The p53 tumor suppressor gene is critically involved in cell cycle regulation, DNA repair, and programmed cell death. Several lines of evidence suggest that p53 death signals lead to caspase activation; however, the mechanism of caspase activation by p53 still is unclear. Expressing wild type p53 by means of an adenoviral expression vector, we were able to induce apoptotic cell death, as characterized by morphological changes, phosphatidylserine externalization, and internucleosomal DNA fragmentation, in p53null Saos-2 cells. This cell death was accompanied by caspase activation as well as by cleavage of caspase substrates and was preceded by mitochondrial cytochrome c release. The addition of the broad-spectrum caspase inhibitor benzoxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk) directly after transduction almost completely prevented p53-induced apoptotic cell death but did not inhibit mitochondrial cytochrome c release. In contrast, N-acetylcycteine, even at high concentrations, could not prevent induction of programmed cell death by p53 expression. Cytosolic extracts from Saos-2 cells transduced with p53, but not from Saos-2 cells transduced with the empty adenoviral vector, contained a cytochrome c-releasing activity in vitro, which was still active in the presence of zVAD-fmk. When Bax was immunodepleted from the cytosolic extracts of p53-expressing cells before incubation with isolated mitochondria, the in vitro cytochrome c release was abolished. Thus, we could demonstrate in cells and in vitro that p53 activates the apoptotic machinery through induction of the release of cytochrome c from the mitochondrial intermembrane space. Furthermore, we provide in vitro evidence for the requirement of cytosolic Bax for this cytochrome c-releasing activity of p53 in Saos-2 cells.

The p53 tumor suppressor gene is the central integrator of the cellular response to DNA damage, oncogenic transformation, and growth factor withdrawal (1). The cell cycle regulatory and the DNA repair functions of p53 are largely executed by transactivation of p53-response genes such as p21/WAF1/CIP1 (2–5) or GADD45 (6), thus relying on the ability of p53 to act as a sequence-specific transcription factor. In contrast, the tumor-suppressing activity of p53 does not depend on its transcriptional function (7–10).

It has been shown in several cell types that wild type (wt) p53 is required for the apoptotic cell death as induced by γ-irradiation or a variety of anticancer drugs (11, 12). Yet, the pathways whereby p53 leads to execution of the apoptosis program are not well characterized. Possible mechanisms include transcriptional activation of the proapoptotic Bcl-2 family member Bax (13, 14), the generation of reactive oxygen species (15), and transcriptional up-regulation of death receptors such as CD95/Fas/APO-1 or DR5/KILLER (16–19). However, several lines of evidence imply that the proapoptotic activity of p53 is independent of its function as a transcription factor (20–22).

The release of cytochrome c from mitochondria is a central event in the death receptor-independent, “intrinsic,” apoptotic pathway (23, 24). Cytochrome c together with ATP and Apaf-1 facilitates activation of caspase 9 of the effector caspases (25–28), which then cleave their substrates, finally leading to the apoptotic cell death. Furthermore, cytochrome c release can also occur in death receptor-dependent, “extrinsic,” apoptotic pathways by cleavage and activation of the proapoptotic Bcl-2 family member Bid through caspase 8 (29–31), possibly serving as an amplification loop. Several studies were undertaken to establish the involvement of caspase activation in p53-mediated cell death (18, 32–35). Recently, the requirement of Apaf-1 or caspase 9 for the p53-dependent apoptosis of oncogene-transformed murine embryonic fibroblasts has been conclusively demonstrated (36).

The present study was undertaken to address the hypothesis that p53 might induce apoptosis by a death receptor-independent pathway involving the release of mitochondrial cytochrome c. We show in Saos-2 cells that p53 evokes cytochrome c release prior to caspase activation and prior to the occurrence of apoptotic cell membrane changes. Furthermore, we biochemically demonstrate the requirement of cytosolic Bax protein for the cytochrome c-releasing activity of p53. Thereby, in our experimental system, we provide a link between p53 and the death receptor-independent activation of the apoptotic machinery downstream of mitochondria.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Antibodies, and Reagents**—The human osteosarcoma cell line Saos-2 cells was obtained from ATCC (Manassas, VA) and was maintained in McCoy’s 5A medium containing 15% fetal calf serum, penicillin, and streptomycin.

