The role of p53 and cell death by apoptosis and necrosis in 4-hydroperoxycyclophosphamide-induced limb malformations

S. Adel Moallem and Barbara F. Hales*

Department of Pharmacology and Therapeutics, McGill University, Montréal, QC, H3G 1Y6, Canada

*Author for correspondence (e-mail: BHALES@pharma.mcgill.ca)

Accepted 26 May; published on WWW 21 July 1998

SUMMARY

The exposure of embryonic murine limbs in vitro to an activated analog of cyclophosphamide, 4-hydroperoxycyclophosphamide (4OOH-CPA), induced limb malformations and apoptosis. The purpose of this study was to investigate the role of the tumor suppressor/cell cycle checkpoint gene, p53, and of cell cycle arrest in the response of the limbs to cyclophosphamide. Limbs, excised on day 12 of gestation from wild-type, heterozygous or homozygous p53-knockout transgenic murine embryos, were treated with vehicle (water) or 4OOH-CPA (0.3, 1.0 or 3.0 µg/ml) and cultured for 6 days. Exposure of wild-type (+/+) limbs to 4OOH-CPA resulted in limb malformations, and reduced limb areas and developmental scores. The homozygous (−/−) limbs were dramatically more sensitive to the effects of 4OOH-CPA, as assessed by limb morphology, area and score. Heterozygous limbs exposed to the drug were intermediate for each parameter. Apoptosis, as assessed by the formation of a DNA ladder, was increased in drug-exposed wild-type limbs, but not in the drug-exposed homozygous limbs. Light and electron microscopy examination of the limbs revealed that drug treatment of wild-type limbs induced the morphological changes typical of apoptosis, particularly in the interdigital regions. In contrast, there was no evidence of apoptosis in homozygous limbs exposed to 4-OOH-CPA; morphological characteristics of necrosis such as cell membrane breakdown, mitochondrial swelling and cellular disintegration were evident throughout these limbs. Heterozygous limbs had cells dying with the characteristics of both apoptosis and necrosis. Fragments of poly(ADP-ribose) polymerase characteristic of necrosis predominated in the drug-treated heterozygous and homozygous limbs, 4-OOH-CPA-treatment of limbs from wild-type embryos led to arrest of the cell cycle at the G1/S phase. No cell cycle arrest was observed after drug treatment of homozygous limbs, in which populations of cells in S and G2/M phases, as well as a population of sub G1 cells, were found. Thus, the presence of p53 and of p53-dependent apoptosis protect organogenesis-stage limbs from insult with a teratogen. The absence of p53 may decrease DNA repair capacity and contribute to the accumulation of DNA damage in limb cells and their daughter cells; the failure of apoptosis to eliminate cells with DNA damage may result in increased cell death by necrosis and major limb malformations.

Key words: p53, Limb development, Malformations, Apoptosis, Necrosis, Mouse, Cyclophosphamide, Cell cycle, Teratogen

INTRODUCTION

Apoptosis is a form of active cell death that is distinct from necrosis or passive cell death (Kerr et al., 1972; Schwartzman and Cidlowski, 1993). It is characterized morphologically by chromatin condensation, cytoplasmic shrinkage and membrane blebbing. During apoptosis, an endogenous endonuclease is activated that catalyses internucleosomal DNA cleavage to multiple 180-200 bp fragments (Wyllie et al., 1980; English et al., 1989). Apoptotic cell death can be initiated by a number of external signals including cytotoxic anti-cancer drugs (Hickman, 1992), glucocorticoids, irradiation, heat shock and the withdrawal of some growth factors or hormones (Sen, 1992). When the regulation of apoptosis is disturbed, either by inhibition or inappropriate induction, disease can result (Bursch et al., 1992). Apoptotic cell death plays a crucial role in embryogenesis (Sanders and Wride, 1995). In the developing embryo, the spatiotemporal occurrence of apoptosis is precisely controlled and occurs in the neural tube, heart, palate, duodenal mucosa and limb buds (Gerschenson and Rotello, 1992; Wyllie, 1993).

