The Rac1/MKK7/JNK pathway signals upregulation of Atg5 and subsequent autophagic cell death in response to oncogenic Ras

Joo-Yun Byun1, Chang-Hwan Yoon1, Sungkwon An2, In-Chul Park3, Chang-Mo Kang4, Min-Jung Kim1,5 and Su-Jae Lee1,5,∗

1Laboratory of Molecular Biochemistry, Department of Chemistry, Hanyang University, 17 Haengdong-Dong, Seongdong-Ku, Seoul 133-791, Korea, 2Department of Microbiological Engineering, KonKuk University, Seoul 143-701, Korea, 3Division of Radiation Biology and 4Division of Radiation Cancer Biology, Korea Institute of Radiological and Medical Sciences, Seoul 139-706, Korea and 5Research Institute of Natural Sciences, Hanyang University, 17 Haengdong-Dong, Seongdong-Ku, Seoul 133-791, Korea

∗To whom correspondence should be addressed. Tel: +82 2 2220 2557; Fax: +82 2 2299 0762;
Email: sj0420@hanyang.ac.kr

Correspondence may also be addressed to Min-Jung Kim. Tel: +82 2 2220 4554;
Fax: +82 2 2299 0762;
Email: kimnj74@hanyang.ac.kr

To prevent the development of malignancies, mammalian cells activate disposal programs, such as programmed cell death, in response to deregulated oncogene expression. However, the molecular basis for regulation of cellular disposal machinery in response to activated oncopgenes is unclear at present. In this study, we show that upregulation of the autophagy-related protein, Atg5, is critically required for the oncogenic H-ras-induced autophagic cell death and that Rac1/mitogen-activated kinase kinase (MKK) 7/c-Jun N-terminal kinase (JNK) signals upregulation of Atg5. Oxexpressed H-rasV12 induced marked autophagic vacuole formation and cell death in normal fibroblasts, which remained unaffected by a caspase inhibitor. Pretreatment with Baflomycin A1, an autophagy inhibitor, completely attenuated H-rasV12-induced cell death as well as autophagic vacuole formation. Selective production of Atg5 was observed in cells overexpressing H-rasV12, and small interfering RNA (siRNA) targeting of Atg5 clearly inhibited autophagic cell death. Interestingly, inhibition of JNK or c-Jun by specific siRNA suppressed Atg5 upregulation and autophagic cell death. Moreover, inhibition of MKK7, but not MKK4, effectively attenuated H-rasV12-induced JNK activation. In addition, ectopic expression of RacN7 or Rac1-siRNA effectively inhibited MKK7/JNK activation, Atg5 upregulation and autophagic cell death. These data support the notion that upregulation of Atg5 is required for the oncogenic H-ras-induced autophagic cell death in normal fibroblasts and that activation of Rac1/MKK7/JNK-signaling pathway leads to upregulation of Atg5 in response to oncogenic H-ras. Our findings suggest that in cells acquiring deregulated oncogene expression, oncogenic stress triggers autophagic cell death, which protects cells against malignant progression.

Introduction

Oncoproteins of the Ras family have been extensively analyzed because of their involvement in human cancer. Uncontrolled Ras activation is one of the most common genetic alterations associated with human cancer. Point mutations in the ras gene occur at high frequency in mammalian cells, resulting in malignant transformation and further progression to cancer (1). In fact, oncogenic Ras mutations are found in >30% of all human malignancies (2). Ras guanosine triphosphatases act as molecular switches to transduce extracellular signals to the nucleus, where they regulate an overlapping set of cellular responses. These proteins participate primarily in cell proliferation and suppression of apoptosis, as evident from Ras activation in the signal transduction pathways triggered by various stimuli (3). However, Ras functions have been extended in recent years to include the initiation of programmed cell death in signal transduction pathways (4–7). This pro-apoptotic activity has been confirmed in a number of experimental systems, both for Ras and Ras effectors (8–10). Despite multiple reports demonstrating a requirement for Ras in cell death processes, relatively limited information is available on the molecular and biochemical mechanisms of Ras action in the regulation of programmed cell death.

