Pivotal Role of the Cyclin-dependent Kinase Inhibitor p21WAF1/CIP1 in Apoptosis and Autophagy*

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Programmed cell death (PCD) is involved in a variety of biologic events. Based on the morphologic appearance of the cells, there are two types of PCD as follows: apoptotic (type I) and autophagic (type II). However, the molecular machinery that determines the type of PCD is poorly defined. The purpose of this study was to show whether the presence of the cyclin-dependent kinase (CDK) inhibitor p21WAF1/CIP1, a modulator of apoptosis, determines which type of PCD the cell undergoes. Treatment with C2-ceramide was associated with both the cleavage of caspase-3 and poly(ADP-ribose) polymerase and the degradation of autophagy-related Beclin 1 and Atg5 proteins, without a change in the cyclin-CDK activity, which culminated in apoptosis in p21WAF1/CIP1−/− MEFs. On the other hand, C2-ceramide did not cleave caspase-3 or poly(ADP-ribose) polymerase and kept Beclin 1 and Atg5 proteins stable in p21WAF1/CIP1−/− MEFs, events that this time culminated in autophagy. When expression of the p21 protein was inhibited by small interfering RNA or when the overexpression of Beclin 1 or Atg5 was induced, autophagy rather than apoptosis was initiated in the p21+/+ MEFs treated with C2-ceramide. In contrast, the exogenous expression of p21 or the silencing of Beclin 1 and Atg5 with small interfering RNA increased the number of apoptotic cells and decreased the number of autophagic cells among C2-ceramide-treated p21−/− MEFs. γ-Irradiation, which endogenously generates ceramide, induced a similar tendency in these MEFs. These results suggest that p21 plays an essential role in determining the type of cell death, positively for apoptosis and negatively for autophagy.

*p21WAF1/CIP1, which was originally identified as a universal inhibitor of cyclin-dependent kinases (CDKs) that belongs to the CIP/KIP family of CDK inhibitors (14–17). Recently, accumulating evidence has indicated that p21 has functions in addition to CDK inhibition (18, 19). In particular, p21 positively or negatively regulates the apoptotic signaling pathway that ultimately determines cell death (20). However, the association between p21 and autophagy is not understood. Therefore, in this study, we assessed the p21-dependent kinase; MEF, mouse embryonic fibroblast; PARP, poly(ADP-ribose) polymerase; siRNA, small interfering RNA; RT, reverse transcription; Z, benzoxyloxyarbonyl; FMK, fluoromethyl ketone; FACS, fluorescence-activated cell sorter; DMEM, Dulbecco’s modified Eagle’s medium; AVO, acidic vesicular organelle; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gy, gray; GFP, green fluorescent protein; LC3, light chain 3.
role of p21 in inducing apoptosis and autophagy using p21\(^{+/+}\) and p21\(^{-/-}\) mouse embryonic fibroblasts (MEFs). C\(_2\)-Ceramide, a cell-permeable ceramide analogue that induces apoptosis or autophagy in some types of cells (21–25), was used to induce cell death.

**MATERIALS AND METHODS**

**Cell Lines**—Two types of MEFs, p21\(^{+/+}\) and p21\(^{-/-}\), were kindly provided by Dr. James M. Roberts (Fred Hutchinson Cancer Research Center, Seattle, WA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen), 4 mM glutamine, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin at 37 °C in a 5% CO\(_2\) incubator.

**Reagents**—C\(_2\)-ceramide (\(N\)-acetyl-\(N\)-erythrospHINGosine) (Calbiochem) was prepared as a 50 mM stock solution in dimethyl sulfoxide (Sigma). Benzyloxy carbonyl-valyl-alanyl-aspartic acid (O-methyl)-fluoromethyl ketone (Z-VAD-FMK) was purchased from Promega (Madison, WI). DNA-binding Hoechst 33258 and acridine orange were purchased from Sigma and Polysciences (Warrington, PA). Anti-CDK2 and -CDK4 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Viability Assay**—The effect of C\(_2\)-ceramide on the cell viability of MEFs was determined by using a WST-1 reagent (Roche Applied Science), as described previously (26). MEFs (2 \(\times\) 10\(^4\) cells/well) were seeded in 96-well flat-bottomed plates. After 24 h, cells were fed with culture medium containing 0.1% fetal bovine serum and treated with C\(_2\)-ceramide at concentrations ranging from 0 to 100 \(\mu\mM\) for 24 h, as described previously (23). The cells were then exposed to 10 \(\mu\)l of WST-1 reagent for 1 h at 37 °C. Absorbance at 450 nm was measured in a microplate reader. The viability of cells treated with vehicle alone was considered to be 100%.