p53, a positive regulator of apoptosis, was one of the first tumor suppressor genes identified (Oren, 1992). While it is not essential in most cells for normal cell cycle regulation, it acts as a cell cycle checkpoint gene that is induced in response to DNA damage. DNA damage causes a rapid increase in total p53 concentrations. This is achieved by increased gene transcription and the stabilization of normally rapidly degraded p53 protein; this increase in p53 leads to the suppression of cell growth or apoptosis (Smith and Fornace, 1996). It has been suggested that p53-mediated cell cycle arrest provides more time for DNA repair to take place. However, recent findings...
suggest that p53 is involved actively in the DNA repair process (Smith et al., 1995; Li et al., 1996). It is not clear how a cell decides whether to arrest growth and repair DNA or to induce apoptosis in response to increased p53. Rowan et al. (1996) reported that, in a p53 mutant cell line, p53 had a direct role in mediating cell-cycle arrest despite the loss of the ability to induce apoptosis. The process of apoptosis may be p53-dependent or independent. Thymocytes from p53-deficient mice are resistant to the induction of apoptosis by radiation, but not by anti-CD3 antibody or glucocorticoids; this observation suggests that the induction of apoptosis by radiation, but not other insults, is p53-dependent (Clarke et al., 1993; Lowe et al., 1993a). E1A, the adenovirus early region, is an oncogene that increases cellular susceptibility to apoptosis, including its induction by anticancer agents (Lotem and Sachs, 1993). Parental embryonic fibroblasts are resistant to low doses of irradiation and several DNA-damaging chemotherapeutic agents (Lowe et al, 1993b). However, treatment of fibroblasts expressing E1A, with the same agents rapidly induces apoptosis, whereas there are no effects on the viability of p53-deficient E1A-expressing cells (Lowe et al, 1993b). Therefore, apoptosis caused by DNA damaging agents has a clearly established dependency on p53.

p53 is expressed extensively during mouse embryogenesis; however, the concentration of transcripts declines dramatically in cells undergoing terminal differentiation (Schmid et al., 1991). Although transgenic p53 ‘knockout’ mice survived to birth, a high incidence of developmental abnormalities was found. Sah et al. (1995) reported that a fraction of the p53-deficient embryos displayed defects in neural tube closure and, subsequently, exencephaly. Studies by Armstrong et al. (1995) revealed that these defects were found predominantly in the female embryos. More information is needed on the role of p53 during development. While the role of p53 in mediating drug teratogenicity is not yet clear, with benzo[a]pyrene as the teratogen, p53 had a ‘teratological suppressor’ function (Nicol et al., 1995). In this study, there was an increase in fetal resorptions and other anomalies in p53 heterozygous and homozygous mice. However, two anomalies, omphalocele and hemiangioendothelioma (red nevis of the skin) were decreased in heterozygous mice compared to the wild type; these investigators suggested that these two anomalies might involve a cellular mechanism mediated by apoptosis. Norimura et al. (1996) found that radiation caused an increase in embryonic malformations in p53-homozygous mice; however, wild-type embryos had higher incidence of death. This reciprocal relationship of radiosensitivity to anomalies and to embryonic or fetal lethality supports the notion that embryonic or fetal tissues have a p53-dependent ‘guardian’ that aborts cells bearing radiation-induced teratogenic DNA damage. One explanation for these results is that, in wild-type embryos, p53-dependent apoptosis suppresses DNA damage-induced malformations by removing teratogen-injured cells. However, there is no clear explanation for the higher rate of death in these embryos. In another study, with a different teratogen, the presence of p53 was necessary for 2-chloro-2’dioxyadenosine exposure to induce eye defects (Wubah et al., 1996); p53-dependent apoptosis played a critical role in these eye defects as wild-type (+/+ ) embryos were more susceptible than the homozygous (−/) knockout embryos lacking p53. Taken together, these studies support the suggestion that p53 has as its primary function the coordination of multicellular adaptive responses to diverse environmental stresses (reviewed in Hall and Lane, 1997).

Limb development has been used extensively to study growth, differentiation, patterning and cell death during development (Zakeri and Ahuja, 1994; Cohn and Tickle, 1996; Hurle et al, 1996; Serrano and O’Farrell, 1997). Apoptosis occurs predominantly in the interdigital areas and apical ectodermal ridge during normal limb development (Scott, 1979). Interference with this process by chemicals may be an important mechanism of action of teratogens that induce limb malformations (Alles and Sulik, 1989; Kurishita, 1989; Moallem and Hales, 1995, 1996). Exposure to cyclophosphamide during organogenesis results in a spectrum of malformations that includes exencephaly or hydrocephaly, open eyes, cleft palate, kinky tails and limb anomalies such as phocomelia, adactyly, oligodactyly, syndactyly and polydactyly (Gibson and Becker, 1968; Hales, 1981). These effects are associated with induction of apoptosis by cyclophosphamide in the developing embryo. Although a causal relationship was not established, it is likely that cyclophosphamide disturbs physiological apoptosis, leading to embryo and limb malformations. We have shown that 4-hydroperoxycyclophosphamide (4OHP-CPA), a preactivated analog of cyclophosphamide, induced concentration and time-dependent limb reduction malformations and apoptosis in the interdigital areas of cultured murine limbs (Hales and Jain, 1986; Moallem and Hales, 1995). Although it is well known that DNA, RNA and protein are alkylated by metabolites of cyclophosphamide (phosphoramide mustard and acrolein), DNA is assumed to be the primary target; DNA cross-linking leads to cytotoxicity and may be important also in mediating the action of this drug as a teratogen (Mirkes, 1985).

