Induction of LGR5 by H_2O_2 treatment is associated with cell proliferation via the JNK signaling pathway in colon cancer cells

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Abstract. Recently, the leucine-rich repeat G protein-coupled receptor 5 (LGR5/GPR49) was identified as a potential marker of intestinal stem cells in human. The LGR5 is known as a Wnt signaling target gene, and its expression pattern is related with β-catenin mutation. H_2O_2 is a member of reactive oxygen species (ROS) and regulates metabolism, aging, apoptosis and the intensity of growth factor signaling. In addition, it acts as a negative or positive regulator of Wnt signaling. However, the effect of H_2O_2 on Wnt signaling and its target gene LGR5 is not clear. In this study, we investigated the effects of ROS on cancer stem cells, in colorectal cancer cells. Colorectal cancer cells were treated with exogenous H_2O_2, after which cellular responses and the expression of LGR5 were examined. In SNU-C2A cells, proliferation increased following treatment with 50-300 μM of H_2O_2, whereas cell viability significantly decreased after treatment with 600-900 μM of H_2O_2. Expression of heme oxygenase (HO)-1 and jun, which aid in the reduction of oxidative stress, were induced in the low dose H_2O_2-treated SNU-C2A cells. The LGR5 expression level was significantly increased following 50-300 μM H_2O_2 treatment; in addition, β-catenin was increased in H_2O_2-treated colon cancer cells. However, the increased β-catenin was detected not in the nucleus but in the cytoplasm, which means that β-catenin was stabilized in the cytoplasm and not translocated into the nucleus where it could function as a transcription factor for the expression of LGR5. In addition, there was no direct interaction between LGR5 and β-catenin. In this study, we found that LGR5 expression increased when cancer cells were treated with a low dose of H_2O_2. Our results indicate that the LGR5 increase resulted via activation of the JNK signaling pathway. The induction of LGR5 expression influenced cell proliferation in colorectal cancer cells.

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Introduction

In a recent study, the leucine-rich repeat G protein-coupled receptor 5 (LGR5, also known as GPR49) was identified as a potential marker of intestinal stem cells in human (1-3). LGR5 is expressed mainly in crypt base columnar (CBC) cells at the bottom of intestinal crypts and it is a markers for both cancer and normal intestinal stem cells (1). Cancer stem cells (CSCs) are a small population of cancer cells that have the ability to affect tumor initiation, differentiation and metastasis. The self-renewal and differentiation capabilities of CSCs are similar to those of normal embryonic or adult stem cells (4,5). For isolation of CSCs, some cell surface markers, so-called cancer stem cell markers, can be used. In the intestine, functional stem cells are located at the bottom of the crypts, especially at the +4 position (1-3), and they can be isolated and studied via molecular markers such as CD34, CD44, CD133, and LGR5 (6-8). CSCs play an important role in cancer recurrence after radio- or chemotherapy (9-11), and CSC-mediated resistance to radiation and chemotherapeutic agents is influenced by the cellular level of reactive oxygen species (ROS) (11,12).

Many ROS, including O_2^-, OH^-, and H_2O_2, are produced by physiological intracellular reactions and function not only as a byproduct of cellular metabolisms but also as a mediator of cell signaling pathways (13).

Diehn *et al* demonstrated that CSCs in breast and head/neck tumors have lower ROS levels and higher expression levels of antioxidant genes or proteins, compared to levels in non-tumorigenic progeny, which were similar to levels in normal stem cells (12). However, the effects of ROS on CSCs have not been fully described.

The above-mentioned LGR5 is a Wnt target gene and its expression pattern has been related to β-catenin mutation (2,14,15). It has been shown that H_2O_2 can inhibit the Wnt/β-catenin signaling pathway through decreases in the amount of nuclear β-catenin and Tcf/Lef dependent transcription (16). In contrast, Funato *et al* showed that H_2O_2 induces β-catenin stabilization and increases the expression of endogenous Wnt target genes (17). Despite these studies, little is known about the effect of H_2O_2 on the Wnt/β-catenin signaling pathway.

The purpose of this study is to reveal the effects of ROS on various aspects of CSCs, in colorectal cancer cells. To study this, colorectal cancer cells were treated with exogenous H_2O_2,

Table I. Sequences of specific primers used in the study.

