < 0.05$ compared with control)

RT-PCR Analysis for Interstitial Cells of the Cajal (ICC) Network Using a GHSR1a Antagonist

In a separate experiment, to examine the relationship between ghrelin and neuromuscular marker expression, the rats were administered an intraperitoneal injection of [D-Lys3] GHRP-6, a GHSR antagonist, (6.0 mg/kg; Bachem, King of Prussia, PA, USA) in 1 ml saline ($n = 8$), or saline alone ($n = 8$) and then euthanized 60 min after administration. Then, the mRNA expression levels of c-kit, SCF, and ChAT were analyzed by quantitative RT-PCR, as previously described.

Statistical Analysis

All results are expressed as mean ± SE, and the statistical analysis was performed using the Student t test (two-tailed test) with Stat Mate III (Atoms, Tokyo, Japan). P values less than 0.05 were considered to be statistically significant.

Results

Gastric Emptying Rate in this Rat Model

The gastric emptying rate after 15 min as measured by the PR method was significantly lower in the GOO group than

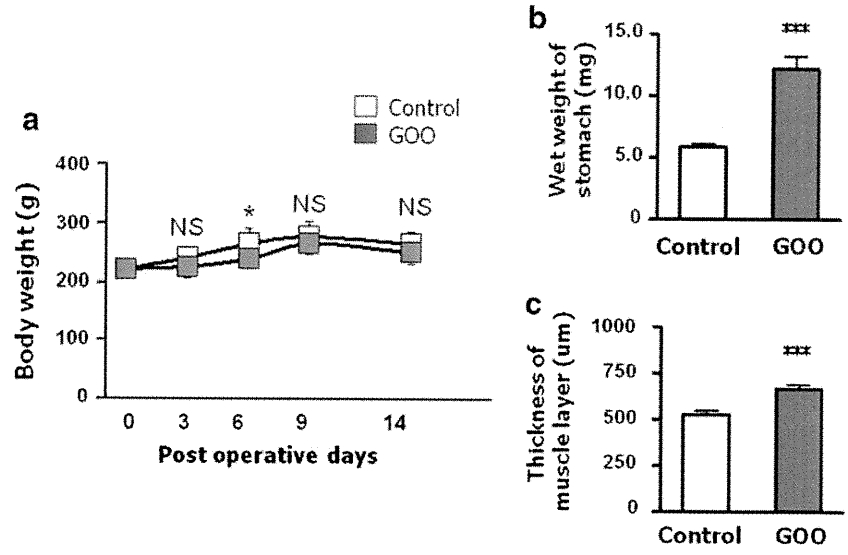

Fig. 2 **a** Body weight was measured 0, 3, 6, 9, and 14 days after the operation. On days 9 and 14 after the operation, no significant differences in the changes of the body weights were observed between the two groups. The mean weight of the rats on the operation day was 220.2 ± 4.5 g in the control group and 218.2 ± 2.0 g in the GOO group. **b** Wet weight of the removed stomach 2 weeks after operation. **c** Thickness of the muscle layer at the antrum using HE stain. (mean ± S.E.M.; $n = 13$ in each group.*$P < 0.05$, ***$P < 0.001$ compared with control.)