The purpose of the present study was to investigate the role of p53 in mediating cyclophosphamide-induced limb malformations. Limbs from p53-transgenic knockout, heterozygous and wild-type mice were cultured in the absence or presence of 4OHP-CPA and examined for limb malformations, the presence of cell death by apoptosis or necrosis and cell cycle stage.

MATERIALS AND METHODS

Limb bud culture and p53-genotype
p53-knockout heterozygous C57BL/6 mice (+/−) were purchased from Taconic Farms, Inc. (Germantown, NY, USA), and bred to form a colony at the McIntyre Animal Centre (McGill University, Montréal, QC). Mice (20-25 grams) were mated between 8 and 10 am (day 0 of gestation). On day 12 of gestation, between 8 and 10 am, females were killed by cervical dislocation, uteri were removed and the embryos were dissected in sterile Tyrode’s saline solution; the embryos at this time were 5-6 mm in length with about 42 somite pairs. Forelimbs were excised just lateral to the somites and cultured in a chemically defined medium, as previously described (Kwasigroch et al., 1984). Time were 5-6 mm in length with about 42 somite pairs. Forelimbs were dissected in sterile Tyrode’s saline solution; the embryos at this time were 5-6 mm in length with about 42 somite pairs. Forelimbs were excised just lateral to the somites and cultured in a chemically defined medium, as previously described (Kwasigroch et al., 1984). Mice (20-25 grams) were mated between 8 and 10 am (day 0 of gestation). On day 12 of gestation, between 8 and 10 am, females were killed by cervical dislocation; uteri were removed and the embryos were dissected in sterile Tyrode’s saline solution; the embryos at this time were 5-6 mm in length with about 42 somite pairs. Forelimbs were excised just lateral to the somites and cultured in a chemically defined medium, as previously described (Kwasigroch et al., 1984). Mice (20-25 grams) were mated between 8 and 10 am (day 0 of gestation). On day 12 of gestation, between 8 and 10 am, females were killed by cervical dislocation; uteri were removed and the embryos were dissected in sterile Tyrode’s saline solution; the embryos at this time were 5-6 mm in length with about 42 somite pairs. Forelimbs were excised just lateral to the somites and cultured in a chemically defined medium, as previously described (Kwasigroch et al., 1984). Mice (20-25 grams) were mated between 8 and 10 am (day 0 of gestation). On day 12 of gestation, between 8 and 10 am, females were killed by cervical dislocation; uteri were removed and the embryos were dissected in sterile Tyrode’s saline solution; the embryos at this time were 5-6 mm in length with about 42 somite pairs. Forelimbs were excised just lateral to the somites and cultured in a chemically defined medium, as previously described (Kwasigroch et al., 1984). Mice (20-25 grams) were mated between 8 and 10 am (day 0 of gestation). On day 12 of gestation, between 8 and 10 am, females were killed by cervical dislocation; uteri were removed and the embryos were dissected in sterile Tyrode’s saline solution; the embryos at this time were 5-6 mm in length with about 42 somite pairs. Forelimbs were excised just lateral to the somites and cultured in a chemically defined medium, as previously described (Kwasigroch et al., 1984). Mice (20-25 grams) were mated between 8 and 10 am (day 0 of gestation). On day 12 of gestation, between 8 and 10 am, females were killed by cervical dislocation; uteri were removed and the embryos were dissected in sterile Tyrode’s saline solution; the embryos at this time were 5-6 mm in length with about 42 somite pairs. Forelimbs were excised just lateral to the somites and cultured in a chemically defined medium, as previously described (Kwasigroch et al., 1984). Mice (20-25 grams) were mated between 8 and 10 am (day 0 of gestation). On day 12 of gestation, between 8 and 10 am, females were killed by cervical dislocation; uteri were removed and the embryos were dissected in sterile Tyrode’s saline solution; the embryos at this time were 5-6 mm in length with about 42 somite pairs. Forelimbs were excised just lateral to the somites and cultured in a chemically defined medium, as previously described (Kwasigroch et al., 1984). Mice (20-25 grams) were mated between 8 and 10 am (day 0 of gestation). On day 12 of gestation, between 8 and 10 am, females were killed by cervical dislocation; uteri were removed and the embryos were dissected in sterile Tyrode’s saline solution; the embryos at this time were 5-6 mm in length with about 42 somite pairs. Forelimbs were excised just lateral to the somites and cultured in a chemically defined medium, as previously described (Kwasigroch et al., 1984).
mouse p53 exon 6 antisense; and ‘57’ primer (5’ TTTACCGAGCCTGGCGCTCGATGT 3’), a polII from the neo cassette, antisense were used with 35 cycles as follows: 94°C, 1 minute; 62°C, 2 minutes; 72°C, 6 minutes; autoextend 10 seconds per cycle. The resulting 1.2 kb or 0.6 kb band correspond to the + or – alleles, respectively.