Gene		Primer sequence	Size (bp)	Refs.
h LGR5	Forward	5'-AGGATCTGGTGAGCCTGAGAA-3'	151	(25)
	Reverse	5'-CATAAGTGATGCTGGAGCTGGTAA-3'		
β-catenin	Forward	5'-TCTTGGCTATTACGACAG-3'	459	(16)
	Reverse	5'-CCTCTATACCACCCACTT-3'		
HO-1	Forward	5'-GCTCAACATCCAGCTCTTTGAGG-3'	282	-
	Reverse	5'-GACAAAGTTCATGGCCCTGGGA-3'		
β-actin	Forward	5'-GACCACACCTTCTACAATGAG-3'	301	(17)
	Reverse	5'-GCATACCCCTCGTAGATGGG-3'		

and then examined for expression of the CSC marker, LGR5, and for cellular responses.

Materials and methods

Cell culture. Thirty of the 32 human colorectal cancer cell lines were maintained in RPMI-1640 media. The CACO-2 cell line was maintained in minimum essential medium and the WiDr cells were maintained in Dulbecco's modified Eagle's medium (cells obtained from Korean Cell line Bank, Seoul, Korea). All media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin. Cells were maintained in humidified incubators at 37˚C, with an atmosphere of 5% CO_2 and 95% air.

Cell proliferation assay by MTT. Cell proliferation was analyzed by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] (Sigma Chemical Co.) assay.

Initially, $3x10^6$ cells were seeded on 96-well plates and cultured for 1 day. Subsequently, media containing 50-900 μM of H_2O_2 were added to the plates and the cells incubated for 24 h before being treated with 50 μl of MTT solution for 4 h at 37˚C. Absorbance at 540 nm was measured by an ELISA reader (Molecular Devices Co., CA, USA).

Total RNA extraction, cDNA synthesis and reverse transcriptase-PCR (RT-PCR). Total RNA was isolated by using easy-BLUE™ kits (Intron Biotechnology, Gyeonggi, Korea). For cDNA synthesis, 2 μg of total RNA were combined with a random primer, dNTP, and 200 U of Superscript II™ reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a final volume of 20 μl. The mixture incubated for 60 min at 42˚C, after that incubated for 15 min at 70˚C for denaturation. Next, 80 μl of distilled water was added to the reverse-transcription reaction mixture. PCR was performed to obtain mRNA expression data. The prepared 15 μl PCR mixtures included 1 μl of cDNA, 10X buffer, 2.5 mM of dNTP, 0.1 pM of primers and 1 U of Taq DNA polymerase (Intron Biotechnology). PCR amplification was conducted using the described primer sets (Table I). The β-actin was used as an endogenous reference for the normalization of expression levels. The following PCR conditions were used: initial denaturation for 5 min at 94˚C, cycling at 94˚C (30 sec), 55˚C (30 sec), and 72˚C (30 sec) for 35 cycles, with final elongation for 7 min at 72˚C. PCR was performed in a thermal cycler (PCR System 9700, Applied Biosystems, Foster City, CA, USA). The PCR products were divided in 2% agarose gel stained with ethidium bromide.

Treatment of hydrogen peroxide. For exogenous H_2O_2 treatment, cells were seeded in 75 cm^2 culture flasks at a rate of $0.5x10^6$ cells per flask. After 1 day, the cells treated with 0, 50, 100, 150, 300, 600, or 900 μM of H_2O_2 (Sigma Chemical Co.) for 24 h. Subsequently, the cells were harvested for protein, RNA isolation, and cell cycle analysis.

Western blotting. For protein analysis, harvested cells were washed in PBS, suspended in Pro-Prep™ kits (Intron Biotechnology) and placed on ice for 30 min. After centrifuging at 13,000 x g for 30 min at 4˚C, the supernatant was collected. Protein quantitative analysis was accomplished by using Bradford's method using BSA (Sigma Chemical Co.). Protein (20 μg) and 4X SDS sample buffer (Invitrogen) mixtures were boiled at 95˚C for 5 min and loaded on 4-12% Bis-Tris gel (Invitrogen) at 100 V for 3 h. Proteins were transferred to a PVDF membrane (Invitrogen) by electroblotting at a constant 270 mA current for 2 h. For membrane blocking, the membrane was incubated for 1 h at room temperature in 1.5% non-fat dry milk and 0.5% Tween-20 TBS buffer containing 1 mM of $MgCl_2$. Primary antibodies against hLGR5 (GPR49) (Abcam, Cambridge, UK) (1:500), heme oxygenase-1 (Abcam) (1:1,000), jun (BD, New Jersey, USA) (1:1,000), PARP (BD) (1:1,000), β-catenin (Abcam) (1:2,000), HSP60 (Abcam) (1:1,000), and Lamin-B (Abcam) (1:1,000) were incubated overnight at 4˚C. For secondary antibody incubation, peroxidase conjugated mouse or rabbit IgG antibody (Jackson Immunoresearch, Baltimore, MD, USA) was diluted 1:5,000. After incubation at room temperature for 1 h, a chemiluminescent working solution, WESTZOL™ (Intron Biotechnology) was decanted to the membrane. The membrane was then covered with a thin plastic wrap and exposed to Fuji RX film for 1-60 min.