**Cell Cycle Analysis**—For the cell cycle analysis, MEFs treated with or without C\(_2\)-ceramide as described above were trypsinized, fixed with 70% ethanol, and stained with propidium iodide by using the cellular DNA flow cytometric analysis reagent set (Roche Applied Science), as described previously (23). Samples were analyzed for DNA content with the FACScan using CellQuest software (BD Biosciences).

**Detection of Apoptosis**—Nuclei were stained with Hoechst 33258 to detect the chromatin condensation or nuclear fragmentation characteristic of apoptosis, as described previously (23). MEFs treated with or without C\(_2\)-ceramide, as described above, were fixed with 4% paraformaldehyde and stained with Hoechst 33258 (1 \(\mu\)g/ml) for 15 min. Cells were counted and scored for the incidence of apoptotic chromatin changes under a fluorescence microscope.

**Electron Microscopy**—MEFs, grown on gelatinized plastic coverslips, were treated with or without C\(_2\)-ceramide, as described above, fixed for 2 h with 2.5% glutaraldehyde (EM Science, Hatfield, PA) in 0.1 M cacodylate buffer, pH 7.4, postfixed in 1% OsO\(_4\) in the same buffer, and then subjected to electron microscopic analysis as described previously (23). Representative areas were chosen for ultra-thin sectioning and viewed with a Hitachi 7600 electron microscope.

**Quantification of Acidic Vesicular Organelles (AVOs) with Acridine Orange Staining**—Autophagy is the process of sequestering cytoplasmic proteins into the lytic component and is characterized by the development of AVOs. To detect and quantify AVOs in C\(_2\)-ceramide-treated MEFs or MEFs at 2 h after amino acid deprivation with Earle’s balanced salt solution (Invitrogen) as described previously (27), we performed the vital staining with acridine orange, followed by FACScan analysis, as described previously (23).

**Assessment of the Involvement of Microtubule-associated Prote1 Light Chain 3 (LC3)—LC3, a mammalian homologue of yeast Atg8, is recruited to the autophagosome membrane during autophagy (28), which makes LC3 a marker for autophagy. In an earlier study, cells transfected with the expression vector of the green fluorescent protein-linked LC3 (GFP-LC3) showed a diffused distribution of GFP-LC3 under control conditions, whereas a punctate pattern of GFP-LC3 expression (GFP-LC3 dots) was induced (29). Therefore, we transiently transfected MEFs with the GFP-LC3 expression vector using the FuGENE6 transfection reagent (Roche Applied Science) for 24 h, treated them with or without C\(_2\)-ceramide, fixed the cells with 4% paraformaldehyde, and determined the proportion of cells expressing GFP-LC3 dots (\(\geq 10\) dots/cell) under a fluorescence microscope, as described previously (23). The GFP-LC3 vector was kindly supplied by Dr. Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan).

**Western Blotting**—MEFs treated with or without C\(_2\)-ceramide, as described above, were lysed using lysis buffer (10 mM Tris-HCl, pH 7.8, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA) for 10 min and centrifuged (15,000 \(\times\) g, 20 min) at 4 °C, as described previously (23). Equal amounts of protein (40 \(\mu\)g) from the supernatant were separated by 10 or 15% SDS-PAGE. Membranes were incubated with each primary antibody and horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). The bound antibody complex was detected by an enhanced chemiluminescence (ECL) or ECL-Plus reagent (Amersham Biosciences). The antibodies used were anti-p21 (Santa Cruz Biotechnology), anti-PARP, and caspase-3 (Cell Signaling, Beverly, MA), anti-Beclin 1 (Santa Cruz Biotechnology), and anti-Atg5 antibodies (kindly supplied by Dr. N. Mizushima). For a loading control, the membranes were reprobed with \(\beta\)-actin antibody (Sigma).

