p53 Activates the CD95 (APO-1/Fas) Gene in Response to DNA Damage by Anticancer Drugs

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Summary

Chemotherapeutic drugs cause DNA damage and kill cancer cells mainly by apoptosis. p53 mediates apoptosis after DNA damage. To explore the pathway of p53-dependent cell death, we investigated if p53-dependent apoptosis after DNA damage is mediated by the CD95 (APO-1/Fas) receptor/ligand system. We investigated hepatoma, gastric cancer, colon cancer, and breast cancer cell lines upon treatment with different anticancer agents known to act via p53 accumulation. Cisplatin, mitomycin, methotrexate, mitoxantrone, doxorubicin, and bleomycin at concentrations present in the sera of patients during therapy led to an upregulation of both CD95 receptor and CD95 ligand. Induction of the CD95 ligand occurred in p53 wild-type (wt), p53 mutant (mt), and p53 deficient (p53−/−) cell lines and at wt and mt conformation of temperature-sensitive p53 mutants. In contrast, upregulation of the CD95 receptor was observed only in cells with wt p53, not in cells with mt or without any p53. Restitution of inducible wt p53 function restored the ability of p53−/− Hep3B cells to upregulate the CD95 receptor in response to anticancer drugs. This rendered the cells sensitive to CD95-mediated apoptosis. In an attempt to understand how CD95 expression is regulated by p53, we identified a p53-responsive element within the first intron of the CD95 gene, as well as three putative elements within the promoter. The intronic element conferred transcriptional activation by p53 and cooperated with p53-responsive elements in the promoter of the CD95 gene. wt p53 bound to and transactivated the CD95 gene, whereas mt p53 failed to induce apoptosis via activation of the CD95 gene. These observations provide a mechanistic explanation for the ability of p53 to contribute to tumor progression and to resistance of cancer cells to chemotherapy.

Key words: apoptosis • CD95 (APO-1/Fas) • p53 • cancer therapy • drug resistance
has been known that these diverse drugs can induce apoptosis (1, 2). However, the mechanism of apoptosis induced by the drugs was not known. Recent evidence (3–6) suggests that genes that regulate apoptotic cell death may play an important role in determining the sensitivity of tumor cells to chemotherapy.

Friesen et al. (3) and Müller et al. (6) have shown that death induced in tumor cells by anticancer treatment is an active program of the cell which involves the CD95 system, one of the key regulatory systems of apoptosis. Treatment of leukemic and hepatocellular carcinoma cell lines, respectively, in vitro with chemotherapeutic agents causes upregulation of the CD95 ligand (CD95L), activating both an autocrine suicide and a fratricide death system. CD95L is expressed in a membrane form or is released by the tumor cells exposed to the drug. Binding of CD95L to the CD95 receptor then initiates the apoptotic signal in chemosensitive cells. Furthermore, we have shown for hepatoma cells that in addition to the expression of CD95L, cells treated with anticancer drugs upregulate CD95 receptor expression (6).

The presence of functional wild-type (wt) 1 p53 is closely coupled with efficient induction of CD95-mediated apoptosis in many (6–8) but not all (9, 10) cell types. Upregulation of the CD95 receptor—after anticancer therapy—inside hepatocarcinomas was preceded and directed by upregulated p53 and, thus, only occurred in hepatoma cell lines that express wt p53 (6). p53 has multiple functions (11–13), including cell cycle control in response to DNA damage (14, 15), induction of apoptosis (16–19), and DNA repair (20–22). Anticancer therapy causes damage of the DNA in the treated cells. p53 modulates cellular responses to DNA damage in mammals, affecting cell cycle progression and/or programmed cell death. Although the cyclin-dependent kinase inhibitor CDKN1A, also known as p21, appears to be the major effector of p53-mediated G1 cell cycle arrest after DNA damage (23–26), the mechanism by which p53 signals influence the apoptotic machinery after DNA damage remains unclear. p53 also acts as a sequence-specific DNA binding protein which activates the transcription of target genes. The proapoptotic protein BAX appears to be transcriptionally induced by p53 after DNA damage in certain cell types (27). However, BAX appears to contribute only in part to p53-mediated cell death (28).

Based on our observation that the CD95 receptor was only upregulated in cancer cells carrying wt p53 after DNA damage and on the fact that forced overexpression of wt p53 can stimulate CD95 gene transcription (29), we investigated if the CD95 system is activated and/or regulated by p53 in a variety of human solid cancer cell lines. These cell lines are representative of the "major killers" among human tumors, in which p53 mutations are known to play an important role. Thus, colon, stomach, hepatoma, and breast cancer cell lines with different p53 status were evaluated. We applied anticancer drugs with different mechanisms of action but concentrated on drugs that use the p53 pathway. Our data demonstrate that drug-induced p53 upregulation is involved in CD95 gene induction and apoptosis. Induction of CD95 gene transcription by p53 is mediated through a strong p53-responsive element located within the first intron of the gene. This element cooperates with sequences in the CD95 promoter to achieve maximal transactivation by wt p53.

