ANALYSIS OF P53 FUNCTION IN CELLULAR RESPONSES TO MICROTUBULE-ACTING DRUGS

by

Jennifer Sue Lanni

Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

at the
Massachusetts Institute of Technology

June, 1998

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ABSTRACT

The p53 tumor suppressor gene product is a key regulator of cell cycle arrest and apoptosis in vertebrate organisms. Mutations in the p53 gene are commonly found in human cancers, which may contribute to chemotherapeutic resistance. These experiments have addressed two distinct aspects of p53 function relating to its effect on chemotherapeutic resistance and its role as a cell cycle checkpoint.

The effect of p53 genotype of tumor cells on their response to treatment with the chemotherapeutic agent paclitaxel (trade name Taxol), which stabilizes cellular microtubules, was tested. Although paclitaxel is broadly effective against human tumor xenografts in mice, including some known to carry p53 mutations, it was observed that p53-containing mouse tumor cells were significantly more sensitive to direct treatment with paclitaxel. In order to reconcile these data, it was tested whether there was a requirement for p53 in the cytotoxic effects of tumor necrosis factor-α (TNF-α), a cytokine released from murine macrophages upon paclitaxel treatment. TNF-α was seen to induce apoptosis in tumor cells independently of p53 genotype. These data suggest that the efficacy of paclitaxel against p53-deficient tumors could be due in part to its ability to induce local release of TNF-α.

In a separate project, a proposed role for p53 as a checkpoint at mitosis was investigated. Wild-type and p53 -/- murine fibroblasts were treated with the spindle inhibitor nocodazole and observed using time-lapse videomicroscopy. Cells of both genotypes were seen to undergo transient mitotic arrest, indicating that p53 does not have a checkpoint function at mitosis. However, p53 was found to prevent nocodazole-treated cells which had adapted out of mitotic arrest from reentering the cell cycle and initiating another round of DNA synthesis. Despite having a 4N DNA content, adapted cells are similar to G1 cells in that they have upregulated cyclin E expression and hypophosphorylated Rb protein. Also, the mechanism of the p53-dependent arrest in adapted cells requires the cyclin-dependent kinase inhibitor p21. Taken as a whole, these data suggest that the p53-dependent checkpoint following spindle disruption functionally overlaps with the p53-dependent checkpoint following DNA damage.

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ACKNOWLEDGMENTS

I would like to thank my advisor, Tyler Jacks, for the wonderful mentoring he has given to me. He has always been ready to provide good scientific advice and encouraging moral support. Also, I would like to thank all the members of the Jacks Lab, present and former, who made the lab such a great place in which to work.

The M.I.T. biology community has been extremely helpful in discussing experiments and providing protocols. In particular, I would like to thank Jackie Lees, Frank Solomon, Jun Liu, and the many other members of the M.I.T. Center for Cancer Research who so generously gave their time and knowledge whenever I asked.

I give my heartfelt thanks to my family, who have unfailingly supported me at every stage of my career. I am especially grateful to my parents, who rearranged their lives in order to provide me with day care and make it possible for me to write this thesis.

None of this work would have been possible without the loving support of my husband Frank; none of it would have been finished on time without the arrival of my son Dominic. To give them the acknowledgment they deserve would take up more pages than this thesis. They both have my thanks and love.

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Chapter 1

Overview of p53 and microtubule-acting drugs

The p53 tumor suppressor gene is a key regulator of cell cycle arrest and apoptosis in vertebrate organisms. In order to divide successfully and maintain the fidelity of their DNA, cells must be able to arrest their progression through the cell cycle to wait for more favorable growth conditions or to carry out necessary repairs. Furthermore, when cells have sustained extensive genetic or structural damage, they must be able to activate the cell's intrinsic suicide program, known as apoptosis. Such cells may have gained oncogenic mutations, so that failure to eliminate them could lead to the onset of tumors in the organism. The p53 tumor suppressor gene product is a component of cellular signalling pathways resulting in cell cycle arrest and apoptosis, and therefore plays an important role in governing cell behavior in response to many different environmental stimuli.

In this chapter, I intend to review current understanding of the role of p53 in human cancer, the known regulatory functions of p53, and the mechanism of action of the p53 protein. I will then provide background information for the two distinct research projects described in Chapters 2 and 3 of this document. As background for Chapter 2, I will discuss relevant literature on the chemotherapeutic agent paclitaxel, and how p53 may affect the response of cells to treatment with this drug. Furthermore, in preparation for Chapter 3, I will discuss mitotic checkpoints, and review literature regarding p53's recently proposed role as a cell cycle checkpoint at mitosis.

I. The tumor suppressor gene p53

The p53 gene has been extensively studied because of its role in tumor development. Much is known about p53 mutations and the tumor types in which they are likely to occur. Below, I have summarized the effect of germline and somatic p53 mutations in human cancer. Additionally, I briefly review information gained from mouse models of p53 inactivation.

p53 mutations in human cancer

The p53 gene has been identified as the most frequently mutated gene in human cancer, with mutations detected in over 50% of all tumors analyzed (Hollstein *et al.*, 1994). Absence of functional p53 protein through either inherited or spontaneous somatic mutation is associated with increased tumor predisposition in humans. In a rare heritable condition known as Li-Fraumeni syndrome, a p53 allele containing a point mutation is passed through the germline of affected individuals (Malkin *et al.*, 1990). Families in these pedigrees exhibit very high incidences of tumors of several different tissue types, including breast carcinomas, soft tissue sarcomas, brain tumors, and osteosarcomas. Analysis of tumor DNAs from Li-Fraumeni patients reveals retention of the point-mutated p53 allele accompanied by loss of the remaining wild-type p53 allele (Malkin *et al.*, 1990). Thus, p53 acts as a tumor suppressor gene, because inactivation of both copies of the gene is necessary in order to promote tumorigenesis.

A far more common means by which p53 inactivation promotes tumorigenesis is via spontaneous mutation in somatic tissues. Consecutive sporadic mutations of both p53 alleles in the same somatic cell effectively eliminate p53's tumor suppressor function, in accordance with the two-hit model originally described by Knudson (Knudson 1971). This mechanism appears to be a common occurrence in

tumor development, given the high percentage of sporadic tumors which contain p53 mutations. Interestingly, p53 mutations have been detected in tumors of many different tissue types, including lung, breast, colon, bladder, brain, and hematopoietic tissue (Hollstein *et al.*, 1991). Thus, the specific mechanism of the p53 protein allows it to act as a global suppressor of tumorigenesis.

