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NAG-1 up-regulation mediated by EGR-1 and p53 is critical for quercetin-induced apoptosis in HCT116 colon carcinoma cells

J. H. Lim · J.-W. Park · D. S. Min · J.-S. Chang · Y. H. Lee · Y. B. Park · K. S. Choi · T. K. Kwon

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Abstract Quercetin, a flavonoid molecule ubiquitously present in nature, has multiple effects on cancer cells, including the inhibition of cell proliferation and migration. However, the responsible molecular mechanisms are not fully understood. We found that quercetin induces the expression of NAG-1 (Non-steroidal anti-inflammatory drug activated gene-1), a TGF-β superfamily protein, during quercetin-induced apoptosis of HCT116 human colon carcinoma cells. Reporter assays using the luciferase constructs containing NAG-1 promoter region demonstrate that early growth response-1 (EGR-1) and p53 are required for quercetin-mediated activation of the NAG-1 promoter. Overexpression of NAG-1 enhanced the apoptotic effect of quercetin, but suppression of quercetin-induced NAG-1 expression by NAG-1 siRNA attenuated quercetin-induced apoptosis in HCT116 cells. Taken together, the present study demonstrates for the first time that quercetin induces apoptosis via NAG-1, providing a mechanistic basis for the apoptotic effect of quercetin in colon carcinoma cells.

Keywords Quercetin · NAG-1 · Apoptosis · Sp1 · EGR-1 · p53 · Colon carcinoma cells

J. H. Lim · J.-W. Park · T. K. Kwon (✉)
Department of Immunology, School of Medicine,
Keimyung University,
194 DongSan-Dong, Taegu 700-712, Korea
e-mail: kwontk@dsmc.or.kr

D. S. Min
Department of Molecular Biology, College of Natural Science,
Pusan National University,
Korea

J.-S. Chang
Department of Life Science, Daejin University,
Pochon-gun, Kyeonggido,
Korea

Y. H. Lee
Division of Molecular & Life Science, College of Science &
Technology, Hanyang University,
Ansan, Korea

J. H. Lim · Y. B. Park
Department of Genetic Engineering, College of Natural Sciences,
Kyungpook National University,
Taegu, Korea

K. S. Choi
Institute for Medical Science, School of Medicine,
Ajou University,
Suwon, Korea

Introduction

Many natural dietary phytochemicals found in fruits, vegetables, spices, and tea have been shown to be protective against cancer in various animal models [1, 2]. Quercetin (3,3′,4′,5,7-pentahydroxy-flavone) is one of the most widely distributed flavonoids in the plant kingdom [3]. Previous studies have shown that quercetin possesses a broad range of biological properties. Quercetin inhibits leukemic cell growth without suppressing normal hematopoiesis [4]. It blocks the cell cycle at the G_1/S boundary in human colon cancer cells and in human leukemic T-cells, but in G_2/M phase in human breast cancer cells [5–9]. In addition, quercetin inhibits kinase activity, suppresses tumor invasive behavior by the inhibition of secretion of matrix metalloproteinases, and induces apoptosis [10]. These activities of quercetin make it a promising candidate for treatment and prevention of various cancers.

Nonsteroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1) was identified from indomethacin (a cyclooxygenase (COX) inhibitor)-induced gene library [11].

NAG-1 is a member of TGF β superfamily and is also known as macrophage inhibitory cytokine-1 (MIC-1), placental transforming growth factor β (PTGF-β), or prostate derived factor (PDF) [12–14]. Purified recombinant NAG-1 is able to inhibit lipopolysaccharide-induced TNF-α production in macrophages, suggesting that NAG-1 acts as an autocrine regulatory molecule [15]. *In vitro* and *in vivo* NAG-1 has anti-tumorigenic and pro-apoptotic activities independent of COX inhibition [11, 16]. Moreover, ectopic expression of NAG-1 induced apoptosis and induction of death receptor-4 (DR4) and DR-5 expression in gastric cancer cells [17]. Not only NSAIDs but also several antitumorigenic compounds with chemopreventive activities, including resveratrol, genistein, catechins, and peroxisome proliferators-activated receptor-γ ligands, regulate NAG-1 expression in a prostaglandin-independent manner [18–22]. Thus, pro-apoptotic activity of NAG-1 may provide a molecular basis to explain chemopreventive agents-mediated antitumorigenesis.

In this study, we examined the regulation of NAG-1 expression during quercetin-induced apoptosis in HCT116 colon carcinoma cells. Quercetin-induecd NAG-1 upregulation is regulated by EGR-1 and p53. In addition, we found that NAG-1 siRNA-mediated inhibition of NAG-1 expression attenuated quercetin-induced apoptosis. These data suggest that the induction of NAG-1 may provide a novel mechanism for understanding the downstream effectors for quercetin-induced apoptosis.

Materials and methods

Cells and materials

HCT116, HT29, MDA231, Huh-7 and Hep 3B cells were obtained from the American Type Culture Collection (ATCC: Rockville, MD), whereas HCT116 p53(−/−) cells were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). The culture medium used throughout these experiments was Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum (FCS), 20 mM HEPES buffer and 100 μg/ml gentamicin. The quercetin was directly added to cell cultures at the indicated concentrations, while untreated cells contained the solvent alone. For establishment of the Huh-7 cells overexpressing NAG-1, Huh-7 cells were transfected with pcDNA3.1-NAG-1 plasmid containing a full-length NAG-1 cDNA (kindly provided by Dr Baek, University of Tennessee, Knoxville, TN). Transfected cells were selected with fresh media containing G418 (500 μg/ml) and overexpression of NAG-1 was analyzed by Western blotting using anti-NAG-1 antibody. Antibody for NAG-1 was purchased from Upstate biotechnology (Lake Placid, NY). Anti-HSC 70, anti-PARP, anti-Sp1, anti-EGR-1, anti-p53 antibodies and Sp1 siRNA were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Quercetin was obtained from Biomol (Plymouth Meeting, PA).

Western blotting

Cellular lysates were prepared by suspending 1×10^6 cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, 100 μM phenylmethylsulfonyl fluoride, and 20 μM leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA). Detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

Cell count and flow cytometry analysis

Cell counts were performed using a hemocytometer. Approximately 1×10^6 HCT116 cells were suspended in 100 μl of PBS, and 200 μl of 95% ethanol were added while vortexing. The cells were incubated at 4°C for 1 h, washed with PBS, and resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μg of RNase. Incubation was continued at 37°C for 30 min. The cellular DNA was then stained by applying 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed by fluorescent activated cell sorting (FACS) on a FACScan flow cytometer for relative DNA content based on red fluorescence.