Materials and Methods

Cell Lines. The following cell lines were used: (a) HepG2 cells derived from a human hepatoblastoma (30) only expressing small amounts of wt p53 (31); (b) our unpublished sequence data; (b) HepG2 cells derived from a hepatocellular carcinoma (32) and shown to express p53 with increased half-life as a result of a point mutation at codon 220 (33); (c) Hep3B cells (30) deficient of p53 (31); (d) Hs746T gastric cancer cells expressing wt p53; (e) HT29 colon cancer cells with mt p53 (34, 35); (f) AGS colon cancer cells with wt p53; (g) MCF7 breast cancer cells expressing wt p53 (36, 37); and (h) H1299 human lung adenocarcinoma cells deficient of p53 (38). HepG2, Hep4, and Hs746T cells were maintained in DME (GIBCO BRL, Eggenstein, Germany) containing 10% FCS, 5 mM 1-glutamine, and 100 μg/ml penicillin (GIBCO BRL). Hep3B cells were grown in EM (Laboratoires Eurobio, Ruanheim, Germany). HT29, MCF7, and H1299 cells were cultured in RPMI 1640 medium (GIBCO BRL) supplemented with 10% heat-inactivated FCS, 10 μg/ml streptomycin (GIBCO BRL). MCF7 breast cancer cells expressing wt p53 (36, 37); and (h) H1299 human lung adenocarcinoma cells deficient of p53 (38). HepG2, Hep4, and Hs746T cells were maintained in DME (GIBCO BRL, Eggenstein, Germany) containing 10% FCS, 5 mM 1-glutamine, and 100 μg/ml penicillin (GIBCO BRL). Hep3B cells were grown in EM (Laboratoires Eurobio, Ruanheim, Germany). HT29, MCF7, and H1299 cells were cultured in RPMI 1640 medium (GIBCO BRL) supplemented with 10% heat-inactivated FCS, 10 μg/ml streptomycin (GIBCO BRL), and 2 mM 1-glutamine (GIBCO BRL). AGS cells were propagated in Ham's F-12 medium (GIBCO BRL) supplemented with 10% heat-inactivated FCS, 10 μg/ml streptomycin (GIBCO BRL), and 2 mM 1-glutamine.

Transfections. Before transfection, H1299 and Hep3B cells were seeded at 0.6 × 10^6 cells/6-cm dish. The medium was changed to DME supplemented with 10% FCS, and cells were transfected by the calcium phosphate method with the precipitate left on cells for 16 h. Next, cells were washed with 1 min and placed in RPMI medium supplemented with 10% FCS. Cells were harvested 24 h later and assayed for luciferase activity as described previously (39).

For preparation of cell extracts for immunoselection assays, H1299 cells were plated at a density of 1.2 × 10^6/10-cm dish and transfected as above with 5 μg pCMVp53wt (40) DNA.

Generation of Stable Hep3B-derived Clones. Stable Hep3B-derived clones were generated in which wt p53 activity can be deliberately induced. Hep3B cells are particularly appropriate for our studies because they lack p53 (30, 31, 41). Stable Hep3B clones were generated expressing either puromycin resistance alone (control cell line: BT-2E) or in conjunction with either the temperature-sensitive mutant p53ala143 (42) or a p53 (modified)-estrogen receptor chimera in which p53 activity is induced posttranslationally by addition of the specific ligand 4-OH tamoxifen (cell line: BT-4P) (43). These cell lines have been described (41).

A third type of stably transfected Hep3B clones was established expressing the temperature-sensitive mutant p53ala143 (44). Transfection was performed by calcium phosphate precipitation with p53ala143. 2 d later G418 (Sigma Chemical Co., St. Louis, MO) was added to the cell culture. Colonies were isolated, and drug-resistant cells were expanded. These drug-resistant colonies were sequenced to determine whether they expressed the p53ala143 mutation (45). Colony growth was determined by an automated colony counter (CAMBEC, Carson, CA) and by the number of colonies in culture. Drug-resistant colonies were used and cloned for all experiments reported here.

Abbreviations used in this paper: IGF-IR, insulin-like growth factor I receptor; MANOVA, multivariate analysis of variance; mt, mutated; wt, wild-type.
intact cells by forward/side scatter analysis, and 10
twice again, and assayed. Upon data acquisition a gate was set on
anti–APO-1. After 30 min incubation, cells were washed twice,
and stored at –20° and expanded.

Treatment with Apoptic agents. The different cell lines were
treated with bleomycin (H einrich M ack N achf., Illertissen, Ger-
many) at a dose range of 60 ng/ml to 6 mg/ml, or with doxorubi-
cin (Röhne-Poulenc R erör Gmbh, Köln, Germany) at a dose
range of 0.4 ng/ml to 4 ng/ml for 3–72 h. Additionally, cisplatin
was at a dose range of 0.1 ng/ml to 0.1 mg/ml or methotrexate at a
dose range of 0.1 ng/ml to 1 mg/ml was applied. The concentra-
tions relevant for therapy are 1.5–3 μg/ml for bleomycin (45), 0.4–
1.6 μg/ml for cisplatin (46, 47), 7–12 μg/ml for methotrexate (48),
and 0.001–0.02 μg/ml for doxorubicin (49) in patients’ sera.

Treatment with Anti APO-1. The CD95 receptor was stimu-
lated according to the formula (% CD95
1
C as a sterile solution of 1 mg/ml in water.

Detection of CD95L mRNA Expression by PCR. The CD95
receptor was expressed by FACScan
analysis carried out in a FACScan flow cytometer (Beckton Dickin-
son Gmbh, Heidelberg, Germany) using CellIQ ues software.

Quantitation of DNA fragmentation was performed by FACScan
analysis of propidium iodide–stained nuclei as described previously (53). Hepatocytes floating in the culture medium were
collected by centrifugation at 200 g. Adherent hepatocytes were
harvested by incubation with 1% trypsin for 1 min. The cells
were washed with PBS, suspended in hypotonic lysis buffer (0.1%
sodium citrate; M erck, Darmstadt, Germany), 0.1% Triton X
Serva Feinbiochemica, Heidelberg, Germany), and 50 ng/ml propidium iodide (Sigma Chemical Co.) and incubated at 4°C for 6
h. Cells were then analyzed for DNA content by flow cytometry.