**Reverse Transcription (RT)-PCR**—Two micrograms of total RNA was isolated from cells using the RNeasy mini kit (Qiagen) and subjected to reverse transcription using the ThermoscriptTM RT-PCR system (Invitrogen) according to the manufacturer’s instructions. Random hexamer was used in the RT reactions. The primer kit for Beclin 1 was purchased from SuperArray (Frederick, MD). The primers for GAPDH were 5′-CCTGGAGAAACTGCCAAATG-3′ and 5′-AGATGGTGGGTGTTGGAAG-3′ (Sigma). The amplification program for Beclin 1 was 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C for 40 cycles after denaturing at 95 °C for 4 min, and for GAPDH it was 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C for 30 cycles after denaturing at 95 °C for 4 min, as described previously (30). These cycles were followed by a 10-min elongation step at 72 °C. The PCR products were analyzed by 2% agarose gel electrophoresis. The level of Beclin 1 mRNA (normalized to
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**Figure 1. Induction of apoptosis in p21−/− MEFs but not in p21+/− MEFs by C2-ceramide.** A, reduced cell viability of p21−/− and p21+/− MEFs treated with C2-ceramide. After exposure of the cells to C2-ceramide for 24 h, cell viability was measured by the WST-1 assay. Values shown are means ± S.D. *, p < 0.02. B, increase in sub-G1 phase of p21−/− MEFs but not p21+/− MEFs treated with C2-ceramide. After exposure of the cells to 25 μM C2-ceramide for 24 h, cell cycle analysis was performed. C, induction of apoptosis in p21−/− MEFs but not p21+/− MEFs treated with C2-ceramide. After exposure of the cells to 25 μM C2-ceramide for 24 h, nuclei were stained with Hoechst 33258 to determine the percentage of apoptotic cells. Values shown are means ± S.D. *p < 0.0005. D, cleavage of caspase-3 and PARP in p21−/− MEFs but not p21+/− MEFs treated with C2-ceramide. After exposure of the cells to 25 μM C2-ceramide for 3, 6, or 24 h, immunoblotting for caspase-3, PARP, and β-actin (control) was performed. E, increased cell viability produced by a pan-caspase inhibitor, Z-VAD-FMK, in p21−/− MEFs but not p21+/− MEFs treated with C2-ceramide. After exposure of the cells to 20 μM Z-VAD-FMK for 24 h, cell viability was measured by the WST-1 assay. Values shown are means ± S.D. F, no development of AVOs in p21−/− MEFs treated with C2-ceramide and Z-VAD-FMK. After exposure of the cells to 25 μM C2-ceramide for 24 h in the presence of 10 or 20 μM Z-VAD-FMK, development of AVOs was detected by FACES analysis in acridine orange-stained MEFs. FL1-H, green fluorescence intensity; FL3-H, red fluorescence.

GAPDH (Scion Corp., Frederick, MD) was quantified by using Scion imaging software (Scion Corp., Frederick, MD) and averaged over three experiments.

**Immunoprecipitation**—MEFs were treated with or without 25 μM C2-ceramide for 1 h. Proteins were extracted using lysis buffer (10 mM Tris-HCl, pH 7.8, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 0.035 mg/ml aprotinin). Equal amounts of protein (200 μg) were immunoprecipitated with 1 μg of anti-Beclin 1 antibody or anti-p21 antibody at 4 °C overnight, and the immunoprecipitated protein was pulled down with protein A-agarose beads (Roche Applied Science) and separated by 15% SDS-PAGE. Membranes were probed with anti-CDK2 and -CDK4 antibodies.

**Plasmid**—The p21 expression vector (14) was kindly provided by Dr. Bert Vogelstein (The Johns Hopkins Oncology Center, Baltimore). The Beclin 1 expression vector (31) was kindly provided by Dr. Beth Levine (University of Texas Southwestern Medical Center, Dallas). The Atg5 expression vector was used as described previously (32). As a control, a β-galactosidase expression vector (Invitrogen) was used. The FuGENE 6 transfection reagent was used to transfect cells with these plasmids 24 or 48 h before C2-ceramide treatment.

**Small Interfering RNA (siRNA)**—To inhibit specifically the expression of p21, Beclin 1, or Atg5 protein, MEFs were transfected with siRNA directed against each target gene. All siRNAs were purchased from Dharmacon (Lafayette, CO). The sequences of siRNAs were as follows (numbers in parentheses indicate nucleotide positions within the respective open reading frame): mouse p21 siRNA, 5'-AGACCAGCCUCAGAAGUUU-
3’ (515–533); mouse Beclin 1 siRNA, 5’-GGACAGUUUGGC-ACAAUA-3’ (951–969); and mouse Atg5 siRNA, 5’-ACCGGAAACUAGGAUA-3’ (506–524). Nontargeting siRNA (Dharmacon) was used as a control. These siRNAs (100 nM) were transfected with DharmaFECT3 (Dharmacon) for 48 or 72 h.

Ionizing Radiation—Radiation treatment was administered using a cesium 137 irradiator (model E-0103; U. S. Nuclear Corp., Burbank, CA) at a dose rate of 3.216 Gy/min, as described previously (33).

Statistical Analysis—All the experiments were repeated at least three times. Values are expressed as means ± S.D. Statistical analysis was performed by using Student’s t test (two-tailed). The criterion for statistical significance was p < 0.05.