Mouse knockout model

A variety of vertebrate organisms contain homologues of the p53 gene, including Mus musculus (Lane and Crawford 1979; Linzer and Levine 1979), chicken (Soussi *et al.*, 1988), and Xenopus laevis (Soussi *et al.*, 1987). The tumor suppression function of p53 appears to be conserved in at least one of these species, the mouse. Knockout mouse models of p53 have been constructed in which p53 has been inactivated in the germline using gene replacement techniques. Mice which are homozygous for the disrupted p53 allele all show greatly increased tumor frequency, rapidly developing thymic lymphomas and dying by age six months, one quarter of their normal lifespan (Donehower *et al.*, 1992; Jacks *et al.*, 1994). Heterozygous animals are also predisposed to tumor development, and develop thymic lymphomas and a variety of different sarcomas by nine months of age. In addition to the information gained from the knockout animals themselves, fibroblasts generated from knockout animals have been an extremely useful reagent for dissecting p53 function at the cellular level. These cells, which are devoid of functional p53, can be analyzed for their growth characteristics in different environments to identify circumstances in which p53 function is required for normal growth regulation. These cells and clones derived from them have been the primary reagent used in this work, as described in chapters 2 and 3.

p53 and cell cycle checkpoints

p53 inhibits cellular proliferation by two general pathways, cell cycle arrest and apoptosis. In its arrest function, p53 acts to induce cell cycle arrest in G1 in cells which have experienced DNA damage. The role of p53 in the cellular response to DNA damage was initially suggested by the observation that levels of p53 protein increased in myeloid cells following ionizing radiation (Kastan *et al.*, 1991). The p53 protein was subsequently demonstrated to be a necessary component of the G1 checkpoint activated after DNA damage in normal fibroblasts, in experiments utilizing p53-deficient fibroblasts generated from knockout mice. After treatment with ionizing radiation, the percentage of wild-type fibroblasts still in S phase was found to drop nearly to zero, while the fraction of p53 -/- fibroblasts in S phase remained virtually unchanged (Kastan *et al.*, 1992). These data indicate that p53 is a primary upstream component of the G1 arrest pathway. Such a checkpoint is believed to provide cells which have sustained DNA damage with an opportunity to repair their DNA before initiating DNA synthesis (Donehower and Bradley 1993). Cells which lack p53 would not pause for repairs and could fix radiation-induced alterations in their genome by progressing into S phase with damaged DNA. For this reason, the ability of the p53 protein to help implement G1 arrest has been described as a means of ensuring genomic stability (Lane 1992). In support of this view, cells lacking p53 have been shown to undergo gene amplification when placed under selective pressure (Livingstone *et al.*, 1992). The p53 protein has also been found to induce arrest in G0 when levels of ribonucleoside triphosphate pools fall below a critical threshold (Linke *et al.*, 1996). In this pathway, p53 again appears to maintain genomic stability by regulating S phase entry, as cells which began DNA replication without adequate supplies of nucleotides could suffer catastrophic DNA damage.

In addition to its regulatory role in G1, the p53 protein has been proposed to regulate checkpoints at other phases of the cell cycle, including G2 and M. Several groups have utilized conditional expression of p53 protein in cells using temperature-sensitive or tetracycline-inducible constructs to analyze its function during different phases of the cell cycle (Agarwal *et al.*, 1995; Guillouf *et al.*, 1995; Stewart *et al.*, 1995). Depending on the timing of p53 induction, such cell lines were observed to arrest in the G2 phase of the cell cycle, indicating that p53 is capable of inducing arrest in G2. However, it cannot yet be definitively concluded that p53 is a physiological component of a G2 checkpoint, because these experiments required the expression of high levels of p53 protein from nonhomologous promoters. Other data suggestive of a role for p53 in G2 include the observation that the p53 protein is phosphorylated *in vitro* by CDKs specific to the S and G2 phases of the cell cycle, resulting in stimulation of sequence-specific DNA binding by p53 (Wang and Prives 1995). It has further been suggested that p53 is a component of a checkpoint at mitosis, based on the arrest phenotype of p53-deficient fibroblasts following disruption of the mitotic spindle (Cross *et al.*, 1995). The evidence for a p53-dependent checkpoint at mitosis is discussed in detail later in this chapter; experimental evidence addressing this question is presented in Chapter 3.

<u>p53 and apoptosis</u>

p53 also regulates cell proliferation via a second pathway, activation of apoptosis. The role of p53 in apoptosis was first defined by Yonish-Rouach and colleagues, who observed that myeloid leukemic cells transfected with wild-type p53 protein rapidly underwent apoptosis (Yonish-Rouach *et al.*, 1991). Subsequently, it was demonstrated that the p53 protein was required for induction of cell death in a physiological setting, in immature thymocytes following DNA damage (Clarke *et al.*, 1993; Lowe *et al.*, 1993b). Later research suggests that this may reflect a role for p53 in

eliminating thymocytes with faulty or incomplete antigen receptor gene rearrangements, which could produce oncogenic mutations (Guidos *et al.*, 1996; Nacht *et al.*, 1996). Subsequently, p53 has also been found to be necessary for induction of apoptosis in abnormally proliferating fibroblasts. Normal fibroblasts which have been transformed by the addition of oncogenes such as c-*myc*, adenovirus E1A, and activated H-*ras* undergo apoptosis when grown in medium depleted of growth factors. However, similarly transformed p53-deficient fibroblasts do not undergo apoptosis in identical conditions (Hermeking and Eick 1994; Lowe *et al.*, 1994b). Thus, one aspect of p53's function as a tumor suppressor may be to suppress tumorigenesis by inducing apoptosis in transformed cells which fail to arrest under growth-limiting conditions. Interestingly, oncogenically transformed cells growing in a solid tumor, where they are in conditions of limiting oxygen, also undergo p53-dependent apoptosis (Graeber *et al.*, 1996). These observations suggest that a physiological selective pressure exists for p53 mutations during tumor development. Cells which have gained oncogenic mutations and are proliferating inappropriately are eliminated by apoptosis, unless they have also inactivated the apoptotic pathway.

p53-dependent apoptosis has also been observed to occur in abnormally proliferating fibroblasts following treatment with certain chemotherapeutic agents or ionizing radiation (Lowe *et al.*, 1993a). This result is noteworthy, because it implies that p53 status may affect the response of tumors to therapy (discussed at greater length in <u>p53 and cancer treatment</u>, below). However, it is important to note that not all forms of apoptosis require p53. For instance, apoptosis in immature thymocytes following treatment with glucocorticoids does not require p53 (Clarke *et al.*, 1993; Lowe *et al.*, 1993b), nor does interdigital apoptosis during embryonic development, nor apoptosis during mammary involution, as these processes occur normally in

the p53 -/- mouse (Donehower *et al.*, 1992; Jacks *et al.*, 1994). Thus, while p53 is an upstream molecule in several pathways leading to cell death, it is not a required component of the apoptotic machinery.