Early apoptotic changes were identified by using Annexin-V–
Fluos (Boehringer Mannheim GmbH, Mannheim, Germany),
which binds to phosphatidylserine exposed on the outer leaflet
of apoptotic cell membranes. Propidium iodide was used for the dis-
identification of necrotic cells from the annexin V positively
stained cell clusters. Cells were trypsinized, washed with PBS,
centrifuged at 200 g for 5 min, and resuspended in 100 μl An-
nexin-V–Fluos labeling solution containing 20 μl Annexin-V–
Fluos labeling reagent in 1,000 μl H epes buffer (10 mM H epes/
N aOH, pH 7.4, 140 mM NaCl, 5 mM CaCl2) and 20 μl propid-
ium iodide. Cells were incubated for 10–15 min and analyzed on
a flow cytometer using CellIQ ues software. A 488-nm excitation
and a filter >560 nm for propidium iodide detection were used.

Detection of the CD95 (APO-1/Fas) Receptor. Cell surface
expression of the CD95 receptor was assessed by FACScan.
Anti–APO-1 (IgG3, κ) was used as purified biotinylated antibody.
Quantum Red streptavidin (Sigma Chemical Co.) was used as
secondary reagent for indirect immunofluorescence. Hepatoma
cells were incubated in 50 μl culture medium with biotinylated
anti–APO-1. After 30 min incubation, cells were washed twice,
incubated for 30 min with Quantum Red streptavidin, washed
again twice, and assayed. Upon data acquisition a gate was set on
intact cells by forward/side scatter analysis, and 104 viable cells
were analyzed. Percent enhanced CD95 expression was cal-
culated according to the formula (% CD95+– treated cells – %
Quantum Red+– treated cells) – (% CD95– control cells – %
Quantum Red+– control cells).

Detection of CD95 mRNA Expression by PCR. Total cellular
RNA was prepared from 3 × 106 cells treated with different anti-
cancer drugs, using the R N a eKy Kit (QIAGEN GmbH, Hilden,
Germany) according to the manufacturer’s instructions. Expression
of β-actin (MWG Biotech Gmbh, Ebersberg, Germany) was
used as an internal standard for R NA integrity and equal gel
loading. 1 μg of total cellular R NA was retrotranscribed after
heat denaturation (3 min, 60°C) and annealing with oligo (dT)
primers (16–mer; Perkin Elmer, W eiterstadt, Germany) in the
presence of 75 U M nLV RT (Perkin Elmer), 67 μM MgCl2, and
63 μM of each dNTP in 20 μl for 45 min at 42°C. Reactions
were stopped by heat inactivation for 5 min at 90°C. Aliquots of
10 μl of the cDNA were then amplified in a DNA thermoden-
cycler (Stratagene Inc., Heidelberg, Germany) with 2.5 U of AmpI Taq
DNA polymerase AS (Perkin Elmer), 10 μM of both upstream
and downstream APO-1 primers, and 2.5 μM of both upstream
and downstream human β-actin primers in a 50 μl vol. Each
PCR cycle consisted of a denaturation step (94°C, 1 min), an
annealing step (56°C, 1 min), and an elongation step (72°C, 1 min).
The primers used for amplification of the CD95L have been de-
scribed (54). The PCR products were analyzed on a 1.4% T BE
(Tris-borate–EDTA) agarose gel.

Cytoxicity Assay: MTT Assay. The MTT assay is a colori-
metric assay based on the ability of the viable cells to reduce a sol-
uble yellow tetrazolium salt (MTT) to blue formazan crystals. A
100-μl suspension of 7 × 105 cells was added to each well of 96-
well plates 24 h before the assay. Various concentrations of the
different anticancer drugs, IgG3 anti–APO-1, and F(ab)2 anti-
APO-1 fragments were added to the cells. After 3–72 h, 5 mg/ml of
MTT dye was added and the plates were incubated for 12 h. OD
was determined by eluting the dye with isopropanol/formic
acid, and absorbance was measured at 540 nm.

Results are shown as the mean of data from six independent
wells ± SD. To rule out that drug-treated cells may simply arrest
growth, making it difficult to determine what percentage of the
reduced MTT is due to apoptosis, all cytotoxicity assays were
verified by FACScan analysis using the method of N icoli et al.
(53) to assess the subdiploid DNA content and specific apoptosis.

Immunoselection of p53-binding DNA Fragments. DNA of cosm id
cap-1 (55) was digested to completion with Sau3A1. The mix-
ture of small DNA fragments was ligated into the BamH I site of
pBlueScript II KS+, and the ligated products were transformed
into E. coli strain DH1. Transformants were selected in liquid culture
containing ampicillin, and DNA was prepared for the drug-resis-
tant culture. The DNA was taken through three consecutive rounds of p53 immunoselection. The procedure was essentially as
described in Zauberman et al. (56), except that the extracts used
for selection were prepared from H1299 cells transiently trans-
fected with a p53 expression plasmid (pCMVp53twt) and har-
vested 40 h after transfection. At the end of the third selection
cycle, digestion of the enriched plasmid DNA revealed only a single
insert band of 0.7 kb. Single colonies were obtained from the en-
riched plasmid population, the corresponding plasmids were ex-
tracted, and each was confirmed for the presence of the expected
0.7-kb insert. The insert was then subjected to DNA sequencing,
and used for construction of luciferase-based reporter plasmids.

