Resveratrol enhances the expression of non-steroidal anti-inflammatory drug-activated gene (NAG-1) by increasing the expression of p53

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Dietary phenolic substances including resveratrol, a stilbene compound, are found in several fruits and vegetables, and these compounds have been reported to have anti-oxidant, anti-inflammatory and antitumorigenic activities. However, the molecular mechanisms underlying the antitumorigenic or chemopreventive activities of these compounds remain largely unknown. The expression of NAG-1 [non-steroidal anti-inflammatory (NSAID) drug-activated gene-1], a member of the transforming growth factor-beta (TGF-β) superfamily, has been shown to be associated with pro-apoptotic and antitumorigenic activities. Here, we have demonstrated that resveratrol induces NAG-1 expression and apoptosis in a concentration-dependent manner. Resveratrol increases the expression of p53, tumor suppressor protein, prior to NAG-1 induction, indicating that NAG-1 expression by resveratrol is mediated by p53 expression. We also show that the p53 binding sites within the promoter region of NAG-1 play a pivotal role to control NAG-1 expression by resveratrol. Derivatives of resveratrol were examined for NAG-1 induction, and the data suggest that resveratrol-induced NAG-1 and p53 induction is not dependent on its anti-oxidant activity. The data may provide linkage between p53, NAG-1 and resveratrol, and in part, a new clue to the molecular mechanism of the antitumorigenic activity of natural polyphenolic compounds.

Introduction

Several non-nutritive components in fruits, vegetables, herbs and spices have been found to inhibit tumor formation in experimental animals (1). Epidemiological studies have also suggested that nutrition plays an important role in carcinogenesis. Approximately 30% of cancer morbidity and mortality can potentially be prevented with the proper adjustment of diets. Many of the investigations studying the relationship between diet and cancer development have focused on resveratrol as this phenolic chemical has potent antitumorigenic and anti-inflammatory properties (2). Resveratrol is found in many plants, particularly in grape skin, and a significant amount of resveratrol is present in red wine. Resveratrol inhibits the development of pre-neoplastic lesions in mammary glands of carcinogen-treated mice and reduces tumor formation as measured by the two-stage model of skin cancer (2,3). In addition to antitumorigenic activity, resveratrol is thought to be responsible for the reduced risk of cardiovascular disease associated with moderate consumption of red wine (4). Resveratrol has anti-inflammatory activity as it suppresses carragegen-induced pedal edema, an effect attributed to suppression of prostaglandin synthesis via inhibition of cyclooxygenase (COX) activity (5). Furthermore, resveratrol inhibits TPA-induced COX-2 transcription (6). The mechanism by which resveratrol exerts anti-inflammatory and antitumorigenic activities may be related to the inhibition of either COX transcription or inhibition of COX activity, but further evidence is required to support this hypothesis.

The molecular mechanism of the tumor inhibition by resveratrol is not clear, but it appears to alter several biological processes of potential importance to tumor development. For example, resveratrol inhibits ribonucleotide reductase (7), DNA polymerase (8) and COX-2 transcription (6) and acts as an agonist for the estrogen receptor (9). Therefore, the molecular mechanism of resveratrol in antitumorigenesis is probably due to multiple actions. Recently, resveratrol was reported to trigger apoptosis (10,11), and many reports suggest that resveratrol induces apoptosis in cell culture (11–14). We have investigated the stimulation of apoptosis in cultured cells by some inhibitors of prostaglandin H synthase (COX). These COX inhibitors induced apoptosis and the expression of a protein that we called NAG-1 [non-steroidal anti-inflammatory (NSAID) drug-activated gene-1; also known as PTGF-β], which is a transforming growth factor-beta (TGF-β) superfamily protein. NAG-1 has antitumorigenic activity and stimulates apoptosis in colorectal and other cancer cell lines (15). NAG-1 basal expression is upregulated by Sp1, Sp3 and Coup-TF1 transcriptional factors (16), and by activators of the p53 tumor suppressor gene (17,18). Therefore, we decided to investigate the relationship between NAG-1, p53 and resveratrol.