Asp-Glu-Val-Asp-ase (DEVDase) activity assay

To evaluate DEVDase activity, cell lysates were prepared after their respective treatment with quercetin. Assays were performed in 96-well microtiter plates by incubating 20 μg of cell lysates in 100 μl of reaction buffer (1% NP-40, 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 10% glycerol) containing the caspases substrate [Asp-Glu-Val-Asp-chromophore-p-nitroanilide (DVAD-pNA)] at 5 μM. Lysates were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

Plasmids, transfections, and luciferase gene assays

Chromosomal DNA was prepared from HCT116 cells using the DNAzol^{TM} reagent (Gibco-BRL, Gaithersburg, MD). Human NAG-1 promoter was amplified from chromosomal DNA with the following synthetic primers: 5′-TGTGCGGTACCCCTCA TTTGACCACCT (sense), and 5′-CCACGAAAGCTTCAGCAACACCAGGAG

(antisense). The PCR product was digested with *Kpn*I and *Hind*III (−514 to + 89) and cloned upstream of the firefly luciferase gene of pGL2-basic (Promega). PCR products were confirmed by their size, as determined by electrophoresis and DNA sequencing. Point mutations of the Sp1 binding sites to the NAG-1 promoter were generated by a two-step PCR method using the following primers: mSp1A (sense) 5′-CCCAGACCCCGAACAGCTGT GGTC, and (antisense) 5′-GACCACAGCTGTTC GGGGTCTGGG; mSp1B (sense) 5′-AGGGGGAGGAGTTCGGGACTGAGC, and (antisense) 5′-GCTCAGTCCCGAAC TCCTCCCCCT; mSp1C (sense) 5′-GGGACTG AGCATTCGGAGACG GAC, and (antisense) 5′-GTCCGTCTCCGAATGCTCA GTC CC. Clones representing each point mutation were sequenced to ensure the accuracy of the PCR amplification procedure. For transfection, in brief, cells were plated onto 6-well plates at a density of 5×10^5 cells/well and grown overnight. Cells were co-transfected with 2 μg of various plasmid constructs and 1 μg of the pCMV-β-galactosidase plasmid for 5 h by the Lipofectamine method. After transfection, cells were cultured in 10% FCS medium with vehicle (DMSO) or drugs for 24 h. Luciferase and β-galactosidase activities were assayed according to the manufacturer's protocol (Promega). Luciferase activity was normalized for β-galactosidase activity in cell lysate and expressed as an average of 3 independent experiments.

RNA isolation and reverse transcriptase–polymerase chain reaction (RT-PCR)

NAG-1 mRNA expression was determined by RT-PCR. Total cellular RNA was extracted from cells using the TRIzol reagent (Life Technologies). A cDNA was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD). The cDNA for NAG-1 and actin were amplified by PCR with specific primers. The sequences of the sense and anti-sense primer for NAG-1 were 5′-CAGTCGGACCAACTGCTGGCA-3′ (493 to 513) and 5′-TGAGCACCATGGGATT GTAGC-3′ (833 to 853), respectively. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

Small interfering RNA (siRNA)

The 21-nucleotide siRNA duplexes used in this study were purchased from Invitrogen (Calsbad, CA) and had the following sequences: NAG-1, ACA UGC ACG CGC AGA UCA A (755 to 733); GFP, AAG ACC CGC GCC GAG GUG AAG. Cells were transfected with siRNA oligonucleotides using LipofectAMINE 2000 (Invitrogen, Calsbad, CA) according to the manufacturer's recommendations.

Chromatin immunoprecipitation assay (ChIP assay)

Chromatin Immunoprecipitation assays were done as followed. Briefly, asynchronously growing HCT116 cells were incubated with formaldehyde to cross-link protein-DNA complexes. The cross-linked chromatin was then extracted, diluted with lysis buffer, and sheared by sonication. After preclearing with 1:2 mix of protein A/protein G-agarose beads Upstate), the chromatin was divided into equal samples for immunoprecipitation with anti-Sp1, anti-EGR-1, and anti–immunoglobulin G (negative control) polyclonal antibody (Santa Cruz Biotechnology). The immunoprecipitates were pelleted by centrifugation and incubated at 65°C to reverse the protein-DNA cross-linking. The DNA was extracted from the eluate by the phenol/chloroform method and then precipitated by ethanol. Purified DNA was subjected to PCR with primers specific for a region (–138 to –1) in the NAG-1 promoter spanning two putative Sp1 binding sites. The sequences of the PCR primers used are as follows: PF1 (–138 to –121) 5′-CTAAATACACCCCCAGAC-3′, PR1 (–18 to –1), 5′-GACCAGATGCTGCCGGAC-3′

Results

Quercetin increases NAG-1 protein levels

NAG-1 expression is linked to cell growth arrest and apoptosis, and is responsible for the chemopreventive effects of several dietary natural compounds or nonsteroidal anti-inflammatory drugs (NSAIDs) in a variety of carcinoma models [11]. To investigate whether quercetin-induced growth arrest and apoptosis is associated with NAG-1 expression, HCT116 colon carcinoma cells were treated with various concentrations of quercetin and the protein levels of NAG-1 were measured by Western analysis. As shown in Fig. 1(A), quercetin induced the expression of NAG-1 protein in a dose-dependent manner. Quercetin-induced NAG-1 up-regulation was also observed in a variety of tumor cell types (other colon carcinoma HT29, breast cancer cells MDA231 and hepatocellular carcinoma Hep3B) (Fig. 1(B)). These results suggest that NAG-1 up-regulation may be a common response of cancer cells to quercetin treatment.

The Sp1 site is required for quercetin-induced transactivation of NAG-1 in HCT116 cells

To understand the molecular mechanisms underlying quercetin-induced NAG-1 up-regulation, we first examined whether quercetin regulates NAG-1 expression at the transcriptional level. RT-PCR analysis demonstrated that quercetin induced the expression of NAG-1 mRNA in a dose-dependent manner (Fig. 2(A)). To further elucidate the