Cell cycle analysis. For cell cycle distribution analysis, cells treated with H_2O_2 were fixed overnight in 70% ethanol at -20˚C. Subsequently, cellular DNA was stained with 100 μg/ml of propidium iodide (PI) (Sigma Chemical Co.) for 30 min on ice. After staining, cells were subjected to fluorescence-activated

cell sorter (FACs CantoII™, BD, NJ, USA) analysis of DNA content to determine the percentage of the cells in different cell phases and in apoptosis.

Immunocytochemical staining. For visual cell examination, $0.5x10^5$ cells were grown on cover slips in 12-well culture plates. After 1 day, the cell-covered slips were treated with H_2O_2 for 24 h, then rinsed briefly in PBS and fixed with 3.7% formaldehyde (Sigma Chemical Co.) in PBS for 15 min at room temperature. For permeabilization, samples were incubated for 10 min with 0.25% Triton X-100 (Merck, Darmstadt, Germany) in PBS at room temperature. Samples were then incubated with 1% BSA (Invitrogen) in PBS-T for 30 min at room temperature for blocking, and then treated with 1:1,000 diluted LGR5 and β-catenin primary antibody in PBS-T overnight at 4˚C. Subsequently, the samples were rinsed and incubated with 1:500 diluted goat anti-rabbit secondary antibody for 2 h at room temperature in the dark. 4',6-diamidino-3-phenylindole dihydrochloride hydrate (DAPI, 100 μg/ml) (Sigma Chemical Co.) was used for counter staining. Cells were visualized using a Pascal confocal microscope (Carl Zeiss, Oberkochen, Germany).

Preparation of nuclear extraction. The cells were removed from the culture flask by scraping and washed in PBS. For cytoplasmic fraction collection, cells were resuspended in 500 μl of 1X hypotonic buffer and 25 μl of detergent (Active Motif, Shinjuku, Japan). Following centrifuging at 14,000 x g for 30 sec at 4˚C, the supernatant (the cytoplasmic fraction), to a new tube. Remaining nuclear pellet was resuspended in 50 μl of complete lysis buffer (Active Motif) and incubated for 30 min on ice. Following which it was centrifuged for 10 min at 14,000 x g at 4˚C. The supernatant (the nuclear fraction), was then transferred to a new tube.

Co-immunoprecipitation of LGR5. LGR5 primary antibody (5 μg) was added to the microcentrifuge tube that contained the SNU-C2A lysate (200 μg); the mixture was then incubated overnight at 4˚C. Subsequently 20 μl of anti-rabbit IgG beads (eBioscience, Iceland, UK) were added and the mixture incubated for 1 h at 4˚C. Next, the tube was centrifuged for 1 min at 10,000 x g at 4˚C. The supernatant was then removed and the bead pellet resuspended using Pro-Prep™ (Intron Biotechnology). After washing, add 20 μl of 2X SDS sample buffer (Invitrogen) were added and the mixture boiled at 95˚C for 10 min. While avoiding loading of anti-rabbit IgG beads, the supernatant was loaded on to 4-12% Bis-Tris gel.

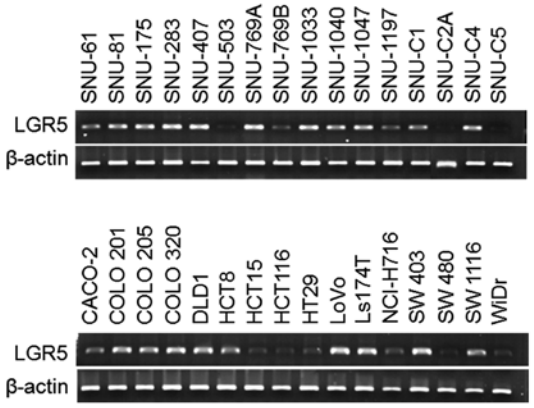

Figure 1. Analysis of LGR5 expression level. LGR5 expression levels in 32 human colorectal cancer cell lines were analyzed by RT-PCR. Each sample was normalized to β-actin levels.