Plasmids. Plasmid pCMVp53wt, encoding mouse wt p53, has
been described previously (40). Plasmid CD95(Ps)-luc was con-
structed by ligating a 1.71-kb HindIII–SacII fragment of cosm id
cap-1 (55), containing the 1.43-kb 3′ end of the human CD95
gene promoter and the 5′ end of exon 1 (0.28 kb) (57), into
pGL3-Basic (Promega Corp., Madison, WI) or into pTATA-Luc (a gift of T. Wirth, Institut für Medizinische Strahlen- und Zell-
forschung, Würzburg, Germany). Plasmid CD95(P)-luc, contain-
ing an extended 5′ version of the CD95 promoter (1.9 kb) and
the 5’ end of exon 1 (0.28 kb) (57), was constructed by cloning a 450-bp PCR fragment of the 5’ part of the CD95 promoter (58) in the HindIII site of CD95(Ps)-luc. Orientation of the PCR fragment and sequence was checked by sequencing. Plasmid CD95(I+SV)-luc was constructed by ligating a 0.7-kb CD95 gene fragment of intron 1 (excised from the pBluescript vector with SacI + SalI) between the SacI and XhoI sites of pGL3-Pro-

**Table 1.** Induction of CD95 and A poptosis by A nticancer D rugs in C eell Lines with wt p53

<table>
<thead>
<tr>
<th>Drug*</th>
<th>M echanism of ac tion</th>
<th>Concentration tested for CD95 receptor induction</th>
<th>CD95 receptor induction, increase above controls</th>
<th>Increased responsiveness towards induction of apoptosis by CD95 receptor stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorouracil</td>
<td>Pyrimidine antagonist</td>
<td>2.5–2,500 μg/ml %</td>
<td>5–62§</td>
<td>46–55</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Folic acid antagonist</td>
<td>0.25–2,500 μg/ml %</td>
<td>8–65§</td>
<td>28–70</td>
</tr>
<tr>
<td>M itomycin</td>
<td>Alkylation</td>
<td>0.01–100 μg/ml %</td>
<td>3–56§</td>
<td>0–53</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Alkylation</td>
<td>0.1–100 μg/ml %</td>
<td>0–60**</td>
<td>0–64</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Alkylation</td>
<td>0.001–100 μg/ml %</td>
<td>28–40**</td>
<td>39–45</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>Intercalation</td>
<td>0.0001–1 μg/ml %</td>
<td>8–52‡‡</td>
<td>43–51</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Intercalation</td>
<td>0.002–2 μg/ml %</td>
<td>– §§</td>
<td>18–43</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Inhibits topoisomerase II</td>
<td>0.001–1 μg/ml %</td>
<td>22–55‡‡</td>
<td>45–62</td>
</tr>
<tr>
<td>Bleomycin**</td>
<td>Inhibits DNA polymerase</td>
<td>0.3–3,000 μg/ml %</td>
<td>15–78‡‡</td>
<td>27–73</td>
</tr>
</tbody>
</table>

* Anticancer drugs with different mechanisms of action were analyzed for their ability to induce the CD95 receptor. Different solid human cancer cell lines (HepG2, Hs746T, AGS, MCF7, Huh7, and HT29) were treated with the specific anticancer drug for 48 h.  
†A wide range of drug concentrations were tested. Clinically relevant concentrations of the chemotherapeutic drugs are marked with an asterisk in Fig. 2.  
‡Treatment with 5-fluorouracil, methotrexate, mitomycin, cisplatin, mitoxantrone, doxorubicin, etoposide, cyclophosphamide, and bleomycin led to upregulation of the CD95 receptor in HepG2 cells (wt p53), in AGS cells (wt p53), in Hs746T cells (wt p53), and in MCF7 cells (wt p53). No induction or only weak induction of the CD95 receptor was observed in cell lines with mutant p53 (Huh7 and HT29) and in cell lines lacking p53 (Hep3B). Cell surface expression of the CD95 receptor was assessed by FACScan®. Percent CD95 induction was calculated as (% CD95+ treated cells – % CD95+ control cells – % CD95+ control cells).  
§The upregulation of the CD95 receptor by cytostatic treatment is functional. It provides the cell with an increased responsiveness towards CD95-mediated apoptosis. Hepatoma cells with different p53 mutational status were treated with diverse anticancer drugs alone (for 48 h) and in combination with or without the agonistic apoptosis-inducing antibody IgG3 anti–APO-1, 100 ng/ml, for an additional 24 h. The rate of cell death was assessed by cytotoxicity assay (MTT). Data are expressed as 100 – % of the living cells treated with the specific anticancer drug only. A balanced two-way ANOVA revealed a synergistic interaction between anti-APO-1+ and chemotherapy-induced cell death for all the drugs tested (P < 0.0001, tested in HepG2).  
¶Range of data from tests in HepG2, Hs746T, AGS, and MCF7.  
**Could not exactly be quantified by FACScan® due to autofluorescence of doxorubicin.  
††Range of data from tests in HepG2.  
‡‡These data have been published in part (for HepG2) in reference 6.
phosphamide, and bleomycin led to upregulation of CD95 mRNA (6) and of the CD95 receptor protein in HepG2 cells (wt p53), in AGS cells (wt p53), in HST46T cells (wt p53), and in MCF-7 cells (wt p53) (Table 1, and Figs. 1 and 2). In contrast, no induction or only weak induction of the CD95 receptor was observed in Huh7 and HT29 cells with mt p53, and in Hep3B cells lacking p53 altogether. All drugs tested induced the CD95 receptor in cells with wt p53 independent of their mechanism of action (Table 1). A wide range of drug concentrations were tested, and it is of note that clinically relevant concentrations were effective in upregulation of the CD95 receptor with all drugs tested.

