Small interfering RNA targeting of Recepteur d'Origine Nantais induces apoptosis via modulation of nuclear factor-κB and Bcl-2 family in gastric cancer cells

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Abstract. The abnormal accumulation and activation of the receptor tyrosine kinase, Recepteur d'Origine Nantais (RON), has been implicated in tumorigenesis and metastasis in epithelial tumors including gastric cancer. This study examined whether the sequence-specific small interfering RNA (siRNA) suppression of the RON expression could induce apoptotic cell death, and investigated the involved molecular mechanisms. Sequence-specific siRNA effectively suppressed the RON expression at both the mRNA and protein levels. Silencing of the RON expression significantly inhibited gastric cancer cell proliferation and induced apoptosis in a time-dependent manner. The induction of apoptosis was confirmed by the ladder-patterned DNA fragmentation, the presence of cleaved and condensed nuclear chromatin and the increased number of annexin V-positive cells. RON-targeted siRNA effectively inhibited the constitutive nuclear factor-κB (NF-κB) activation as revealed by an altered electrophoretic mobility shift. In agreement with this, silencing of the RON expression resulted in a decrease in the nuclear level of the p65 subunit of NF-κB. The transfection of siRNA, which blocked the RON expression, also caused a change in the ratio of Bax/Bcl-2 in a manner that favored apoptosis. The siRNA silencing of RON induced cytochrome c release and the activation of caspase-8 and caspase-9. These results indicate that RON-targeted siRNA could be therapeutically efficacious by inducing cell apoptosis through the modulation of the NF-κB and Bcl-2 family in gastric cancer cells.

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Introduction

Although the incidence of gastric cancer has decreased over the last few decades, it is still the most frequent cancer of the digestive tract. It has a poor prognosis and a high mortality rate. Approximately 24,000 and 700,000 new cases of gastric cancer are diagnosed each year in the United States and worldwide, respectively (1,2). Due to local tissue invasion and metastasis, radiation therapy or chemotherapy does not significantly affect the length or quality of life of patients with advanced gastric cancer. Thus, novel therapies are required in order to target the molecular alterations that lead to gastric cancer development and progression. The Recepteur d'Origine Nantais (RON), a member of the c-MET family of scatter factor receptors, can be activated through ligand-dependent or -independent mechanisms (3), which lead to responses important for tumorigenesis and metastasis (4). The macrophage-stimulating protein (MSP) is the only ligand that has been identified for RON (5). Upon ligand binding, RON dimerizes, autophosphorylates, and transduces a variety of signals that regulate different downstream pathways including the Ras/mitogen-activated protein kinase (MAPK), the phosphatidylinositol 3-kinase (PI3K), the c-Jun N-terminal kinase (JNK), ß-catenin, and the nuclear factor-κB (NF-κB) (6,7). Several human tumor tissues show the aberrant expression and activation of RON, including tumors of the breast, colon, and prostate (8). The altered RON expression is also accompanied by the production of splice variants such as RONΔ160, which possesses tumorigenic activities *in vivo* (6). RON and its splice variant play an important role in the occurrence, progression, and metastasis of gastric carcinoma (9). Besides its role in tumorigenesis and metastasis, RON is also involved in cell apoptosis. Silencing of the RON gene expression results in increased apoptotic death in colon cancer cells (10). The abrogated RON expression in cells treated with RON-specific small interfering RNA (siRNA) is associated with increased numbers of apoptotic cells with nuclear condensation in thyroid cancer cells (11). However, the detailed mechanisms involved in this process have not been fully elucidated. Recent studies have suggested that the regulation of NF-κB activity could be important in cell survival (12). NF-κB exists in a latent form in the cytoplasm

bound to the inhibitory protein, IκB. The released NF-κB dimer from IκB translocates to the nucleus and then activates target genes by binding to the promoter/enhancer region. The known target genes of NF-κB include a number of anti-apoptotic genes, such as the Bcl-2 family. There is growing evidence to support the role of NF-κB in promoting cell survival and also in the protection against apoptosis (13). In order to study the biological role of RON in gastric cancer, we inhibited the expression of RON via RNA interfering. The application of siRNA to RON inhibited constitutive NF-κB activation and caused a change in the ratio of Bax/Bcl-2 in a manner that favors apoptosis in gastric cancer cells.