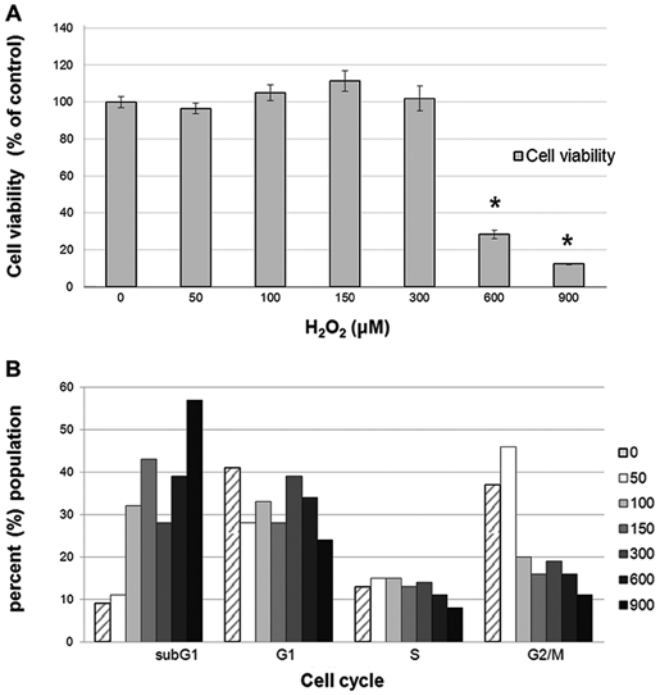

Figure 2. Increased cell proliferation following low dose H_2O_2. (A), Cell proliferation assay results using the MTT method. SNU-C2A cells treated with 50, 100, 150, 300, 600, or 900 μM of H_2O_2 for 24 h before determining cell viability. To validate the data, the experiment was repeated three times (*$p<0.05$). (B), Cell cycles analyzed by using fluorescence-activated cell sorter (FACs). Cells treated with H_2O_2 were fixed in 70% ethanol before staining DNA with 100 μg/ml of PI.

Results

Increased apoptotic cell death at high dose of H_2O_2. For the analysis of expression level of LGR5, we investigated expression in 32 human colorectal cancer cell lines by RT-PCR. LGR5 was highly expressed in most of cell lines (29/32 cell lines; 90.63%) and expressed at a low level in 3 cell lines (SNU-503, SNU-C2A, and SNU-C5) (Fig. 1). To determine whether ROS treatment would affects the cell proliferation in SNU-C2A, which has wild-type β-catenin (18), SNU-C2A cells were treated with 50, 100, 150, 300, 600, or 900 μM of H_2O_2 and incubated for 24 h. As shown in Fig. 2A, the number of SNU-C2A cells increased when treated with a low dose of H_2O_2, but the relationship dosage and cell increase had no significant correlation. However, SNU-C2A cell viability was significantly decreased when treated with 600 or 900 μM of H_2O_2 ($p<0.005$). Park *et al* suggested that a low level of H_2O_2 promotes cancer cell proliferation, compared to that in the control cells, while at a higher H_2O_2 concentration the cancer cells exhibited an 80% growth inhibition with apoptosis triggered via activation of the AMP-activated protein kinase (AMPK) pathway (19). Here, since SNU-C2A cell viability increased at the low concentration of H_2O_2, the percentage of

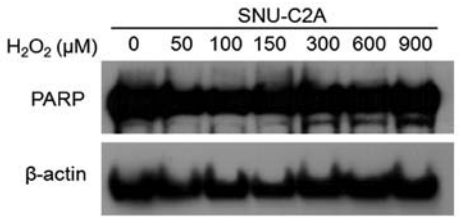

Figure 3. Western blotting of PARP for apoptosis assessment. Intact PARP was identified as a 116-kDa band (upper band). During apoptosis, PARP is cleaved from the intact form into 85 kDa (lower band). When treated with H_2O_2, the cleaved PARP form increased in dose-dependent manner.

subG1, G1, S, and G2/M cell cycle phases were determined by FACs in order to investigate the effect of H_2O_2 on the cell cycle. As shown in Fig. 2B, the percentage of cells in the G1 stage increased under treatment with 50-300 μM of H_2O_2, which indicates that the cell cycle was arrested to evade cell death by the ROS. However, at higher H_2O_2 concentration the percentage of cells in the G2/M stage decreased, and that in the subG1 stage increased. The increase in the subG1 stage is mediated through cell death at the higher concentration of H_2O_2.