**Limb morphology**

After 6 days, the cultures were terminated and limbs (six to seven per bottle) were fixed and stained as previously described (Hales and Jain, 1986). The morphology of the limbs was examined with a dissecting microscope. Two-dimensional limb area was measured by image analysis with Micro-Plan II (Nikkon, Montréal, QC) as an indication of limb growth; limbs were scored for the extent of development in vitro according to the scoring system of Neubert and Barrach (1977). The data were analyzed by two-way ANOVA using the SYSTAT for Windows version 5.05 software program (Systat Inc., Evanston, IL). The level of significance was P≤0.05.

Other limbs were cultured for 24 hours, fixed and processed for light or electron microscope examination. For examination with the light microscope (Leica, Montréal, QC), limbs were fixed with buffered formalin; semithin sections were cut and stained with methylene blue. For the electron microscope studies, limbs were fixed with 3.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours, then washed with buffer and postfixed with 1% osmium tetroxide in cacodylate buffer for 1 hour at 4°C. The samples were dehydrated with ethanol and embedded in Epon at 55°C for 24 hours. Ultrathin sections were cut and examined using a Philips EM410 electron microscope.

**DNA electrophoresis**

DNA was extracted and labeled with 32P for gel electrophoresis (Tilly and Hsueh, 1993). Briefly, limbs were homogenized in liquid nitrogen and digested in a buffer containing proteinase-K. Samples were extracted with phenol:chloroform:isomyl alcohol (25:24:1) and DNA was precipitated with ethanol. RNA was digested with RNase (Boehringer Mannheim Canada, Laval, QC) and the DNA re-extracted. DNA was quantified by absorbance at 260 nm. The 3’ end labeling was carried out by terminal transferase reaction (Pharmacia Biotech Inc., Baie d’Urfé, QC) in a medium containing DNA, [γ32P]-dATP and cobalt chloride. The labeled DNA was precipitated in 0.2× volume 10 M ammonium acetate and 3× volume cold 100% ethanol, washed, air-dried and resuspended in buffer. DNA samples (1 μg each) were loaded onto a 2% agarose gel and separated by electrophoresis for 3.5 hours at 50 V. Gels were dried and autoradiographed.

**Western blot analysis of poly(ADP-ribose) polymerase (PARP) fragmentation**

At the designated times, three to four limbs per group were placed in liquid N2 and stored at −80°C. Limbs were homogenized in the presence of liquid N2 and dissolved in sample loading buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 6M urea; 10% glycerin; 0.003% β-mercaptoethanol). Samples (10 μg protein per lane as determined by Bio-Rad protein spectrophotometric assay) were fractionated by SDS-PAGE in 8% acrylamide gels (Towbin et al., 1979). The blots were blocked with 5% nonfat dried milk in PBS-T (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 7H2O, 1.4 mM KH2PO4, pH 7.4; 0.1% Tween 20) at room temperature for 1 hour and then incubated with 422 PARP antibody (1/1000, kindly provided by Dr Guy Poirier) or the preimmune serum overnight. The blots were incubated with peroxidase-conjugated second antibody for 1 hour, developed with a ECL kit (Amersham Canada Ltd., Oakville, ON) and autoradiographed for <1 hour.

**Flow cytometric analysis**

Limbs were homogenized gently to minimize cellular breakdown and cell suspensions were made by filtering the homogenates through nylon meshes of 100 μm, 50 μm and 10 μm. Approximately 1×106 cells were collected by low speed centrifugation at 23°C and the cell pellet was resuspended in 0.1% sodium citrate and 0.1% Triton X-100. Cells were washed and fixed in 2% paraformaldehyde in PBS for 2 hours. After washing, they were treated with RNase (Boehringer Mannheim Canada, Laval, QC) and stained with propidium iodide (10 μg/ml). Samples were filtered through a 10 μm nylon mesh immediately before analysis by flow cytometry. Fluorescence >570 nm emitted from the propidium iodide-DNA complex in each cell nucleus was measured with a FACScan flow cytometer. (Becton Dickinson Canada Inc., Mississauga, ON). Approximately 10,000 cells were examined for each sample. The percentages of cells within the G1, S and G2/M phases of the cell cycle were determined by analysis with the LYSIS II system. Three replicates were done for each experiment. Data were analyzed by ANOVA and Bonferroni T-test (Glanitz, 1992).

**RESULTS**

**Limb morphology after teratogen exposure is dependent on p53 genotype**

Limbs from wild-type p53 (+/+) and heterozygous (+/-) embryos developed to a similar extent in the control medium (Fig. 1, first row); scapula, long bones and paw skeleton were observed. The majority (80%) of the limbs from p53-deficient homozygous (−/−) embryos also developed normally in the control medium, but approximately 20% were malformed. A malformed p53 (−/−) limb is shown in Fig. 1 (top right panel). Treatment with 4OOH-CPA caused concentration-dependent limb reduction malformations in wild-type limbs (Fig. 1). The effects of 4OOH-CPA on the development of both the long bones and paw skeleton in the +/- limbs was concentration dependent with a dramatic reduction in the formation of these cartilaginous anlagen at the highest drug concentration (3 μg/ml). In the limbs from p53-deficient (−/−) embryos, there was a significant response to 4OOH-CPA even at the lowest concentration, while higher concentrations of 4OOH-CPA (≥1 μg/ml) caused dramatic limb reduction malformations (Fig. 1). Interestingly, the heterozygous limbs (+/−) had an intermediate response to 4OOH-CPA treatment (Fig. 1).