Antibodies against cytochrome c (clone 7H8.2C12), caspase 3 (polyclonal rabbit antiseraum), poly(ADP-ribos)e polymerase (clone C2–10),

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p53 (clones Do-7 and PAb122), p21/WAF1/CIP1 (clone SX118), and Bax (polyclonal rabbit antiserum) were purchased from Pharmingen (San Diego, CA). Polyclonal rabbit antisera against protein kinase C-δ and JNK1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and an antibody against actin (clone C4) was obtained from ICN Pharmaceuticals (Costa Mesa, CA). A rabbit antiserum against caspase 9 has been described previously (37, 38).

The broad-spectrum caspase inhibitor benzoxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk) and the fluorochrome caspase substrate benzoxycarbonyl-Asp-Glu-Val-Asp aminofluoromethylcoumarin (DEVD-afc) were purchased from Enzyme System Products (Livermore, CA).

Vectors and Transduction—Replication-deficient adenoviral vectors were generous gifts from Drs. D. C. Manvel and I. Atencio, Canji Inc., San Diego, CA. The vectors either encoded the complete human wt p53 cDNA (Ad.p53) or green fluorescent protein (Ad.GFP) under the control of the cytomegalovirus immediate/early gene promoter (39, 40). The Ad.GFP vector was applied to determine infectibility and maximal tolerated dose of Saos-2 cells, which was found to be 10^9 particles/ml. For further experiments, the Ad.p53 vector was used for p53 gene transfer, and the empty adenoviral vector (Ad) served as control. Approximately 18 h before transduction, cells were passaged in 10-cm culture dishes or 12-well plates (Fisher) at a density of 200,000/ml or 50,000/ml, respectively. Vectors were diluted in serum-free medium (OptiMEM I, Life Technologies, Inc.) at the appropriate dose (10^9 particles/ml). Cells were pulsed with the vectors for 60 min, washed, and supplemented with fresh growth medium containing fetal calf serum.

Flow Cytometry—For flow cytometry, cells were harvested by mild trypsinization, followed by washing with growth medium and phosphate-buffered saline. Cell death was determined by two-color analysis of fluorescein isothiocyanate-labeled annexin V (CLONTECH Laboratories) binding and propidium iodide (PI) uptake using a Becton Dickinson FACSDiVa apparatus, followed by transfer on nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech). Antibody binding was detected by enhanced chemiluminescence (Super Signal; Pierce).

Isolation of Mitochondria and in Vitro Cytochrome c Release Assay—Mitochondria were freshly isolated from the liver of 2-month-old mice as described previously (41, 42). Aliquots of the mitochondrial preparation were incubated with cytosolic extracts at 37 °C for various time periods. After centrifugation at 20,000 × g for 15 min, the supernatants were analyzed for the presence of cytochrome c by Western blotting. Mitochondrial pellets were resuspended in lysis buffer and were also analyzed by Western blotting.

Western Blotting—The protein content of cytosolic or whole cell extracts was determined by the Bradford assay (Bio-Rad). For each time point, 25 µg of total protein were boiled in Laemmli buffer for 5 min followed by centrifugation. The supernatants were subjected to electrophoresis in 8% or 15% SDS-polyacrylamide gels using a Bio-Rad mini gel apparatus, followed by transfer on nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech). After blocking with phosphate-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20, the membranes were exposed to the primary antibodies overnight at 4 °C. The membranes were washed extensively using phosphate-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20, the supernatants were stored at −70 °C until further analysis. Extracts of the pellets as well as whole cell extracts were obtained by dissolving in lysis buffer, followed by repetitive vortexing and freeze-thawing. After centrifugation at 16,000 × g, the supernatants were stored at −70 °C.

Isolation of Mitochondria and in Vitro Cytochrome c Release Assay—Mitochondria were freshly isolated from the liver of 2-month-old mice as described previously (41, 42). Aliquots of the mitochondrial preparation were incubated with cytosolic extracts at 37 °C for various time periods. After centrifugation at 20,000 × g for 15 min, the supernatants were analyzed for the presence of cytochrome c by Western blotting. Mitochondrial pellets were resuspended in lysis buffer and were also analyzed by Western blotting.