As PARP [poly(ADP-ribose) polymerase] cleavage is considered a marker of apoptosis (20), we performed western blotting to detect the presence of PARP cleavage. The level of the 85 kDa cleavage form of PARP, was increased at the higher concentrations of H_2O_2 (Fig. 3, lower bands).

These results suggest that ROS treatment of over 300 μM of H_2O_2, will arrest the cell cycle, with the cells then falling into apoptosis in the SNU-C2A cell line. As a result, the SNU-C2A proliferation rate significantly decreased when treated with 600 or 900 μM of H_2O_2.

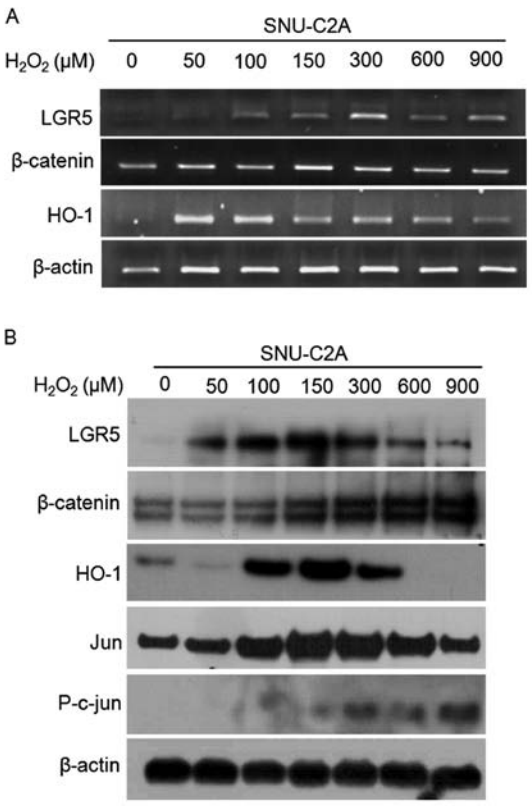

Figure 4. Induction of β-catenin and LGR5 expression following treatment with H_2O_2. (A), RT-PCR was performed after treatment with H_2O_2. SNU-C2A cancer cells were treated with 50, 100, 150, 300, 600, or 900 μM of H_2O_2 and mRNA expressions of LGR5, β-catenin, and HO-1 were assessed. Each sample was normalized by β-actin level. (B), Protein levels determined by western blotting after treatment with H_2O_2. In each lane, 20 μg of protein was loaded.

JNK signaling pathway regulates the expression level of LGR5 by ROS. To investigate whether ROS affected expression of a cancer stem cell marker, we examined LGR5 expression level after H_2O_2 treatment of SNU-C2A cells for 24 h, by using RT-PCR, western blotting, and immunocytochemical staining (Figs. 4 and 5A).

To clarify the effect of ROS in these cells, we assessed the expression level of HO-1, which is induced by various stimuli and is known to play a significant role in protection of cells against oxidative injury, heat shock, and ROS. In addition, HO-1 may be involved in anti-apoptotic activity through activation of the Akt pathway (21). We detected overexpression of HO-1 at treatment of 100-300 μM H_2O_2 in SNU-C2A cells (Fig. 4). We also investigated jun expression, considered to be another indicator that affected by ROS. The expression pattern of jun, a JNK signaling pathway component, was similar to HO-1. Jun expression resulted from activation of the JNK signaling pathway, which is also involved in H_2O_2 induced upregulation of HO-1 (22). When jun is activated by phosphorylation, the JNK signaling pathway is stimulated (23). Here, phospho-jun increased in a dose-dependent manner with H_2O_2 treatment (Fig. 4B), indicating that JNK signaling is activated by H_2O_2. Our results support previous MTT results (Fig. 2) and suggest that JNK signaling has a protective effect against ROS (22). In addition, the result indicate that ROS induce cell proliferation through upregulation of jun and via cross-talk with the PI3-K/PKB pathway (24). Furthermore, a recent study has shown that LGR5 is a novel target of c-jun/Mbd3 (25). Based on our results, we can confirm a similar expression pattern in LGR5 and jun, leading to the suggestion that activation of JNK signaling is associated with alteration of LGR5 expression by ROS.

Induction of β-catenin and LGR5 as treated with H_2O_2. LGR5 expression increased following cell treatment with 50-300 μM of H_2O_2; a finding that is similar to the proliferation increase noted in Fig. 2. In contrast, LGR5 expression decreased at doses higher than 600 μM of H_2O_2, which were the same level at which apoptosis was induced (Figs. 4 and 5A).