Programmed cell death, an important means to exclude abnormalities and prevent emerging malignancies, is a prominent mechanism for tumor suppression (11). This process is subdivided into at least two major subtypes, specifically, apoptotic and autophagic cell death. Apoptosis is a well-defined cell death pathway that is conserved from nematodes to humans. The caspase family of cysteine proteases plays a central role in apoptotic signaling and cell execution. Autophagic cell death is a non-apoptotic form of programmed cell death, an evolutionally conserved genetically controlled process that regulates the normal turnover of proteins and organelles and removes those with compromised function to maintain homeostasis (12). Apoptosis and autophagic cell death are alternative pathways that lead to the same end and act as backup mechanisms for each other under conditions where cell death is inevitable (13).

Autophagy is a dynamic process that prolongs survival for a short time under starvation conditions. A membrane forms around the targeted cytoplasm, and organelles fuse with a lysosome or vacuole, resulting in subsequent degradation of organelle contents and recycling to maintain cell viability (14,15). Certain forms of cell death are prevented either with autophagic inhibitors or by reduced Atg gene expression, implying that autophagy triggers and participates directly in the cell death mechanism (13,16,17). Autophagy-related cell death is characterized by the accumulation of autophagic vesicles, autophagosomes and autolysosomes, often occurring when extensive cell elimination is required (18,19). Consistently, large-scale induction of autophagy destroys severely damaged cells as a defense mechanism against cancers (20–22). Based on these findings, it is proposed that defects in this pathway confer a selective survival advantage to cells, resulting in cancer.

To prevent the development of malignancies, mammalian cells activate cellular disposal programs, such as programmed cell death, in response to deregulated oncogene expression. However, the molecular basis for regulation of cellular disposal machinery in response to activated oncopgenes is unclear at present. In this study, we show that upregulation of the autophagy-related protein, Atg5, is critically required for the oncogenic H-ras-induced autophagic cell death and that Rac1/mitogen-activated kinase kinase (MKK) 7/c-Jun N-terminal kinase (JNK) signals upregulation of Atg5. Oxexpressed H-rasV12 induced marked autophagic vacuole formation and cell death in normal fibroblasts, which remained unaffected by a caspase inhibitor. Pretreatment with Baflomycin A1, an autophagy inhibitor, completely attenuated H-rasV12-induced cell death as well as autophagic vacuole formation. Selective production of Atg5 was observed in cells overexpressing H-rasV12, and small interfering RNA (siRNA) targeting of Atg5 clearly inhibited autophagic cell death. Interestingly, inhibition of JNK or c-Jun by specific siRNA suppressed Atg5 upregulation and autophagic cell death. Moreover, inhibition of MKK7, but not MKK4, effectively attenuated H-rasV12-induced JNK activation. In addition, ectopic expression of RacN7 or Rac1-siRNA effectively inhibited MKK7/JNK activation, Atg5 upregulation and autophagic cell death. These data support the notion that upregulation of Atg5 is required for the oncogenic H-ras-induced autophagic cell death in normal fibroblasts and that activation of Rac1/MKK7/JNK-signaling pathway leads to upregulation of Atg5 in response to oncogenic H-ras. Our findings suggest that in cells acquiring deregulated oncogene expression, oncogenic stress triggers autophagic cell death, which protects cells against malignant progression.

Materials and methods

Cell culture and transfection

Rat2, NIH3T3 and WI38 cells were obtained from American Type Culture Collection (Manassas, VA). Rat2 cells were grown in RPMI 1640 supplemented with 5% fetal bovine serum, and NIH3T3 and WI38 were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (1000 U/ml) and streptomycin (1000 µg/ml) in a humidified 5% CO2 atmosphere. The dominant-negative MKK4 and MKK7 were cloned into the pcDNA3 vector and transiently delivered into lipofection.
Chemical reagents and antibodies
Pan-caspase inhibitor, actinomycin D, propidium iodide (PI), dimethyl sulfoxide and N-acetyl-cysteine were purchased from Sigma (St. Louis, MO). Caspase 3 inhibitor, cyclohexamide, Bafilomycin A1, PD98059, SB203580 and SP600125 were purchased from Calbiochem (San Diego, CA), LysoTracker-Green DND-26 was purchased from Molecular Probe (L-7526, Invitrogen, Carlsbad, CA). Polyclonal antibodies to anti-phospho-extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), caspase 8, caspase 9, caspase 3, c-jun and Atg6 (beclin 1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-Atg5, LC3 and lysosomal associated membrane protein-1 (LAMP-1) were obtained from Abgent (San Diego, CA). Polyclonal antibodies to poly adenosine diphosphate-ribose polymerase, ERK, JNK, phospho-JNK, phospho-p38 MAPK, MKK4, phospho-MKK4, MKK7, phospho-MKK7 and phospho-c-jun were obtained from Cell Signaling Technology (Beverly, MA). Monoclonal anti-β-actin was obtained from Sigma.