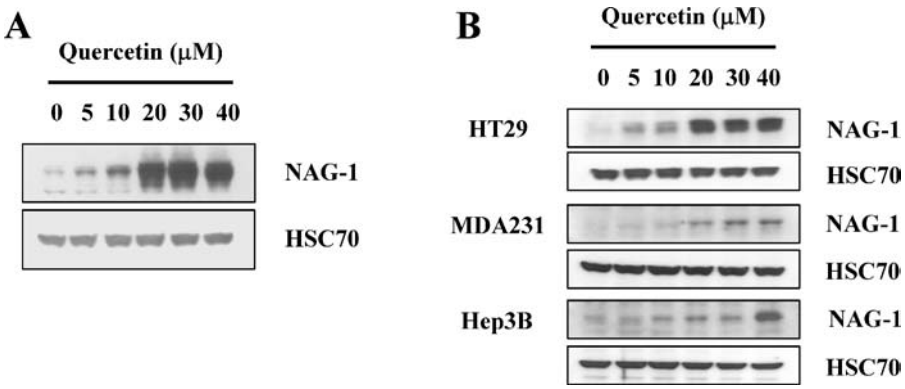

Fig. 1 Quercetin dose-dependently increases NAG-1 protein levels in a variety of cancer cell lines. (A) HCT116 cells were treated with the indicated concentrations of quercetin for 24 h. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for NAG-1 or anti-HSC70 antibody to serve as control for the loading of protein level. (B) HT29, MDA231 and Hep3B cells were treated with the indicated concentrations of quercetin. Expression of NAG-1 was determined by Western blot analysis

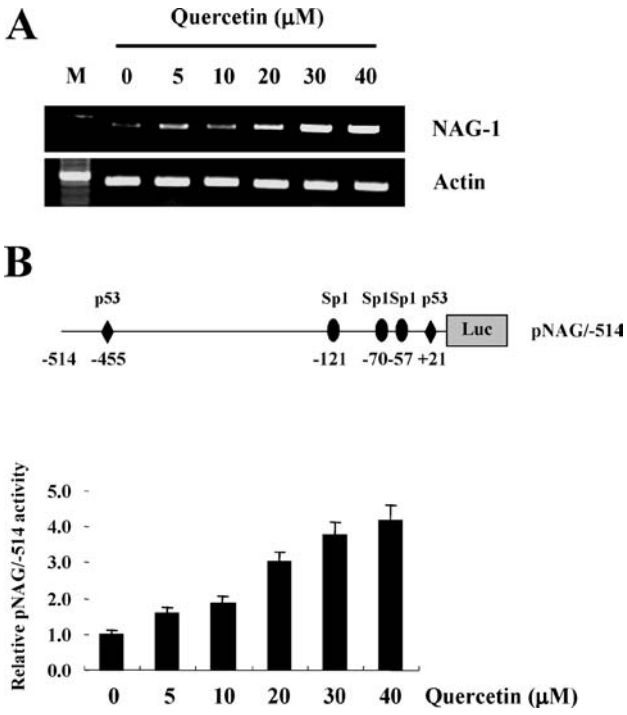

Fig. 2 Effects of quercetin on the mRNA levels and the promoter activity of NAG-1. (A) HCT116 cells were treated with the indicated concentrations of quercetin. Total RNA was isolated and RT-PCR analysis was performed. A representative study is shown; two additional experiments yielded similar results. (B) Schematic structure of NAG-1 promoter constructs used for testing luciferase activity. pNAG/–514 promoter plasmid was transfected, and treated with varying concentrations of quercetin. The cells were lysed and luciferase activity measured. Data represent the mean ± SD of at least 3 independent experiments

transcriptional machinery involved in quercetin-mediated NAG-1 up-regulation, we employed the luciferase gene expression system. The 0.6 kb of the promoter region of human NAG-1 gene was cloned into the pGL2-basic luciferase reporter vector, and the resultant was named as pNAG/-514. HCT116 cells were transfected with pNAG/-514 and the luciferase activity was assayed after 24 h of the various concentrations of quercetin treatment. Quercetin significantly increased pNAG/-514 promoter activity in a dose-dependent manner (Fig. 2(B)). Since the region spanning –514 to +90 contains three Sp1 sites and two p53 binding sites (Fig. 2(B)), we first examined whether Sp1 is involved in quercetin-induced transcriptional up-regulation of NAG-1. When we transfected HCT116 cells with Sp1 cDNA and then treated with quercetin, ectopic expression of Sp1 significantly increased the promoter activity in pNAG/-514 (Fig. 3(A)). To decipher which Sp1 site plays a critical role in quercetin-mediated activation of pNAG/–514 promoter, several Sp1 mutants of the promoter were made and tested in the transfection and luciferase assay (Fig. 3(B)). Introduction of mSp1A (mutated at –121 Sp1 site), mSp1B (mutated at –70 Sp1 site) or mSp1-3 (mutated at –57 Sp1 site) construct significantly decreased not only the basal NAG-1 promoter activity but also quercetin-mediated NAG-1 promoter activity, compared with the wild-type (pNAG/–514) construct. Quercetin-induced activation of Sp1 transcription factor was further confirmed from the experiments using the Sp1 reporter vector, an artificially constructed plasmid containing three Sp1 binding sites and luciferase-coding gene. Transcriptional activity of this promoter was also significantly increased by treatment with quercetin in a dose-dependent manner (Fig. 3(C)). We next tested whether inhibition of Sp1 actually affects the quercetin-induced NAG-1 promoter activity. HCT116 cells were transfected with pNAG/–514 and treated with mithramycin A, which interferes with the binding of the Sp family of transcription factors to GC-rich promoter regions [23]. Mithramycin A significantly decreased pNAG/–514 promoter activity in a dose-dependent manner. Interestingly, western blotting analysis using these cell extracts demonstrates that both the basal NAG-1 protein levels and the quercetin-induced up-regulation of NAG-1 protein were significantly reduced