Since LGR5 is a Wnt target gene (2,14,15), we analyzed the correlation between LGR5 and β-catenin, a Wnt signaling component. As shown in Fig. 4, the expression level of β-catenin increased with H_2O_2 treatment in a dose-dependent manner. Immunocytochemistry was performed in SNU-C2A cells that were treated with the same dosage levels of H_2O_2 as shown in Fig. 4 (Fig. 5). The expression patterns of LGR5 and β-catenin were found to be similar (Fig. 4).

To determine whether induction of LGR5 is the result of β-catenin-mediated Wnt signaling, the location of β-catenin within the cells was determined. As shown in Fig. 5, β-catenin was mainly present in the cytoplasm. To confirm the immunocytochemistry results, we separated and isolated cytoplasmic and nuclear proteins, and performed western blotting. The β-catenin levels were lower in the nucleus of H_2O_2-treated cells than in the cytoplasm (Fig. 6). For activation of β-catenin-

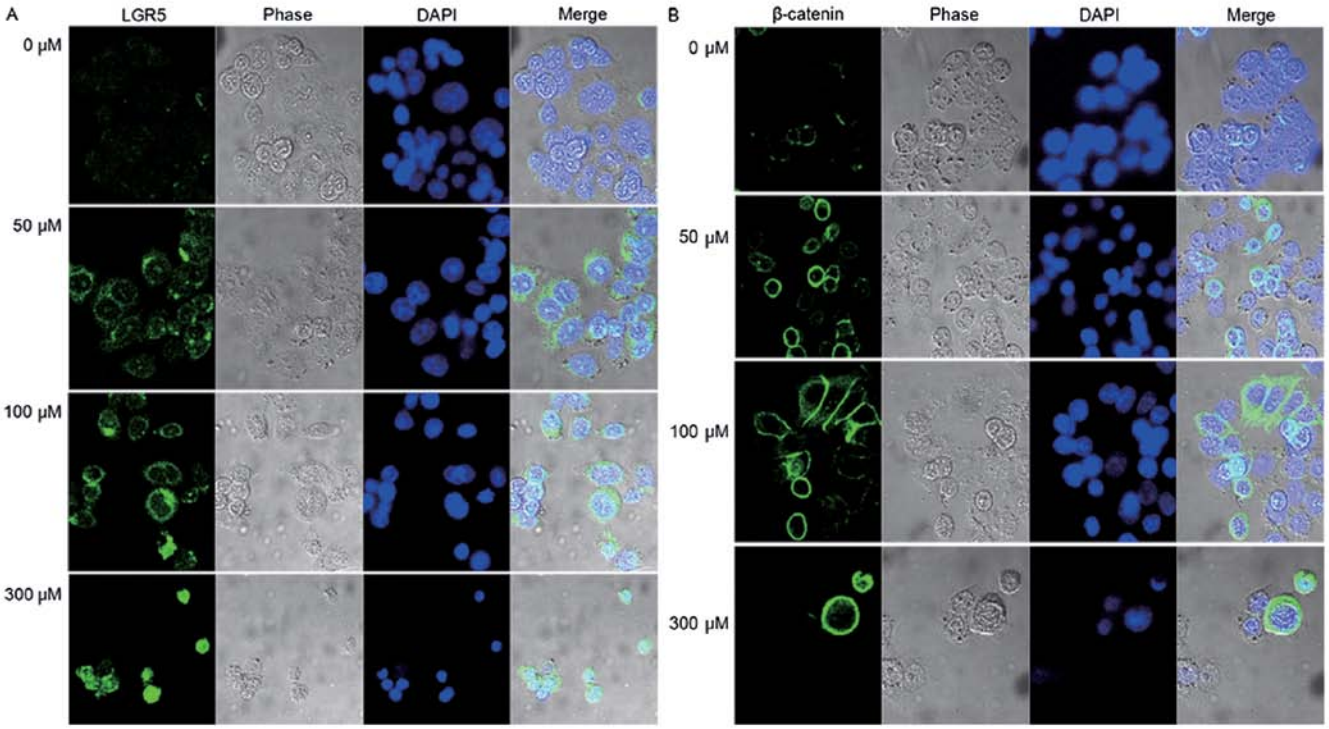

Figure 5. Immunostaining of LGR5 and β-catenin. SNU-C2A cancer cells were treated with H_2O_2 for 24 h. LGR5 (A) and β-catenin (B) expression levels were detected by staining FITC-conjugated antibody and nucleus by DAPI (100 μg/ml).