Quantification of cell death
Flow cytometric analysis using PI (2.5 μg/ml) staining detects cell death by means of the dye entering the cells along with changes in the target cell membrane and DNA damage. For the cell death assessment, the cells were plated in 60 mm dish with cell density of 1 x 10^5 cells per dish and infected with H-rasV12. At indicated time points, cells were harvested by trypsinization, washed in phosphate-buffered saline and then incubated in PI for 5 min at room temperature. Then, (10 000 per sample) were analyzed on a flow cytometric scan flow cytometer, using Cell Quest software.

Transmission electron microscopy
Cells incubated as described above were pelleted and fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 120 min, washed three times and postfixed in osmium tetroxide 1% for 60 min. The cells were dehydrated in an ascending ethanol battery ranging from 20 to 100% and were later placed in 3:1, 2:1, 1:1, 1:2 and 1:3 ratios of propylene oxide or epon-812 resin for 1 h at room temperature, respectively. Ultrathin sections of 70 nm were made and impregnated with 2% uranyl acetate and Reynolds’s lead citrate. The sections were visualized in a Zeiss EM-900 transmission electron microscope at 50 kV and C2/1881 Chemical reagents and antibodies transfection–polymerase chain reaction reactions were performed with SuperScript III (Invitrogen) according to the manufacturer’s instructions. Primer sequences and amplification conditions are described as follows: atg5 (forward, 5'-CCATGGAGAAGGCTGGGG-3'), (reverse, 5'-CAAAGTTGTATTCAGC-3'), (reverse, 5'-GAAGCAGAAAGGCAGCATA-3') and gapdh (forward, 5'-CTGCRGCCTTGGAACATCACA-3'), (reverse, 5'-AGGGTATGCAGCTGTCATCG-3'), (reverse, 5'-GGGGCCACCCCATGGCAG-3'), (reverse, 5'-GGGGCCACCCCATGGCAG-3'), (reverse, 5'-GGGGCCACCCCATGGCAG-3'), (reverse, 5'-GGGGCCACCCCATGGCAG-3'), (reverse, 5'-GGGGCCACCCCATGGCAG-3'), (reverse, 5'-GGGGCCACCCCATGGCAG-3') and (reverse, 5'-GGGGCCACCCCATGGCAG-3').

Small interfering RNA transfection
Small interfering RNA (siRNA) targeting of Atg5, p38 MAPK, ERK1, JNK1 and c-jun were performed using 21 bp (including a 2-deoxynucleotide overhang) and purchased from Ambion (Austin, TX). A control siRNA specific for green fluorescent protein (CCACTACCTGAGCACCCAG) was used as the negative control. Cells were plated on 100 mm dishes at 30% confluence, and siRNA duplexes (50 nM) were introduced into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) by following the procedure recommended by the manufacturer.

Western blot analysis
Cell lysates were prepared by extracting proteins with lysis buffer (40 mM Tris–HCl (pH 8.0), 120 mM NaCl, 0.1% Nonidet-P40) supplemented with protease inhibitors. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham Biosciences, Arlington Heights, IL). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline and incubated with primary antibodies for overnight at 4°C. Blots were developed with a peroxidase-conjugated secondary antibody and proteins visualized by enhanced chemiluminescence procedures (Amersham), using the manufacturer’s protocol.