The limb areas and limb developmental scores for each genotype from each treatment group are presented in Fig. 2. The limb areas (Fig. 2A) represent the extent of limb growth; the limb scores (Fig. 2B) describe the degree of limb differentiation (Neubert and Barrach, 1977). The p53 genotype did not affect the limb area in control cultures; a significant (P≤0.05; ANOVA and Bonferroni T-test) 4OOH-CPA concentration-dependent decrease in the areas of limbs from +/- embryos was found. While the areas of limbs from heterozygous embryos were not significantly different from controls, exposure of the limbs from homozygous (−/−) embryos to each concentration of 4OOH-CPA resulted in limb areas that were approximately half of those from the +/- embryos.

As observed for limb area, p53 genotype did not affect the limb scores in control cultures. A 4OOH-CPA concentration-dependent decrease was observed in the scores of limbs from +/- embryos; for each drug concentration, the scores of −/−
limbs exposed to 4OOH-CPA were significantly lower than those from +/+ embryos. The response of the +/− limbs was intermediate, indicating that the presence of a functional wild-type allele of the p53 gene in these limbs has an impact.

**DNA fragmentation pattern**

While previous studies have shown that 4OOH-CPA induces apoptosis in organogenesis-stage murine limbs (Moallem and Hales, 1995), it is not clear whether this apoptosis is p53 dependent. If p53-dependent apoptosis plays a critical role in limb malformations, the p53-deficient limbs should have a decreased, rather than an increased, susceptibility to limb malformations. To determine the p53 dependence of apoptosis in the limb, DNA was extracted from the limbs, quantified by spectrophotometric measurement, labeled with 32P-labeled nucleotide and electrophoresed (Fig. 3). A smear of radioactivity, probably indicative of a low amount of DNA fragmentation, was observed in control limbs from embryos of all genotypes. An increase in ‘DNA ladder’ formation, typical of apoptotic cell death, was observed in 4OOH-CPA-exposed +/+ limbs and in +/− limbs, but not in drug-exposed −/− limbs. Thus, the apoptosis induced by the exposure of limbs during organogenesis to 4OOH-CPA is p53-dependent; 4OOH-CPA failed to induce apoptosis in the limbs from the p53-deficient (−/−) embryos.

**Morphological analysis of the type of cell death in the limb**

To investigate the type of cell death in drug-treated limbs of each genotype, limb sections were examined with the light and electron microscope. Light microscope examination of control limbs showed very few, if any, dying cells (Fig. 4A,C,E); p53 genotype did not affect the occurrence of cell death in these limbs. 4OOH-CPA treatment (3 μg/ml) of the +/+ limbs resulted in an increase in the number of pyknotic cells compared to the control limbs, especially in the interdigital regions (Fig. 4B). More dying cells were found in the limb after drug treatment of the heterozygous limbs (Fig. 4D). Many pyknotic and fragmented cells appeared in 4OOH-CPA-treated −/− limbs (Fig. 4F), not only in the interdigital areas, but throughout the limbs.

The morphology of cells in the limbs from each genotype and treatment group was examined in more detail with the electron microscope (Fig. 5). Most of the cells in the sections of limbs from +/+ embryos cultured in control medium had the condensed chromatin and withdrawn cytoplasm typical of apoptosis (Fig. 5A), after exposure to 3 μg/ml 4OOH-CPA (Fig. 5B) numerous cells had the morphological features of apoptosis; some of these cells were still intact while others were in the process of undergoing phagocytosis. Drug treatment of the heterozygous (+/−) limbs induced a further increase in the numbers of dying cells (Fig. 5D), compared to their control counterparts (Fig. 5C). Some cells had the condensed chromatin and intact plasma membrane typical of apoptosis while others had disintegrated cellular structures, suggesting that they were dying by necrosis as well as by apoptosis. Examination of sections from the 4OOH-CPA-treated p53-deficient (−/−) limbs revealed many cells with ruptured cell membranes and disintegrated organelles, confirming the occurrence of a high level of necrosis (Fig. 5F). Apoptosis was not observed. Thus, the type of cell death in the developing limb in response to 4OOH-CPA is p53-genotype dependent. Moreover, the type of cell death is affected by the p53 gene dosage. Finally, in the absence of the p53 gene dying cells are not localized to specific interdigital areas.