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RESULTS

Expression of wt p53 Induces Apoptosis in p53null Saos-2 Cells—First we assessed whether adenovirus-mediated p53 expression resulted in apoptotic cell death and caspase activation in Saos-2 cells. Transduction with Ad.p53 induced phosphatidylinerne externalization as detected by annexin V binding at 24 h following by membrane disruption, leading to PI uptake starting at 36 h. No such effects were observed upon transduction with the same particle number of the empty Ad vector cassette (Fig. 1A). Similarly, an increase in subdiploid DNA content, indicating internucleosomal DNA fragmentation, was only observed in Saos-2 cells transduced with Ad.p53 but not in Saos-2 cells transduced with Ad (Fig. 1B).

Caspase activity leading to cleavage of DEVD-afc was detected in extracts from Saos-2 cells obtained at 24, 36, and 48 h after transduction with Ad.p53 (Fig. 2A). Immunoblotting demonstrated loss of procaspase 9, cleavage of procaspase 3, and cleavage of the caspase substrates poly(ADP-ribose) polymerase and protein kinase C-δ. Expression of p53 protein as well as induction of the p53 target gene p21/WAF1/CIP1 could be detected as early as 12 h after transduction (Fig. 2B).

p53-mediated Apoptosis in Saos-2 Cells Is Prevented by zVAD-fmk but Not by N-Acetylcyesteine—Next, testing was conducted to determine if the apoptotic cell death observed after p53 expression in Saos-2 cells was dependent on caspase activity. The addition of the broad spectrum caspase inhibitor zVAD-fmk at a concentration of 100 µm almost completely abrogated cell death (Fig. 3A) and DNA fragmentation (Fig.
3B), as induced by expression of wt p53 in Saos-2 cells. Several mitochondrial genes potentially generating reactive oxygen species were reported to be induced by p53 overexpression (15), implicating a role of reactive oxygen species as downstream effectors of p53-mediated apoptosis. However, in Saos-2 cells, the addition of N-acetylcysteine up to concentrations of 10 mM had no protective effect against p53-induced cell death (Fig. 3A). Moreover, N-acetylcysteine at concentrations of 20 mM or above induced necrotic cell death in Saos-2 cells (data not shown).

Cytochrome c Is Released from Mitochondria upon p53 Expression—To assess whether p53 activates caspases by inducing the release of cytochrome c from the mitochondrial intermembrane space into the cytosol (26, 43), subcellular fractionation of Saos-2 cells was performed at various time points following transduction with Ad.p53 or with Ad. Cytochrome c release into the cytosolic fraction of Saos-2 cells could be detected as early as 12 h after transduction with Ad.p53; this corresponded with a depletion of the mitochondria-containing pellet fraction from cytochrome c (Fig. 4A). No such effect could be observed after transduction of Saos-2 cells with vector alone (Fig. 4B). Similar kinetics of mitochondrial cytochrome c release were observed after adenoviral expression of wt p53 in the p53null human lung cancer cell line Calu-6 (data not shown). The proapoptotic Bcl-2 family member Bax, which induces mitochondrial cytochrome c release (44, 48), is reported to be transcriptionally activated by p53 in some human cell lines (13). In concordance, an increase of Bax protein expression was found in Saos-2 cells following p53 overexpression, but significantly elevated Bax levels were detected only after the onset of cytochrome c release (Fig. 4A).

Ligation of death receptors such as CD95/Fas/APO-1 or tumor necrosis factor receptor 1 also can result in cytochrome c release via activation of caspase 8, followed by cleavage and activation of Bid. Cleaved Bid can then target mitochondria and induce the release of cytochrome c (29–31). However, Bid
cleavage in death receptor-independent apoptosis as induced by etoposide has been shown to occur downstream of mitochondrial cytochrome c release and was prevented by the caspase inhibitor zVAD-fmk (46). To address whether the p53-mediated cytochrome c release observed in our system was dependent on the caspase 8 cleavage of Bid, we again applied the broad spectrum caspase inhibitor zVAD-fmk, which at a concentration of 1 mM is equally potent against effector caspases such as caspases 3 and 7 and against activator caspases such as caspases 8, 9, and 10 in vitro (47); the addition of 100 μM zVAD-fmk directly after transduction with Ad.p53 did not prevent mitochondrial cytochrome c release (Fig. 4B). Since caspase activity is required for Bid cleavage and activation via the death receptor FADD-caspase 8 pathway, this rules out a role for this pathway in p53-induced cytochrome c release from mitochondria in Saos-2 cells.