Mechanism of the p53 protein

The biochemical function of the p53 protein is a transcription factor. The protein was shown to bind DNA in a sequence-specific manner, and the p53 binding site has been mapped to two copies of the 10 base pair motif 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3' (Kern *et al.*, 1991; Vogelstein and Kinzler 1992). Wild-type p53 protein is able to activate transcription *in vitro* from reporter constructs which contain the p53 consensus sequence, while mutant p53 protein fails to do so (Farmer *et al.*, 1992; Kern *et al.*, 1992; Zambetti *et al.*, 1992). *In vivo*, p53 activates transcription from a number of different target genes, which are described in detail below.

Structure of the p53 protein

The p53 protein itself consists of three domains: an N-terminal transactivation domain, a central DNA-binding domain, and a C-terminal oligomerization domain. The N-terminal domain of the protein has been demonstrated to bind several general transcription factors, including TATA-box binding protein (TBP) (Horikoshi *et al.*, 1995), the p62 subunit of TFIIH (Xiao *et al.*, 1994), and several TBP-associated factors (TAFs) (Lu and Levine 1995; Thut *et al.*, 1995). p53 increases transcription of its target genes by localization of these molecules to the promoter region, as mutations which disrupt the interactions between p53 and its TAFs render the protein incapable of activating transcription (Lin *et al.*, 1994). The central portion of the p53 protein contains the sequence-specific DNA binding domain. This domain is the most frequent site of point mutations in the gene, indicating that sequence-

specific binding is crucial to its activity (Hollstein *et al.*, 1994). Crystal structure analysis of the p53 protein-DNA complex has revealed that the point mutations disrupt DNA binding interactions (Cho *et al.*, 1994). Thus, the spectrum of p53 mutations in tumors strongly supports the link between its tumor suppression activity and its function as a transcription factor. It has also been observed that the p53 protein can act as a repressor of transcription when cotransfected with a number of different genes (Donehower and Bradley 1993, and references therein). This repression only occurs with promoters containing a TATA box (Mack *et al.*, 1993); therefore, it may be due to sequestration of TBP and other transcription factors by the large quantities of p53 protein expressed in these experiments.

Structurally, the active form of the p53 protein consists of a tetramer. The molecule is formed of two homodimers, which are linked by the C-terminal oligomerization domain (Clore *et al.*, 1994). The C-terminal portion of the protein may help regulate p53 activity, as p53 appears to require a conformational change in order to sequence-specifically bind DNA. Deletion of the C-terminal domain of p53 results in activation of DNA binding, as does interaction with short single strands of DNA (Hupp and Lane 1994; Jayaraman and Prives 1995). The mechanism by which the C-terminal domain regulates the DNA binding domain of the protein has not yet been fully elucidated.

The p53 protein contains multiple phosphorylation sites in both the N-terminal transcriptional activation domain and the C-terminal oligomerization domain (rev. in Ko and Prives 1996). A number of protein kinases have been shown to phosphorylate p53 *in vitro*, including double-stranded DNA-activated protein kinase (DNA-PK), cyclin-dependent kinases, casein kinases I and II, and protein kinase C (rev. in Meek 1994). Historically, the importance of these phosphorylation

sites in the p53 protein has been unclear, as mutation of individual or multiple phosphorylation sites was seen to affect the transcriptional activity of p53 in some instances but not in others (rev. in Ko and Prives 1996). However, recent reports demonstrate a direct link between phosphorylation of p53 and activation of the protein following DNA damage. Shieh *et al* found that after DNA damage, p53 became phosphorylated by DNA-PK at serine residue 15 in the N-terminus (Shieh *et al.*, 1997). This phosphorylation decreased the interaction between p53 and its negative regulator MDM2, resulting in increased transactivation by p53. Siliciano and colleagues also identified serine 15 as a residue which became phosphorylated after DNA damage, and reported that this modification was associated with enhanced transcription of p53 target genes (Siliciano *et al.*, 1997). Thus, phosphorylation of a residue in the N-terminus of p53 by DNA-PK may regulate its activity following DNA damage.

p53-responsive genes

p53 activates transcription from a number of different target genes, at least some of which appear to function in cell cycle arrest and apoptosis. One major transcriptional target of p53 is the p21 gene, which was identified by at least three different screening techniques (El-Deiry *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993). The p21 protein is an inhibitor of cyclin-dependent kinase molecules (CDKs). Upon induction of expression by p53, p21 inhibits cell cycle progression by binding to CDKs which are expressed in G1. The ability of p53 to cause cell cycle arrest in G1 is dependent in large part on its ability to induce expression of p21 as knockout fibroblasts lacking p53 or p21 both fail to arrest in G1 following DNA damage (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). However, at least one additional, unidentified p53 target gene must contribute to the p53-dependent G1 arrest, as the

arrest phenotype of p21-deficient cells is slightly less pronounced than that of p53 deficient cells.