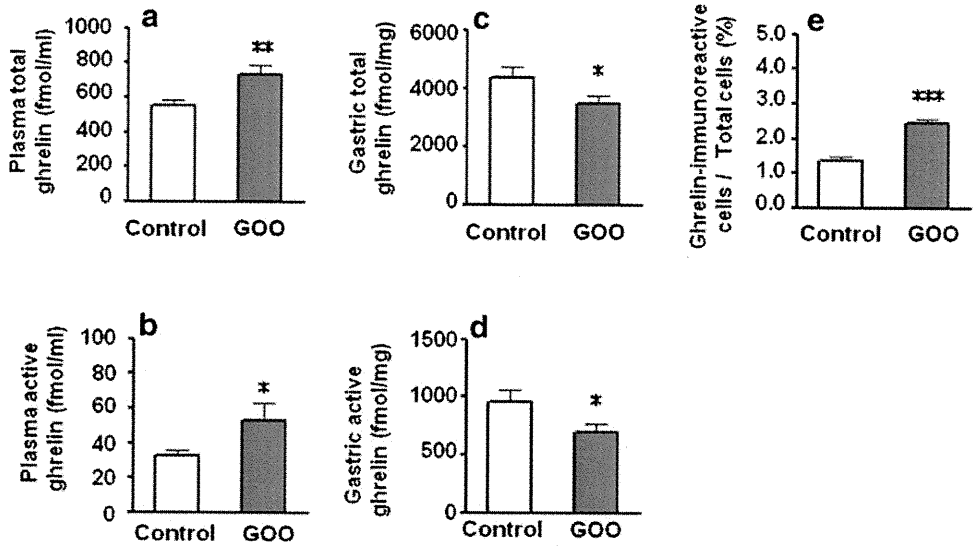

Fig. 3 Fasting levels of total (**a**) and active (**b**) plasma ghrelin concentrations and of the total (**c**) and active (**d**) gastric ghrelin contents were measured (**a–d**). The plasma ghrelin levels were increased in the GOO group, whereas gastric ghrelin levels decreased. **e** Immunohistochemistry for ghrelin. The density of the ghrelin-immunoreactive cells in the gastric corpus (mean ± S.E.M.; $n = 13$ in each group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control.)

in the control group (21.8 ± 7.8% vs. 61.0 ± 12.0%, $P = 0.011$; Fig. 1b).

Gastric Wet Weight and Thickness After Outlet Obstruction

In this model of GOO, 2-weeks survival in the GOO group was 92.9% ($n = 13/14$); survival in the control group was 92.9% ($n = 13/14$). The changes in the mean weights of the rats are shown in Fig. 2a. After 14 days of obstruction, the gastric wet weight in the GOO group was increased compared with that in the control group (12.2 ± 3.3 g vs. 5.8 ± 1.1 g, $P < 0.001$; Fig. 2b). The thickness of the gastric antral muscle layer was significantly higher in the GOO group than in the control group (675.4 ± 24.6 μm vs. 558.5 ± 20.8 μm, $P < 0.005$; Fig. 2c). Similarly, the thickness of the gastric antral mucosal layer was also significantly increased in the GOO group compared with that in the control group (322.0 ± 26.4 μm vs. 196.8 ± 7.9 μm, $P < 0.001$). In contrast, there was no significant difference in the fasting intraluminal pH of the stomach between the two groups (control group, pH 1.70 ± 0.13 vs. GOO group, pH 1.70 ± 0.26).

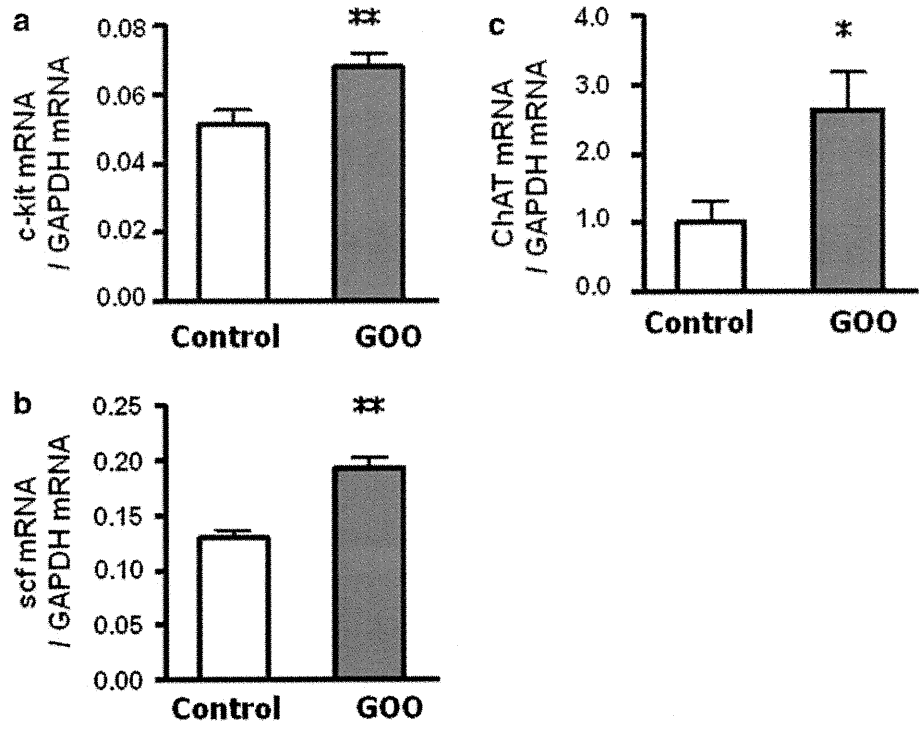

Fig. 4 Gastric c-kit, membrane-bound SCF, ChAT mRNA expression was measured by RT-PCR. **a** gastric c-kit mRNA; **b** membrane-bound SCF mRNA; **c** ChAT mRNA. (mean ± S.E.M.; $n = 13$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control)

