Activation of Caspases in p53-induced Transactivation-independent Apoptosis

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Though p53-induced apoptosis plays an important role in tumor suppression, the mechanism(s) by which p53 induces apoptosis is still unclear. To elucidate the p53-induced apoptotic pathway, we examined the role of p53 transactivation activity and caspase in J138V5C cells carrying a human temperature-sensitive (ts) p53 mutant (138Ala → Val). The results showed that p53-induced apoptosis was not blocked by cycloheximide, which effectively prevented the expression of p53 target genes, indicating that transactivation was not essential for p53-induced apoptosis in this system. Western blot analysis showed that PARP, CPP32 and ICH-1 precursors were cleaved during apoptosis. The CPP32-preferential tetrapeptide inhibitor Ac-DEVD-CHO blocked the cleavage of ICH-1 and PARP precursors, suggesting that CPP32 or some other DEVD-sensitive caspase(s) is the upstream activator of ICH-1. We also examined the role of the Fas pathway by using Fas and Fas ligand-neutralizing antibodies. Both antibodies failed to block p53-induced apoptosis, suggesting that the Fas pathway was not essential for p53-induced apoptosis in this system. Taken together, our results indicate that p53-induced, transactivation-independent apoptosis in Jurkat cells involves sequential activation of CPP32 or some other DEVD-sensitive caspase(s) and ICH-1, via a Fas-independent pathway.

Key words: p53 — Apoptosis — Caspase — Fas — Jurkat

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system, partly due to the sequence difference between human p53 and mouse p53,26 and also due to different genetic backgrounds even within the human cells used for analysis. The inability of Fas and Fas ligand-neutralizing antibodies to block p53-induced apoptosis suggests that the Fas pathway is not essential for p53-induced apoptosis in this system.

MATERIALS AND METHODS

Cell culture and treatment of caspase inhibitor J138V5C and Jneo were established previously from a human Jurkat T-cell line.24 The cells were grown in RPMI1640 medium containing 10% fetal calf serum (FCS) at 37.5°C for growth, or at 32.5°C for induction of apoptosis. For inhibitor treatment, Ac-DEVD-CHO (Pep-tonic Institute, Osaka) was added to the culture medium at a final concentration of 100 μM and incubated at 37.5°C for 1 h before temperature shift-down.

DNA fragmentation analysis DNA was extracted as previously described. Briefly, cells (2×10⁶) were collected and washed with cold phosphate-buffered saline (PBS). The cell pellets were suspended in 40 μl of 0.2 M Na₂HPO₄/0.1 M citric acid (192:8, v/v) buffer and incubated at room temperature for 60 min. The suspensions were centrifuged at 2,000×g for 30 min, then the supernatants were transferred to new tubes and treated with 0.02% Nonidet P-40 and RNase A (1 μg/ml) at 37°C for 30 min. Proteinase K (1 μg/ml) was added and the mixture was incubated at 50°C for 30 min. Aliquots (10 μl) were loaded on 1.5% agarose gel and electrophoresis was carried at 5 V/cm for 1 h in 1× TBE [89 mM Tris-borate, 2 mM EDTA (pH 8.0)] buffer. The DNA was stained with ethidium bromide and visualized under a UV illuminator.

Western blot analysis Cells cultured at permissive or non-permissive temperature were collected by centrifugation at low speed, washed with PBS, and lysed in RIPA buffer [1× PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate and 1% sodium dodecyl sulfate (SDS)] in the presence of protease inhibitors [0.1 μg/ml phenylmethanesulfonyl fluoride (PMSF) and 0.1 μg/ml aprotinin] and phosphatase inhibitor (1 mM sodium orthovanadate). DNAs were sheared by passage through 21 gauge needles and centrifuged at 15,000×g for 20 min. The supernatant equivalent to 50 μg total protein was fractionated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF filter (Nihon Millipore, Tokyo) by using a semi-dry electroblotting system. The membrane was blocked in 1× TTBS [20 mM Tris-HCl (pH 7.6), 137 mM NaCl and 0.1% Tween-20] containing 5% skim milk and bands were detected with the following antibodies: anti-PARP (C-2-10) (Enzyme System Products, Dublin, OH), anti-CPP32 (clone 19) (Transduction Lab., Lexington, KY), ERK-2 (K-23), ICH-1 (H-19) and p21 (C-19) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The horseradish peroxidase-conjugated second antibody with the ECL detecting system (Amersham, Tokyo) was used to visualize immunoreactive proteins.