![Fig. 1. p53 wild-type (+/+), heterozygous (+/−), and homozygous (−/−) limbs cultured for 6 days with vehicle (water) or 4-hydroperoxycyclophosphamide (0.3, 1 or 3 μg/ml). Limbs were fixed, stained and photographed. Bar, 0.3 mm.](image-url)
Poly(ADP-ribose) polymerase (PARP) protein fragmentation

To further differentiate between cell death by apoptosis and necrosis in the limb, the poly(ADP-ribose) polymerase (PARP) cleavage pattern was investigated by western blot analysis. Shah et al. (1996) reported that PARP is cleaved to different fragments in response to apoptosis (89 kDa fragment) or necrosis (50, 40 and 35 kDa fragments). No fragmented PARP was detected in wild-type limbs cultured in control medium (Fig. 6). The 89 kDa fragment of immunoreactive PARP, characteristic of apoptosis, was detected in +/- limbs after 4OOH-CPA treatment; the small molecular weight fragments of PARP, characteristic of necrosis, were not observed in these limbs. While the predominant PARP fragment observed in the control heterozygous (+/−) and homozygous (−/−) limbs in the absence of drug treatment was the 89 kDa fragment, lower amounts of the smaller molecular weight fragments (50, 40 and 35 kDa) characteristic of necrosis appeared also to be present, especially in the homozygous (−/−) limbs. The relative proportion of these small molecular weight fragments to total immunoreactive PARP increased dramatically in both the heterozygous (+/−) and homozygous (−/−) limbs exposed to 4OOH-CPA. However, the 89 kDa apoptotic fragment was still detected in the treated homozygous limbs, which may indicate the presence of p53-independent apoptosis. Both morphological (subcellular morphology/electron microscope) and biochemical criteria (PARP fragmentation pattern and DNA ladder formation), provide evidence that cell death in the 4OOH-CPA-exposed limbs from p53-deficient homozygous (−/−) and heterozygous (+/−) embryos occurs predominantly by necrosis. While the PARP cleavage pattern may indicate a low rate of apoptosis in the limbs from homozygous (−/−) embryos, it is conceivable that an increase in the drug concentration may completely switch the type of cell death from apoptosis to necrosis.

Cell cycle analysis

Flow cytometry was done to analyze the effects of exposure to 4OOH-CPA on cell cycle regulation in limbs derived from embryos with each p53 genotype. A normal distribution of cell phase populations was found in suspensions from control wild-type (+/+) limbs. Interestingly, there was a significant difference (P<0.05) in the percentage of cells in S-phase among limbs from the three genotypes cultured in control...
medium. While this observation has been supported by other studies (Harvey et al., 1993), the biological importance of this observation is unclear. Further studies are needed to elucidate why this phenomenon occurs and how significant it is.

Exposure of limbs from wild-type embryos to 4OOH-CPA caused a cell cycle block at the G1/S phase; this block was evident from the absence of cell populations at the S and G2/M phases (Fig. 7). Similarly, the cell cycle was blocked at the G1/S phase in response to 4OOH-CPA treatment of limbs from heterozygous (+/−) embryos. While there were fewer cells from drug-treated homozygous (−/−) limbs in the S and G2/M phases of the cell cycle compared to the vehicle-treated samples (P < 0.05), exposure to 4OOH-CPA did not lead to a cell cycle block in limbs from p53-deficient (−/−) embryos. Indeed, more cells were in the S and G2/M phases of the cell cycle (increased 20-fold and 3-fold, respectively; P < 0.001) in the −/− limbs compared to the wild-type (+/+) drug-treated limbs. Thus, in the absence of p53, cells with DNA damage continued to cycle and failed to show a complete block at the G1/S phase.

Interestingly, there was an increase in the number of hypodiploid cells represented by the sub G1 fraction in response to 4OOH-CPA treatment; this was most pronounced in the homozygous (−/−) limbs. This observation is consistent with previous findings (Smith et al., 1995; Li et al., 1996; Smith and Fornace, 1996) in which p53 acts as a cell cycle checkpoint gene to ensure efficient DNA repair in response to DNA damage; the absence of p53 leads to continuous cell cycling with insufficient time for DNA repair.

**DISCUSSION**

The absence of p53 rendered embryonic limbs more susceptible to alkylating agent-induced malformations, demonstrating that p53 plays a protective role against teratogenic insults during embryonic development. This is consistent with previous studies with benzo[a]pyrene as the teratogen, in which Nicol et al. (1995) demonstrated a ‘teratological suppressor’ function for p53. However, investigations of other insults during development have led to opposite conclusions. Norimura et al. (1996) showed that irradiation of p53+/