RNA isolation and reverse transcription–polymerase chain reaction
RNA was isolated using the RNeasy kit (QIAGEN, Valencia, CA). RNA was treated with DNase I (Ambion) to eliminate any contaminating DNA. Reverse transcription–polymerase chain reaction reactions were performed with SuperScript III (Invitrogen) according to the manufacturer’s instructions. Primer sequences and amplification conditions are described as follows: atg6 (forward, 5'-CTCAGCCCTTGGGACATACA-3'), (reverse, 5'-AGGGTATGACGCTGTCATCG-3'), (reverse, 5'-AGGGAAGGUGGCCAGCAGA-3'), (reverse, 5'-UGGCAACACCCCUUUGCCG-3'), (reverse, 5'-CAAGACCTCTCAAATTCAGC-3'), (reverse, 5'-GAAGCAGAAGGCCCACGAT-3') and (reverse, 5'-GAGGGCCACCCCATGGCAG-3').

Phase-contrast and fluorescence microscopy
Cells were seeded 60 mm dish and 24 h later, cells were transfected with MFG-H-rasV12 or MFG control and dimethyl sulfoxide or inhibitors are added. And 48 h after, cells were imaged by phase-contrast microscopy. (Leica Microsystems, Bannockburn, IL). To visualize acidic vacuole in H-rasV12-overexpressed cells or control cells at 48 h after infection, cells were stained with 50 nM LysoTracker-Green DND-26 (L-7526, Molecular Probe) fluorescence dye for 10 min and washed with phosphate-buffered saline and then changed to culture media. Cells were imaged by fluorescence microscopy (Leica Microsystems).

Statistical analysis
All experimental data are reported as mean and the error bars represent the experimental standard error. Statistical analysis was performed by the non-parametric Student’s t-test.

Results
Oncogenic Ras induces caspase-independent cell death in normal fibroblasts
To determine whether oncogenic Ras might induce cell death in normal fibroblasts, we infected cells with oncogenic H-ras (H-rasV12) using a retroviral expression vector and analyzed cell death by flow cytometric analysis. Infection of three different normal fibroblasts, Rat2, NIH3T3 and WI38 with H-rasV12 caused notable levels of cell death after 72 h (Figure 1A). Approximately 40% of Rat2 cells underwent cell death by 72 h in response to H-rasV12 expression. Moreover, the caspase inhibitors were unable to attenuate oncogenic H-ras-induced cell death (Figure 1A). Furthermore, western blot analysis failed to reveal cleavage of pro-caspases to their active counterparts or appearance of the caspase substrate, poly adenosine diphosphate-ribose polymerase, at any time after transfection (Figure 1B). Thus, it appears that oncogenic H-ras-induced cell death in normal fibroblasts occurs in a caspase-independent manner. On the other hand, overexpression of oncogenic H-ras was associated with massive vacuole formation in all three different normal fibroblasts (Figure 1C). A time-course light microscopy study revealed that vacuolated cells accumulated at 24 and 48 h before cell death in response to oncogenic H-ras. At 48 h after infection, >50% of cells contained at least one vacuole filling most of the cellular compartment, followed by a marked reduction in vacuolated cells at 72 h, consistent with caspase-independent cell death.

Autophagic vacuole formation is related to oncogenic H-ras-induced caspase-dependent cell death
To determine whether vacuolation in oncogenic H-ras-infected cells was associated with autophagy, we first performed LysoTracker-Green staining. As shown in Figure 2A, oncogenic H-ras induced extensive LysoTracker-positive acidic vacuole formation in normal fibroblasts 48 h after infection (Figure 2A). Moreover, transfection of cells with green fluorescent protein (GFP)-tagged LC3 showed that punctuate GFP-LC3 positive cells were increased by oncogenic H-ras (Figure 2B). Oncogenic H-ras also induced increase in LC3-II, a pro-form of LC3. A time-course light microscopy study revealed that GFP-LC3 positive cells containing at least one vacuole filling most of the cellular compartment underwent cell death by 72 h in response to oncogenic H-ras (Figure 2A). Moreover, western blot analysis revealed cell death by 72 h in response to oncogenic H-ras (Figure 2A). Furthermore, the caspase inhibitors were unable to attenuate oncogenic H-ras-induced cell death (Figure 1A). Thus, it appears that oncogenic H-ras-induced cell death in normal fibroblasts occurs in a caspase-independent manner. On the other hand, overexpression of oncogenic H-ras was associated with massive vacuole formation in all three different normal fibroblasts (Figure 1C). A time-course light microscopy study revealed that vacuolated cells accumulated at 24 and 48 h before cell death in response to oncogenic H-ras. At 48 h after infection, >50% of cells contained at least one vacuole filling most of the cellular compartment, followed by a marked reduction in vacuolated cells at 72 h, consistent with caspase-independent cell death.
completely blocked oncogenic H-ras-induced autophagy (Figure 2F and supplementary Figure S1A is available at Carcinogenesis Online) and cell death (Figure 2G and supplementary Figure S1B is available at Carcinogenesis Online) in three different fibroblasts. These results indicate that overexpression of oncogenic H-ras induces substantial autophagy, which is related to caspase-independent cell death.