Fas and Fas ligand-neutralizing antibodies analysis Cells (5×10⁶) were cultured in normal medium or medium containing apoptotic neutralizing anti-Fas antibody (clone ZB4) or anti-Fas ligand antibody (clone 4H9) (MBL Inc., Nagoya) at a concentration of 500 ng/ml for 1 h. Then the cells were either transferred to 32.5°C to induce apoptosis or maintained at 37.5°C as a control. The DNA fragmentation and cell viability were analyzed at different time points.

RESULTS

p53-induced apoptosis in Jurkat cells does not require de novo protein synthesis p53 can exert either transcriptional activation-dependent or -independent activity to induce apoptosis, depending on the cell types or experimental conditions. To elucidate the pathway by which p53 induces apoptosis in Jurkat cells, we analyzed whether the translation inhibitor cycloheximide could block p53-induced apoptosis. As cycloheximide itself induced apoptosis at high concentration in J138V5C cells, we treated

Fig. 1. p53-induced chromatin condensation in the absence of translation of p53-responsive genes. J138V5C cells (1×10⁶) were treated with vehicle or cycloheximide (0.5 μg/ml, Sigma, St Louis, MO) for 2 h before the culture was shifted to 32.5°C or maintained at 37.5°C. After 18 h of temperature shift-down, the cells were collected. They were fixed in 4% formaldehyde for 30 min, then stained with Hoechst 33342 (10 μM in PBS) and observed under a fluorescence microscope.

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J138V5C cells with cycloheximide at a concentration of 0.5 \( \mu \text{g/ml} \) for 2 h before and during temperature shift-down for 18 h. The treatment failed to block \( p53 \)-induced apoptosis at permissive temperature (32.5°C) as judged from the appearance of chromatin condensation and nuclear fragmentation (Fig. 1). At 18 h after temperature shift-down, 70% of cycloheximide-treated cells and 68.5% of untreated cells showed chromatin condensation and nuclear fragmentation. More than 90% of cells cultured at non-permissive temperature (37.5°C) were normal. DNA fragmentation in cycloheximide-treated cells was as extensive as in untreated cells (Fig. 2, upper panel) and protein level of \( p53 \)-responsive gene products, MDM2 and p21 (lower panel), as described in “Materials and Methods.”

**Activation of caspase is essential for \( p53 \)-induced apoptosis**

To investigate the involvement of caspases in \( p53 \)-induced apoptosis, we first examined the effect of the CPP32-preferential tetrapeptide inhibitor Ac-DEVD-CHO. Upon temperature shift-down, J138V5C cells cultured without inhibitor showed extensive apoptosis as judged by trypan blue exclusion test and DNA fragmentation analysis. Treatment of cells with 100 \( \mu \text{M} \) DEVD at 37.5°C for 1 h, then either shifted to 32.5°C (L) or left at 37.5°C (H). The cell viability was determined by means of the trypan blue exclusion test at different time points. X H+, ○ H−, □ L+, Δ L−. B: Cells (2×10⁶) were treated with the caspase inhibitor DEVD as in A. DNA fragmentation was analyzed before (0) or 18 h (18) after temperature shift-down. \( p53 \)-induced DNA fragmentation (18−) was blocked by caspase inhibitors (18+). No DNA fragmentation was detected in cells cultured at 37.5°C with (0+) or without (0−) inhibitors.