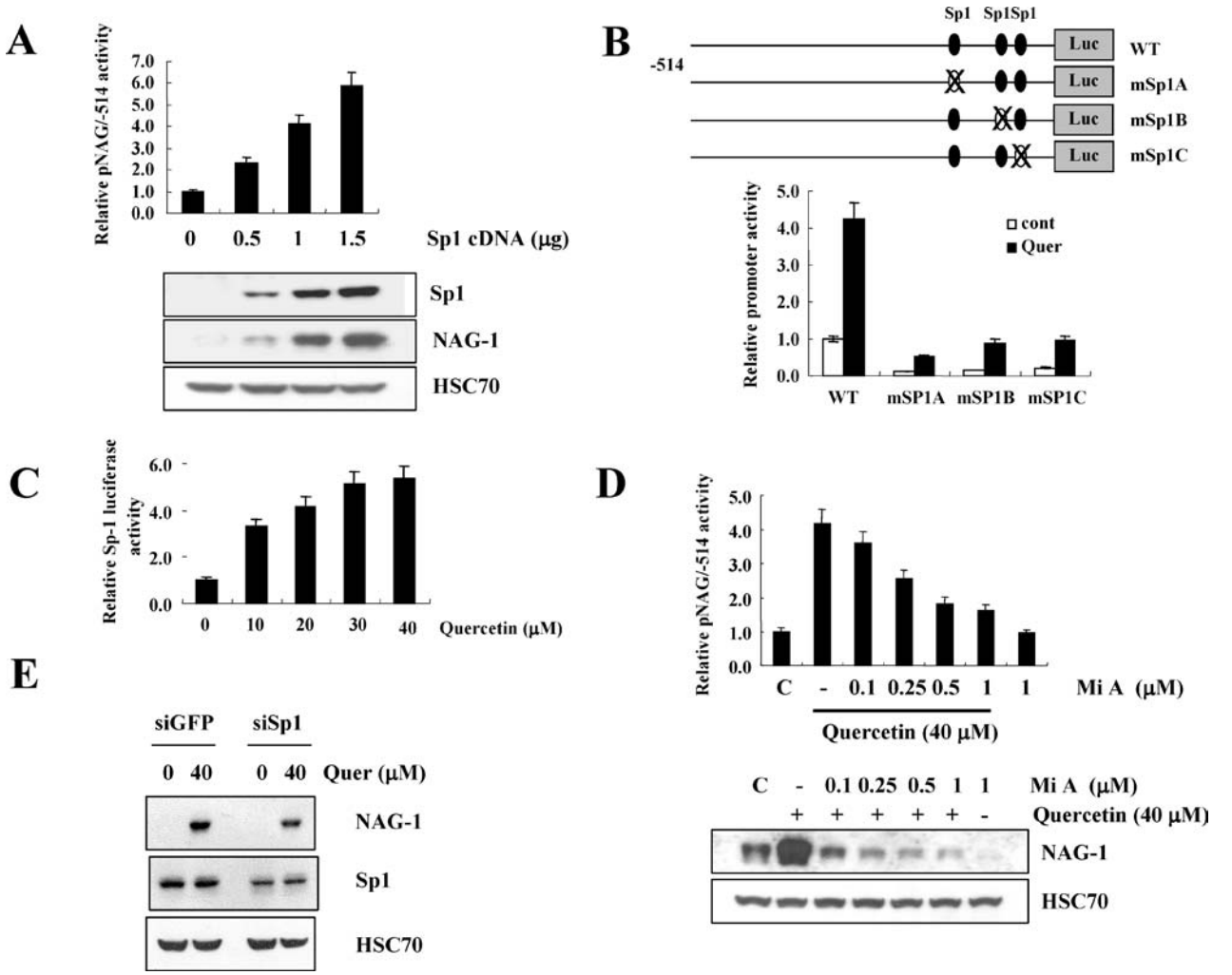

Fig. 3 Sp1 is involved in quercetin-induced NAG-1 up-regulation. (A) Effect of ectopic expression of Sp1 on NAG-1 expression. HCT116 cells were cotransfected with Sp1 cDNA and NAG-1 promoter plasmids. The cells were lysed and luciferase activity measured. The expression levels of Sp1 and NAG-1 protein in transfected cells were determined by Western blot analysis. (B) Nucleotides of the Sp1 consensus site in the promoter region were substituted as described in Material and Methods. HCT116 cells were transfected transiently with clones in bottom panel. The extracts from cells treated with or without quercetin were assayed for luciferase activity. Data represent the mean ± SD of at least 3 independent experiments. (C) Effects of quercetin on the activities of Sp1. To elucidate the effects of quercetin on Sp1 activities, a reporter vector (pSp1-luc) that has three Sp1–binding sites was transfected. The cells were treated with or without varying concentrations of quercetin, and lysed and luciferase activity measured. (D) HCT116 cells were transfected with pNAG/-514 promoter plasmid and further cultured with quercetin (40 μM) in the presence or absence of mithramycin A. The cells were lysed and luciferase activity measured. Data represent the mean ± SD of at least 3 independent experiments. The expression levels of NAG-1 protein in transfected cells were determined by Western blot analysis. (E) HCT116 cells were transfected with Sp1 siRNA or GFP siRNA. Post-transfection, after 24 h, the cells were treated with 40 μM quercetin for 24 h. Equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with specific antibodies, anti-NAG-1, anti-Sp1 or anti-HSC70 to serve as control for the loading of protein level

by mithramycin treatment (Fig. 3(D)). To further confirm the functional significance of quercetin-induced NAG-1 up-regulation, we employed the siRNA duplex against Sp1 mRNA. HCT116 cells transfected with the control GFP or Sp1 siRNA were treated with or without quercetin for 24 h and subjected to immunoblot analysis. As shown in Fig. 3(E), transfection of siRNA against Sp1 resulted in a suppression of NAG-1 expression induced by quercetin in HCT116 cells, compared with cells transfected with control GFP siRNA. Taken together, these results suggest that multiple Sp1 binding sites in the proximal promoter region are critical not only for the basal transcription of NAG-1 but also for quercetin-mediated transactivation of NAG-1 promoter.

Expression of EGR-1 is induced by quercetin

EGR-1 contains a zinc finger motif that shares a DNA-binding site with Sp1 [24–26]. To determine whether EGR-1 plays a role in quercetin-induced NAG-1 expression, we