Fig. 4. Light micrographs of limbs from each p53 genotype (A,B wild-type; C,D heterozygote; E,F homozygote) after culture for 24 hours in the control medium (A,C,E) or in the presence of 3 μg/ml 4OOH-CPA (B,D,F). Arrows point to dying cells with pyknotic nuclei. Bar, 12 μm.
In exploring the mechanism by which p53 ‘suppressed’ the response of the organogenesis-stage limb to insult, we found that the major type of cell death in the limbs exposed to 4OOH-CPA in the absence of p53 was necrosis. Initially, apoptosis and necrosis were considered as morphologically and biochemically different modes of cell death. However, there is increasing evidence that the two types of cell demise can occur simultaneously in tissues exposed to the same stimulus, and often the intensity of the stimulus decides the prevalence of either apoptosis or necrosis. Thus, both types of cell death may be triggered by the same signals and share early events. Many signals as diverse as heat shock (Schrek et al., 1980), viruses (Subramanian et al., 1995), protein synthesis inhibitors (Lennon et al., 1991), oxidative stress (Dypbukt et al., 1994), hypoxia (Shimizu et al., 1996b), Ca²⁺ ionophores, radiation (Fukuda et al., 1993), glutamate (Ankarcrona et al., 1995) and nitric oxide (Bonfoco et al., 1995) can induce both apoptosis and necrosis (Leist and Nicotera, 1997). Second messengers and downstream steps such as ATP (Leist et al., 1997) and bcl-2 (Shimizu et al., 1996a) have been shown to be involved in both apoptosis and necrosis. Although it is unlikely, this study does not exclude completely the possibility that early massive apoptosis was followed by failure of complete phagocytosis, leading to ‘secondary necrosis’ (Vermes et al., 1997; review by Slater et al., 1995).

Previous studies from our laboratory and others have implicated cell death by apoptosis in both normal and teratogen-induced abnormal limb development (Alles and Sulik, 1989; Kurishita, 1989; Moallem and Hales, 1995). Nevertheless, p53-deficient (~/-) limbs were resistant to drug-induced apoptosis; examination with the electron microscope clearly revealed the presence of necrotic cells in ~/- limbs. It may be significant that the specific localization of dying cells to the interdigital regions was lost in the ~/- limbs; necrotic cells appeared throughout the limb. The basis for differences in the response of cells in the interdigital areas versus those in the cartilaginous anlagen to stimuli is not well understood. In response to insult, p53 may direct interdigital cells to undergo death by apoptosis, while cells in the digital areas may undergo growth arrest and DNA repair. The loss of p53 may block this regulatory function and lead to sustained cell division or necrosis, depending on the extent of DNA damage. Further investigation of the p53-dependent switch in the type of cell death from apoptosis to necrosis in the limb after insult may contribute to our understanding of the role of p53 and of cell death in limb pattern formation.

The amount of stress or insult (i.e., dose and time of exposure) can affect the type of cell death (Lennon et al., 1991; Dypbukt et al., 1994; Sato et al., 1995). Boyle and Hickman (1997) recently suggested that the threshold for drug-induced apoptosis is determined not only by the extent of cytotoxic damage but also by the trophic environment and the ability of a cell to modulate survival signals that attenuate toxicity. The p53 protein may serve to ‘modulate’ the response of the limbs to insult. The absence of p53 may increase the impact of an insult on cell function in the limbs of the p53-deficient animals. The presence of lower amounts of p53 in the heterozygous mice (gene dosage) resulted in an intermediate response, suggesting that an insult may ‘overwhelm’ the p53 defensive mechanisms to arrest the cell cycle and/or induce apoptosis.
**p53** has an important function as a cell cycle ‘checkpoint’ gene (Lane, 1992). The cause and effect relationship between expression of wild-type **p53** and **G1** arrest after irradiation was established by Kastan et al. (1992) and Kuershitz et al. (1992). According to the model that has been proposed, **p53** helps to maintain genetic stability by initiating a transient **G1** cell cycle arrest; the cell cycle arrest allows time for the repair of damaged DNA before entry into **S** phase. Recently, an additional role for **p53** as a **G2** cell cycle checkpoint was discovered (Cross et al., 1995; Paules et al., 1995). Our results are in accordance with the above studies and indicate that, even in embryonic tissues, **p53** functions to block the cell cycle at the **G1** phase in response to insult with a DNA-damaging agent. Although it is not clear which molecule(s) **p53** targets to induce cell cycle arrest in the limb, it is attractive to speculate that one target may be insulin-like growth factor-1 (**IGF-1**). **p53** inhibited the activity of insulin-like growth factor-1 (**IGF-1**) by direct transcriptional induction of insulin-like growth factor binding protein 3 (**IGF-BP3**) (Buckbinder et al., 1995); it was demonstrated that the cellular **p53** induced **IGF-BP3** gene expression in fibroblasts in response to DNA damage (Buckbinder et al., 1995). Consequently, the induced **IGF-BP3** inhibited the **IGF-1** mitogenic signal. **IGF-1** is a strong candidate as a major mitogen involved in the morphogenesis of developing limbs (Geduspan et al., 1992; Dealy and Kosher, 1995); in addition, **IGF-BP3** is present in the developing limb (Bhaumick, 1993).