Another gene whose transcription is upregulated in response to p53 expression is the bax gene (Miyashita and Reed 1995). Bax has been shown to promote apoptosis in a number of different cell types. The Bax protein has homology to the survival factor Bcl-2, which inhibits apoptosis (Oltvai and Korsmeyer 1994). Bax and Bcl-2 are capable of forming homo- and heterodimers; thus, by increasing Bax levels, p53 can alter the ratio of the two proteins and effectively shift a cell's fate towards induction of apoptosis. A mouse knockout model for Bax has been constructed, and the mice display no increased tumor predisposition, unlike p53-deficient mice (Knudson *et al.*, 1995). These results could be interpreted to mean that Bax does not significantly contribute to the ability of p53 to induce apoptosis. However, other research would seem to indicate that Bax does play an important role in p53-dependent apoptosis. McCurrach *et al* found that E1A-expressing fibroblasts, which undergo p53-dependent apoptosis when subjected to DNA damage, displayed partial resistance to apoptosis when the Bax protein was not present (McCurrach *et al.*, 1997). Additionally, Yin and colleagues reported that absence of Bax in a transgenic mouse tumor model led to accelerated tumor growth and decreased levels of apoptosis (Yin *et al.*, 1997). Transgenic mice which retained functional Bax displayed p53-dependent expression of the Bax gene in tumors, suggesting that in this model system, the p53 protein inhibits tumor progression by inducing apoptosis through Bax.

Several other genes have been identified as p53-responsive genes, with functions that may also affect control of proliferation. The cyclin G gene, which is induced in a p53-dependent manner following DNA damage, encodes a novel cyclin with no

identified CDK partner (Okamoto and Beach 1994). The GADD45 gene is also induced after DNA damage (Kastan *et al.*, 1992), and appears to play a role in the DNA repair process (Smith *et al.*, 1994). The death receptor-5 gene (DR5) has recently been identified as a p53-inducible gene following DNA damage (Wu *et al.*, 1997). DR5 is one of several receptors for the apoptosis-inducing ligand TRAIL, a member of the tumor necrosis factor ligand family (Sheridan *et al.*, 1997). Another p53 response gene is insulin-like growth factor binding protein 3 gene (IGF-BP3), which is induced after DNA damage (Buckbinder *et al.*, 1995). The IGF-BP3 protein may inhibit cell proliferation by repressing signalling by insulin-like growth factors. Finally, Polyak and colleagues have identified a group of transcripts induced in p53-expressing tumor cells undergoing apoptosis (Polyak *et al.*, 1997). Interestingly, many of these genes were found to encode proteins involved in oxidative stress. These results suggested a model in which activation of p53 could lead to apoptosis by generation of reactive oxygen species, which cause degradation of mitochondrial components and cell death. A number of other genes have been proposed to be transcriptional targets of p53, as screens for p53-response genes continue.

Transcriptional activity of p53 and apoptosis

One area in which the mechanism of activity of the p53 protein remains unclear is induction of apoptosis. Although its transcriptional activity is clearly important to its function in inducing growth arrest, there have been conflicting reports as to whether p53-mediated transcription is required for induction of the cell death pathway. The p53 protein has been shown to induce apoptosis in cells treated with transcriptional and translational inhibitors, suggesting that no new transcription or protein synthesis is needed for p53-dependent apoptosis to occur (Caelles *et al.*, 1994; Wagner *et al.*, 1994). Also, truncated and point-mutated versions of the p53 protein which lacked transcriptional activity were found to be capable of inducing cell death

when transfected into HeLa cells, although at a slower rate than when transactivation-competent p53 was added (Haupt *et al.*, 1995). Interestingly, a mutant p53 sequence derived from a human tumor was found to be competent for transcriptional activation and induction of G1 arrest, yet unable to induce apoptosis when transformed into rodent cells, possibly indicating that the point mutation affected some other aspect of p53 function (Rowan *et al.*, 1996).

In contrast to these data, Sabbatini *et al.* reported that a double-point mutant version of p53 which lacked the ability to activate or repress transcription from promoters containing p53-binding sequences was compromised in its ability to induce apoptosis (Sabbatini *et al.*, 1995). Attardi *et al.* also found transcriptional activation to be essential for p53 to cause apoptosis (Attardi *et al.*, 1996). In their experimental system, wild-type and mutant p53 constructs were microinjected into p53-deficient E1A-expressing fibroblasts, which were subsequently assayed for apoptosis. Versions of the p53 protein which lacked the N-terminal transactivation domain or the C-terminal oligomerization domain or which contained point mutations commonly found in human tumors did not induce cell death in this assay. Moreover, the ability of p53 deletion constructs to induce apoptosis could be reconstituted by addition of a nonhomologous transcriptional activation domain or oligomerization domain. Further research will be necessary in order to resolve the question of how transcriptional activation affects the ability of the p53 protein to induce apoptosis.

p53 and cancer treatment

In addition to providing cells with a selective advantage during tumor formation, p53 mutations have also been found to affect the response of tumors to therapy. In general, the frequency of p53 mutations in various tumor types correlates with their therapeutic responsiveness (Lowe 1995). For example, cancers of the lung, colon,

prostate and bladder all have a high incidence of p53 mutations (Takahashi *et al.*, 1989; Fearon and Vogelstein 1990; Isaacs *et al.*, 1991; Sidransky *et al.*, 1991). These tumor types often respond poorly to treatment with chemotherapeutic drugs or radiation (Lowe *et al.*, 1994c). Other kinds of tumors have been noted to have a very low frequency of p53 mutations, such as testicular teratocarcinomas and childhood acute lymphoblastic leukemias (Jonveaux and Berger 1991; Heimdal *et al.*, 1993; Lutzker and Levine 1996). In contrast to the previous group of tumor types, these tumors are often extremely responsive to therapy. For instance, every testicular teratocarcinoma examined appears to have wild-type p53 protein; 90-95% of these tumors are cured by treatment with the chemotherapeutic cisplatin (Levine 1997). Furthermore, while lymphoblastic leukemias generally are responsive to treatment, a certain fraction do undergo relapse. In these cases, failure of therapy can be correlated with the occurrence of mutations in the p53 gene (Felix *et al.*, 1992; Yeargin *et al.*, 1993).

The significance of p53 mutations in tumor responsiveness has also been examined by determining the p53 status of individual tumors and correlating it with their response to therapy. These studies confirm and extend the more general correlation between p53 status, tumor type, and therapeutic efficacy described above. In one clinical study, aberrant expression of p53 protein, an indicator of the presence of missense mutations in p53, was found to be associated with resistance to cisplatin therapy in non-small cell lung cancer (Rusch *et al.*, 1995). At least two groups have addressed the effect of p53 mutations in individual breast cancer patients. Bergh *et al.* observed that chemotherapy and local radiotherapy were less effective against breast cancers which contained p53 mutations (Bergh *et al.*, 1995). Also, Aas *et al.* have demonstrated that specific mutations in the p53 gene predict primary resistance to therapy with doxorubicin and early relapse in breast cancer patients

(Aas *et al.*, 1996). A final tumor type in which p53 status appears to affect therapeutic response is Wilms' tumor, a childhood cancer of the kidney. Most Wilms' tumors do not contain p53 mutations and usually are successfully treated. However, Wilms' tumors which displayed anaplastic morphology were found to have frequent p53 mutations and a significantly worse prognosis and response to treatment (Bardeesy *et al.*, 1994).