Materials and methods

Cell culture and viability assay. Human gastric cancer MKN28, AGS, and MKN45 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). SNU638 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured with RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37˚C in an atmosphere containing 5% CO_2. In order to examine the effect of transfected siRNA on cell viability, the cells were incubated in RPMI with low serum (1% FBS) containing 20 nM oligonucleotide (Sigma-Aldrich, St. Louis, MO, USA) for 72 h, and then cell respiration was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich) assay.

siRNA transfection. Gene silencing was performed using scrambled (sc-37007) and human RON sequence-specific duplex siRNA (sc-36434; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Briefly, for each transfection reaction in two separate tubes, 20 nM siRNA oligonucleotides and 2 μl Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) were mixed with 100 μl of the serum-free medium Optimem (Hyclone, Logan, UT, USA) and incubated for 5 min at room temperature. Then the contents of the two tubes were combined and allowed to form siRNA-Lipofectamine complexes for 30 min at room temperature. A 900 μl volume of AGS cells cultured in serum-free medium was combined with the siRNA-Lipofectamine mix, plated in wells of a 6-well tissue culture dish and placed in a 37˚C, 5% CO_2 incubator for 5 h. The medium was then replaced with a normal growth medium.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from MKN28 cells and used for RT-PCR analysis. The following oligonucleotides were used for amplification: 5'-TCGCCTCGATGGAGCTCCTC-3' and 5'-CATGTGTGCCACTGTGACGT-3' for RON and 5'-TCAACGGATTTGGTCGTATT-3' and 5'-CTGTGGTCAT GAGTCCTTCC-3' for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The amplification conditions for RON were 94˚C for 5 min, followed by 28 cycles at 94˚C for 40 sec, 58˚C for 40 sec, 72˚C for 1 min 30 sec and one cycle at 72˚C for 10 min. The conditions for GAPDH were 94˚C for 5 min, followed by 28 cycles at 94˚C for 20 sec, 59˚C for 30 sec, 72˚C for 40 sec and one cycle at 72˚C for 10 min. The PCR products for RON and GAPDH were analyzed by electrophoresis on a 1.2% agarose gel containing ethidium bromide.

Nuclear staining with Hoechst 33342. MKN28 cells ($1x10^6$/ml) were cultured in 60-mm diameter dishes in RPMI containing 1% FBS in the absence or presence of transfected RON siRNA (20 nM). The fixed cells were stained with the DNA-specific fluorochrome bis-benzimide (Hoechst 33342; Molecular Probes, Eugene, OR, USA) for 30 min. The adhered cells were washed with PBS, air dried, and then observed by fluorescence microscopy.

DNA fragmentation assay. MKN28 cells cultured in RPMI containing 1% FBS in the presence or absence of transfected RON siRNA for 3 and 6 days, were trypsinized and centrifuged. The pellet was incubated with lysis buffer [10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% sodium dodecyl sulfate (SDS) and 80 μg/ml proteinase K] at 37˚C overnight. After extraction with phenol/chloroform, the DNA was precipitated with 100% ethanol and then dissolved in a Tris-EDTA buffer (pH 8.0) with RNase A at 37˚C overnight. The absorbance was then read at 260 and 280 nm, and DNA was resolved in a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide in a 1X Tris-borate-EDTA buffer. The bands were visualized using an ultraviolet transilluminator.

Flow cytometry. An annexin V FLUOS staining kit (Sigma-Aldrich) was used to measure the level of annexin V binding according to the manufacturer's instructions. Briefly, the cells were trypsinized in phosphate-buffered saline, collected by centrifugation, and resuspended in a binding buffer 100 μl containing annexin V. After incubation at room temperature for 15 min in the dark, the cells were analyzed by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA). Several controls were used to optimize the instrument settings and determine the gating for the Windows-based platform.

Caspase activity. Caspase-8 and -9 activities were determined using a commercially available kit according to the manufacturer's protocol (Calbiochem, San Diego, CA, USA).