RESULTS

Inhibitory Effect of C2-ceramide on the Cell Viability of p21<sup>+/+</sup> and p21<sup>−/−</sup> MEFs—p21<sup>+/+</sup> and p21<sup>−/−</sup> MEFs were treated with C2-ceramide at concentrations of 0, 5, 10, 25, 50, and 100 μM in DMEM with 0.1% fetal bovine serum for 24 h, and then cell viability was determined. Treatment with C2-ceramide reduced the viability of p21<sup>+/+</sup> and p21<sup>−/−</sup> MEFs in a dose-dependent manner (Fig. 1A). We found that p21<sup>+/−</sup> MEFs were more resistant to C2-ceramide at concentrations of 10, 25, and 50 μM than were p21<sup>+/+</sup> MEFs (p < 0.02). Because treatment with 25 μM C2-ceramide for 24 h reduced the viability of both types of MEF to 40–60% of the control cells treated with vehicle alone for 24 h, we used this concentration of C2-ceramide for the following experiments.

Induction of Apoptosis by C2-ceramide in p21<sup>+/+</sup> MEFs but Not in p21<sup>−/−</sup> MEFs—To determine the type of cell death caused by C2-ceramide, we first performed apoptosis detection assays using cell cycle analysis and Hoechst 33258 DNA staining. The DNA flow cytometric analysis showed that treatment with C2-ceramide for 24 h increased the proportion of the p21<sup>+/+</sup> MEFs in the sub-G<sub>1</sub> phase from 5.3–23.3%, which is characteristic of apoptosis; in contrast, there was no significant increase in the percentage of p21<sup>−/−</sup> MEFs (Fig. 1B). Nuclei of MEFs treated with or without C2-ceramide were then stained with Hoechst 33258 to determine the percentage of cells with apoptotic morphology, such as chromatin condensation or nuclear fragmentation. The percentages of p21<sup>+/+</sup> MEFs showing apoptosis significantly increased 24 h after exposure to C2-ceramide (p < 0.0005), whereas there was no significant increase in the percentages of p21<sup>−/−</sup> MEFs showing apoptosis in response to C2-ceramide (Fig. 1C). Both caspase-3 and PARP were cleaved in p21<sup>+/+</sup> MEFs 6 and 24 h after C2-ceramide treatment, but these cleavages were undetected in treated p21<sup>−/−</sup> MEFs (Fig. 1D). Interestingly, C2-ceramide inhibited the expression of PARP to an undetectable level in p21<sup>−/−</sup> MEFs 6 h after the treatment but did not affect the expression level of caspase-3. The viability of p21<sup>+/+</sup> MEFs inhibited by C2-ceramide treatment was restored in a dose-dependent manner by Z-VAD-FMK, a caspase inhibitor with broad specificity (Fig. 1E), confirming the role of caspases in C2-ceramide-induced apoptotic cell death in p21<sup>+/+</sup> MEFs. Protection of Z-VAD-FMK was incomplete in cells treated with 25 μM C2-ceramide, suggesting that Z-VAD-FMK leads to death by autophagy under these conditions. However, Z-VAD-FMK did not induce autophagy in p21<sup>+/+</sup> MEFs (Fig. 1F), which therefore excluded this possibility. These results indicate that C2-ceramide cleaves caspase-3 and then PARP, thereby inducing apoptosis in p21<sup>+/+</sup> MEFs, whereas no significant apoptosis was detectable in p21<sup>−/−</sup> MEFs treated with C2-ceramide.