One potential explanation for the correlation between p53 status and tumor responsiveness has been provided by experiments using p53 knockout cells in conjunction with murine tumor models. Wild-type murine fibroblasts transformed with the oncogenes E1A and Ras undergo apoptosis when treated with anticancer agents (Lowe *et al.*, 1993a). However, p53-deficient fibroblasts transformed with the same oncogenes fail to die after treatment. Since p53 is known to regulate initiation of apoptosis, this raised the possibility that chemotherapeutic agents and radiation might be acting by inducing apoptosis. This hypothesis was tested *in vivo* using a genetically controlled system, in which p53 +/+ and p53 -/- tumors were generated in nude mice and then treated with radiation or the chemotherapeutic adriamycin (Lowe *et al.*, 1994a). Upon treatment, p53 +/+ tumors regressed and contained large numbers of apoptotic cells, while p53 -/- tumors failed to respond and had a low apoptotic index. Furthermore, when several p53 +/+ tumors which had initially responded to treatment underwent a relapse, these tumors were found to have acquired p53 mutations rendering them resistant to apoptosis. Thus, the effectiveness of chemotherapeutic agents and radiation treatment may depend in part on the ability of these treatments to activate p53-dependent apoptosis in tumor cells. In this model, tumors which have acquired p53 mutations are resistant to undergoing apoptosis and therefore survive such treatments.

The concept of p53-dependent apoptosis as a component of therapeutic effectiveness reflects a change in understanding of how anticancer treatments work. Many anticancer therapies, including ionizing radiation and several common antineoplastic drugs, induce DNA damage or interfere with DNA replication. For example, the chemotherapeutic cisplatin causes DNA damage by intercalating between DNA strands, while the drug adriamycin prevents the topoisomerase enzyme from separating DNA strands during DNA replication. Historically, the mechanism of these drugs has been assumed to be via genotoxic damage to cancer cells, which were assumed to be more sensitive to these agents due to their escalated rate of proliferation. However, emerging data suggests that these drugs may act in part by induction of apoptosis in tumor cells, which initiate apoptosis more readily because of their transformed status (Fisher 1994). As the p53 status of a tumor impacts strongly on its ability to undergo apoptosis, this new model may explain in part why p53 mutation is so strongly correlated with poor response to treatment (Lowe 1995). Of course, the p53 status of a tumor is not the sole determinant of successful treatment. Other genetic factors that could affect tumor responsiveness to therapy include amplification of the multidrug resistance gene, mdm2, and mutations in other components of the apoptotic signalling pathway and machinery. However, because the p53 status of a tumor may be one of the major factors affecting therapeutic success, one of the high priorities of biomedical research is finding therapies which are effective against tumors with mutated p53.

p53 and microtubule-acting drugs

As discussed earlier, the p53 protein clearly plays a central role in cellular pathways which trigger responses to DNA damage. One interesting question is whether p53 can act as a sensor for disruptions in the cell other than DNA damage, such as microtubule damage. Given that p53 is also a component of signalling pathways

which detect more general environmental stresses, such as hypoxia and inadequate nutrient supply (Lowe *et al.*, 1994b; Graeber *et al.*, 1996), it is not an unreasonable hypothesis that the stress of microtubule damage might activate a p53-dependent signalling pathway.

The microtubule cytoskeleton plays a vital role during cell division and in interphase functions of the cell, such as intracellular transport, maintenance of cellular shape, and cell motility. Microtubules are formed of tubulin dimers, each of which is composed of an α–tubulin and a β–tubulin molecule. Microtubules exist in a dynamic equilibrium with their tubulin subunits, enabling the microtubule network to undergo flexible rearrangement by assembling and disassembling microtubules. This property of dynamic instability is crucial to completing normal cellular division. During mitosis, the microtubule network is remodelled to form the mitotic spindle, which serves to align chromosomes at metaphase and separate sister chromatids during anaphase and telophase. A number of drugs exist which inhibit mitosis by binding to tubulin subunits or intact microtubules, thus shifting the equilibrium towards depolymerization or stabilization of microtubules. These drugs disrupt the microtubules which comprise the mitotic spindle, thereby inhibiting cell division. Depending on the cell type treated and dosage, such drugs can induce cell cycle arrest or cell death.

One primary goal of this work was to determine whether microtubule-acting drugs function via p53-dependent mechanisms. If this were the case, it could indicate a link between p53 and the microtubule cytoskeleton, which would identify a new p53-dependent signalling pathway. Because the upstream signals leading to p53 activation have not been elucidated clearly, identification of a novel upstream stimulus would be a useful tool for further research. Alternatively, if microtubule-

acting drugs were found to be wholly p53-independent in their action, they would have enormous potential as a novel class of antiproliferative agent. As mentioned above, many chemotherapeutic drugs act by causing DNA damage or inhibiting DNA metabolism, which leads to activation of p53-dependent pathways. Identification of antiproliferative drugs which act by p53-independent mechanisms would be potentially valuable for biomedical research, as these drugs might prove effective against tumors containing p53 mutations.

II. The chemotherapeutic agent paclitaxel

Paclitaxel (trade name Taxol) was identified in 1971 following a screen by the National Cancer Institute for compounds with cytotoxic activity. The original source of the drug was from the bark of the Pacific yew tree (*Taxus brevifolia*). However, scarcity of this natural resource and the difficulty in purification led to the development of chemical synthesis protocols (Nicolaou *et al.*, 1994). Among chemotherapeutic agents, paclitaxel has a unique activity in that it binds to and stabilizes cellular microtubules. Other microtubule-acting drugs have been widely used as anticancer agents, such as vincristine and vinblastine, members of the *Vinca* alkaloid family. However, these drugs all act by binding to free tubulin subunits and thus promoting disassembly of microtubules. The novel mechanism of paclitaxel raised hopes that it would prove effective against tumors which were refractory to other therapies.