Ghrelin Dynamics

The ghrelin dynamics 2 weeks after the operation are shown in Fig. 3. The plasma total and active ghrelin levels were higher in the GOO group than in the control group (plasma total ghrelin; $P = 0.002$, plasma active ghrelin; $P = 0.024$; Fig. 3a, b). In contrast, the gastric total and active ghrelin levels were lower in the GOO group than in the control group (gastric total ghrelin; $P < 0.001$, gastric active ghrelin; $P < 0.001$; Fig. 3c, d). The results of the immunohistochemical analysis to determine the density of the ghrelin-immunoreactive cells in the gastric corpus are shown in Fig. 3e. Increase in the cell count ratio in the GOO group compared with that in the control group was observed ($P < 0.001$).

Gastric Neuromuscular Marker Expression

The mRNA expression levels of these markers as assessed by quantitative RT-PCR analysis are shown in Fig. 4. Significant increases of the expression levels of ChAT mRNA (263.9 ± 54.0% compared with control $P = 0.019$), c-kit mRNA (132.5 ± 7.2% compared with control, $P = 0.008$), and SCF mRNA (149.4 ± 7.8% compared with control, $P < 0.001$) were observed in the GOO group compared with expression levels in the control group.

Effects of [D-Lys3] GHRP-6 on c-Kit and SCF Expression

Significant decreases of expression of c-kit mRNA (54.8 ± 10.3%, $P = 0.024$) and membrane-bound SCF mRNA (51.8 ± 7.5%, $P = 0.009$) were observed in the GOO group after pretreatment with [D-Lys3] GHRP-6 compared with those in the control group (Fig. 5a, b).The expression of ChAT mRNA also tended to decrease to the control level.

Discussion

This study showed experimentally that GOO induced an increase of the fasting plasma ghrelin levels and hyperplasia of the gastric muscle layers. Associated with these pathological processes, expression of ChAT, a marker of vagal efferent fibers in the stomach, c-kit, a marker of the interstitial cells of Cajal (ICC), and SCF, a c-kit ligand on the gastric muscles, were all significantly enhanced.

Two signal transmission pathways from secreted ghrelin to the myenteric plexus in the stomach have been reported [12, 13]. One is a direct route in which ghrelin directly stimulates the GHSR on the surface of the myenteric neurons [12]; the other is an indirect route in which ghrelin signaling stimulates GHSR on the vagal afferent fibers, with the vagal signal traveling through the central nervous system and then to the vagal efferent nerve fibers, finally

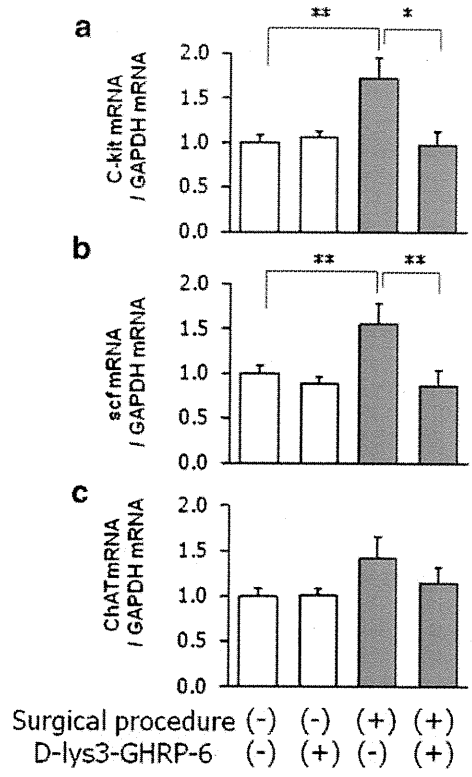

Fig. 5 Effect of synthetic GHS-R1a antagonist ([D-Lys3]-GHRP-6 6.0 mg/kg) on mRNA expression of gastric c-kit (**a**), membrane-bound SCF (**b**) and ChAT (**c**) at 2 weeks after the operation. (mean ± S.E.M.; $n = 8$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control)