Atg5 is involved in autophagy and caspase-independent cell death triggered by oncogenic Ras

Expression patterns of autophagy-related (Atg) genes under specific conditions provide key information about the autophagic process (13,16,17). The transcriptional inhibitor, actinomycin D, and protein synthesis inhibitor, cycloheximide, significantly attenuated oncogenic H-ras-induced autophagy and subsequent cell death (Figure 3A), indicating a requirement for de novo protein synthesis. Consequently, we examined alterations in the expression patterns of key Atg proteins in response to oncogenic H-ras and analyzed whether changes in Atg expression contributed to autophagy and subsequent cell death. As shown in Figure 3B, cells expressing oncogenic H-ras displayed a selective increase in Atg5 messenger RNA and protein levels. Moreover, siRNA targeting of Atg5 effectively attenuated oncogenic H-ras-induced autophagy (Figure 3C and supplementary Figure S2A is available at Carcinogenesis Online) as well as cell death (Figure 3D and supplementary Figure S2B is available at Carcinogenesis Online) in normal fibroblasts. The data strongly suggest that upregulation of Atg5 expression is required for autophagic vacuole formation and non-apoptotic cell death induced by oncogenic H-ras.

JNK activation is critical for Atg5 upregulation, autophagy and subsequent cell death in response to oncogenic H-ras

MAPK is an essential downstream effector of Ras and mediates Ras-induced diverse cellular events (23). To investigate the potential involvement of MAPK in oncogenic H-ras-induced autophagy and cell death (24), we initially analyzed the activation status of ERK, JNK and p38 MAPK by immunoblotting with antibodies specific for the phosphorylated forms of these kinases. As expected, infection of cells with oncogenic H-ras resulted in a dramatic increase in the phosphorylated forms of all three MAPKs, implying activation in response to H-ras (Figure 4A). To further ascertain whether active MAPK was required for autophagy and subsequent cell death, cells were pretreated with a specific chemical inhibitor or siRNA, prior to infection with oncogenic H-ras. Inhibition of JNK with SP600125 or specific siRNA prevented autophagy (Figure 4B) and cell death (Figure 4C) induction by oncogenic H-ras. Inhibition of JNK also clearly attenuated oncogenic H-ras-induced upregulation of Atg5 messenger RNA and protein expression (Figure 4D). However, pretreatment with a chemical inhibitor or siRNA-targeting ERK or p38 MAPK did not affect autophagy, cell death and Atg5 expression. Based on these findings, we propose that JNK activation acts as a signal for upregulation of Atg5, autophagy and subsequent non-apoptotic cell death in response to oncogenic H-ras expression. We additionally showed that...
c-Jun is activated by JNK in response to oncogenic H-ras expression. The phosphorylated c-Jun level was markedly increased following oncogenic H-ras infection and suppressed upon pretreatment with SP600125, a specific inhibitor of JNK (supplementary Figure S3A is available at Carcinogenesis Online). Moreover, siRNA-targeting c-Jun clearly inhibited oncogenic H-ras-induced Atg5 messenger RNA and protein expression (supplementary Figure S3B is available at Carcinogenesis Online), autophagy on (supplementary Figure S3C