Induction of Autophagy by C2-ceramide in p21<sup>−/−</sup> MEFs but Not in p21<sup>+/+</sup> MEFs—Recent investigations have demonstrated that C2-ceramide triggers nonapoptotic cell death with autophagic features in some types of cells (22–25). Therefore, we analyzed the ultrastructure of p21<sup>+/+</sup> and p21<sup>−/−</sup> MEFs treated with C2-ceramide with electron microscopy. In p21<sup>−/−</sup> MEFs, no autophagic features were observed in p21<sup>−/−</sup> MEFs. Representative micrographs showing the ultrastructure of MEFs are shown. Bars, 1.0 μm. A, electron micrographs showing the ultrastructure of MEFs treated with C2-ceramide. After exposure of the cells to 25 μM C2-ceramide for 24 h, nuclear fragmentation was observed in p21<sup>+/+</sup> MEFs, whereas numerous autophagic vacuoles were observed in p21<sup>−/−</sup> MEFs. B, effect of C2-ceramide on the morphology of p21<sup>+/+</sup> and p21<sup>−/−</sup> MEFs. Representative photographs of p21<sup>+/+</sup> and p21<sup>−/−</sup> MEFs treated with or without 25 μM C2-ceramide for 0, 6, or 24 h and examined by phase-contrast microscopy are shown. Bars, 50 μm. C, development of AVOS in p21<sup>−/−</sup> MEFs but not in p21<sup>+/+</sup> MEFs treated with C2-ceramide. After exposure of the cells to 25 μM C2-ceramide for 24 h, development of AVOS was detected by FACS analysis (FL1-H, green fluorescence intensity; FL3-H, red fluorescence). D, and E, punctate GFP-LC3 fluorescence (GFP-LC3 dots) in p21<sup>−/−</sup> MEFs but not in p21<sup>+/+</sup> MEFs treated with C2-ceramide. MEFs transfected with GFP-LC3 were treated with or without 25 μM C2-ceramide for 24 h. Representative photographs of p21<sup>+/+</sup> and p21<sup>−/−</sup> MEFs. Bars, 20 μm. F, percentage of cells with GFP-LC3 dots was calculated relative to all GFP-positive cells. Values shown are means ± S.D., *p < 0.001. F, induction of autophagy by amino acid deprivation in both p21<sup>+/+</sup> and p21<sup>−/−</sup> MEFs. After culture with Earle’s balanced salt solution for 2 h, development of AVOS was detected by FACS analysis (FL1-H, green fluorescence intensity; FL3-H, red fluorescence).
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![Image](49x478 to 408x733)

FIGURE 3. Effect of silencing p21 on C2-ceramide-induced apoptosis in p21+/+ MEFs. A, expression of p21 protein in p21+/+ MEFs transfected with p21 siRNA. p21+/+ MEFs were transfected with 100 nm control siRNA or p21 siRNA for 72 h, and expressions of p21 and β-actin (control) were analyzed by immunoblotting. B, reduced number of apoptotic cells among C2-ceramide-treated p21+/+ MEFs after transfection with p21 siRNA. After transfection with 100 nm control siRNA or p21 siRNA for 24 h, p21+/+ MEFs were treated with or without 25 μM C2-ceramide for 24 h, followed by Hoechst 33258 staining. Values shown are means ± S.D., *p < 0.02. C, increased autophagic cells among C2-ceramide-treated p21+/+ MEFs after transfection with p21 siRNA. After transfection with GFP-LC3 for 24 h, p21+/+ MEFs were transfected with 100 nm control siRNA or p21 siRNA for 24 h and treated with or without 25 μM C2-ceramide for 24 h. Values shown are means ± S.D., *p < 0.02. D, no effect of siRNA p21 on cytotoxicity of C2-ceramide in p21+/+ MEFs. After transfection with 100 nm control siRNA or p21 siRNA for 24 h, p21+/+ MEFs were treated with or without 25 μM C2-ceramide for 24 h and cell viability was measured by the WST-1 assay.

Interestingly, however, p21+/+ and p21−/− MEFs showed a similar potential to stimulate autophagy in response to the classic autophagic inducer, amino acid deprivation (Fig. 2F). These results suggest that C2-ceramide induces apoptosis or autophagy in MEFs, depending on the presence or absence of p21, respectively.

Effect of Silencing p21 on C2-ceramide-induced Apoptosis in p21+/+ MEFs—On the basis of the above data, we speculated that p21 plays a key role in inducing apoptosis instead of autophagy in MEFs after exposure to C2-ceramide. To test our hypothesis, we first determined whether the inhibition of p21 by siRNA affects the induction of apoptosis in C2-ceramide-treated p21+/+ MEFs. Expression of p21 protein was remarkably suppressed by transfection with 100 nm siRNA directed against p21 for 72 h, but not by transfection with 100 nm nontargeting (control) siRNA (Fig. 3A). After siRNA transfection, p21+/+ MEFs were exposed to 25 μM C2-ceramide for an additional 24 h. As shown in Fig. 3, B and C, compared with control siRNA transfection, p21 knockdown significantly decreased the percentage of apoptotic cells (p < 0.02) and increased the percentage of autophagic cells expressing GFP-LC3 dots (p < 0.02) after a 24-h treatment with 25 μM C2-ceramide. These results indicate that inhibition of the p21 protein suppresses the ability of p21+/+ MEFs to undergo apoptosis and increases their sensitivity to apoptosis caused by C2-ceramide.