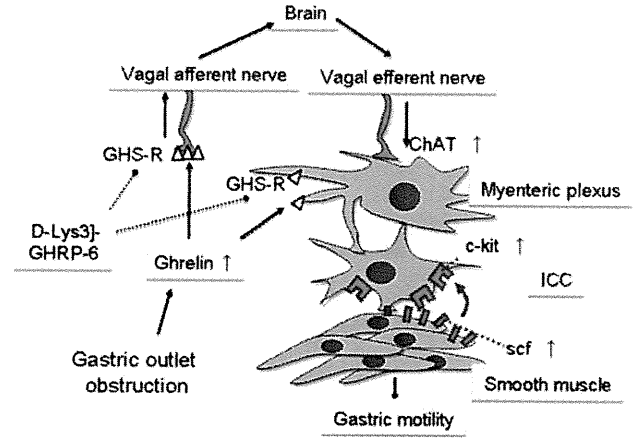

Fig. 6 Model of the association of ghrelin with the ICC network in the present GOO model. Signal of enhanced levels of plasma ghrelin in the GOO model is transmitted to the brain via vagal afferent nerves. Enhancement of ChAT mRNA might be induced via vagal efferent nerves from the central nervous system. Sustained enhanced ghrelin secretion might be associated with the activated ICC network in this animal model. The compensative ghrelin secretion and production are enhanced by gastric outlet obstruction, and enhanced ghrelin activates the ICC network either through the vagal nerve or the direct effect of ghrelin

activating the myenteric plexus [13]. In this study, because not only c-kit and SCF expression but also expression of ChAT were enhanced (Fig. 4), the ghrelin signal might be transmitted not only through the direct route, but also via the indirect route (Fig. 6).

Because [D-Lys3] GHRP-6, a receptor antagonist of GHSR1a, normalized the enhanced expression levels of c-kit and SCF (Fig. 5), it seems that ghrelin signaling might be an upstream event in relation to other neuromuscular activation markers, for example vagal efferent choline acetyl transferase (ChAT), c-kit (ICC), and SCF (gastric smooth muscle).

Both the increase in the plasma ghrelin levels and in the number of ghrelin-immunoreactive cells in the gastric corpus clearly indicates the activation of ghrelin production under the state of GOO (Fig. 3a, b, e). On the other hand, the decreased gastric ghrelin content in GOO (Fig. 3c, d) might be because of emptying (degranulation) of ghrelin from the A-like cells of the stomach in response to fasting. A similar phenomenon has already been reported in the Mongolian gerbil model of *Helicobacter pylori* infection [14].

Whereas the rat model of diabetic gastroparesis induced by STZ showed vagal denervation [15] and the rat model of ischemia–reperfusion induced transient gastroparesis showed vagal and c-kit damage [16], this method of induction of mechanical GOO was superior to the above-mentioned methods, because it involved simple obstruction of gastric outflow without vagal denervation or drug administration. Therefore, we could observe the neurological or hormonal feedback in simple outlet obstruction under the condition of intact gastric mucosa, vagal nerve, and gastric nerve plexus.

In conclusion, this study provides the first evidence to suggest that the production and secretion of gastric ghrelin is increased in rats with GOO, implying that dysregulation of gastric motility may alter the ghrelin dynamics, as reported in clinical settings [7, 17]. This experimental rat model is not only useful for study of GOO, but also for that of chronic gastric emptying disorders, for example gastric paresis or FD, especially postprandial distress syndrome.

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Reduced ghrelin production induced anorexia after rat gastric ischemia and reperfusion

Sachiko Mogami,[1,2] Hidekazu Suzuki,[1] Seiichiro Fukuhara,[1] Juntaro Matsuzaki,[1] Kenji Kangawa,[3] and Toshifumi Hibi[1]

[1]*Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan;* [2]*Tsumura Research Laboratories, Tsumura & Co., Ibaraki, Japan;* [3]*National Cardiovascular Center Institute, Osaka, Japan*