In this study, the effect of resveratrol on the induction of NAG-1 expression and apoptosis in colorectal and other cell lines was investigated. We report that resveratrol enhances NAG-1 expression, induces apoptosis and suppresses cell growth. Furthermore, resveratrol induces p53 protein, which regulates NAG-1 expression in the promoter region. We propose a novel mechanism that NAG-1 may mediate the antitumorigenic activity of resveratrol.

Materials and methods

Cell lines and reagents

Cell lines were purchased from ATCC (Rockville, MD). Human colorectal carcinoma cells, HCT-116, HCT-15 and human osteosarcoma cells, U2OS, were maintained in McCoy’s 5A medium supplemented with 10% FBS and gentamicin. A549 lung epithelial carcinoma cells were grown in RPMI 1640 medium supplemented with 10% FBS and gentamicin. T-Rex™-U2OS cells were purchased from Invitrogen (Carlsbad, CA) and maintained in DMEM with antibiotics. Resveratrol was purchased from Sigma (St Louis, MO) and dissolved in dimethylsulfoxide (DMSO). All
resveratrol derivatives were generously provided by Dr Sang Kook Lee (Ewha Womans University, Seoul, Korea).

RNA isolation and northern blot analysis

When reaching 60–80% confluence in 10 cm plates, the cells were treated at indicated concentrations and times with different compounds or DMSO in the absence of serum. Total RNAs were isolated using TRIzol reagents (Life Technologies, Rockville, MD) according to the manufacturer’s protocol. For northern blot analysis, 10 µg of total RNA was denatured at 55°C for 15 min and separated in a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to Hybond-N membrane (Amersham, Piscataway, NJ). After fixing the membrane by UV, the blots were pre-hybridized in hybridization solution (Rapid-hyb buffer, Amersham) for 1 h at 65°C followed by hybridization with cDNA labeled with [32P]dCTP by random primer extension (DECAprimeII kit, Ambion, Austin, TX). The probe used was full-length NAG-1 fragment (15). After 4 h incubation at 65°C, the blots were washed once with 2X standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) at room temperature and twice with 0.1X SSC/0.1% SDS at 65°C. Messenger RNA abundance was estimated by intensities of the hybridization bands of autoadiographs using Scion Image (Scion Image, Frederick, MD). Equivalent loading of RNA samples was confirmed by hybridizing the same blot with a 32P-labeled β-actin probe, which recognizes RNA of ~2 kb.

Western blot analysis

The level of NAG-1 expression was evaluated using western blot analysis with anti-human-NAG-1 antibody (15). Cells were grown to 60–80% confluence in 10 cm plates following by 48 h treatment in the presence of indicated compounds. The media was harvested and concentrated ~15-fold using 10 cm plates. The cells were treated at 65°C, the blots were washed once with 2X standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) at room temperature and twice with 0.1X SSC/0.1% SDS at 65°C. Messenger RNA abundance was estimated by intensities of the hybridization bands of autoadiographs using Scion Image (Scion Image, Frederick, MD). Equivalent loading of RNA samples was confirmed by hybridizing the same blot with a 32P-labeled β-actin probe, which recognizes RNA of ~2 kb.

Cell proliferation assay

Cell proliferation assay was performed by CellTiter 96 Aqueous Non-Radioactive cell proliferation assay kit (Promega, Madison, WI). The assay was carried out as described in the manufacturer’s protocol. In 96 well plates, cells were split into 500 cells/well in 100 µl. After 16 h, cells were treated with various compounds in a 96 well plate and incubated at different time points. MTS/PSMS solution (20 µl/well) was added and incubated for 1 h. Absorbance at 490 nm was measured using an ELISA plate reader (Molecular Dynamics, Menlo Park, CA).