Increased Responsiveness Towards Induction of Apoptosis by Anti-APO-1 Antibodies

Upregulation of CD95 receptor expression after cytostatic treatment resulted in increased responsiveness towards CD95-mediated apoptosis. Hepatoma cells with different p53 mutational status were treated with diverse anticancer drugs alone or in combination with the agonistic apoptosis-inducing antibody IgG3 anti-APO-1. In HepG2 cells, treatment with anticancer drugs for 48 h followed by stimulation of the CD95 receptor with anti–APO-1 resulted in induction of apoptosis in up to 70% of the cells, dependent on the concentration and the particular drug applied (Fig. 3). Thus, a statistically significant (P < 0.0001) synergy in induction of apoptosis by anticancer drugs and by anti–APO-1 was seen for all the drugs tested (Table 1).

Induction of CD95L by Anticancer Drugs

CD95L mRNA was induced upon cytostatic treatment in HepG2 cells (wt p53) as well as in Hep3B (p53−/−) and Huh7 cells (mt p53). Thus, cytostatic treatment stimulated CD95L mRNA expression in different hepatoma cell lines independent of the p53 status of the cells. The ability to induce CD95L mRNA expression occurred regardless of the...
mechanism of action of the anticancer drug (Fig. 4). However, it is of note that there was a high variability in the extent of CD95L induction dependent on the chemotherapeutic agent used and the cell line tested. Furthermore, anticancer drugs with clinically known therapeutic effectiveness for the specific tumor were observed to be most effective in CD95L induction, e.g., mitoxantrone in hepatomas and hepatoblastomas (Fig. 4) or 5-fluorouracil in cell lines from colorectal cancer (data not shown).

These data are supported by the fact that CD95L mRNA was highly elevated upon bleomycin treatment of Hep3B cells stably transfected with the temperature-sensitive mutants p53ala143 or p53val135. CD95L mRNA expression was induced in HepG2 (wt p53), Hep3B (p53<sup>−/−</sup>), and Huh7 cells (mt p53). Densitometry was performed to analyze CD95L expression in relation to β-actin expression. CD95L mRNA induction was independent of the p53 status of the cells. The extent of CD95L expression upon chemotherapeutic treatment showed variability dependent on the agent and the cell line tested.

The data suggest that cytostatic drugs upregulate both CD95 and CD95L, the former dependent and the latter independent of the p53 status.

**Figure 3.** Increased responsiveness towards induction of apoptosis by CD95 receptor stimulation after treatment with anticancer drugs. Cytotoxicity assay with MTT staining of viable cells. HepG2 cells (wt p53, inverted triangles), Huh7 cells (mt p53, squares), and Hep3B cells (p53<sup>−/−</sup>, diamonds) were treated with different doses of 5-fluorouracil, methotrexate, mitomycin, cisplatin, mitoxantrone, doxorubicin, etoposide, and cyclophosphamide alone for 48 h and in combination with or without IgG3 anti–APO-1, 100 ng/ml, for an additional 24 h. Data are expressed as the fraction of living cells treated with specific anticancer drug only (mean ± SD, n = 6 wells). Only HepG2 cells with wt p53 exhibited an increased responsiveness towards induction of apoptosis by agonistic anti–APO-1 antibodies after cytostatic treatment. Anti–APO-1 treatment did not induce further toxicity in Huh7 cells (mt p53) or Hep3B cells (p53<sup>−/−</sup>). *By MANOVA, between-subject effect P < 0.0001 compared with Hep3B, P < 0.0001 compared with Huh7.

**Figure 4.** Induction of the CD95L by cytostatic drugs with different mechanisms of action. Semiquantitative PCR analysis of CD95L mRNA expression in HepG2, Hep3B, and Huh7 cells upon treatment with cyclophosphamide (cyclo), 5-fluorouracil (5-FU), doxorubicin (doxo), mitomycin (mitom), mitoxantrone (mitox), and actinomycin (actino). CD95L mRNA expression was induced in HepG2 (wt p53), Hep3B (p53<sup>−/−</sup>), and Huh7 cells (mt p53). Densitometry was performed to analyze CD95L expression in relation to β-actin expression. CD95L mRNA induction was independent of the p53 status of the cells. The extent of CD95L expression upon chemotherapeutic treatment showed variability dependent on the agent and the cell line tested.

**Figure 5.** Induction of the CD95L by bleomycin (bleo) in the temperature-sensitive (ts) mutants p53ala143 and p53val135. Semiquantitative PCR analysis of CD95L mRNA expression in Hep3B cells stably expressing the temperature-sensitive mutant p53ala143 or p53val135. CD95L mRNA expression was induced in Hep3B cells (mt p53) at both temperatures, independent of their p53 mutational status.
Restitution of wt p53 Induces the CD95 Receptor

Our data show a strong correlation between wt p53 status and induction of CD95 receptor expression in cancer cells exposed to chemotherapeutic drugs. This relationship was further characterized using Hep3B cells transfected (a) transiently with wt p53 cDNA, (b) stably with a tamoxifen-regulated p53–estrogen receptor chimera, (c) stably with the temperature-sensitive mutant p53val135, and (d) stably with the temperature-sensitive mutant p53ala143.