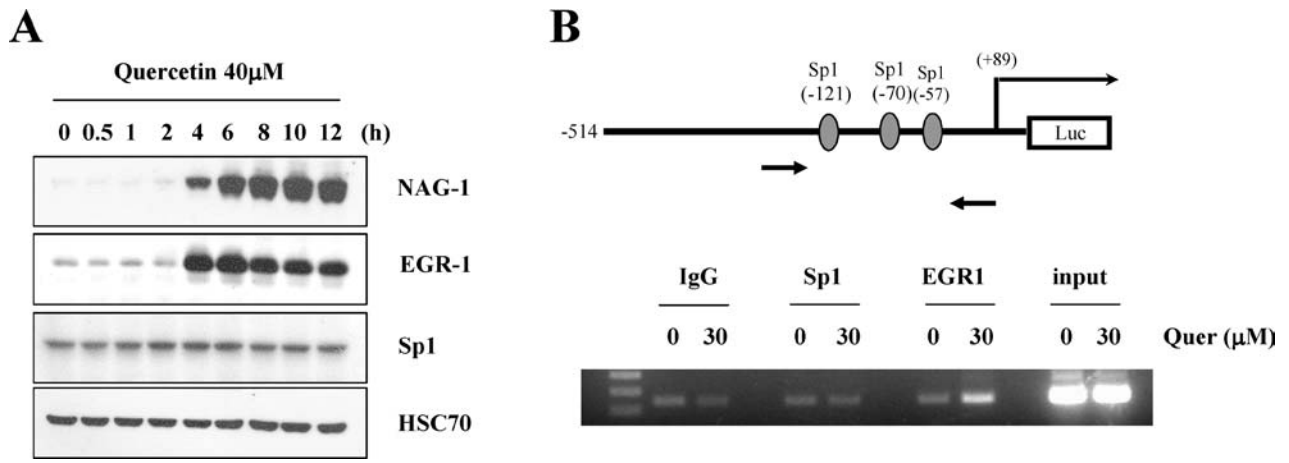

Fig. 4 EGR-1 is associated with quercetin-mediated up-regulation of NAG-1. (A) HCT116 cells were incubated for the periods indicated in the presence or absence of quercetin. Expression levels of NAG-1, EGR-1, and Sp1 were determined by Western blot analysis. (B) Chromatin immunoprecipitation analyses were performed with anti-Sp1 antibody and anti-EGR-1 antibody, as described in Materials and Methods. The DNAs were extracted from total sonicated nuclei (Input), protein A without antibody (–), protein A with antibody (anti-Sp1 or anti-EGR-1), or with pre-immune serum (anti-IgG). Specific promoter regions of the NAG-1 genes were amplified by PCR, separated on 1.5% agarose gels, and stained with ethidium bromide. Also indicated is the relative position of the PCR product generated in ChIP assay

assessed the EGR-1 expression levels in cells treated with quercetin. HCT116 cells were treated with 40 μM quercetin at the indicated times, and expression levels of EGR-1, Sp1 and NAG-1 were determined by Western blot analysis. As shown in Fig. 4(A), EGR-1 expression was increased with treatment with quercetin. After treatment with quercetin for 4 h, the EGR-1 was significantly increased and then gradually decreased. In contrast, Sp1 protein expression was not altered during the quercetin treatment. NAG-1 expression was also increased after 4 h of treatment.

To explore the functional influence of EGR-1 on the NAG-1 promoter and its interplay with Sp1, ChIP assays were performed with Sp1 or EGR1 specific antibody and PCR primers encompassing the three putative Sp1-binding sequences present in NAG-1 promoter region (Fig. 4(B)). As shown in Fig. 4(B), EGR1 significantly increased direct binding of NAG-1 promoter compared with the Sp1, providing the evidence that EGR1 directly binds the NAG-1 promoter. Taken together, these data suggest that the expression of EGR-1 is critical for quercetin-induced NAG-1 expression, whereas Sp1 may work as basal transcription factor.

Effect of p53 on quercetin-induced NAG-1 expression

Previous studies reported that NAG-1 expression was regulated by the tumor suppressor protein p53 at the transcriptional level by other chemopreventive polyphenolic agents including genistein and resveratrol [19, 20]. We first examined whether quercetin treatment affects the expression or activity of p53. In HCT116 cells treated with various concentrations of quercetin, the protein and phosphorylation (Ser15 and Ser20) levels of p53 were measured by Western analysis. As shown in Fig. 5(A), quercetin treatment resulted in an increase in the phosphorylation levels but not in total protein levels of p53 in a dose-dependent manner of quercetin. These results suggest a possibility that p53 may be involved in quercetin-mediated NAG-1 regulation, since p53 phosphorylation is one of the key regulatory steps in its activation. To determine whether p53 is required for quercetin-induced NAG-1 up-regulation, we compared the effect of quercetin on NAG-1 expression using isogenic human colon carcinoma cell lines differing only in the presence or absence of p53 (HCT116 p53(+/+) and HCT116 p53(–/–)). Quercetin-induced up-regulation of NAG-1 was not significant in p53 null HCT cells, compared that in HCT116 cells with wild type p53 (Fig. 5(B)). These results suggest that the presence of wild type p53 is critical for quercetin-induced NAG-1 up-regulation. Next, we assessed the effect of p53 on NAG-1 expression by transfecting HCT116 cells with mutant p53 (aa 248; Arg–> Trp). This p53 mutant has been shown to inhibit the transactivation of wild-type p53 acting as a dominant-negative mutant [27]. HCT116 cells were co-transfected with p53 mutant expression vector and the luciferase construct containing NAG-1 promoter. Quercetin-induced enhancement of NAG-1 promoter activity was significantly attenuated by the co-expressed mutant p53 in a dose-dependent manner (Fig. 5(C)). These results indicate that the p53 activity as a transcription factor is associated with quercetin-induced NAG-1 expression. We confirmed that quercetin-induced enhancement of NAG-1 protein levels was also reduced by ectopic expression of the mutant p53 (aa 248; Arg–> Trp) (Fig. 5(D)). In addition, to further