**p53 Induces Mitochondrial Cytochrome c Release by a Pathway Requiring Cytosolic Bax**—To further characterize the mechanism of p53-induced cytochrome c release, cytosolic extracts from Saos-2 cells either transduced with Ad.p53 or with the empty Ad vector were prepared and incubated with freshly isolated murine liver mitochondria. Only extracts from cells expressing wt p53 contained a cytochrome c-releasing activity in vitro, which was not inhibited by addition of zVAD-fmk (Fig. 5A), also ruling out the requirement of caspase activity for the in vitro cytochrome c release by p53. When p53 was immunodepleted from the cytosolic extract, the cytochrome c-releasing activity was still retained (Fig. 5B). This argued against a direct effect of p53 on mitochondria in our in vitro system.

As p53 has been described to transactivate the proapoptotic Bcl-2 family member Bax in some cell types (13), which was shown to induce cytochrome c release when added to isolated mitochondria (36, 48), the role of Bax in this in vitro system of p53-mediated cytochrome c release was further investigated. Immunodepletion of Bax, but not of p53 or of the control antigen JNK1, abolished the cytochrome c-releasing activity from the extract of p53 overexpressing cells. (Fig. 5B). Thus, the cytochrome c-releasing activity of p53 was dependent on the presence of Bax in the cytosol.

**DISCUSSION**

From current understanding, there are two pathways transducing a death signal to the apoptotic machinery. The “extrinsic” pathway involves trimerization of death receptors such as CD95/Fas/APO-1 or TNF receptor 1 by binding of their respective ligands, which leads to recruitment of the activator caspase 8 via adapter molecules like FADD and TRADD and to its autoactivation (49). Activated caspase 8 either can directly cleave and activate the effector caspases (50, 51), or it can cleave Bid to induce the release of mitochondrial cytochrome c, which also leads to activation of effector caspases via oligomerization with Apaf-1 and caspase 9 in the presence of ATP (29–31). This caspase-dependent activation of the mitochondrial pathway may be important in cells with low concentrations of death receptors or caspase 8 and might act as an amplification loop (42). There is evidence for a role of p53 in
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regulation of membrane expression of some death receptors in several cell types (16–19).

In contrast, the death receptor-independent, intrinsic, apoptotic pathway is directly activated by a death signal, leading to the release of cytochrome c from the mitochondrial intermembrane space into the cytosol, which then, in the presence of ATP, facilitates oligomerization and activation of Apaf-1 and caspase 9, leading to activation of caspase 3 and other effector caspases (24). The release of cytochrome c is regulated by the various pro- and anti-apoptotic members of the Bcl-2 family (23, 44, 48, 52–54).

Many inducers of apoptosis, such as γ-irradiation, anticancer drugs, staurosporine, and growth factor withdrawal, were shown to activate apoptotic cell death independent of death receptor pathways, thus presumably directly activating this intrinsic apoptotic pathway (34, 46, 55–57). Furthermore, mice deficient in Apaf-1 or caspase 9, which in the intrinsic pathway act downstream of mitochondrial cytochrome c release, show severe developmental defects leading to embryonic lethality, and their cells are resistant to death receptor-independent induction of apoptosis by radiation and a variety of anticancer drugs (58–60).

Whereas the mitochondrial and the downstream events in the intrinsic pathway are well elaborated, the understanding of the events upstream of mitochondria in this pathway is incomplete. A number of studies implies a central role of the p53 tumor suppressor gene in the integration of a death signal to the intrinsic apoptotic pathway (11, 12, 15, 61). Recently, Soengas et al. (36), using murine embryonic fibroblasts, provided evidence that apoptotic cell death as induced by oncogenic transformation requires wt p53 as well as Apaf-1. Thus, p53 appears to transduce a signal to the intrinsic apoptotic pathway (11, 12, 15, 61).

Upon activation, p53 induces the release of mitochondrial cytochrome c by a pathway involving cytosolic Bax. Cytosolic cytochrome c facilitates the activation of caspase 3 and other effector caspases by caspase 9 oligomerization with the adapter molecule Apaf-1 in the presence of ATP. Whether p53 induces cytochrome c release by transcriptionally increasing cytosolic Bax levels, which was observed in some human cell lines (13), or whether p53 leads to conformational changes and mitochondrial targeting of Bax by a pathway involving other Bcl-2 family members (54, 63) or yet unidentified mediators requires further analysis.

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