Protein extraction and Western blot analysis. At the end of transfection, the cells were suspended in ice-cold RIPA-M buffer with 1% NP-40 and cell lysates were prepared essentially as described above. The cell lysate protein (100 μg) was resolved by 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The blots were blocked for at least 1 h at room temperature in a blocking buffer (5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20; TBST). The anti-RONß (Santa Cruz Biotechology), anti-Bcl-2, anti-Bax, anti-cytochrome C, and NF-κB p65 antibodies (Cell Signaling Technology, Beverly, MA, USA) were diluted in a blocking buffer and incubated with the blots overnight at 4˚C. The bound antibodies were detected with a 1:3000 dilution of horseradish peroxidase-conjugated secondary antibody according to the instructions provided with the ECL kit (Amersham, Franklin Lakes, NJ, USA).

Extraction of nuclear protein. Eighty to ninety percent confluent MKN28 cells were incubated in a medium containing 1% FBS in the presence or absence of transfected RON siRNA for 3 and 6 days. The cells were resuspended in 500 μl of

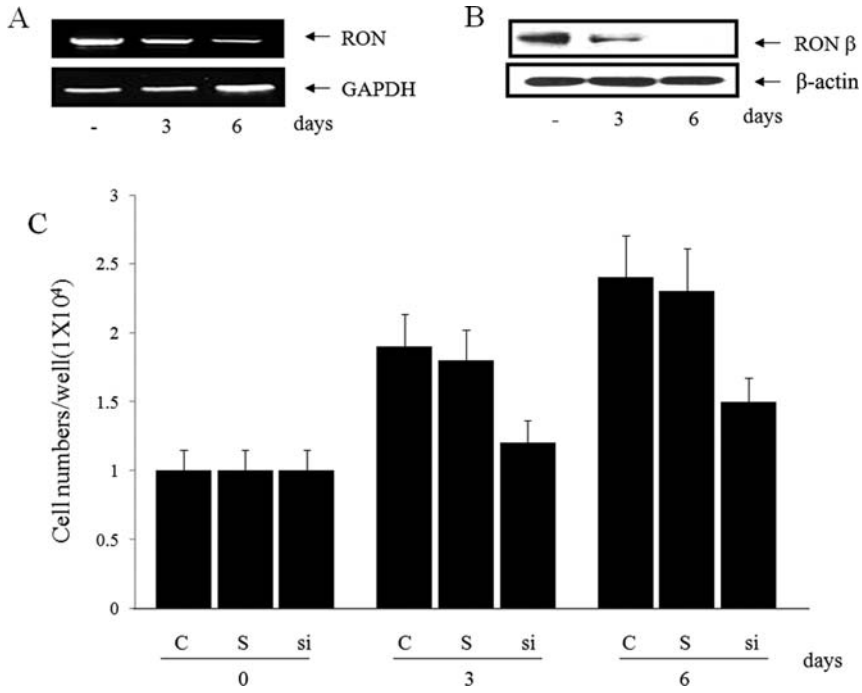

Figure 1. RON siRNA reduces RON expression and cell survival. MKN28 cells were transfected with siRNAs against RON for 3 and 6 days, and the mRNA and protein levels of RON were determined by RT-PCR (A) and Western blot analysis (B). (C) Cells were transfected with scrambled (S) or RON siRNA (si) for 3 and 6 days, and cell viability was determined by an MTT assay. The number of viable cells in the well is expressed as mean ± SD.

cold buffer A [50 mM Tris (pH 7.4), 150 mM NaCl, 0.2 mM EDTA, 3% (v/v) glycerol, and 1.5 mM $MgCl_2$]. The cells were allowed to swell for 5 min on ice. Subsequently, they were lysed with 500 μl of buffer B (identical to buffer A but containing 0.05 % Nonidet P-40; Sigma-Aldrich). The cell lysates were gently layered onto an equal volume cushion of buffer C [10 mM Tris (pH 7.4), 25% (v/v) glycerol, and 1.5 mM $MgCl_2$] and centrifuged for 5 min at 200 x g. The white nuclear pellet was resuspended in 75 μl of a cold high-salt lysis buffer [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride]. This suspension was agitated for 30 min at 4˚C and microcentrifuged for 15 min at 4˚C. The resulting supernatant was stored in aliquots at -80˚C. The protein was quantified spectrophotometrically using the BCA assay (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard.