Preclinical and clinical testing

In the National Cancer Institute screen, paclitaxel was found to have cytotoxic effects against a panel of different tumor cell lines, including murine L1210, P388, and P1534 leukemias, Walker 256 carcinosarcoma, sarcoma 180, and Lewis lung tumor (National Cancer Institute, 1983). However, the compound was not selected for

clinical development at that time because it did not appear to be significantly more effective against experimental tumors than other agents under development. Paclitaxel was later tested against murine B16 melanoma and displayed significant activity, providing impetus for its further development as a clinical drug (Rowinsky *et al.*, 1990). As further characterization of its activity, paclitaxel was tested against several xenografts of human tumors in the DCT screening tumor panel. It was found to induce regression or significant growth delay in a number of tumors, including human CX-1 colon tumors, LX-1 lung tumors, MX-1 mammary tumors, and intraperitoneally implanted murine P388 and L1210 leukemias (National Cancer Institute, 1983). In addition, paclitaxel was tested against other tumor xenografts in studies by independent investigators. Riondel *et al.* found that paclitaxel demonstrated activity against xenografts of primary tumors of the breast, ovary, endometrium, lung, tongue, and brain (Riondel *et al.*, 1986). Paclitaxel was also effective against a different panel of human tumors transplanted into mice, including A431 vulva, A2780 ovarian, H2981 and L2987 lung, and RCA and HCT-116 colon carcinomas (Rose 1993). The drug was seen to be ineffective in some instances, such as against xenografts of pancreatic carcinoma and M5076 sarcoma (Sternberg *et al.*, 1987; Rose 1993).

Early clinical trials of paclitaxel were carried out in the mid-1980s against a number of different human solid tumors and leukemias. In phase 1 trials, several groups reported problems with acute hypersensitivity reactions in patients, and development of the drug was nearly halted. However, paclitaxel did demonstrate antineoplastic activity against melanomas, ovarian carcinomas, non-small cell lung carcinomas, and several other tumor types (Rowinsky *et al.*, 1990). Paclitaxel appeared more promising during phase 2 trials, when it was found to be effective against ovarian carcinomas and melanomas (Rowinsky and Donehower 1995). In

particular, the efficacy of the drug against refractory ovarian carcinomas caused excitement. Paclitaxel was observed to have an overall response rate of 37% against advanced ovarian cancers, many of which were resistant to treatment with the chemotherapeutic cisplatin (Thigpen *et al.*, 1994). Paclitaxel was also found to display substantial antitumor activity against metastatic breast cancer, with a response rate of over 50% in women who had previously received other forms of chemotherapy (Holmes *et al.*, 1991). Currently, paclitaxel is used effectively against a number of different tumor types, including cancers of the ovary, breast, head and neck, and lung (Rowinsky and Donehower 1995).

Cellular effects of paclitaxel

The best known activity of paclitaxel is its ability to bind to and stabilize microtubules. Schiff *et al.* first characterized the action of paclitaxel *in vitro*, and reported that it was able to promote microtubule assembly (Schiff *et al.*, 1979). Paclitaxel was observed to decrease the lag time required for microtubule assembly and shift the equilibrium for assembly in favor of microtubules, effectively decreasing the concentration of tubulin needed to promote assembly. Also, microtubules formed in the presence of taxol were more stable, and were resistant to undergoing depolymerization when placed in the cold (4°C) or in $CaCl_2$ (4 mM). Paclitaxel was shown in photoaffinity studies to have a binding site on the β-tubulin subunit of the tubulin dimer in microtubule polymers, although it did not bind free tubulin (Rao *et al.*, 1992). In a later report, an electron crystallography study of paclitaxel bound to microtubules showed that one molecule of paclitaxel was bound per tubulin dimer (Nogales *et al.*, 1995). Furthermore, it was observed that the binding site of paclitaxel spanned the region where interactions occurred between protofilaments of the microtubule. This finding was consistent with the preferential binding of paclitaxel to polymerized tubulin.

Paclitaxel was also characterized by examining its effect on cells in tissue culture. Schiff and Horwitz treated human and mouse fibroblasts with the drug, and reported that cells accumulated in the G2 and M portions of the cell cycle when grown in medium containing 10 μM paclitaxel (Schiff and Horwitz 1980). The treated cells were seen to contain bundles of cytoplasmic microtubules which were unusually stable, and had large increases in their total microtubule mass. It was concluded that the cells were unable to depolymerize these cytoplasmic microtubules to form the mitotic spindle, resulting in their arrest just prior to mitosis. De Brabander *et al.* also observed that when present at micromolar concentrations, paclitaxel induced massive assembly of free microtubules in cells (DeBrabander *et al.*, 1981). Thus, when present at high concentration, paclitaxel induces polymerization of cytoplasmic tubulin and prevents assembly of the mitotic spindle. More recent studies have focused on the effects on paclitaxel at low (nanomolar) concentrations. Jordan *et al.* found that at a dose of 8 nM, paclitaxel did not cause any increase in microtubule polymer mass, but was still capable of inhibiting cell proliferation in HeLa cells (Jordan *et al.*, 1993). Long and Fairchild made a similar observation, reporting that 4 nM concentrations of paclitaxel were sufficient to inhibit mitotic progression in human colon carcinoma cells (Long and Fairchild 1994). These results suggest that at low concentrations, paclitaxel prevents cell proliferation by stabilizing the microtubules of the mitotic spindle. This inhibition of dynamic instability has also been observed to occur when cells are treated with low concentrations of microtubule depolymerizing agents, such as vincristine, vinblastine, and nocodazole (Jordan *et al.*, 1991; Vasquez *et al.*, 1997). Thus, paclitaxel may affect cellular microtubules by different mechanisms at different concentrations.

In addition to its effects on cellular microtubules, paclitaxel has an additional activity which is potentially relevant to its antineoplastic properties - specifically, the ability to mimic bacterial lipopolysaccharide (LPS) and trigger an immune response in macrophages. LPS is a major component of the coat of Gram-negative bacteria. When macrophages are exposed to LPS, they respond by releasing a number of different inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-1, interleukin-6, interferon-α, interferon-β, and reactive oxygen and nitrogen intermediates (Vogel *et al.*, 1995). Ding *et al.* discovered that like LPS, paclitaxel could elicit release of TNF-α from murine macrophages (Ding *et al.*, 1990). This effect was mediated via the same pathway as LPS signalling, as macrophages derived from LPS-hyporesponsive mice failed to secrete TNF-α in response to either paclitaxel or LPS. It was subsequently shown that paclitaxel induced expression of a group of genes known to be upregulated by LPS (Manthey *et al.*, 1992). Also, paclitaxel induced tyrosine phosphorylation of the erk1 and erk2 proteins, which are phosphorylated upon LPS stimulation.