Effect of Exogenous Expression of p21 on C2-ceramide-induced Autophagy in p21−/− MEFs—As a next step in testing our hypothesis about the role of p21 in PCD, we attempted to induce p21 protein expression in p21−/− MEFs. Immunoblotting revealed the exogenous expression of p21 in p21−/− MEFs transfected with the p21 expression vector, but not with the β-galactosidase construct (Fig. 4A). To determine whether p21 expression affects the induction of apoptosis or autophagy in p21−/− MEFs treated with C2-ceramide, the cells were co-transfected with GFP-LC3 and p21 or β-galactosidase expression vectors, followed by exposure to 25 μM C2-ceramide for 24 h and Hoechst 33258 staining. As shown in Fig. 4B, apoptotic cells with nuclear condensation but not GFP-LC3 dots were detected in p21-transfected cells, whereas autophagic cells exhibiting GFP-LC3 dots but not an apoptotic morphology were observed in β-galactosidase-transfected cells. The exogenous expression of p21 significantly increased the percentage of apoptotic cells (p < 0.004) (Fig. 4C) and decreased the extent of autophagy in p21−/− MEFs treated with C2-cera-
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Beclin 1 protein was suppressed even 1 h after exposure to 25 μM C2-ceramide. At that time point, p21 expression remained stable. Immunoprecipitation with anti-Beclin 1 antibody followed by immunoblotting with anti-p21 antibody revealed that Beclin 1 was not directly associated with p21, when Beclin 1 was gradually degraded by the C2-ceramide. This finding was also supported by another immunoprecipitation with anti-p21 antibody and then Western blot with anti-Beclin 1 antibody (Fig. 5B). Furthermore, we determined whether change in cyclin-CDK activity was required. As shown in Fig. 5B, in p21+/+ MEFs after treatment with or without 25 μM C2-ceramide for 3 h, the expressions of CDK2 and CDK4 proteins immunoprecipitated with anti-p21 antibody were not changed. These results suggest that the induction of apoptosis in C2-ceramide-treated p21+/+ MEFs is because of inhibition of the autophagic pathway through the destabilization of autophagy-related proteins but not because of a change in the cyclin-CDK activity.

We next knocked down the expression of Beclin 1 or Atg5 in p21−/+ MEFs using siRNA (Fig. 5C) to determine whether inhibition of their expressions affected C2-ceramide-induced autophagy in p21−/+ MEFs. After siRNA transfection, we performed Hoechst 33258 staining and the GFP-LC3 dot assay. Compared with control siRNA transfection, Beclin 1 or Atg5 knockdown significantly increased the percentage of cells showing apoptosis (p < 0.002) (Fig. 5D) and suppressed the induction of autophagy in p21−/+ MEFs treated with C2-ceramide (p < 0.002) (Fig. 5E). In contrast, the expression of Beclin 1 and Atg5 proteins was down-regulated in p21+/+ MEFs during C2-ceramide treatment. Therefore, we concluded that the inhibition of Beclin 1 or Atg5 protein in p21+/+ MEFs might increase the frequency of apoptosis more than might control siRNA.

Moreover, we reintroduced Beclin 1 or Atg5 expression in p21+/+ MEFs to determine whether the restoration of these proteins affected the induction of apoptosis or autophagy triggered by C2-ceramide. Overexpression of Beclin 1 or Atg5 significantly reduced the percentage of apoptotic cells (p < 0.05) and increased the percentage of autophagic cells among the C2-ceramide-treated p21+/+ MEFs (p < 0.005) (Fig. 6, A and B). These collective results indicate that C2-ceramide-induced apoptosis requires a reduction in the expression of Beclin 1 and Atg5 proteins, whereas C2-ceramide-induced autophagy requires Beclin 1 or Atg5 protein expression.

It was also important to determine whether our findings are unique to C2-ceramide or apply to other apoptosis inducers that endogenously generate ceramide. Therefore, we treated p21+/+ MEFs and p21−/+ MEFs with γ-irradiation, which has been shown to generate the second-messenger ceramide and mediate apoptosis (21). As shown in Fig. 7A, apoptosis was induced in p21+/+ MEFs by γ-irradiation at 10 Gy more than in p21−/+ MEFs. On the other hand, γ-irradiation caused autophagy in p21−/+ MEFs more than in p21+/+ MEFs (Fig. 7B). These results suggest that our findings do apply to other apoptosis inducers.