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Mogami S, Suzuki H, Fukuhara S, Matsuzaki J, Kangawa K, Hibi T. Reduced ghrelin production induced anorexia after rat gastric ischemia and reperfusion. *Am J Physiol Gastrointest Liver Physiol* 302: G359–G364, 2012. First published December 8, 2011; doi:10.1152/ajpgi.00297.2011.—The gastrointestinal (GI) tract is one of the most susceptible organs to ischemia. We previously reported altered gastric motility after gastric ischemia and reperfusion (I/R). However, there have also been few reports of alterations in the eating behavior after gastric I/R. Ghrelin is a GI peptide that stimulates food intake and GI motility. Although ghrelin itself has been demonstrated to attenuate the mucosal injuries induced by gastric I/R, the endogenous ghrelin dynamics after I/R has not yet been elucidated. The present study was designed to investigate the relationship between food intake and the ghrelin dynamics after gastric I/R. Wistar rats were exposed to 80-min gastric ischemia, followed by 12-h or 48-h reperfusion. The food intake, plasma ghrelin levels, gastric preproghrelin mRNA expression levels, and the histological localization of ghrelin-immunoreactive cells were evaluated. The effect of exogenous ghrelin on the food intake after I/R was also examined. Food intake, the plasma ghrelin levels, the count of ghrelin-immunoreactive cells corrected by the percentage areas of the remaining mucosa, and the expression levels of preproghrelin mRNA in the stomach were significantly reduced at 12 h and 48 h after I/R compared with the levels in the sham-operated rats. Intraperitoneal administration of ghrelin significantly reversed the decrease of food intake after I/R. These data show that gastric I/R evoked anorexia with decreased plasma ghrelin levels and ghrelin production, which appears to be attributable to the I/R-induced gastric mucosal injuries. The decrease in the plasma ghrelin levels may have been responsible for the decreased food intake after gastric I/R.

food intake; ghrelin; mucosal injury

GASTROINTESTINAL (GI) TRACT is one of the most susceptible organ systems to ischemia. Various investigations have demonstrated that ischemia and reperfusion (I/R) contribute significantly to the gastric mucosal injuries caused by stress, such as burn stress (17) or hemorrhagic shock (35), nonsteroidal anti-inflammatory drugs (30), and *Helicobacter pylori* (*H. pylori.*) infection (26, 27). We previously demonstrated, not only postischemic mucosal injury, but also transient delay in gastric emptying in a rat model of gastric I/R (28). These changes were found to be associated with disruption of the network of the interstitial cells of Cajal and decrease in neuronal nitric oxide synthase-positive neurons in the smooth muscle layer. On the other hand, there have been no reports on alterations in eating behavior after gastric I/R, at least to our knowledge.

Ghrelin, a 28-residue octanoylated peptide, is an endogenous ligand of the growth hormone secretagogue receptor (18) and is produced and secreted from the A-like cells found mainly in the oxyntic glands of the gastric fundus (8). Gastric ghrelin accounts for the major part of circulating ghrelin, as an ~80% reduction in the circulating levels of ghrelin has been demonstrated after gastrectomy or fundectomy (10). Ghrelin is now known to play a role, not only in growth-hormone release, but also in stimulating gastric motility and food intake (1, 21, 32). Recent studies have also reported the gastroprotective effect of ghrelin; ghrelin has been demonstrated to reduce ethanol-induced gastric ulceration (23), acetic acid-induced chronic gastric and duodenal ulceration (6), and I/R-induced gastric ulceration (11) in rats. Although changes in the plasma ghrelin levels and association with various GI diseases have been reported such as in functional dyspepsia (22), chronic gastritis and gastric ulcer (14), the ghrelin dynamics after gastric I/R has yet to be elucidated.

The present study was designed to investigate the influences of gastric I/R injuries on the food intake and ghrelin dynamics in a rat model of gastric I/R injury.

MATERIALS AND METHODS

I/R. Six-week-old male Wistar rats were purchased from Japan SLC (Shizuoka, Japan). All rats were handled according to the guidelines of the Keio University Animal Research Committee (approved protocol No. 078086) and the Experimental Animal Ethics Committee of Tsumura & Co. (approved protocol No. 09–155, 09–157, 10–096, 10–110, 10–156). All rats were used after acclimation for 1 wk and denied access to food for 22–24 h (but allowed free access to water) before the operation. The rats were anesthetized with pentobarbital sodium (50 mg/ kg ip) during the surgery. The abdomen was opened by a midline incision, and the celiac artery was occluded with a small clamp for 80 min. Reperfusion was established for 12 h or 48 h by removal of the clamp. For comparison, some rats were subjected to a sham operation (surgery, but no clamping). Rats were supplied with food after the surgery (returned to normal feeding). Food intake was measured at 12 h after I/R (when gastric emptying was delayed compared with sham-operated rats) and at 48 h after I/R (when gastric emptying was restored) (Fig. 1*A*). In the fasting condition, food deprivation was continued after the surgery when reperfusion was established for 12 h. When reperfusion was established for 48 h, the rats were fed after the operation (normal feeding), but were again deprived of food for 24 h before euthanasia to establish the fasted condition (Fig. 1*B*). To measure plasma ghrelin levels in the fed condition at 48 h after I/R, I/R rats were fed ad libitum after the surgery. Sham-operated rats were given the same amount of food as

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