Electrophoretic mobility shift assay (EMSA). EMSA was performed using a gel shift assay system (Promega, Madison, WI, USA). Briefly, the oligonucleotide with the consensus sequence for NF-κB (5'-AGTTGAGGGGACTTTCCCAGG-3') was end-labeled with [γ-^{32}P]-adenosine triphosphate (3 Ci/mmol; Amersham Pharmacia Biotech, Buckinghamshire, UK) using T4 polynucleotide kinase. The labeled oligonucleotide was then purified in Microspin G-25 columns (Sigma-Aldrich) and used as a probe for EMSA. The nuclear extract proteins (10 μg) were pre-incubated with the binding buffer [10 mM Tris (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 4% (v/v) glycerol, and 0.05 mg/ml poly(deoxyinosine-deoxycytidine)] for 5 min, and then incubated with the labeled probe for 15 min at 37˚C. Each sample underwent electrophoresis in a 5% non-denaturing polyacrylamide gel with a 0.5X Tris-borate-EDTA buffer (pH 7.4) at 150 V for 4 h. The gel was dried and subjected to autoradiography. In the competition study, a 50-fold excess of the unlabeled oligonucleotide was included in the reaction mixture with the radiolabeled probe.

Results

RON siRNA silences RON expression and reduces cell survival. In order to determine whether siRNAs regulate RON expression in human gastric cancer MKN28 cells that express RON endogenously (14), siRNAs against RON were transiently transfected into the cells. As shown in Fig. 1A and 1B, siRNA inhibited the RON expression at both the RNA and protein level in a time-dependent manner. Considering that RON is a member of the MET proto-oncogene family, the effects of siRNA-targeted RON on the expression of MET, were examined. Western blot analysis confirmed that RON siRNA had no inhibitory effect on the MET expression (data not shown). In order to study whether the silencing of the RON expression affected cell survival, we determined the effect of siRNA on cell proliferation by an established MTT assay. The siRNA introduction resulted in the significant inhibition of cell proliferation compared to the scrambled siRNA (control) in human gastric cancer MKN28 cells (Fig. 1C).

RON siRNA induces cell apoptosis. We then examined if the reduced cell survival by RON siRNA results from cell apoptosis. Cells undergoing apoptosis showed profound structural changes, including nuclear disintegration and membrane blebbing. After a 3- and 6-day treatment with RON-targeted siRNA, the nuclei were stained with Hoechst 33342 in order to examine the changes in nuclear morphology. The nuclei of the cells transfected with siRNAs were clearly fragmented