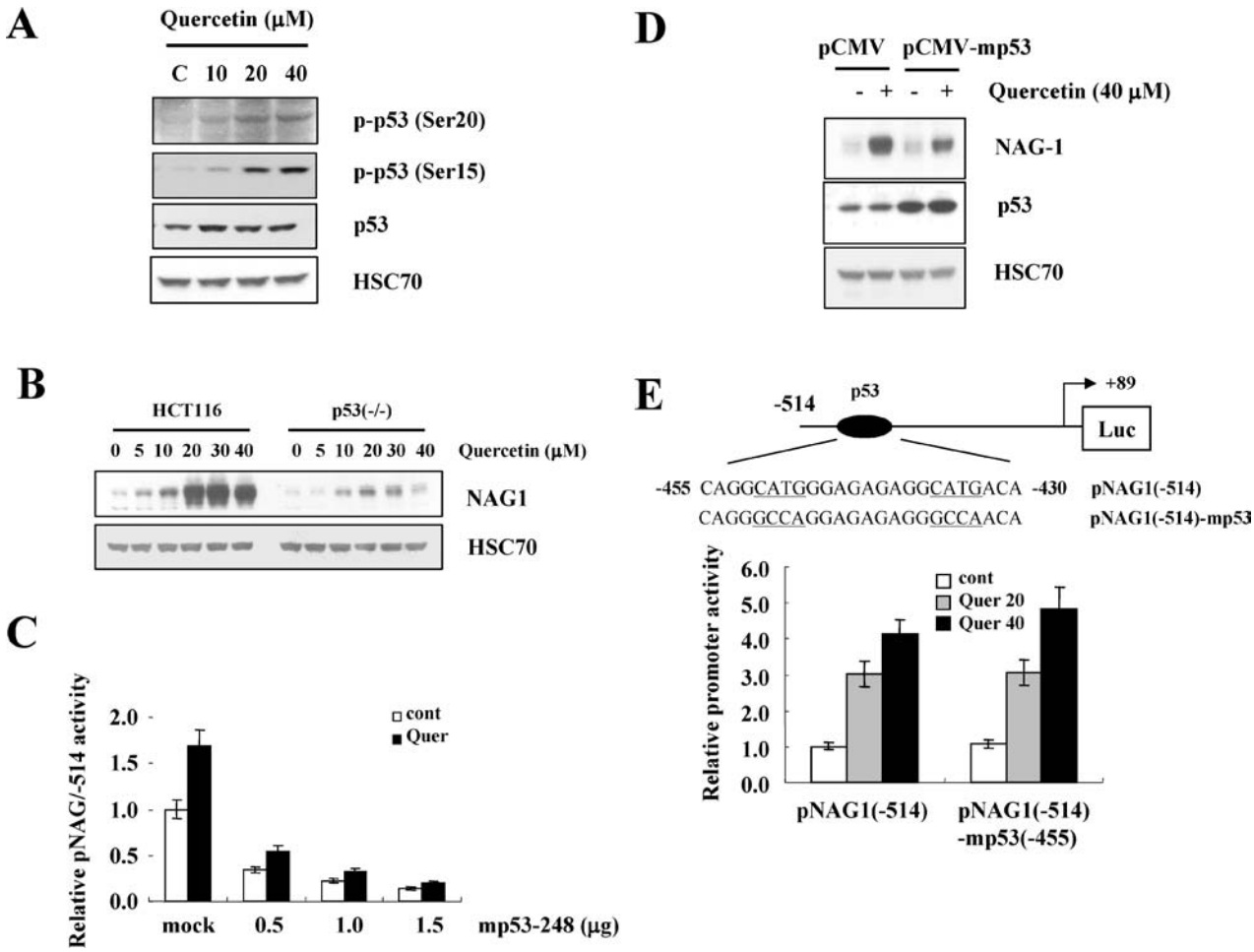

Fig. 5 Effect of p53 on quercetin-induced NAG-1 up-regualtion. (A) Effect of quercetin on the expression and activity of p53. Immunoblot detection of the expression and phosphorylation status (phospho-p53 Ser20 or phosphor-p53 Ser15) of p53 in HCT116 cells after 24 h exposure to quercetin. (B) Quercetin-induced NAG-1 expression in p53 wild type and p53 null HCT116 cells. p53 wild type and p53–/– HCT116 cells were treated with the indicated concentrations of quercetin for 24 h. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blot for NAG-1 or anti-HSC70 antibody to serve as control for the loading of protein level. (C) HCT116 cells were cotransfected with pCMV-mp53 cDNA and NAG-1 promoter plasmids. The cells were lysed and luciferase activity measured. (D) The extracts from cells treated with or without quercetin were assayed for NAG-1 expression levels by western blot analysis. (E) pNAG1(–514) promoter and p53 mutated pNAG1(–514)mp53 plasmids were transfected, and treated with or without quercetin. The cells were lysed and luciferase activity measured. Data represent the mean ± SD of at least three independent experiments

clearly determine whether the p53 is directly associated with quercetin-mediated transcriptional activation of NAG-1, we mutated the potential p53 site (–455 to –430) of the NAG-1 gene. As shown in Fig. 5(E), this mutation did not alter the activation of NAG-1 promoter by quercetin, demonstrating that p53 is indirectly involved in the quercetin-mediated upregulation of NAG-1. Thus, these results collectively indicate that p53 plays a key role in quercetin-induced NAG-1 up-regulation.

Suppression of NAG-1 expression attenuates quercetin-induced apoptosis, but enforced NAG-1 expression promotes it

Next, we attempted to clarify the significance of NAG-1 up-regulation in quercetin-induced apoptosis. First, we examined the effects of quercetin on apoptosis in HCT116 cells. Flow cytometric analysis to determine the sub-G1 population after quercetin treatment demonstrates that accumulation of the sub-G1 phase was markedly increased in a dose-dependent manner (Fig. 6(A)). In addition, treatment of HCT116 cells with quercetin stimulated DEVDase activity, a key effector of apoptosis in a dose-dependent manner. Consistently, a reduction of the protein levels of 32 kDa precursor was observed together with a concomitant cleavage of PARP, a substrate protein of caspases. We then examined whether suppression of NAG-1 expression could modulate quercetin-induced apoptosis. HCT116 cells were transfected with the indicated siRNA were treated with or without quercetin for 24 h. Immunoblot analysis demonstrated that transfection of siRNA against NAG-1 resulted in a suppression of NAG-1