The ability of paclitaxel to activate the LPS signalling pathway was shown to be distinct from its microtubule-stabilizing activity. As part of attempts to increase the supply of the drug, a number of paclitaxel-related compounds and partial structures were purified. Burkhart *et al.* performed experiments using several of these paclitaxel analogs and demonstrated that the two activities of paclitaxel could be separated (Burkhart *et al.*, 1994). For instance, the paclitaxel-related compound taxotere was more effective at stabilizing microtubules than paclitaxel, but did not induce any expression of TNF-α. Similar analysis of a number of different analogs supported the conclusion that these effects of paclitaxel occurred via distinct pathways, as no correlation was found between the ability of a compound to stabilize microtubules and its ability to induce release of TNF-α. One intriguing possibility

raised by these results was that the activity of paclitaxel as a chemotherapeutic agent against human cancers might depend in part on its ability to induce TNF-α secretion. However, it is important to note that the LPS-mimetic effects of paclitaxel were found to differ between human and murine macrophages. In murine macrophages, treatment with paclitaxel alone stimulated TNF-α secretion. In human macrophages, paclitaxel enhanced TNF-α secretion when given in conjunction with very low doses of LPS, but when given alone was not capable of inducing TNF-α secretion (Allen *et al.*, 1993).

Tumor necrosis factor-α

The release of TNF-α from macrophages upon paclitaxel treatment is particularly interesting given the historic ability of this molecule to induce cell death and tumor regression. In the late 1800s, Dr. William Coley utilized the antitumor properties of TNF-α by administering LPS-containing bacterial extracts to his cancer patients (Vogel *et al.*, 1995). The resulting TNF-α release caused tumor regression in some patients, but was accompanied by toxic side effects from severe inflammatory responses. Because of its ability to induce tumor regression, TNF-α has undergone considerable testing as an anticancer agent in various animal tumor models and in patients during the past two decades (Spriggs and Yates 1992). It has shown limited success in these systems, as systemic administration has caused severe side effects and relatively poor inhibition of tumor growth. However, some studies utilizing local administration of TNF-α have had more encouraging results, showing cytotoxicity against tumors and reduced severity of side effects. For instance, TNF-α was effective in melanoma patients when administered regionally in combination with chemotherapy (Lejeune *et al.*, 1994). TNF-α also demonstrated antiproliferative activity in combination therapies against xenografts of human

renal cell carcinoma, L1210 leukemia, and murine sarcoma (Baisch *et al.*, 1990; Asher *et al.*, 1991; Lasek *et al.*, 1995).

TNF-α has been shown to be capable of inducing apoptosis in a number of cell types, which may contribute to its antitumor activity. The molecular signalling pathway activated by TNF-α has been rapidly elucidated (rev. in Cleveland and Ihle 1995, and Baker and Reddy 1996). A member of the Fas ligand family, TNF-α is a trimer, and binds to a trimeric transmembrane receptor molecule. The TNF-α receptor contains a 'death domain', a unique cytoplasmic motif found in both the TNF-α receptor and the Fas receptor that is responsible for generating cytotoxic death signals. This domain is capable of activating an apoptotic pathway by interacting with a number of different downstream adaptor molecules, including TRADD, FADD, and RIP. Modulation of the activity of these signalling molecules by the TNF-α receptor ultimately leads to activation of cysteine proteases, resulting in apoptosis.

Paclitaxel efficacy and p53 status

When considering the efficacy of paclitaxel as a chemotherapeutic agent, one important question to resolve is whether it functions via a p53-dependent pathway, as many tumors contain p53 mutations. A body of data would seem to suggest that the activity of paclitaxel is independent of p53. First, the antiproliferative mechanism of paclitaxel appears to be unrelated to the p53 pathway, as paclitaxel affects cellular microtubules, while p53 is understood primarily for its role in detecting DNA damage. Additionally, data obtained from preclinical and clinical testing of paclitaxel would suggest that it does not rely on functional p53 protein to cause tumor regression. In particular, the efficacy of paclitaxel against cisplatin-resistant tumors could reflect activity in a clinical setting where cells contained mutated p53 (Thigpen *et al.*, 1994). Because cisplatin is known to cause DNA

damage, thereby activating a p53-dependent pathway, tumors which proved resistant to cisplatin therapy are likely to contain p53 mutations. Successful treatment of these tumors with paclitaxel could potentially be due to p53-independent effects of the drug. In preclinical testing, paclitaxel was demonstrated to have widespread activity against a number of different xenografts of human tumors in mice, few of which have been screened for their p53 status but at least some of which are likely to contain p53 mutations (National Cancer Institute, 1983; Riondel *et al.*, 1986; Rose 1993). In several instances, tumor cell lines used in xenograft experiments were actually demonstrated to contain mutated p53. For instance, paclitaxel was found to be effective in preclinical testing against the human tumors CX-1 (colon), A431 (vulva), and FaDu (hypopharynx) (National Cancer Institute, 1983; Rose 1993; Joschko *et al.*, 1994), all of which are documented to have p53 mutations (Baker *et al.*, 1989; Reiss *et al.*, 1992). Finally, wild-type and p53-heterozygous human lymphoblastoid cells were found to have similar sensitivities to treatment with paclitaxel *in vitro* (Delia *et al.*, 1996).