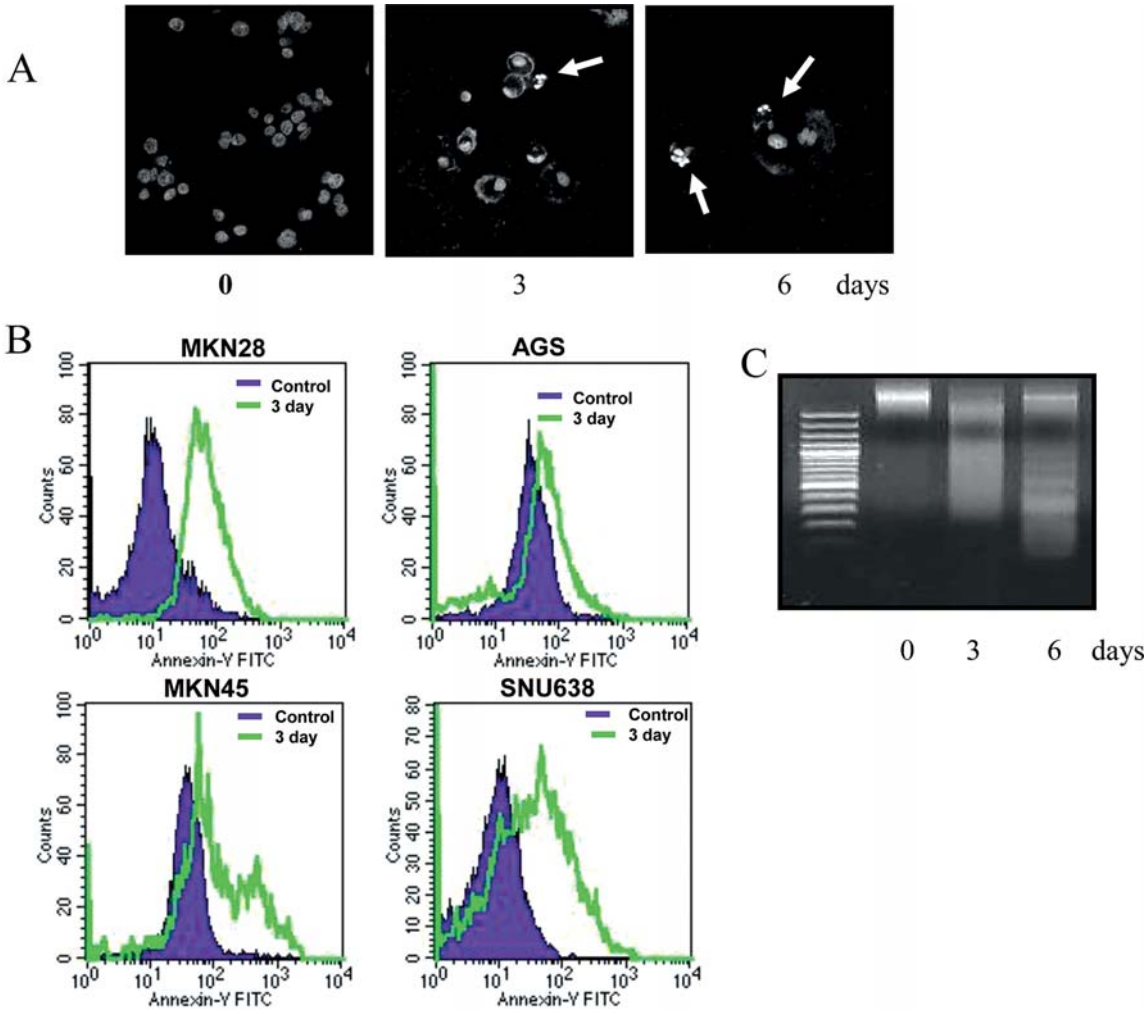

Figure 2. The induction of apoptosis by RON siRNA. (A) MKN28 cells were transfected with RON siRNA for 3 and 6 days, and fixed in paraformaldehyde. The DNA was stained using bis-benzimide (Hoechst 33342) to show the presence of nuclear fragmentation and chromatin condensation. (B) Flow cytometric histograms of cells transfected with RON siRNA for 3 and 6 days. The vertical axes (counts) represent the number of cells and the horizonal axes (Annexin-Y FITC) represent the labeling with annexin V. (C) The cells transfected with RON siRNA for 3 and 6 days. The level of the DNA fragmentation was analyzed by electrophoresis in a 1.5% agarose gel.

with condensed chromatin (Fig. 2A), suggesting that apoptotic changes had occurred in the gastric cancer MKN28 cells. The RON siRNA-induced apoptosis of gastric cancer cells was further analyzed by testing for the presence of Annexin V-conjugated cells using fluorescence analysis. As shown in Fig. 2B, the transfection with RON-targeted siRNA induced the number of Annexin V-positive cells in different gastric cancer cells (MKN28, AGS, MKN45 and SNU638). One of the important hallmarks of apoptosis is DNA fragmentation into multiples of 180-200 bp, which appears as a typical 'DNA laddering' pattern on DNA electrophoresis. The DNA laddering was observed in the gastric cancer MKN28 cells transfected with siRNA (Fig. 2C).

RON siRNA alters the ratio of Bax/Bcl-2 proteins. The ratio of the pro- and anti-apoptotic Bcl-2 is critically balanced during cell proliferation and apoptosis. Therefore, we hypothesized that RON siRNA could alter the level of Bcl-2 family members in a manner favoring apoptosis. The changes in the protein level of Bax and Bcl-2 in MKN28 cells transfected with RON siRNA were examined by Western blot analysis. The RON-targeted siRNA induced an increase in the Bax level with a significant decrease in the Bcl-2 level (Fig. 3A). As the expression of the anti-apoptotic Bcl-2 family is positively regulated by NF-κB, the effects of RON siRNA on NF-κB, were examined. An EMSA study showed that RON siRNA reduced the DNA-binding activity of NF-κB in MKN28 cells in a time-dependent manner (Fig. 3B). The protein level of the p65 subunit of NF-κB in the nuclear lysates of the cells was also found to be decreased upon RON siRNA transfection (Fig. 3C).