Fig. 2. Autophagic vacuole formation is required for oncogenic H-ras-induced caspase-independent cell death. Rat2 cells were infected with MFG-control or MFG-H-rasV12. (A) At 24 and 48 h after infection, cells were stained with LysoTracker-Green and imaged by fluorescence microscopy. Results from three independent experiments are presented as means ± SEMs. (B) At 48 h after infection, cells were stained with GFP-LC3 to identify autophagosome and imaged by fluorescence microscopy. Percentage of autophagic cells with punctate GFP-LC3 fluorescence was calculated relative to all GFP positive cells. Results from three independent experiments are presented as means ± SEMs. (C) At the indicated times, cell lysates were subjected to immunoblot analysis with anti-LC3 antibody. β-Actin was used as a loading control. (D) At 48 h after infection, cells were stained with GFP-LC3 (green) and lysosomal associated membrane protein (LAMP)-1 (red) to identify autophagosome. (E) Transmission electron micrograph of Rat2 cells infected with MFG-control or MFG-H-rasV12 at 48 h. Arrows depict autophagosomes in cells containing recognizable cellular materials. (F) Cells were pretreated with dimethyl sulfoxide, Bafilomycin A1 (1 nM) or 3-MA (1 mM) and then infected with MFG-control or MFG-H-rasV12. After 48 h, cells were stained with LysoTracker-Green. Percentage of vacuolated cells was calculated under fluorescence microscope. Results from three independent experiments are presented as means ± SEMs; *P < 0.001. (G) Cells were harvested at 72 h after infection and stained with propidium iodide (PI). Cell death was measured as the percentage of PI-positive cells using flow cytometric analysis. Results from three independent experiments are presented as means ± SEMs; *P < 0.001.
MKK7 is an upstream regulator of JNK-mediated Atg5 and autophagic cell death

The JNK-activating kinases, MKK4 and MKK7, are differentially required for JNK activity in response to extracellular stimuli (25–28). To explore any requirement for MKK4 or MKK7 in JNK-mediated autophagic cell death in response to oncogenic H-ras, we used dominant-negative forms of the proteins. Interestingly, phosphorylated MKK7, but not phosphorylated MKK4, was increased in cells expressing oncogenic H-ras, signifying activation of MKK7 (Figure 5A). Ectopic expression of dominant-negative MKK7 effectively prevented JNK and c-Jun activation (Figure 5B) and upregulation of Atg5 (Figure 5C). Moreover, inhibition of MKK7 attenuated autophagy (Figure 5D) and cell death (Figure 5E), whereas inhibition of MKK4 via use of a dominant-negative form of MKK4 had no significant effects. These results suggest that MKK7 selectively acts as an upstream regulator of the JNK-mediated Atg5 induction pathway in oncogenic H-ras-stimulated autophagic cell death.
Fig. 4. Activation of JNK is critically required for the Atg5 upregulation, autophagy induction and subsequent cell death in response to oncogenic H-ras. (A) Rat2 cells were infected with MFG or MFG-H-rasV12 and harvested after indicated time. Cell lysates were subjected to immunoblot analysis with indicated antibodies. β-Actin was used as a loading control. The data represent a typical experiment conducted three times with similar results. (B) (Upper) cells were pretreated with SP600125 (10 μM), SB203580 (10 μM) or PD98059 (25 μM) and infected with MFG-control or MFG-H-rasV12. After 48 h, cells were stained with LysoTracker-Green. Percentage of vacuolated cells was calculated under fluorescence microscopy. Significantly different from control; *P < 0.01. (Lower) cells were transfected with or JNK1, p38 MAPK, ERK2 siRNA and then infected with MFG-control or MFG-H-rasV12. After 48 h, cells were stained with LysoTracker-Green. Percentage of vacuolated cells was calculated under fluorescence microscopy. Significantly different from control; *P < 0.01. (Lower right) after 48 h, cell lysates were subjected to immunoblot analysis with indicated antibodies. β-Actin was used as a loading control. (C) At 72 h after infection, cell death was measured as the percentage of propidium iodide-positive cells using flow cytometric analysis. Results from three independent experiments are presented as means ± SEMs. Significantly different from control; (upper) *P < 0.005, (lower) *P < 0.01. (D) Cellular messenger RNA was isolated 24 h after MFG or MFG-H-rasV12 infection with MAPK inhibitors. (Upper) messenger RNA was examined by reverse transcription–polymerase chain reaction toward atg5, gapdh served as an internal standard. And total cell lysates were subjected to immunoblot analysis with anti-Atg5 antibody. β-Actin was used as a loading control. The data represent a typical experiment conducted three times with similar results. (Lower) relative messenger RNA expression was measured by Bio-Rad Gel doc software. Results from three independent experiments are presented as means ± SEMs significantly different from control; *P < 0.01.
However, the molecular mechanism underlying this oncogene-induced cellular disposal process remains unclear. Here, we show that oncogenic H-ras induces autophagy and subsequent caspase-independent cell death through upregulation of Atg5, a key autophagy protein, in normal fibroblasts and that activation of Rac1/MKK7–JNK–c-Jun-signaling pathway is necessary for Atg5 induction. Although oncoproteins of the Ras family are perhaps best known for their role in initiating cell proliferation and suppressing apoptosis, oncogenic Ras can, under certain conditions, initiate cell death (4,6,30,31). In this study, we provided further evidence that overexpression of oncogenic H-ras triggers cell death in normal cells through a caspase-independent mechanism. We also found that oncogenic H-ras leads to massive autophagic vacuole formation. Recent studies show that certain forms of cell death are prevented either with autophagic inhibitors or by reduced Atg gene expression, implying that autophagy initiates and participates directly in the death process (13,16,17). Consistent with these findings, our results show that chemical inhibitors of autophagy completely blocked oncogenic H-ras-induced cell death, suggesting that oncogenic H-ras induces autophagic cell death in normal fibroblasts. Recently, it has been also shown that autophagy is involved in cellular senescence, another tumor suppressor mechanism, induced by oncogenic Ras (30). However, in the current study, we found no evidence for the induction of senescence in response to oncogenic H-ras in Rat2 normal fibroblasts (data not shown). We further observed selective Atg5 expression in cells overexpressing oncogenic H-ras. siRNA targeting of Atg5 clearly attenuated autophagy and cell death in response to oncogenic H-ras, signifying that upregulation of Atg5 is a critical event for oncogenic Ras-induced autophagy and cell death. These results are in agreement with recent studies showing that JNK is actively involved in autophagy induced by a variety of stimuli, including endoplasmic reticulum stress (35), caspase inhibition (36), insulin-like growth factor-1 treatment and exposure to tumor necrosis factor-alpha (37). Evidence for extensive cooperation and cross talk between guanosine triphosphatases and other signaling pathways is well documented. Rac1 is one of the well-characterized small guanosine...
These results suggest that Rac1 is essentially involved in activation of MKK7–JNK-signaling pathway that induces Atg5 upregulation and autophagic cell death in response to oncogenic H-ras.