Cloning of NAG-1 promoter

The luciferase constructs containing the NAG-1 promoter were generated by polymerase chain reaction (PCR) methods using human genomic DNA (Promega). The following primers were used to generate each construct for pNAG966+/+70 clone, sense primer, 5'-TCTAGAATCTTCTAGCTCATGATCTAC3'; antisense, 5'-TGAGAGCTTATTCCCACTGCTCTTTG-3'; pNAG966+/+41 clone, sense, 5'-TCTAGAATCTTCTAGCTCATGATCTAC3'; antisense, 5'-TCTAGAATCTTCTAGCTCATGATCTAC3'; pNAG133+/41, sense primer, 5'-CACCACCCAGACCCCGACGCTCTTGGAGC-3'; pNAG133+/41, sense primer, 5'-CACCACCCAGACCCCGACGCTCTTGGAGC-3'; 5'-TCTAGAATCTTCTAGCTCATGATCTAC3'; antisense, 5'-TGAGAGCTTATTCCCACTGCTCTTTG-3'; pNAG133+/41, sense primer, 5'-CACCACCCAGACCCCGACGCTCTTGGAGC-3'; 5'-TCTAGAATCTTCTAGCTCATGATCTAC3'; antisense, 5'-TGAGAGCTTATTCCCACTGCTCTTTG-3'. After PCR, each fragment was cloned into TA vector (Invitrogen), sequenced and further cloned into pGLBasic3 vector digested with XhoI/HindIII restriction enzymes.

Transfection using the luciferase reporter system

U2OS and HCT-15 cells were plated in 6 well plates at 2×105 cells/well in McCoy’s 5A media supplemented with 10% fetal bovine serum. After growth for 16 h, plasmid mixtures containing 1 µg of NAG-1 promoter linked to luciferase and 0.1 µg of pRL-null (Promega) were transfected by lipofectamine (Life Technologies) according to the manufacturer’s protocol. After 24 h, the media was changed to serum-free media and resveratrol was added. Cells were harvested in 1X luciferase lysis buffer after 48 h treatment with resveratrol, and luciferase activity was determined and normalized to the pRL-null luciferase activity using the Dual Luciferase Assay Kit (Promega).

Inducible expression of NAG-1 system in U2OS cells

For the generation of stable cell lines with controlled expression of NAG-1, the T-Rex system was utilized. T-Rex™-U2OS cells were purchased from Invitrogen, containing pCDNA6/TR that expresses the Tet repressor protein. The PCR fragment was amplified from pCDNA3.1/NAG-1 (15) using the following primers: 5'-GGAAATTCTACACTGAGCTTACCTCTGGC-3' and 5'-GCTCTAGATATGCAGTGCCTTTTGG-3'. The fragment was digested with EcoRI and XhoI restriction enzymes and cloned into pcDNA/TTOmyc-HisA. The plasmid was sequenced for verification and transfected into T-Rex™-U2OS cells using LipofectAMINE™ (Gibco-BRL, Rockville, MD) according to the manufacturer’s protocol. Cells were grown with 200 µg of zeocin (Invitrogen) until colonies formed (3–4 weeks). Single colonies were isolated using cloning cylinders, and two clonal cell lines were expanded and identified by western blot analysis to over express NAG-1 protein. For induction, cells were washed in PBS and cultured in the absence or presence of the indicated amounts of tetracycline. Cells were maintained in complete media with 100 µg/ml zeocin, 50 µg/ml hygromycin (Calbiochem, La Jolla, CA) and tet-free serum.

Colony formation

For colony formation assays of U2OS cells, 1000 cells/10 cm dish were plated and grown until colonies formed. The selected cells were stained with Giemsa and photographed.

Aptosis assay

The DNA content for sub-G1 population was determined by fluorescence-activated cell sorter (FACS). HCT-116 and U2OS cells were plated at 3×104 cells/well in 6 well plates, incubated for 16 h and then treated with different compounds in the presence of serum. The cells (attached and floating cells) were then harvested, washed with PBS, fixed by the slow addition of cold 70% ethanol to a total of 1 ml and stored at 4°C overnight. The fixed cells were pelleted, washed with 50%, 30% ethanol, followed by PBS and stained in 1 ml of 20 µg/ml propidium iodide, 1 mg/ml RNase in PBS for 20 min. 7500 cells were examined by flow cytometry using Becton Dickinson (Franklin Lakes, NJ) FACSort equipped with CellQuest software by gating on an area versus width dot plot to exclude cell debris and cell aggregates. Apoptosis was measured by the level of subdiploid DNA contained in cells following treatment with compounds using CellQuest software.