Transient transfection with wt p53 cDNA. Cells transfected with wt p53 cDNA responded with upregulation of the CD95 receptor upon bleomycin treatment. In contrast, bleomycin treatment did not lead to an upregulation of the CD95 receptor in mock-transfected Hep3B cells (Fig. 6).

Stable transfection with a tamoxifen-regulated p53–estrogen receptor chimera. Hep3B cells stably transfected with a p53–estrogen receptor chimera (BT-4P) were treated with 100 nM 4-OH tamoxifen for 24 h to activate p53. BT-2E control cells transfected with the puromycin resistance gene only were treated with bleomycin for 48 h. Induction of p53 upon tamoxifen treatment led to upregulation of the CD95 receptor from 0–8% of the cells. When bleomycin treatment was also included, the CD95 receptor expressing cells increased to 30% (Fig. 7A). In contrast, BT-2E cells did not exhibit CD95 receptor expression either before or after tamoxifen treatment (Fig. 7B). Significantly, upregulation of CD95 receptor expression after reconstitution of wt p53 activity was of functional consequence. Thus, stimulation of the upregulated CD95 receptor in BT-4P cells with the agonistic antibody IgG3 anti–APO-1 resulted in greatly enhanced apoptosis (Fig. 7C).

Stable transfection with the temperature-sensitive p53 mutants. At 37°C p53val135 and p53ala143 are in an mt conformation and can transform cells through a negative dominant mechanism, whereas at the permissive temperature of 32°C they become transcriptionally active and regain wt activity. 40% of Hep3B cells stably transfected with the temperature-sensitive mutant p53val135, exhibited CD95 receptor expression at 32°C (wt p53 conformation). In contrast, at 37°C (mt p53 conformation) no CD95 receptor expression could be detected. Addition of bleomycin led to a further increase in CD95 receptor expressing cells up to 70% (Fig. 8A). Likewise, Hep3B cells stably transfected with the temperature-sensitive mutant p53ala143 only showed upregulation of the CD95 receptor at the permissive temperature. Upon addition of bleomycin, CD95 receptor-expressing cells increased to 30% (Fig. 8B). Taken together, these data support the conclusion that wt p53 activity is essential for drug-induced CD95-mediated apoptosis. Furthermore, these data prompted us to investigate the molecular basis for the regulation of CD95 gene expression by p53.

A analyse of putative p53-response Elements in the CD95 Gene

To further investigate whether p53 interacts directly with the CD95 gene, we first performed a computer search for potential p53-responsive elements within the promoter of the CD95 receptor gene. This search indicated the presence of three putative elements within the CD95 promoter which showed limited homology with the p53 consensus binding site (63). However, it is of note that each of these putative elements diverges from the consensus in at least one of the positions corresponding to critical DNA-protein contact residues, questioning their ability to function as effective p53 response elements (Fig. 9, A and C). Therefore, a more comprehensive, experimental approach was undertaken to search for possible p53-binding elements within the CD95 gene. A recombinant cosmid comprising the entire human CD95 locus (cAP0-1 [55]) was subjected to an immunoselection protocol, which specifically enriches DNA fragments containing high-affinity p53-binding sites (56). This selection procedure resulted in efficient enrichment of a single p53-binding plasmid clone, carrying a human genomic DNA insert of 0.7 kb (data not shown); no other clones were enriched, suggesting that the isolated
clone may represent the only high-affinity p53-binding element within the CD95 genomic cosm id.

The sequence of the first 266 nucleotides of the cloned 0.7-kb insert is shown in Fig. 10A. It is derived from within the first intron of the human CD95 gene; its 5' end is located 142 nucleotides downstream of the 3' end of exon 1 (Fig. 10B). The cloned DNA fragment contains a stretch of 20 contiguous nucleotides (underlined) exhibiting a high degree of homology (18/20, including full conservation of all critical core nucleotides) with the p53 consensus binding site (63; Fig. 10C).

The CD95 p53-binding element confers transcriptional activation by p53, and cooperates with the CD95 promoter to test whether the intronic p53-binding fragment of the CD95 gene can mediate p53-dependent transcriptional activation, several luciferase-based reporter plasmids were constructed and assayed by transient transfection. A schematic drawing of the relevant plasmids used for this purpose is presented in Fig. 11. In plasmid CD95(Ps)-luc the luciferase gene is preceded by the 1.43-kb CD95 promoter and the 5' end of exon 1 (57; HindIII-SacII fragment, see Fig. 10B). In plasmid CD95(I1SV)-luc, the 0.7-kb CD95 intronic DNA fragment is positioned in front of a minimal SV40 promoter. CD95(Ps1I)-luc contains the CD95 promoter upstream of the CD95 intronic region.