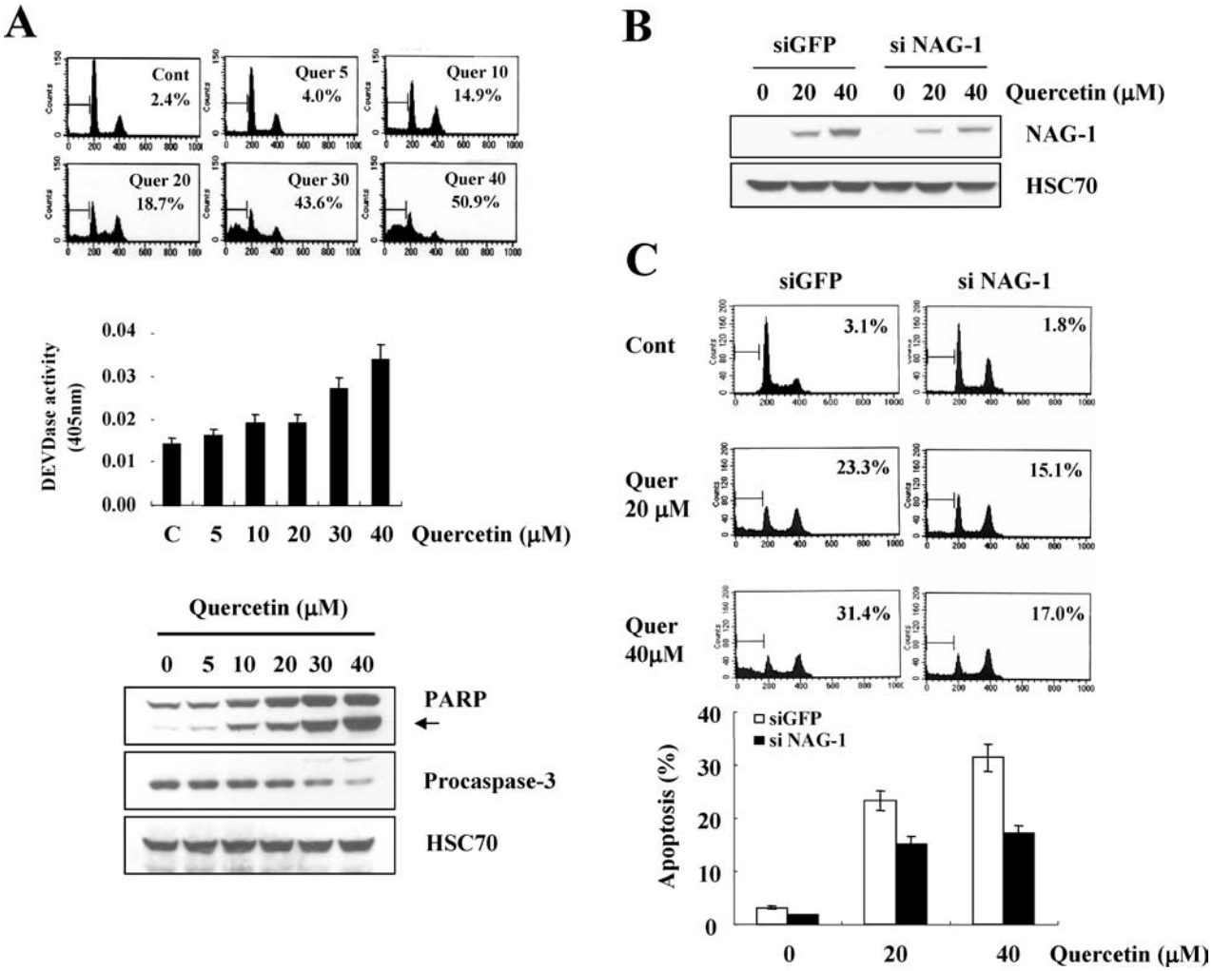

Fig. 6 Suppression of NAG-1 expression attenuates quercetin-induces apoptosis in HCT116 cells. (A) Quercetin induces apoptosis HCT116 cells in a dose-dependent manner. Flow cytometric analysis of apoptotic cells. HCT116 cells were treated for 24 h with the indicated concentrations of quercetin, and their DNA content was measured after propidium iodide staining. The proportion of apoptotic cells is indicated. Enzymatic activities of DEVDase were determined by incubation of 20 μg of total protein with 200 μM chromogenic substrate (DEVD-pNA) in a 100 μl assay buffer for 2 h at 37°C. The release of chromophore *p*-nitroanilide (pNA) was monitored spectrophotometrically (405 nm). Data shown are means ± SD. After treatments, cell lysates were prepared. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for procaspase-3, PARP and HSC70. The proteolytic cleavage of PARP was indicated by arrow. (B) Down-regulation of NAG-1 by transfection with siRNA against NAG-1. HCT116 cells were transfected with NAG-1 siRNA or GFP siRNA. Twenty-four hours after transfection, cells were treated with 20 or 40 μM quercetin for 24 h. Equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies, anti-NAG-1 or with anti-HSC70 antibody to serve as control for the loading of protein level. (C) Down-regulation of NAG-1 reduces quercetin-induced apoptosis in HCT116 cells. Apoptosis was analyzed as a sub-G1 fraction by FACS

expression induced by quercetin in HCT116 cells, compared with cells transfected with control GFP siRNA (Fig. 6(B)). Under these conditions, accumulation of sub-G1 cell population induced by quercetin was significantly attenuated in cells transfected with NAG-1 siRNA when compared with GFP siRNA-transfected cells (Fig. 6(C)). Next, we investigated the effect of NAG-1 overexpressioin on quercetin-induced apoptosis. Since we failed to establish the stable HCT 116 cell lines overexpressing NAG-1 despite the repeated trials, we employed Huh-7 hepatocellular carcinoma cells for overexpression of NAG-1. Compared with HCT116 cells, higher doses of quercetin were required to induce quercetin-induced apoptosis in Huh-7 cells (Fig. 7(A)). As shown in Fig. 7(A), treatment of Huh-7 cells overexpressing NAG-1 (Huh-7/NAG-1) with 80 μM quercetin resulted in a markedly increased accumulation of sub-G1 phase, compared with control Huh-7 cells (Huh-7/vector) treated with the same dose of quercetin. Consistently, ectopic expression of NAG-1 significantly enhanced the reduction of the protein levels of 32 kDa precursor and cleavage of PARP, a substrate protein of caspases (Fig. 7(B)). In parallel with the degradation of caspase-3, Huh-7/NAG-1 cells exhibited a significant