Results

Effects of resveratrol on growth and apoptosis of HCT-116 cells

To investigate the effects of resveratrol on the growth of colorectal cancer cells in culture, resveratrol at various concentrations was added into the culture medium. In the presence of different concentrations of resveratrol, a significant inhibition of cell growth was observed (Figure 1A). Concentrations as low as 50 µM resveratrol resulted in a complete growth arrest of HCT-116 cells, whereas the 10 µM concentration showed ~30% reduction of cell growth compared with vehicle-treated cells observed after growing for 4 days. Subsequently, to determine if resveratrol at these concentrations increased apoptosis in HCT-116 cells, flow cytometry was used to estimate apoptosis under these treatment conditions. As shown in Figure 1B, resveratrol also induced apoptosis (sub G1 population, M1) in a concentration-dependent manner. Therefore, in HCT-116 cells, resveratrol treatment resulted in growth arrest and enhanced apoptosis. These results are consistent with previous reports that resveratrol inhibits growth rate and induces apoptosis in cell culture (13,14,19,20).

Resveratrol induces NAG-1 expression in a time- and concentration-dependent manner

As resveratrol induces apoptosis in HCT-116 cells (Figure 1B), and NAG-1 has pro-apoptotic and/or antitumorigenic properties (15), we measured NAG-1 expression after treatment with resveratrol. Northern and western analyses were performed to estimate the expression of NAG-1 in HCT-116 cells treated with resveratrol. Northern and western analyses were performed to estimate the expression of NAG-1 in HCT-116 cells treated with resveratrol. NAG-1 mRNA expression was increased with duration of resveratrol treatment (100 µM), and the highest mRNA expression was observed after 24 h of treatment (Figure 2A). The NAG-1 mRNA expression was also dependent on resveratrol concentration, with a significant increase in expression observed at 10 µM, maximum expres-
Fig. 1. Concentration-dependent growth retardation and apoptosis of HCT-116 cells treated with resveratrol. (A) Effect of resveratrol on HCT-116 cell growth. HCT-116 cells were plated at 500 cells/well in a 96 well plate and incubated with vehicle (DMSO) or various concentrations of resveratrol. Cell growth was measured using PMS cell proliferation kit (Promega). Values are expressed as mean ± SD of four to five replicate experiments. (B) Flow cytometric analysis of resveratrol-treated HCT-116 cells. HCT-116 cells were plated at 3 × 10^4 cells/well in 6 well plates and incubated without or with various concentrations of resveratrol for 48 h and analyzed for apoptosis as described in Materials and methods. ‘M1’ indicates apoptotic cell population (sub G1 population), whereas x-axis indicates DNA content.
Fig. 2. Northern and western analysis of NAG-1 expression in resveratrol-treated HCT-116 cells. (A) Northern blot analysis of NAG-1 expression by resveratrol. HCT-116 cells were treated with 100 µM resveratrol for various time points. Total RNA (10 µg) was loaded in each lane and transferred onto nylon membrane. The blot was hybridized with NAG-1 probe and re-probed with β-actin cDNA. The hybridization signals were quantified using Scion Image software (Scion), and values for the 1.3 kb NAG-1 transcript were normalized to β-actin transcripts levels. A >3-fold induction of NAG-1 was seen at 24 and 48 h time points. (B) Dose–response of NAG-1 expression. HCT-116 cells were grown in different concentrations of resveratrol for 24 h, and northern blot analysis was performed as described above. (C) Western analysis of NAG-1 expression. HCT-116 cells were treated with different concentrations of resveratrol for 48 h, and western analysis was performed with total cell lysate. The arrow indicates ~35 kDa pro-form of NAG-1.
NAG-1 was determined from concentrated media proteins, and into a short peptide and secreted into media. Thus, the secreted expression of NAG-1 is U2OS-NAG#11 and U2OS-NAG#18. The ectopic expression of NAG-1, Tetracycline induced a dramatic increase in NAG-1 expression, a significant reduction in the number of colonies was observed in U2OS-NAG#18 cell lines. A reduction in colony formation efficiency was also dependent on the concentration of tetracycline. No significant reduction in the number of colonies was observed in the parent U2OS cell line (T-Rex™-U2OS) when treated with tetracycline (Figure 4D). The treatment of tetracycline on U2OS-NAG#11 produced a similar result to U2OS-NAG#18 cell lines (data not shown). Taken together, the data are consistent with the previous report that NAG-1 has pro-apoptotic and/or antitumorigenic activities (15).