**Fig. 3. Effect of caspase inhibitor on \( p53 \)-induced apoptosis.**

A: Cells (5×10⁴) were cultured in 24-well plates in the presence (+) or absence (−) of 100 \( \mu \text{M} \) DEVD at 37.5°C for 1 h, then either shifted to 32.5°C (L) or left at 37.5°C (H). The cell viability was determined by means of the trypan blue exclusion test at different time points. X H+, ○ H−, □ L+, Δ L−. B: Cells (2×10⁶) were treated with the caspase inhibitor DEVD as in A. DNA fragmentation was analyzed before (0) or 18 h (18) after temperature shift-down. \( p53 \)-induced DNA fragmentation (18−) was blocked by caspase inhibitors (18+). No DNA fragmentation was detected in cells cultured at 37.5°C with (0+) or without (0−) inhibitors.
To elucidate further which of the caspases was involved in p53-induced apoptosis, we examined the cleavage of caspase precursors and caspase substrate by western blotting. ICH-1 is expressed as a 49 kDa proenzyme which is activated by multi-step cleavages.\footnote{22} Using an antibody against the prodomain, we detected the 49 kDa precursor in cells cultured at 37.5°C. This protein disappeared from cells cultured at 32.5°C for 6 h (Fig. 4A). CPP32 is synthesized as a 32 kDa precursor which is cleaved to 17 kDa and 12 kDa subunits upon activation.\footnote{17} Using a monoclonal antibody against the prodomain and 17 kDa subunit of CPP32, we detected a high level of 32 kDa CPP32 precursor in J138V5C cells at non-permissive temperature. This 32 kDa band decreased and the 17 kDa band appeared as early as 6 h after temperature shift-down (Fig. 4B). The 116 kDa nuclear enzyme PARP, a common substrate for multiple caspases, was almost completely cleaved to the 85 kDa subunit at the same time point (Fig. 4C). These observations suggested that both CPP32 and ICH-1 were activated in Jurkat cells undergoing p53-induced apoptosis.

To investigate the occurrence of the caspase activation cascade during p53-induced apoptosis, we examined the effect of the CPP32-preferential inhibitor Ac-DEVD-CHO on the activation of ICH-1. We found that Ac-DEVD-CHO completely blocked the cleavage of ICH-1 and PARP precursors (Fig. 4D), suggesting that CPP32 or some other DEVD-sensitive caspase was required for the activation of ICH-1.

**Fas pathway is not essential for p53-induced apoptosis**

Activation of caspases via the Fas pathway has been investigated extensively.\footnote{28} The Fas pathway is essential for induction of apoptosis by anticancer drugs and myc, which may also require p53 activation.\footnote{29\textsuperscript{–}31} Although the transactivation of p53-responsive genes was not essential for p53-induced apoptosis, it is possible that p53 might activate the Fas pathway in a transactivation-independent way. Based on this consideration, we examined whether Fas receptor and Fas ligand-neutralizing antibodies could block p53-induced apoptosis in this system. An agonistic anti-Fas antibody (CH11) induced apoptosis in J138V5C cells at non-permissive temperature, indicating that the Fas pathway is intact in this cell line. An antagonistic Fas antibody (ZB4) blocked CH11-induced apoptosis. However, at the same concentration, it failed to block apoptosis induced by ts p53 in this system (Fig. 5). Morphological change, membrane blebbing and DNA fragmentation in ZB4-treated cells were all as extensive as in untreated cells. Furthermore, the anti-Fas ligand antibody (4H9), which had been shown to block Fas

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**Fig. 4. Western blot analysis showing the activation of caspases.** Cleavage of ICH-1 (A), CPP32 (B) and PARP (C). D: Ac-DEVD-CHO blocked the cleavage of ICH-1 and PARP. Western blotting was performed as described in “Materials and Methods.” Time (h) represents the time period after temperature shift-down.
ligand-induced apoptosis, also failed to block p53-induced apoptosis (Fig. 6). These results suggested that the Fas pathway was not essential for p53-induced apoptosis in this system.

DISCUSSION

Overexpression and activation of human ts p53 without other stimuli induce apoptosis in Jurkat cells. The previous experiment showed that several p53-responsive genes were up-regulated upon temperature shift-down. However, the level of bax protein, one of the p53-responsive gene products, did not change significantly before and after temperature shift-down. It has been reported that the temperature-sensitive p53 143Ala mutant can transactivate several p53 target genes, but not apoptosis-associated bax and insulin-like growth factor-binding protein 3 gene promoter. In addition, as shown in this study, the apoptosis was not prevented by a concentration of cycloheximide which effectively blocked the de novo synthesis of proteins, suggesting that transactivation activity was not essential for apoptosis. Although it is not known whether the translation of all known and unknown p53 target genes was blocked to the same extent under our experimental conditions, the result that the number of apoptotic cells showed no significant difference in the presence of cycloheximide, as measured by Hoechst 33342 staining, further supported the possibility that transactivation-independent apoptosis was the major event in the present system. This result is consistent with and extends the earlier findings that UV induces p53-dependent apoptosis in the absence of protein synthesis, and that transcriptionally inactive p53 mutants retained the
ability to induce cell death.12) The different apoptotic pathways induced by p53 in different systems may result from the difference in cell types. The p53 species employed may also be a contributing factor. In spite of the high homology, there are important structural and functional differences between different species of p53.33,34) For example, the murine p53 shares only 40% homology with human p53 in its SH3-binding domain (amino acid residues 62–91), which is important for p53-induced apoptosis.13)