Our results collectively demonstrate that upregulation of Atg5 expression is necessary for autophagic vacuole formation and caspase-independent cell death in response to oncogenic Ras in normal fibroblasts and that activation of Rac1/MKK7/JNK/c-Jun-signaling pathway is critically required for Atg5 upregulation. Our findings suggest that in cells acquiring deregulated oncogene expression, oncogenic stress triggers autophagic cell death, which protects against malignant progression.

Supplementary material

Supplementary Figures S1–S3 can be found at http://carcin.oxfordjournals.org/

Funding

Korea Science and Engineering Foundation; National Nuclear Technology Program (2009-0081812); Ministry of Education, Science and Technology, Korean government; Basic Research Program (R01-2005-10510-0); Ministry of Education, Science and Technology, Korean government.

Acknowledgements

Conflict of Interest Statement: None declared.

References


Fig. 6. Rac1 is involved in MKK7/JNK-mediated Atg induction and autophagic cell death. (A) Rat2 cells were infected with MFG or MFG-H-rasV12 in the presence or absence of RacN17 or Rac1-siRNA. After 48 h, cell lysates were subjected to immunoblot analysis with indicated antibodies. β-Actin was used as a loading control. The data represent a typical experiment conducted three times with similar results. (B) After 48 h, cells were stained with LysoTracker-Green. Percentage of vacuolated cells was calculated under fluorescence microscopy. Significantly different from control; *P < 0.01. (C) Cells were harvested at 72 h after infection and stained with propidium iodide (PI). Cell death was measured as the percentage of PI-positive cells using flow cytometric analysis. Results from three independent experiments are presented as means ± SEMs significantly different from control; *P < 0.005.

Received May 14, 2009; revised August 23, 2009; accepted September 19, 2009.