RON siRNA induces cytochrome c release and caspase activation. Cytochrome c release from the mitochondrial intermembrane space into the cytosol has been shown to be a key event in the activation of the caspase cascade. We investigated the cytochrome c release from the cells transfected with RON siRNA. An immunoblot analysis of the cytosolic fraction of the RON siRNA-transfected MKN28 cells showed a time-dependent increase in the level of cytochrome c (Fig. 4A). Subsequently, changes in the caspase-8 and -9 activities were examined using a fluorogenic peptide substrate.

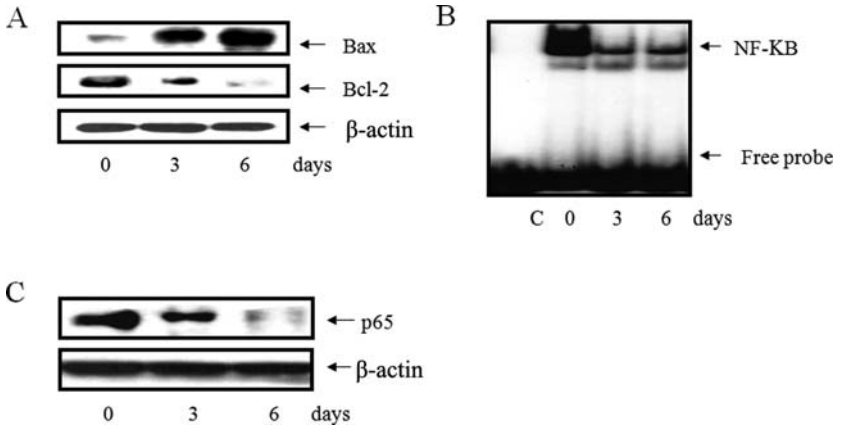

Figure 3. The effects of RON siRNA on the expression of the Bcl-2 family and the activity of NF-κB. (A) After the transfection of the MKN28 cells with RON siRNA for 3 and 6 days, Western blot analyses were performed with the cell lysates using anti-Bax and Bcl-2 antibodies. (B) Nuclear extracts from the cells were analyzed by EMSA for the activated NF-κB using a radiolabeled oligonucleotide probe. (C) RON siRNA-induced a decrease in the level of the p65 subunit of NF-κB. Protein (50 μg) from the above nuclear lysates were subjected to SDS-PAGE and Western blotting using the NF-κB (p65 subunit) antibody.

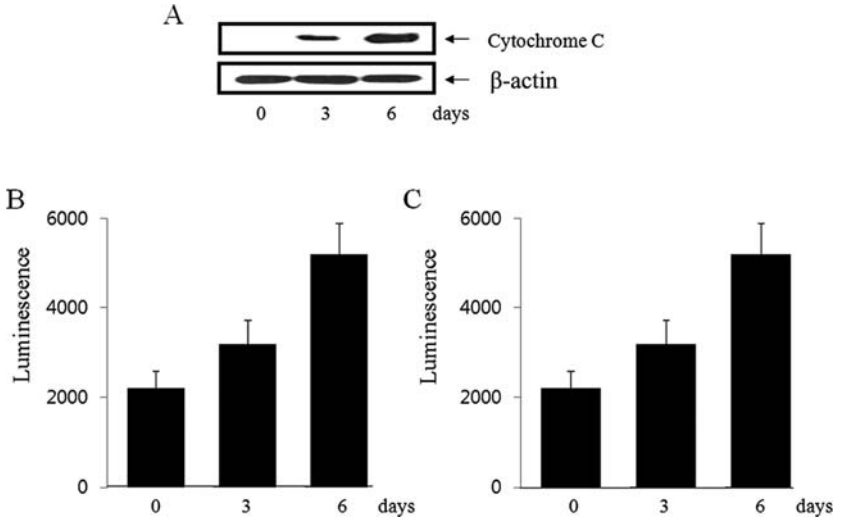

Figure 4. RON siRNA causes the cytochrome c release and caspase activation in cells. (A) After transfection of the MKN28 cells with RON siRNA for 3 and 6 days, Western blot analysis was performed with the cell lysates using the anti-cytochrome c antibody. (B) Caspase-8 and -9 activities were determined using a specific fluorogenic peptide substrate as described in Materials and methods.