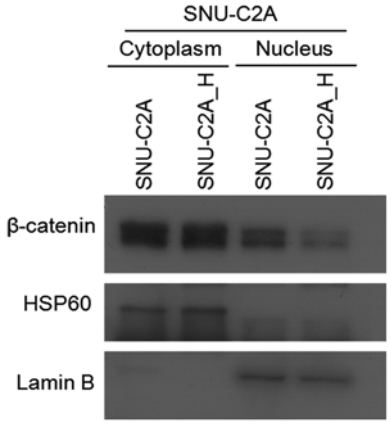

Figure 6. The expression pattern of β-catenin. After treatment of H_2O_2, protein was extracted separately from cytoplasm and nucleus. HSP60 is a mitochondrial chaperon protein that expressed in cytoplasm. Lamin B is an intermediate filament that provides structural function and transcriptional regulation in the cell nucleus (C, SNU-C2A; H, SNU-C2A treated with 150 μM of H_2O_2 for 24 h).

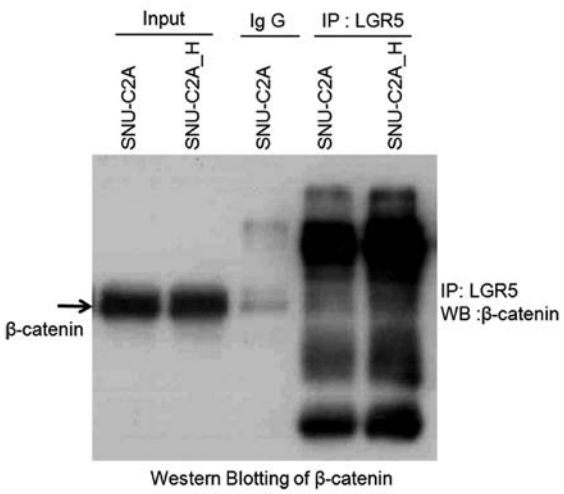

Figure 7. Co-immunoprecipitation of LGR5. For co-immunoprecipitation of LGR5, SNU-C2A lysates that were treated with H_2O_2 were prepared. β-catenin was not detected by western blotting in the immunoprecipitate with LGR5 (WB, western blotting; C, SNU-C2A; H, SNU-C2A treated with 150 μM of H_2O_2 for 24 h).

mediated Wnt signaling, stabilized β-catenin needs to be translocated to the nucleus and not degraded in the cytoplasm. Our results show that nuclear β-catenin levels were low; therefore, the expression of LGR5 could be regulated by factors other than those in Wnt signaling.

To investigate this, we performed co-immunoprecipitation to determine if LGR5 directly interacts with β-catenin. Western blotting with anti-β-catenin was used to analyze the co-immunoprecipitation obtained by using the LGR5 antibody. As shown in Fig. 7, β-catenin was not detected. Therefore, we concluded that LGR5 did not interact with β-catenin.

Discussion

LGR5 (GPR49) has been identified as a marker of tumor initiation (2). The LGR5 gene encodes a G protein coupled receptor that is associated with a glycoprotein ligand. LGR5 gene marks small cells, especially cycling crypt-base columnar (CBC) cells that are interspersed between Paneth cells (1). Moreover, physiological expression of LGR5 is restricted to cells at the base of crypts, a location known as a stem cell niche. LGR5 is

one of the Wnt signaling target genes in colorectal cancer and is critically involved in the development of carcinoma associated with β-catenin mutation (14,15). Wnt signaling regulates intestinal epithelium cell lineage proliferation, differentiation, and maintenance (26). When mutated, such as APC mutation, the Wnt signaling pathway is activated, and it plays a role in tumorigenesis in humans (26). Therefore, it has been suggested that activation of Wnt signaling and expression of Wnt target genes are involved in tumor initiation (27).

In our study, high expression of LGR5 was observed in 90.6% the human colon cancer cell lines tested (Fig. 1). However, in a few cell lines, including SNU-C2A, the LGR5 expression level was low. In order to examine the effect of ROS on colorectal cancer cells and on the cancer stem cell marker LGR5, experiments with the treatment of the H_2O_2 were perform. H_2O_2 is reported to regulate the intensity of growth factor signaling, metabolism, aging and apoptosis (28). In addition, it acts as either a negative or positive regulator in the Wnt signaling pathway. Shin *et al* have shown that H_2O_2 can inhibit Wnt signaling through the down-regulation of β-catenin (16). In contrast, Funato *et al* showed that treatment of cells with a low dose of H_2O_2 induces stabilization of β-catenin and increased expression of endogenous Wnt target genes (17). Even in our results, the effects of H_2O_2 on Wnt signaling and a target gene were not clear. However, based on such findings, LGR5 expression is expected to be affected by H_2O_2.