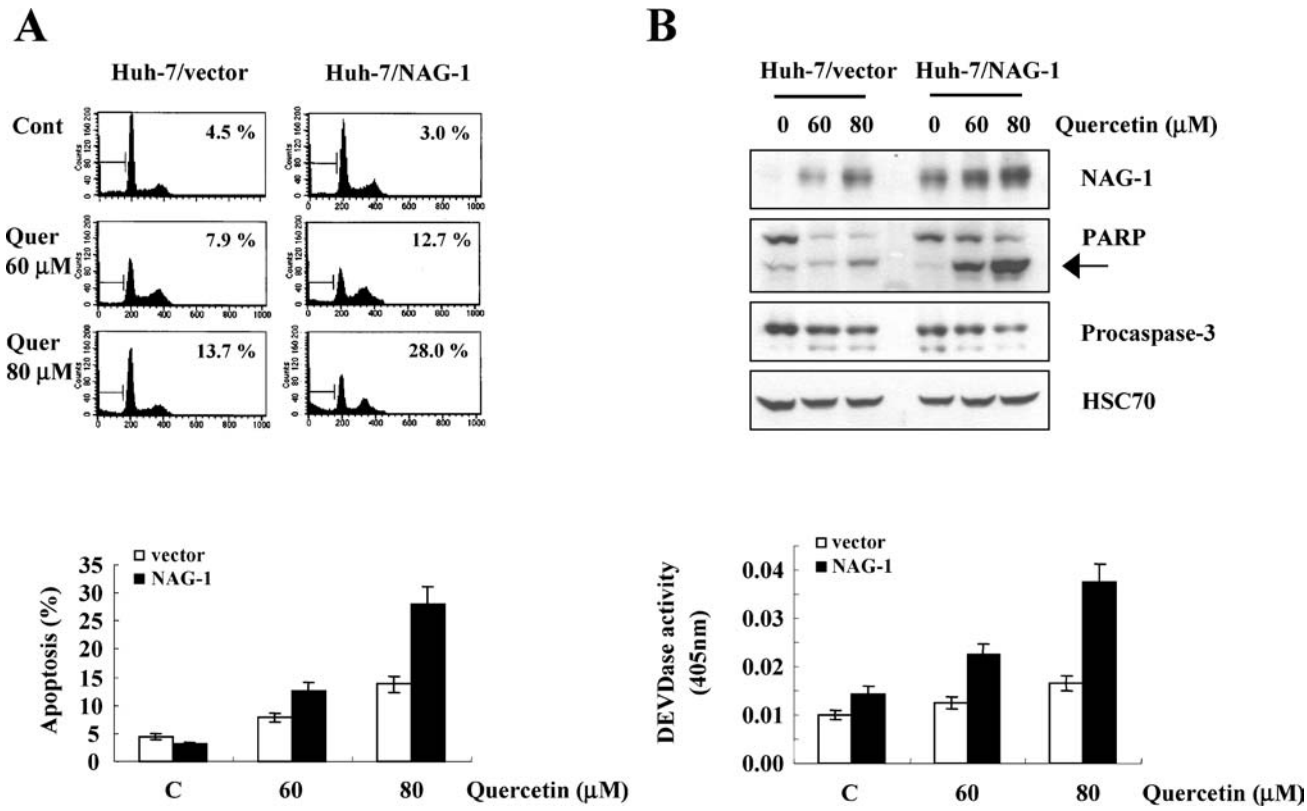

Fig. 7 Enhanced expression of NAG-1 in Huh-7 cells promotes quercetin-induced apoptosis. (A) Huh-7/NAG-1 and Hun-7/vector cells were treated with 60 or 80 μM quercetin for 24 h, and apoptosis was analyzed as a sub-G1 fraction by FACS. The proportion of apoptotic cells is indicated. Data are mean values obtained from three independent experiments and bars represent standard deviations. (B) Equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies, anti-NAG-1, anti-PARP, anti-caspase 3 or with anti-HSC70 antibody to serve as control for the loading of protein level. The proteolytic cleavage of PARP is indicated by an arrow. A representative study is shown; two additional experiments yielded similar results. DEVDase activity was determined as described in Fig. 2(C). Data shown are means $\pm$ SD. ($n = 3$)

increase in DEVDase activity, while only a half level of the activity was observed in Huh-7/vector cells (Fig. 7(B)). Taken together, NAG-1 overepxression in Huh-7 cells enhances quercetin-induced apoptosis. Moreover, these results suggest that quercetin-induced of NAG-1 up-regulation may be one of the underlying mechanisms for quercetin-induced apoptosis.

Discussion

Quercetin is one of the major flavonoids in certain species of plants that have been found to be chemopreventive. Although the molecular mechanisms of quercetin to regulate carcinogenesis are not clear, many different actions are known, including tyrosine kinase inhibition and blocking of cell proliferation [28, 29]. In this report, we clearly show that quercetin is an effective inducer of apoptosis and that NAG-1 up-regulation mediated by the transcription factor Sp1 and p53 is important for quercetin-induced apoptosis.

NAG-1 is regulated by a various *cis*- and *trans*-acting elements present in the promoter sequence [21, 30, 31]. One of the genes involved in the regulation of NAG-1 expression is the p53 tumor suppressor protein. NAG-1 prompter has two p53 binding sites and NAG-1 is induced by signals that activate p53 [32]. Recently, NAG-1 expression is induced by some NSAIDs, PPARγ ligands and some dietary compounds, including resveratrol, indol-3-carbinol and genistein in a p53-dependent/-independent manner [18–21, 33]. In our study, quercetin induces NAG-1 expression in p53-dependent manner. As shown in Fig. 5(A), quercetin increased p53 phosphorylation levels in dose-dependent manner, although it did not up-regulate p53 protein levels. Moreover, the extent of quercetin-induced up-regulation of NAG-1 was much higher in wild type HCT116 cells than those in p53 null HCT116 cells. In this report, we have further found that Sp1 transcription factor may be an important target protein to regulate NAG-1 expression by quercetin. Mithramycin A is known to inhibit tumors by cross-linking GC-rich DNA, thus blocking binding of Sp1-family transcription factors

to gene regulatory elements [23]. Mithramycin A strongly suppressed quercetin-induced promoter activity and protein expression of NAG-1, possibly through inhibition of Sp1-mediated transcriptional activities. Interestingly, Baek *et al*. reported that sulindac sulfide or troglitazone-induced NAG expression is critically regulated by the transcription factor EGR-1 in colon cancer cells [21, 28]. EGR-1 site was found in the NAG-1 promoter that overlaps with the Sp1 binding site [34, 35]. Thus, the transcriptional activity of NAG-1 may be regulated by the balance of Sp1 and EGR-1 family members. In this study, we demonstrated that the putative Sp1 binding sites located at –121, –70 and –57 were functionally active using a combination of the ectopic expression of Sp1 and the luciferase reporter assay. Although we showed that each mutated Sp1 sites significantly decreases quercetin-responsiveness, we cannot rule out the possibility that other *cis*-elements may be also involved in quercetin-dependent activation of the NAG-1. The expression of Sp1 is not changed in the presence of quercetin, whereas EGR-1 expression is increased. Furthermore, we used ChIP analysis to confirm the association of Sp1 or EGR-1 transcription factor with the NAG-1 promoter. Treatment with quercetin significantly increased the association of EGR-1 with NAG-1 promoter, but not Sp1. Thus, EGR-1 expression seems to be critical for quercetin-induced NAG-1 expression in HCT116 cells. In contrast, Sp1 maybe plays role as basal transcription factor in quercetin-induced NAG-1 expression in HCT116 cells.

NAG-1 is a novel TGF-β superfamily gene [13]. Although the precise biological functions of NAG-1 are not yet well understood, its involvement in inflammation, apoptosis or tumorigenesis has been suggested. Overexpression of NAG-1 in colon carcinoma and glioblastoma cells has been shown to inhibit tumorigenicity in nude mice, indicating that NAG-1 exhibits anti-tumor activities [11, 36]. In addition, NAG-1 overexpression by recombinant adenoviral vector results in up to a 50–60% reduction of MDA-468 and MCF-7 breast cancer cell viability [37]. In this study, we demonstrated that NAG-1 is a key regulator for quercetin-induced apoptosis. NAG-1 was induced by quercetin in a dose-dependent manner (Fig. 1), and knockdown of NAG-1 by siRNA attenuated quercetin-induced apoptosis (Fig. 6). Moreover, overexpression of NAG-1 enhanced the apoptotic effect of quercetin (Fig. 7), suggesting that NAG-1 expression is closely associated with apoptosis. The linkage between NAG-1 induction and quercetin revealed in this study provides a new molecular mechanism that may contribute to the anti-tumorigenic activities of quercetin.

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