Expression of NAG-1 and p53 proteins by resveratrol
Resveratrol induces apoptosis in a p53-dependent manner (11) and NAG-1 is regulated by p53 (17,18). Therefore, we examined the expression pattern of NAG-1 and p53 in the presence of resveratrol to determine a possible link between resveratrol and NAG-1 expression, mediated by p53 expression. HCT-116 cells were treated with resveratrol (50 µM), etoposide (25 µM) and sulindac sulfide (30 µM) at different time points. Etoposide was used as a known p53 activator (17), and sulindac sulfide, a COX inhibitor, was used to stimulate NAG-1 expression (15). Total proteins were then isolated and subjected to western analysis. As shown in Figure 5, the expression of p53 protein is increased as early as 3 h after treatment of cells with resveratrol or etoposide. However, NAG-1 expression was seen only after 24 h in both treatments, suggesting that NAG-1 expression might be mediated by p53 proteins. In contrast, sulindac sulfide did not induce p53 protein expression, but did induce NAG-1, indicating that sulindac sulfide induces NAG-1 in a p53-independent manner. The data are consistent with the previous report that NSAIDs induce NAG-1 in a p53-independent manner (15).

Resveratrol induces NAG-1 expression through p53 protein
To investigate whether NAG-1 expression is dependent on p53, NAG-1 promoter activity was examined. We cloned the NAG-1 promoter (~966 to +70) by PCR as described in Materials and methods. The construct, pNAG966/+70, containing two p53 binding sites (p53-A and p53-B), was linked to the luciferase gene and transfected into U2OS cells (Figure 6A). After 48 h treatment with different concentrations of resveratrol or etoposide, luciferase activity was measured. Etoposide treatment increased promoter activity by ~10-fold, whereas resveratrol increased promoter activity by ~3–4-fold, compared with vehicle-treated U2OS cells. The concentration of resveratrol at 50 µM gave the highest luciferase activity. Next, NAG-1 deletion clones, pNAG966/+41, pNAG133/+41 and pNAG133/+70 were further generated by PCR. The pNAG966/+41 clone contained a p53-A site, whereas the pNAG133/+70 clone contained the p53-B site. The pNAG133/
NAG-1 promoter activity in the presence of resveratrol. The +41 clone contained no p53 binding sites (Figure 6B). Transient transfection was performed in U2OS cells (p53 wild-type) and HCT-15 cells (p53 negative) using the above constructs and treated with vehicle or 50 µM resveratrol. As shown in Figure 6B, the constructs lacking two p53 binding sites or having only one p53 site (p53-A) did not significantly induce NAG-1 promoter activity in the presence of resveratrol. The constructs containing the p53-B site (pNAG966/+70 and pNAG133/+70) had significantly enhanced luciferase activity compared with vehicle-treated cells, suggesting that the p53-B site has a more critical role for resveratrol-induced NAG-1 expression. The data are consistent with previous data showing that the p53-B site is more critical for etoposide-induced NAG-1 expression (17). In contrast, the same constructs were transfected into HCT-15 cells (p53 negative cell lines) and showed no induction of luciferase activity with resveratrol treatment.

Finally, to examine whether the transactivation of the NAG-1 promoter by p53 depends on p53 binding sites, we performed co-transfection experiments using p53 cDNA in an expression vector in HCT-15, p53 negative cells. The construct, pNAG133/+70 (p53-B site) and pNAG133/+41 (no p53 sites) clones, were transfected with either empty vector or p53 expression vector. As shown in Figure 6C, the ectopic expression of p53 with the pNAG133/+70 clone in p53 negative cells, enhance NAG-1 promoter activity compared with empty vector transfected cells. In contrast, no induction was observed in the pNAG133/+41 clone, suggesting that NAG-1 expression is mediated by p53 protein expression. In addition, gel-shift assay was performed using oligonucleotide corresponding p53 binding site and shift bands were identified in resveratrol-treated nuclear extracts (data not shown). Taken together, resveratrol activates NAG-1 promoter activity through the p53 binding sites and the activation of NAG-1 promoter activity was observed in HCT-15 cells by co-transfection with wild-type p53 cDNA. These results suggest that p53 has an important role in transactivating the NAG-1 promoter by resveratrol.