The CD95 receptor is inducible only at the permissive temperature, 32°C. In the temperature-sensitive mutant p53val135, temperature down-shift induces the CD95 receptor in up to 40% of the cells; additional treatment with bleomycin leads to CD95 expression in up to 70% of the cells (A). Likewise, in the temperature-sensitive mutant p53ala143, CD95 receptor expression increased to 30% of the cells upon temperature down-shift and additional bleomycin treatment (B).
Figure 11. The p53-binding intronic CD95 region confers p53-dependent transcriptional activation. (Left) Schematic diagram of relevant luciferase reporter constructs used for transcriptional analysis. Ps, 1.43-kb CD95 promoter region (reference 57); P, 1.9-kb CD95 promoter region with the three putative (computer-identified) p53-binding sites (reference 58); I, CD95 0.7-kb intronic region selected for p53 binding; SV, SV40 minimal promoter; Luc, luciferase gene; p53 BE, p53-binding element. Right) Analysis of p53-dependent luciferase activity. Hep3B cells were transfected with 1 μg of each of the indicated reporter plasmids together with 100 ng of either a wt p53 expression plasmid, pCMVp53wt, or an equivalent amount of empty vector. Shown is the fold p53-dependent activation of each reporter plasmid, calculated relative to the value obtained with the same reporter in the absence of p53. These results were supported by data obtained in H1299 human lung cancer carcinoma cells (Fig. 11). These results were supported by data in a different cellular context, in H1299 human lung adenocarcinoma cells (see legend to Fig. 11). A similar lability to confer p53-dependent transcriptional activation was also provided when a shorter version of the intronic region was placed downstream of the CD95 promoter (Ps+Isv, 34-fold activation). In addition, the effect of p53 was specific for CD95, and was not exerted on the RSV promoter, which does not contain any p53-responsive elements (0.95-fold activation). Furthermore, the extent of stimulation by p53 (fold activation) of the CD95 promoter plus intronic region was comparable to that seen with a natural p53-responsive promoter derived from the cyclin G gene (36-fold activation).

Taken together, these data imply that the first intron of the CD95 gene harbors a p53-responsive enhancer element. This element cooperates preferentially with its authentic promoter, and less so with an irrelevant viral promoter. Hence, cooperativity exists between this intronic p53 response element and one or more elements residing within the promoter region of the CD95 gene.

Discussion

The data in this paper show that the CD95 system plays a general role in induction of cytotoxicity by anticancer drugs in a variety of cells of different histotype. Clinically relevant concentrations of diverse anticancer drugs such as cisplatin, bleomycin, methotrexate, doxorubicin, cyclophosphamide, etoposide, and mitoxantrone induce CD95 receptor expression in hepatoma, gastric cancer, colon cancer, and breast cancer cell lines, thereby strongly increasing the sensitivity towards CD95-induced apoptosis. Thus, chemotherapy may sensitize tumor cells by upregulating expression of death regulators such as the CD95 receptor. Most notably, drug-induced upregulation of the CD95 receptor is dependent on the p53 status of the tumor cell. Upregulation of the CD95 receptor occurred only in cell lines with intact p53. This upregulation is dependent on accumulation of endogenous p53 after DNA damage and can be reconstituted in p53 null cell lines by exogenous wt p53 transfection. Caspase-8 cleavage was observed in cell lines of solid human tumors upon treatment with anticancer drugs, irrespective of whether or not apoptosis was dependent on the CD95 system (data not shown). Hence, additional effector pathways besides CD95/C5D95L signaling are likely to contribute to drug-induced apoptosis. We further show for different solid tumors that anticancer drugs at concentrations measured during chemotherapy in patients' sera lead to upregulation of both the CD95 receptor and CD95L. Upregulation of CD95L upon treatment with anticancer drugs was demonstrated in cell lines containing either wt p53, mt p53, or no p53 at all. Thus, the regulation of CD95L clearly involves p53-independent mechanisms.

Upregulation of the CD95 receptor might render a tumor cell chemotherapy sensitive (and sensitive towards CD95L-expressing antitumor T cells). Upregulated CD95L might bind to an increased number of CD95 receptors. On the contrary, drug resistance could result from downregulation of the CD95 receptor. In fact, we have previously demonstrated loss of CD95 receptor and gain of CD95L expression in hepatocellular carcinomas in patients (64).

Constitutive CD95L expression has also been shown for melanoma (65), lung cancer (66), and colon cancer (67). In addition, cytostatic drug treatment can induce production of CD95L. However, drug-induced expression of CD95L in CD95 receptor-negative tumors cannot be effective and, furthermore, could result in selective elimination of antitumor lymphocytes. Thus, chemotherapy might render the tumor an immune-privileged site. Invading T cells, normally engaged in destruction of the tumor tissue, might be eliminated through CD95L produced by the target cells. There are several reports demonstrating that CD95L might be useful for creating immune-privileged tissues in the con-
text of tumor immune privilege and organ and tissue transplantation (68–71). On the other hand, CD95L-expressing fibroblasts transplanted into nude mice were readily rejected as a result of massive neutrophil infiltration of the graft (72). This demonstrates that CD95L expression might not always support survival of the respective tissue. On the other hand, tumor cells that express the CD95 receptor upon induction by anticancer therapy may be turned into susceptible targets for killer cells. Clinical outcome, i.e., death or survival, might be determined by a balance between CD95 receptor and CD95L expression on tumor and immune cells. Upregulation of the CD95 receptor may prepare the tumor cells to be eliminated by the immune system using a CD95-dependent pathway. Thus, in addition to their direct cytotoxic effects, chemotherapeutic drugs sensitize tumor cells to CD95-mediated cytotoxicity and CD95-dependent immune clearance.

Most importantly, our data suggest a potential mechanism by which p53-dependent cell death may be mediated. We show a strong correlation between p53 status and induction of CD95 receptor in response to DNA damage. Both DNA damage and transfection/restitution of wt p53 caused induction of the CD95 receptor, and DNA damage failed to induce the CD95 receptor in tumor cells with mt or deficient p53. The hypothesis that the CD95 gene is p53 regulated was further confirmed in several systems with stably transfected inducible p53.