The result showed that RON siRNA induced the activities of caspase-8 and -9 in a time-dependent manner (Fig. 4B and C).

Discussion

The RON receptor tyrosine kinase, also known as the MSP receptor, is a member of the MET proto-oncogene family (5). Upon the binding of MSP, RON is activated via autophosphorylation within its kinase catalytic domain, and transduces a variety of signals such as Ras/MAPK, PI3K, JNK, ß-catenin and NF-κB (6,7). The pleiotropic effects of RON have been reported, including proliferation, tubular morphogenesis, angiogenesis, cellular motility, invasiveness, and resistance to cell death (anoikis) (15). The aberrant expression and activation of RON has been observed in human cancers and is responsible for various malignant behaviors in cancers of the breast, colon, and ovaries (16). The abnormal RON accumulation is also accompanied by the generation of alternatively spliced RON variants with oncogenic potentials (17). Targeting RON with small chemical inhibitors or monoclonal antibodies has proved effective in the treatment of cancer (18).

The knockdown of the RON gene expression in cancer cells not only impairs cell replication, but also results in increased apoptotic death (7). In this study, we reported that the inhibition of RON by siRNA induces apoptosis in different gastric cancer cells (MKN28, AGS, MKN45 and SNU638). Even though the exact mechanisms of the RON siRNA-induced gastric cancer cell apoptosis are not clear, these findings are important in regards to RON regulating cellular sensitivities towards apoptotic signals in gastric cancer. Recently, it has been reported that the induction of apoptosis in human colon carcinoma cells by the RON knockdown was mediated through the activation of caspases (7).

siRNA is the sequence-specific, post-transcriptional gene silencing method initiated by double-stranded RNAs, which are homologous to the suppressed gene. Therefore, the present study was designed to determine the role of RON in the

apoptosis of gastric cancer cells using siRNA technology *in vitro*. Cells transfected with the RON-targeted siRNA caused cytochrome c release, induction of caspase-8 and -9 activities, and a change in the ratio of Bax/Bcl-2 in a manner that favored apoptosis, suggesting that apoptosis induced by RON siRNA involves a cytochrome c- and caspase-mediated mechanism. We also investigated the role of NF-κB in the RON siRNA-induced apoptotic processing of gastric cancer cells. The cells transfected with RON siRNA showed a clear decrease in the DNA-binding activity of NF-κB and in the protein level of the p65 subunit of NF-κB in nuclear lysates. The involvement of NF-κB in the apoptotic response of the RON siRNA transfected gastric cancer cells was suggested based on the following: i) The known target genes of NF-κB include a number of anti-apoptotic genes such as the Bcl-2 family (19), ii) NF-κB activity is important to cell survival (20), and iii) the inactivation of the endogenous Rel/NF-κB factors by suppressor forms of the inhibitory protein, IκBα, sensitizes the cells to stimulus-induced apoptosis (21).

Certain studies have suggested that RON-transduced survival signals are capable of antagonizing apoptosis elicited through different pathways. Activated RON has the ability to protect different types of epithelial cells from extracellular apoptotic signals elicited by TGF-ß1, Fas-activating agents, or other mechanisms (22). The suggested intracellular signals involved in the RON-transduced survival, are the MAPK and PI-3K/Akt pathways. Avoiding anoikis is another explanation for the anti-apoptotic properties of RON (18). The detachment of cells from the basement membrane or matrix normally leads to anoikis. In contrast to normal epithelial cells, which undergo anoikis upon detachment, cancer cells can avoid anoikis. Metastatic cancer cells must survive during circulation. Therefore, avoiding anoikis is critical for cancer progression. The cells engineered to overexpress RON also avoid anoikis (18).

To our knowledge, this is the first study investigating the effects of the RON knockdown on the apoptosis of gastric cancer cells. A complete understanding of the molecular mechanism(s) involved in the siRNA targeted RON-mediated cancer cell apoptosis could be important for devising better strategies for cancer therapy.

Acknowledgements

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