Structural analysis of resveratrol responsible for NAG-1 and p53 induction

The stilbenic double bonds and the position of hydroxyl groups are important factors to determine the anti-oxidant activity of resveratrol (23), and are associated with the apoptotic gene induction and COX-2 promoter activity (19,24). To study the structural characteristics of resveratrol-induced NAG-1 and p53, several resveratrol derivatives (50 µM each) were used to treat HCT-116 cells for 36 h and western analysis was performed. Changing the para hydroxyl group of resveratrol to a methoxy (MeO) group (SY-002 and SY-040) that greatly diminishes its anti-oxidant activity did not alter NAG-1 or p53 induction, as shown in Table I. However, the removal of the substitutions at the Ring2 position and/or conversion to the Ring2 meta MeO (SY-001 and SY-006) eliminate activity responsible for NAG-1 or p53 induction. Interestingly, the substitution of the para hydroxyl group to a bromide group resulted in the greatest induction of NAG-1 expression. Resveratrol showed the highest induction of p53. The results suggest that the para position at Ring2 is an important factor in NAG-1

Fig. 4. Biological function of NAG-1 in antitumorigenesis. (A) Western analysis of stably transfected U2OS cells. Two individual clones, U2OS-NAG#11 and U2OS-NAG#18, were treated with 2 µg/ml of tetracycline for 48 h in the absence of serum. Total cell lysates were harvested and 30 µg of protein was loaded onto 15% SDS-PAGE. The media was also harvested, concentrated using Centricon and 30 µg of protein was loaded. Arrows indicate ~35 kDa pro-form of NAG-1 and ~12 kDa secreted form of NAG-1. (B) Cell proliferation analysis of U2OS-NAG#18 clone. Cells were grown in the presence or absence of tetracycline for 9 days. The growth rate was measured by PMS cell proliferation kit. The data represent the mean ± SD from different experiments. (C) Apoptotic analysis of U2OS-NAG#18 clone. Cells were treated with or without 2 µg/ml of tetracycline for 2 days, and the sub G1 population was analyzed by FACS. Tet– indicates uninduced, whereas Tet+ indicates induced NAG-1. The data represent mean ± SD from three independent experiments. (D) Inhibition of colony formation by NAG-1. 1000 cells of clone U2OS-NAG#18 and parent U2OS cells (T-Rex™-U2OS) were seeded onto a 10 cm tissue culture dish and cultured for 3 weeks in the presence of the indicated amounts of tetracycline. The media was changed every 3 days with fresh tetracycline. The outgrowing colonies were fixed and stained using Giemsa.
Molecular mechanism of resveratrol in antitumorigenesis

Fig. 5. Expression of NAG-1 and p53 in the presence of resveratrol, etoposide and sulindac sulfide. HCT-116 cells were treated with indicated compounds, and harvested at different time points. Total protein (30 µg) was subjected to 15% SDS–PAGE. The antibodies for p53 (Santa Cruz), NAG-1 and β-actin (Santa Cruz) were probed onto membrane as described in Materials and methods. The blots are representative of three independent experiments.