Caspase family members are expressed as proenzymes, which are cleaved and activated during the apoptotic process.16,17) Apoptosis induced by a given stimulus in a particular cell type may involve a selective activation of several caspases. It has been shown that p53-induced transactivation-dependent apoptosis involves activation of caspases. CPP32 seems to be a common mediator of apoptosis.25–23,35) In this study, we observed that the CPP32-preferential inhibitor Ac-DEVD-CHO effectively prevented p53-induced apoptosis, suggesting that CPP32 or some other DEVD-sensitive caspase(s) was required for p53-induced apoptosis in this system. The cleavage of CPP32 precursor to the 17 kDa active subunit further confirmed the essential role of CPP32 for p53-induced transactivation-independent apoptosis. Jurkat cells express a high level of ICH-1, and activation of ICH-1 has been shown to be involved in Fas and tumor necrosis factor (TNF) α-induced apoptosis in Jurkat cells.36) The ICH-1 precursor was also cleaved during p53-induced apoptosis in our case, suggesting that the activation of ICH-1 is a common event in Jurkat cells undergoing apoptosis induced by various stimuli. The activation of CPP32 and ICH-1 preceded the morphological change and DNA fragmentation, which were relatively early events in p53-induced apoptosis in this system. These results demonstrated that caspases were also essential for p53-induced, transactivation-independent apoptosis. The sequential activation of CPP32-like caspases and ICH-1 had been suggested in Jurkat cells which underwent Fas and TNF-induced apoptosis.36) As the CPP32-preferential peptide inhibitor Ac-DEVD-CHO, but not Ac-YVAD-CHO (data not shown), blocked the cleavage of ICH-1 precursor, we suggest the sequential activation of CPP32 or other DEVD-sensitive caspase(s) and ICH-1 in p53-induced apoptosis. This would be in agreement with the report that ICH-1 was a substrate for CPP32 in vitro.38) To our knowledge, this is the first report showing that caspase is essential for p53-induced transactivation-independent apoptosis.

p53 may mediate the activation of caspase through transcriptional activation of bax in some systems.20) However, in our system, apoptosis was induced by p53 in the absence of protein synthesis from p53 transcriptional target genes, suggesting that p53-induced apoptosis in this system is not initiated by up-regulated bax protein. This result also suggests that induction of other p53-responsive genes such as Fas, IGF-BP3 and p85 is not required for p53-induced apoptosis.10,37) The Fas/Fas ligand pathway is another route leading to activation of caspase.20) To clarify whether p53 may activate Fas/FasL through a pathway other than transcriptional activation, we blocked the Fas pathway with Fas and Fas ligand-neutralizing antibodies: both antibodies failed to block p53-induced morphological change, DNA fragmentation and loss of cell viability. The Fas death pathway was triggered by the binding of Fas ligand to Fas receptor. Both the Fas-neutralizing antibody (ZB4) and Fas ligand-neutralizing antibody (4H9) effectively blocked the activation of the Fas pathway in this and other systems.38) These data indicated that p53-induced apoptosis does not involve the activation of the Fas pathway. This result is consistent with and extends the previous report that cells from Fas-deficient lpr mice were still sensitive to γ-irradiation-induced, p53-dependent apoptosis,25) although γ-irradiation may have additional effects on apoptosis other than via the p53-dependent pathway. Taken together, our results indicate that p53-induced transactivation-independent apoptosis involves sequential activation of CPP32 or some other DEVD-sensitive caspase(s) and ICH-1, which is not mediated by the Fas pathway.

ACKNOWLEDGMENTS

We are very grateful to Dr. A. K. Munirajan for a critical reading of the manuscript. This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Sports of Japan. (Received August 13, 1998/Revised November 6, 1998/Accepted November 11, 1998)

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