DISCUSSION

In this study, we showed that stimulation of the cell death signal by C2-ceramide degrades the autophagy-related proteins...
FIGURE 5. Involvement of Beclin 1 and Atg5 in C2-ceramide-induced cell death. A, reduced expression of Beclin 1 and Atg5 proteins in p21+/+ but not p21−− MEFs treated with C2-ceramide. After exposure of the cells to 25 μM C2-ceramide for 3 h, the expression of Beclin 1, Atg5, and β-actin (control) was analyzed by immunoblotting. Reduced stability of Beclin 1 protein in p21+/+ MEFs treated with C2-ceramide is shown. Subconfluently growing MEFs were labeled with [35S]methionine and then chased with complete nonradioactive medium for the indicated time points. Cells were then lysed and immunoprecipitated (IP) for anti-Beclin-1 antibody. Immunocomplexes were separated by 15% SDS-PAGE. No change in the expression of Beclin 1 mRNA in p21+/+ and p21−− MEFs treated with C2-ceramide was seen. After exposure of the cells to 25 μM C2-ceramide for 3 h, the expression of Beclin 1 and GAPDH mRNA was analyzed by RT-PCR. The level of Beclin 1 mRNA (normalized to GAPDH) was quantified and averaged over three experiments. Values shown are means ± S.D. No significant change was seen. B, relation between Beclin 1 and p21 in C2-ceramide-induced cell death in p21+/+ MEFs. The expression of Beclin 1, p21, and β-actin (control) was analyzed by immunoblotting (IB) in p21+/+ MEFs treated with or without 25 μM C2-ceramide for 1 h by immunoblotting. The direct binding of Beclin 1 and p21 was analyzed by immunoprecipitation with anti-Beclin 1 antibody or anti-p21 antibody followed by immunoblotting with anti-p21 antibody or anti-Beclin 1 antibody, respectively. No change in cyclin-CDK activity was seen. The expression of CDK2 and CDK4 proteins in p21+/+ MEFs after treatment with 25 μM C2-ceramide for 3 h was analyzed by immunoprecipitation with anti-CDK2 antibody followed by immunoblotting with anti-CDK2 and anti-CDK4 antibodies. C, reduced expression of Beclin 1 or Atg5 in p21+/− MEFs transfected with Beclin 1 or Atg5 siRNA, p21+/− MEFs were transfected with 100 nM control siRNA or Beclin 1 or Atg5 siRNA for 72 h, and the expression of Beclin 1, Atg5, and β-actin (control) was analyzed by immunoblotting. D and E, increased percentage of apoptotic cells and reduced percentage of autophagic cells among C2-ceramide-treated p21−− MEFs transfected with Beclin 1 or Atg5 siRNA. D, after transfection with 100 nM control siRNA or Beclin 1 or Atg5 siRNA for 48 h, p21−− MEFs were treated with or without 25 μM C2-ceramide for 24 h and stained with Hoechst 33258. Values shown are means ± S.D., *p < 0.002. E, after transfection with GFP-LC3 for 24 h, p21−− MEFs were treated with 100 nM control siRNA or Beclin 1 or Atg5 siRNA for 48 h and treated with or without 25 μM C2-ceramide for 24 h. Values shown are means ± S.D., *p < 0.002.
Beclin 1 and Atg5, which subsequently induces caspase-dependent apoptosis in p21\(^{+/+}\) MEFs without a change in the cyclin-CDK activity. On the other hand, in p21\(^{−/−}\) MEFs, C2-ceramide drastically disrupts the PARP protein while maintaining the expression levels of Beclin 1 and Atg5 proteins, which then triggers autophagy. Furthermore, the inhibition of p21 or induction of Beclin 1 or Atg5 suppresses the apoptotic pathway and turns on the switch that triggers autophagy in p21\(^{+/+}\) MEFs, whereas the introduction of p21 or inhibition of Beclin 1 or Atg5 increases the sensitivity of p21\(^{−/−}\) MEFs to apoptosis. These findings suggest that, upon the cell death stimuli, p21 triggers apoptosis by inhibiting the autophagic pathway through the suppression of the stability of autophagy-related proteins but not by influencing the cyclin-CDK activity, at least in MEFs. Because the assembly of cyclin D-CDK4 complexes is impaired in p21\(^{−/−}\) MEFs compared with p21\(^{+/+}\) MEFs (35), the intact presence but not change in cyclin-CDK complexes might be required to direct a cell to an apoptotic rather than an autophagic fate.

p21 forms a complex with cyclin and CDK and inhibits their activity as broad acting CDK inhibitors (14–17). p21 can also associate with the proliferating cell nuclear antigen to suppress proliferating cell nuclear antigen-dependent cell growth (36). Besides being an inhibitor of cell cycle progression, p21 acts as a mediator of the apoptotic pathway (20). For example, the enforced expression of p21 induces apoptosis or enhances the apoptotic response to chemotherapeutic agents (37–41). In other situations, however, p21 can function as an anti-apoptotic protein (42). For example, the suppression of p21 renders cells more sensitive to the apoptosis caused by DNA-damaging treatments, including radiation (43–45). p21 is also cleaved by caspase-3, making growth-arrested cells undergo apoptosis (46). However, the precise mechanisms underlying the different roles of p21 in apoptosis remain controversial.