Fig. 6. NAG-1 induction by resveratrol is mediated by p53 protein. (A) Effect of resveratrol on NAG-1 promoter. The pNAG966/+70 clone linked to luciferase was transfected into U2OS cells and treated with different concentrations of resveratrol for 48 h. Etoposide was used as a positive control for NAG-1 promoter activity. The construct (1 µg) was co-transfected with 0.1 µg of pRL-null (Promega) vector, using LipofectAMINE™ (Gibco-BRL), and 48 h later the promoter activities were measured by luciferase activity. Transfection efficiency for luciferase activity was normalized to the Renilla luciferase (pRL-null vector) activity. The y-axis shows fold induction (over relative luciferase activity of vehicle-treated cells as 1.0). The results show the mean ± SD of three independent transfections. (B) Each construct was transiently transfected into HCT-15 cells (left panel) or U2OS cells (right panel), and cell lysates were harvested after 48 h of treatment. The middle panel indicates a schematic diagram of the NAG-1 promoter region. Fold induction refers to ratio of luciferase activity of 50 µM resveratrol-treated cells to vehicle-treated cells. Values are mean ± SD of three independent experiments. (C) Activation of luciferase activity in p53-dependent manner. Each construct was transiently co-transfected with either empty vector (control) or p53 expression vector (p53) into HCT-15, p53-negative cells. The cells were grown for 2 days and harvested for luciferase activity. The y-axis shows fold induction (over relative luciferase activity of empty vector transfected cells as 1.0). Values are mean ± SD of four independent transfections.
Table I. HCT-116 cells were treated with 50 µM of each compound for 36 h and western analysis was performed using NAG-1 and p53 antibodies.

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Induction of protein was indicated by +, whereas no induction was indicated by – signs.

and p53 expression. In addition, the data suggest that the antioxidant activity of resveratrol is not directly linked to NAG-1 expression as all the resveratrol derivatives had lost their anti-oxidant activities.

Discussion

A numerous variety of naturally occurring substances have been shown to protect against experimental carcinogenesis, and it is becoming increasingly evident that certain phytochemicals, particularly those included in our daily diet, have marked cancer chemopreventive properties. Resveratrol is such a dietary chemopreventive phytochemical compound that has recently attracted considerable interest due to its remarkable multi-functional inhibitory effects on multi-stage carcinogenesis. Resveratrol has chemopreventive effects on several cancers (25,26). The mechanism of action of resveratrol has not been elucidated, but may be related to its ability to inhibit ribonucleotide reductase (7), DNA polymerase (8), COX-2 transcription (6) and activate the MAP kinase pathway (27) and the estrogen receptor (7). The molecular mechanism of resveratrol on antitumorigenesis is not clear, but is probably due to multiple actions. The present study was designed to test whether resveratrol may exert antitumorigenic effects through an antitumorigenic protein, NAG-1, and provide a molecular mechanism for regulation of NAG-1 expression.

Our results show that resveratrol is an effective inhibitor of cell growth, and induces apoptosis in HCT-116 cells (Figure 1). Furthermore, resveratrol treatment resulted in an increased S phase in a concentration-dependent manner (data not shown), indicating a growth arrest at the S/G2 phase transition. Resveratrol increases the expression of NAG-1 in colorectal and lung cancer cells as well as in osteosarcoma cells, and NAG-1 has pro-apoptotic and antitumorigenic activities, which may provide, in part, a novel mechanism to explain its antitumorigenic activity.

Recently, we reported that the NAG-1 expression is enhanced by some NSAIDs, in a COX- and p53-independent manner (15). However, as reported here, resveratrol induces NAG-1 expression in a p53-dependent manner. As shown in Figure 5, resveratrol and etoposide induce p53 protein prior to the NAG-1 protein expression. The COX inhibitor, sulindac sulfide, did not induce p53 protein expression, but enhanced NAG-1 expression. Indeed, the biochemical pathway for resveratrol-induced apoptosis appears to require p53 induction (11). In contrast, NSAID-induced apoptosis does not require p53 induction (28). Our data indicate that NAG-1 induction by resveratrol is mediated by p53, and NAG-1 induction by NSAIDs does not require p53 induction. According to previous reports (17,18), NAG-1 contains two p53 binding sites in the promoter region. The second p53 binding site (p53-B) is the more functionally active of the two p53 binding sites, and consistent with the data, resveratrol-dependent NAG-1 induction is mediated by the second p53 binding site (Figure 6B). Interestingly, etoposide is more effective than resveratrol in stimulating NAG-1 promoter activity (Figure 6A), although both compounds induce p53 expression at almost the same magnitude. Recently, She et al. reported that resveratrol affects ERKs and p38 kinase activity, and thus increases p53 phosphorylation within 3 h of resveratrol treatment (27). Thus, it is possible that resveratrol may regulate two different mechanisms: one is an early response (within 3 h) resulting in the phosphorylation of p53, followed by a late response (after 3 h) resulting in p53 protein induction. Further investigations are required to elucidate the detailed mechanism of NAG-1 induction with respect to the involvement of p53 protein. In addition to resveratrol mediation, NAG-1 is induced by several resveratrol derivatives (Table I). The anti-oxidant activity of resveratrol has been linked to apoptotic gene induction and COX-2 promoter activity (19,23,24). We have shown in this report that resveratrol derivatives with weak anti-oxidant activity increase NAG-1 and/or p53 expression. This suggests that resveratrol-induced NAG-1 expression is not associated with the anti-oxidant activity of resveratrol. Furthermore, the substitution at the para position (Ring2) appears to be a critical determinant of NAG-1 expression.