It is intriguing that a ‘subset’ of **p53**/−/ limbs cultured in the control medium did not develop normally. Other investigators have reported that a proportion of **p53**/−/ embryos have developmental abnormalities, namely neural tube defects (Armstrong et al., 1995; Sah et al., 1995). Abnormal limb development was not reported in these studies. The added ‘stress’ of culture in 50% **O2** may disturb limb organogenesis in the **p53**-deficient (−/−) embryos. Oxidative stress occurs during the in vitro culture of whole embryos (Ozolinis and Hales, 1997); high oxidative stress can cause abnormal development (Ishibashi et al., 1997; reviewed in Wells et al., 1997). The appearance of the low molecular weight fragments of **PARP** in the limbs from **p53**/−/ embryos (Fig. 6) is interesting in this context, as is the difference in the distribution of cell populations compared to the control wild-type limbs (Fig. 7). Both **p53**-independent cell death by apoptosis and abnormal cell cycling may contribute to lead downstream to the abnormal development of a proportion of the limbs in this group.

Alternatively, cell-to-cell contacts may be abnormal in the limbs from **p53**-deficient embryos. We found a decrease in cell density and less contact with neighboring cells in sections from the **p53**-deficient, vehicle-treated limbs, compared to sections from the limbs of wild-type embryos. Cano et al. (1996) reported that there was a 70% reduction in the expression of E-cadherin, a calcium-dependent cell-cell adhesion molecule, including focal loss at cell-cell contacts in **p53**-null cells. Also, Kaneko et al. (1996) reported that there was a high frequency of association between **p53** mutations and negative expression of **CD54**, an intercellular adhesion molecule, in Burkitt’s lymphoma. Cells in the **p53**-deficient homozygous limbs may be more susceptible to environmental stress, including in vitro culture, as a consequence of the loss of cell-cell adhesion. There is some evidence that loss of cell-cell contact might trigger apoptosis, suggesting the existence of an anti-apoptotic signal(s) in the extracellular matrix (Peluso, 1997; Levkau et al., 1998). However, another aspect of this relationship is the

---

**Fig. 6.** Western blot analysis of immunoreactive **PARP** in the limbs of wild-type, heterozygous and **p53**-deficient embryos. Protein samples from limbs cultured for 24 hours were electrophoresed, blotted and incubated with **PARP** antibody. **C**, limbs cultured in control medium; **T**, limbs cultured in the presence of 3 μg/ml 40OH-CPA. The size of the molecular weight markers in kDa is indicated on the right of the figure.

**Fig. 7.** Flow cytometric analysis of the effect of **p53** genotype on cell cycle phase in limbs cultured for 24 hours in the presence of vehicle (C) or 3 μg/ml 40OH-CPA (T). Bars are mean±standard errors of the means (n=3). Compare bars depicted with * to their wild-type counterparts (P<0.01).

**Fig. 8.** Schematic diagram of the proposed pathways for limb development after teratogen treatment in the absence and presence of **p53**.
loss of cell contact as a morphologic feature of apoptosis (reviewed in Schwartzman and Cidlowski, 1993). Thus, the cause-effect relationship of loss of cell contact and apoptosis is not fully understood.

We propose that, in wild-type (+/+), limbs, 4OH-CPA induces DNA damage which may lead to arrest of the cell cycle in the digital areas at the G1 phase, allowing DNA repair systems to restore normal DNA structure/function. Some cells in the interdigital areas may have more extensive DNA damage, due to a higher susceptibility of cells to DNA damage and/or to a less efficient DNA repair system. Unrepaired cells in the interdigital regions may be removed by the activation of controlled cell death, namely apoptosis. Thus, insult by a teratogen such as 4OH-CPA leads to an increase in apoptosis, this increased apoptosis removes cells with damaged DNA from the tissue. This increased apoptosis causes moderate limb malformation, but it serves to protect the limb against the greater insult and malformations represented by the survival of the cells with damaged DNA and their subsequent growth and cycling (Fig. 8). The rate of limb malformations after insult is higher in p53-deficient (−/−) embryos exposed to a DNA-damaging teratogen due to the continuity of the cell cycle in surviving cells, which leads to the accumulation of damaged DNA and also the occurrence of massive necrosis. Accumulation of DNA damage and/or necrosis lead to severe limb malformations in the absence of p53.

The authors thank Dr. Bernard Robaire for his thoughtful and helpful discussion of the manuscript, Dr. Guy P. Poirier for providing the PARP antibody, Dr. U. Saragovi for his assistance in flow cytometry and Ms. Marie Ballak for her assistance in the electron microscope studies. This study was supported by the Medical Research Council of Canada. S. A. Moallem is the recipient of a fellowship award from the Faculty of Graduate Studies and Research of McGill University.

REFERENCES