In our study, C2-ceramide induced apoptosis in p21\(^{+/+}\) MEFs, which is associated with the cleavage of caspase-3 and PARP and degradation of Beclin 1 and Atg5. In p21\(^{−/−}\) MEFs,
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however, C2-ceramide induced autophagy without activating the caspase cascade. γ-Irradiation, which generates ceramide, induces similar events in these MEFs. Therefore, as far as ceramide-induced PCD is concerned, p21 functions as an apoptosis inducer. In the greater scheme of things, ceramide has been recognized as an important second messenger molecule involved in the signaling pathways that control cell proliferation, differentiation, and death, especially apoptosis (47, 48). Ceramide-mediated apoptosis, in particular, has been extensively studied (21, 49, 50), and p21 has been found to promote ceramide-induced apoptosis (51). At the same time, ceramide has also been implicated in nonapoptotic autophagic cell death (22–25). For example, in MCF7 breast cancer cells, C2-ceramide stimulated the expression of Beclin 1 and induced autophagy (24). However, whether p21 is involved in ceramide-induced autophagy was not determined in these investigations.

Autophagy is a process in which subcellular membranes sequester proteins and organelles to degrade and recycle these materials (52). Autophagy begins with the isolation of double membrane-bound structures inside a cell. These structures are currently considered either to originate from a pre-existing membrane structure called a phagophore or to be formed de novo (29, 53). These membrane structures elongate, mature, and form autophagosomes to sequester cytoplasmic proteins and organelles. The autophagosomes then fuse with lysosomes and become either autolysosomes or degradative autophagic vacuoles. After that, the sequestered contents are degraded by lysosomal hydrolases. Recent genetic studies have identified at least 16 Atg genes in yeast that are necessary to form autophagosomes (5, 6). For example, Atg5-Atg12 conjugation is required for the formation of autophagosomes (32). To date, more than five mammalian orthologues of Atg genes have been identified. Beclin 1 and LC3 are orthologues of Atg6 and Atg8, which have been implicated in the formation of autophagosomes (28, 31). Autophagy has been observed under various cell conditions, including the degradation of proteins in response to nutrient deprivation, differentiation, aging, and cancer therapy (7–9). However, the molecular machinery that regulates autophagy is not fully understood. In this study, C2-ceramide treatment degraded PARP in p21+/− MEFs while maintaining the expression of Beclin 1 and Atg5 proteins, leading to autophagy rather than apoptosis. Unlike C2-ceramide-induced autophagy in breast cancer MCF7 cells (24), C2-ceramide did not increase the expression of Beclin 1 in p21+/− MEFs. This difference might be because of the fact that MEFs endogenously express enough Beclin 1 or Atg5 to undergo autophagy, whereas endogenous Beclin 1 expression is low in breast cancer cells, including MCF7 cells (31).

In summary, we demonstrated that p21 plays an essential role in deciding the type of PCD, apoptosis or autophagy, MEFs undergo after exposure to C2-ceramide. In other words, in cells with p21, cell death stimuli might degrade autophagy-related proteins while keeping the apoptotic pathway intact, leading to apoptosis. Without p21 expression, the cell death signal for apoptosis might be stopped by the degradation of PARP and the expression of autophagy-related proteins might remain unchanged, resulting in the induction of autophagy instead of apoptosis. When apoptosis is induced, the full-length PARP is cleaved by caspases into the p24 and p89 inactive fragments to prevent excessive NAD consumption and ATP loss (54). On the other hand, DNA-damaging agents such as N-methyl-N′-nitro-N-nitrosoguanidine were observed to cause the expression of the full-length PARP protein to be lost but did not cleave the protein, then activated PARP and depleted ATP, leading to nonapoptotic cell death (55). In addition, an increase in PARP activation and reactive oxygen species production caused caspase-independent autophagic cell death (34). These findings might explain why apoptosis rather than autophagy was induced when PARP was degraded but not cleaved in C2-ceramide-treated p21+/− MEFs. One possible scenario may be that PARP is not cleaved and might be activated, and autophagy is stimulated because of a fall in ATP. Of course, we need to determine whether PARP is actually activated when PARP is degraded because of a fall in ATP. AUTOPHAGY Induced when PARP was degraded but not cleaved in C2-ceramide-treated p21+/− MEFs. Further study will add new knowledge about the PCD pathways.

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