We have shown that the expression of NAG-1 resulted in the induction of apoptosis in vitro and the reduction of tumor size in vivo (15). In this study, we further analyzed the effects of its antitumorigenic activity by an inducible expression system. Our findings demonstrate that functional NAG-1 can be expressed (Figure 4A) and that this protein inhibits cell proliferation (Figure 4B) and induces apoptosis (Figure 4C). However, the induction of apoptosis by NAG-1 is not robust (Figure 4C), suggesting that some additional triggers for resveratrol-induced apoptosis may be required. Furthermore, an unexpected finding was that the effect of NAG-1 was a dramatic inhibition of colony formation, while the growth of an exponential culture was only reduced ~30% (Figure 4B).
One explanation for this difference might be that NAG-1 exerts its function predominantly under restrictive cell growth conditions. Indeed, we have reported that the over expression of NAG-1 resulted in the reduction of tumor size in nude mice experiments (15). These findings are consistent with studies published previously on the function of Mad1. The induction of Mad1 reduced cellular growth but, more profoundly, inhibited colony formation (29).

The biological significance of our findings is implicated in the linkage between resveratrol, NAG-1 expression, mediated by p53 protein and apoptosis. NAG-1 is the most notable p53-induced gene, as determined in a recent investigation using cDNA array technology (30). Resveratrol has well established antitumorigenic and pro-apoptotic activities. Our data shown here indicate that these effects are mediated by p53, and NAG-1 is a critical down stream protein. Although p53 has been well documented as a tumor suppressor, the p53 target protein, produced as a secreted form, has not been studied in detail.

NAG-1 is secreted into the media and has specific antitumorigenic and pro-apoptotic activity, although the induction of p21/WAF-1/CIP1 by resveratrol is also mediated by p53 in HCT-116 cells (data not shown). In addition, p53 expression results in the decrease of COX-2 expression (31).

Thus, resveratrol treatment followed by the induction of the p53 protein enhances apoptosis by up regulating apoptotic proteins including NAG-1, and down regulating anti-apoptotic proteins like COX-2. Although the mechanisms by which resveratrol regulate apoptosis are complex, the data will provide a new pathway to understand how phenolic compounds affect antitumorigenic activity.

Resveratrol concentrations used in this study are comparable with those occurring in wine and grapes (32). These concentrations cause an arrest of HCT-116 cell proliferation and an induction of NAG-1 antitumorigenic protein. It is believed that two glasses of red wine could provide a 10–30 µM concentration of resveratrol in vivo, where most of the pharmacological effects of resveratrol are observed (2). In addition, NAG-1 expression by resveratrol is not restricted to colorectal cells, as resveratrol can induce NAG-1 in A549, and U2OS cells. NAG-1 induction by resveratrol in different cell lines supports the evidence that resveratrol has an antitumorigenic effect in several cancers.

In summary, we have been able to document resveratrol as a potent inducer of NAG-1, a TGF-β superfamily protein that has antitumorigenic activity. Furthermore, we have shown that the expression of NAG-1 results in the reduction of colony formation, induction of apoptosis and is mediated in a p53-dependent manner. NAG-1 expression is correlated with proapoptosis and antitumorigenesis in cell culture systems, and thus resveratrol effects on antitumorigenesis are mediated, in part, by NAG-1. The detailed analysis of biological functions and p53 regulation of NAG-1 expression should shed light on how dietary compounds alter tumorigenesis.

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