To investigate if p53 directly transactivates the CD95 gene in a manner similar to its effects on CDKN1A (23–26), gadd45 (20, 73), mdm2 (56, 74), cyclin G (59, 75), and BAX (27), we looked for p53 response elements in the CD95 gene. Computer analysis suggested three potential p53 response elements within the CD95 promoter. However, the CD95 promoter alone was only minimally stimulated by wt p53, and activation of no more than twofold was obtained. On the other hand, direct cloning of p53-binding elements from the entire human CD95 locus yielded a DNA fragment containing a strong p53-binding site. This site, derived from the first intron of the human CD95 gene, displays a high degree of homology with the p53 consensus binding site (63). When the CD95 promoter was then placed in conjunction with this p53-binding DNA region, transcriptional activity became strongly stimulated (up to 50-fold) in transfected hepatoma and lung carcinoma cells. This suggests a cooperativity between the intronic element and putative p53 response elements within the CD95 promoter. Further evidence for such cooperativity is provided by the observation that the intronic CD95 element is far less potent in conferring p53-responsiveness in conjunction with an irrelevant SV 40 promoter.

Interestingly, there is a remarkably high similarity between the two adjacent 10-mer half-sites of this putative p53-binding element; only the first and tenth nucleotides are not identical between the two half sites. This argues for the possibility that the CD95 intronic p53-binding site may be recognized by a specific form of p53 with distinct binding site preferences.

The observation that the CD95 gene can only be activated by wild-type but not by the p53 mutants tested would also argue for a differential regulation of the CD95 gene by wt and mt p53. We hypothesize that wt p53 can stimulate CD95 gene transcription, whereas the mt p53 protein or specific mutants of p53 protein fail to function properly in transcription of the CD95 gene. A differential regulation by wt and mt p53 is also known for the IGFR (insulin-like growth factor I receptor) promoter. Wt p53 has the potential to suppress the IGFR promoter, whereas mutant versions of the p53 protein can derepress the IGFR promoter (76). Further studies are needed in view of the more than 150 different p53 mutations described in human cancers so far to characterize the kind of mutations that lead to loss of the ability of p53 to act as a transcription factor of the CD95 gene.

The fact that wt p53 has a stimulatory effect on CD95 gene activity, whereas the investigated mt p53 proteins are not capable of activating the CD95 gene, may explain why mutations in p53 contribute to tumor progression and resistance of cancer cells to chemotherapy. Clinical studies will have to show if p53 and CD95 status are correlated in primary human tumors and if treatment response to cancer chemotherapeutic drugs and prognosis are dependent on p53 and the CD95 apoptotic pathway. Preliminary data from ongoing clinical studies in patients with hepatocellular carcinoma show that the majority of the tumors with wt p53 express the CD95 receptor, whereas only a minority of the hepatocellular carcinomas with mutant p53 display CD95 expression (our unpublished observations).

Furthermore, the ability of wt p53 to activate CD95 gene expression and confer sensitivity to CD95-mediated apoptosis in Hep3B cells may have implications for wt p53 gene therapy. Our data suggest that upregulation of the CD95 receptor contributes to the apoptosis-inducing effect of wt p53. These findings may provide an explanation for the tumor regression observed in non-small cell lung cancer patients after wt p53 gene transfer (77). Upregulation of the CD95 receptor in CD95L-positive tumors may account for the increased rate of apoptosis reported to occur in these tumors after wt p53 gene transfer. CD95L expression may have been acquired during the course of tumor development as an immune-privileged site or may have been induced in the tumor cells upon cytostatic treatment. Thus, wt p53 gene transfer and chemotherapy could synergize in their action on CD95 receptor and CD95L expression and induce autocrine and paracrine apoptosis. A synergistic effect of wt p53 gene transfer and chemotherapeutic treatment on induction of apoptosis has already been reported in vitro for lung cancer cell lines (77–79). Our data provide evidence that activation of the CD95 system may be the molecular basis for this enhancement of apoptosis. Thus, reconstitution of p53 function with or without combined chemotherapy is an attractive goal for somatic gene therapy in cancer.

We have shown that p53 directly transactivates the CD95 gene. A critical question is whether p53-induced apoptosis is functionally dependent on the induction of the
CD95 gene. With the identification of killer/DR5 (80) as another pathway of p53-dependent apoptosis after DNA damage, it is evident that p53-dependent apoptosis is not mediated solely by the CD95 system. The observation that p53-dependent cell death after DNA damage is mediated by TNF-R family members could have significant implications for manipulating apoptosis and therapy.

Interference of anticancer drugs with apoptosis pathways can take place at several levels, including accumulation of wt p53 protein, triggering of CD95L–CD95 receptor interaction, triggering of other death receptors, stimulation of signaling cascades, and activation of death effector molecules including caspases, thus influencing the balance of proapoptotic and antiapoptotic programs. Therefore, drug sensitivity and resistance will likely be affected by alterations of any of these components of the apoptosis pathways.

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References


clonal antibody anti-APO-1 class switch variants is dependent on cross-linking of APO-1 cell surface antigens. J. Immunol. 149:3166–3173.


dependent and -independent antigen receptor-induced apoptosis in human T and B cell lines. Int. Immunol. 7:1873–1877.

3062.


57. Wada, N., M. Matsumura, Y. Ohba, N. Kobayashi, T. Taki
zawa, and Y. Nakanishi. 1995. Transcription stimulation of the cyclin G gene contains two distinct p53 binding

dependent and -independent antigen receptor-induced apoptosis in human T and B cell lines. Int. Immunol. 7:1873–1877.


60. O'Connell, J., G.C. O'Sullivan, J.K. Collins, and F. Shana


597.


597.