In our study, SNU-C2A colon cancer cells, which have wild-type β-catenin (17) and a low expression of LGR5, were used to determine the correlation between ROS and LGR5.

To that end, SNU-C2A cells were treated with 50, 100, 150, 300, 600, or 900 μM of H_2O_2 for 24 h to examine the ROS effects. According to Park *et al*, low doses of H_2O_2 resulted in enhanced cell proliferation accompanied by an increase in COX-2 expression, whereas apoptosis was induced by high doses of H_2O_2 and was correlated with AMPK activation (19,29). There were similarities in our results. As shown in Fig. 2A, the number of SNU-C2A cells increased to a greater extent when treated with low doses of H_2O_2, indicating that cell proliferation increased with low dose treatment of H_2O_2; however, there was no statistical significance to the difference. With high doses of H_2O_2, however, cell viability significantly decreased with increased cell death with treatment of 600 and 900 μM of H_2O_2. As cell proliferation changed with different H_2O_2 dosage, we performed cell cycle analysis using the same dosages of ROS. As shown in Fig. 2B, the percentage of cells in the G1 stage increased following treatment with 50-300 μM H_2O_2. At higher H_2O_2 concentrations percentage of the cells in the G2/M phase decreased and those in the subG1 phase increased. The results indicate that, at low levels of H_2O_2 treatment, the cell cycle is arrested at the G1 phase to evade cell death. However, at the higher H_2O_2 dosage the increase in the subG1 stage is mediated through apoptotic cell death, which we confirmed through assessment of the amount of PARP cleavage (Fig. 3, lower bands).

We also investigated whether H_2O_2 affects expression of LGR5. Expression of HO-1 was induced in H_2O_2-treated SNU-C2A in a dose-dependent manner (Fig. 4). The HO-1 is involved in heme catabolism and plays an anti-apoptotic role against various stimuli, including those related to ROS and heat shock (21). Therefore, our result suggest that an increase in HO-1 would indicate that H_2O_2 is a potent inducer of cellular responses to ROS and that HO-1 was expressed in order to reduce oxidative stress in the treated SNU-C2A cells. JNK signaling, related to the promotion of cell survival, was also induced by H_2O_2 treatment (22). Following H_2O_2 treatment, the expression of jun, a JNK signaling component, increased in a pattern similar to that of HO-1 (Fig. 4). This result suggests that an increase in jun expression can be regulated by the cellular ROS level; thus, aiding in the prevention of cell death, as was mentioned for HO-1. The LGR5 expression level significantly increased following low dose treatment of H_2O_2 in colon cancer cells. A recent study showed that LGR5 is a novel target of c-jun/Mbd3 (25), and our results confirm a correlative pattern between LGR5 and jun expression, leading to the suggestion that activation of JNK signaling by ROS is associated with LGR5 expression.

In conclusion, induced HO-1 and jun expression by H_2O_2 treatment results in a reduction of cellular ROS; thereby, LGR5 expression is increased. According to these results, LGR5 is one of the stress induced genes. Since LGR5 is a Wnt target gene (2,14,15), we supposed that LGR5 expression increases through H_2O_2 activation of β-catenin-mediated Wnt signaling. Fig. 4 shows that β-catenin expression levels, associated with the Wnt signaling pathway, also increased in H_2O_2-treated colon cancer cells in a dose-dependent manner. However, an increased level of β-catenin was detected only in cytoplasm, not in the nucleus (Figs. 5 and 6). As a result, Wnt signaling would not be involved in H_2O_2-mediated expression of LGR5; thus, further investigation is needed. Cross-talk between JNK and Wnt signaling has been reported (30) and we speculated that there could be a correlation between β-catenin and jun. However, nuclear β-catenin, which acts as a Wnt signaling transcriptional factor, was not detected. Therefore, there was no relevance to the association between JNK and Wnt signaling. Since induction of LGR5 seemed not to be regulated by Wnt signaling, we performed co-immunoprecipitation in order to determine if LGR5 directly interacts with β-catenin (Fig. 7). However, we detected no direct interaction between LGR5 and β-catenin.

In this study, we demonstrated that LGR5 and β-catenin expression in colorectal cancer cells is increased by H_2O_2 treatment. However, there remain questions as to why the expression levels of LGR5 and β-catenin increased at the same time and what their relative functions may be. In order to answer these questions, further study is necessary; we expect that our data will be fundamental to such a study.

Acknowledgements

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