Other data indicate that the antiproliferative effects of paclitaxel may be affected by p53 status, although the correlation between these two factors is unclear. Wahl *et al.* observed that human fibroblasts with inactivated p53 were more sensitive to paclitaxel treatment than wild-type fibroblasts, with an increased proportion of p53-deficient cells undergoing cell cycle arrest and apoptosis (Wahl *et al.*, 1996). Another *in vitro* study has also found a positive correlation between p53 mutation and increased paclitaxel sensitivity in fibroblasts (Hawkins *et al.*, 1996). Conversely, inactivation of p53 was found to render ovarian teratocarcinoma cells more resistant to undergoing apoptosis following paclitaxel treatment, indicating a correlation between p53 mutation and paclitaxel resistance (Wu and El-Deiry 1996). Also, in a clinical study, Rosell*et al.* looked for a link between paclitaxel sensitivity

and p53 status in non-small cell lung cancer, and hypothesized that p53 mutation possibly could be prognostic of paclitaxel resistance (Rosell *et al.*, 1995). Finally, although disruption of microtubules has not been directly linked to a p53-dependent signalling pathway, it is not unreasonable to suppose that p53 could detect this stimulus, as p53 has been shown to detect other general cellular stresses such as hypoxia and inadequate nutrient supply (Lowe *et al.*, 1994b; Graeber *et al.*, 1996). In Chapter 2, I will provide data describing how the p53 status of tumor cells affects their response to paclitaxel in one specific experimental system.

III. p53 function and mitosis

The p53 protein has been established as an important cell cycle regulator because of its function as a checkpoint in G1. This aspect of p53 function has been well-documented and widely studied. However, p53 has also been proposed to act as part of a cell cycle checkpoint at mitosis after microtubule disruption. Identification of a novel checkpoint regulated by p53 would be significant, because it could relate to p53's role as a tumor suppressor gene. The third chapter of this thesis addresses the role of p53 in a cell cycle checkpoint activated by microtubule-acting drugs, which induce cell cycle arrest at mitosis.

The spindle assembly checkpoint

Proper assembly of the mitotic spindle is an important event in the cell cycle that is monitored by a cell cycle checkpoint (rev. in Rudner and Murray 1996). If the spindle fails to function properly, chromosome loss or gain can result, which is detrimental to the organism. Loss of chromosomes can cause cell death, while gain of additional chromosomes can lead to birth defects or increase the tendency of a cell to become tumorigenic. In order to reduce the occurrence of these errors, cells have evolved a spindle assembly checkpoint, which monitors spindle structure,

attachment of chromosomes to spindle microtubules, and alignment of chromosomes on the spindle. Activation of the spindle assembly checkpoint causes a delay in the onset of chromosome segregation until the problem is corrected.

One convenient way to study the spindle assembly checkpoint is to activate it artificially by treating cells with microtubule-acting drugs. A number of drugs exist which are used for this purpose in the laboratory. In particular, drugs which destabilize microtubules are used commonly in experimental settings, such as benomyl, nocodazole, colcemid, colchicine, and members of the *Vinca* alkaloid family (e.g., vincristine, vinblastine, and vindesine). At high concentrations, these drugs act by binding to free tubulin, preventing it from polymerizing into microtubules and in some cases promoting the disassembly of existing cellular microtubules (Himes *et al.*, 1976; Hoebeke *et al.*, 1976; Owellen *et al.*, 1976). At lower concentrations, these drugs do not alter the amount of total tubulin polymer, but are still capable of inhibiting cell proliferation by altering the dynamic instability of spindle microtubules (Jordan *et al.*, 1991; Vasquez *et al.*, 1997). Specifically, cells treated with such drugs are unable to add and remove tubulin subunits from the ends of spindle microtubules, resulting in an inhibition of spindle function. For example, Jordan *et al.* found that treatment of HeLa cells with low concentrations of vincristine or vinblastine inhibited cell proliferation without causing any detectable microtubule depolymerization or spindle disorganization (Jordan *et al.*, 1991). Treatment of cells with microtubule-destabilizing drugs results in cell cycle arrest at mitosis, indicating that the spindle assembly checkpoint can detect inappropriate depolymerization or stabilization of spindle microtubules. However, this mitotic arrest state is not maintained indefinitely, as some cell types treated with microtubule-acting drugs for a prolonged period of time eventually undergo

adaptation (Kung *et al.*, 1990; Rieder and Palazzo 1992). In these instances, cells return to an interphase state despite the lack of a functional spindle.

A number of genes have been identified which are components of the mitotic checkpoint mechanism. Hoyt *et al.* performed a screen for mutant strains of *S. cerevisiae* which failed to arrest at mitosis following treatment with the microtubule-destabilizing drug benomyl (Hoyt *et al.*, 1991). They isolated several different mutants which continued to progress through the cell cycle, termed *bub* mutants (for "budding uninhibited by benzimidazole"). The phenotype of *bub* mutant strains in the presence of drug was continued budding and DNA replication without cytokinesis, leading to benzimidazole-induced lethality. The three *BUB* genes were later cloned and were found to encode a protein kinase, its target, and a third protein of unknown function (Roberts *et al.*, 1994). A murine homolog of *BUB1* has also been identified, and was found to localize to the kinetochore (Taylor and McKeon 1997). Li and Murray also carried out a screen for checkpoint-defective mutants, by identifying *S. cerevisiae* which did not delay completion of mitosis in the presence of benomyl (Li and Murray 1991). Like the *bub* mutants, the *mad* (mitotic arrest-deficient) mutants isolated in this screen exhibited increased lethality in the presence of drug due to continued cell cycle progression without a functional spindle. The *MAD* gene products are proteins of unknown function which appear to be involved in a signal transduction cascade in combination with the BUB proteins, based on genetic and biochemical studies (Rudner and Murray 1996). Human and *Xenopus* homologs of the *MAD2* gene product have been identified and were shown to localize to free kinetochores (Chen *et al.*, 1996; Li and Benezra 1996).

Evidence for a p53-dependent checkpoint at mitosis

The concept that the p53 protein could be a component of the mitotic spindle checkpoint was first suggested by Cross *et al.* (Cross *et al.*, 1995). They observed that upon treatment of wild-type fibroblasts with microtubule-acting drugs, the cells arrested with a 4N DNA content, presumably because of disruption of the mitotic spindle. However, p53 -/- fibroblasts treated in the same manner failed to arrest, instead proceeding to enter another round of S phase and become 8N. Because the phenotype observed in p53 -/- cells was similar to that observed in yeast strains with mutations in the *BUB* and *MAD* genes, which become polyploid upon treatment with spindle inhibitors, it was proposed that p53 might function at the mitotic checkpoint. Two other groups have also reported that cells without functional p53 protein undergo inappropriate DNA replication in the presence of mitotic spindle inhibitors. Minn *et al.* found that a murine prolymphocytic cell line with inactive p53 protein became polyploid after treatment with nocodazole, while Di Leonardo *et al.* observed that p53-deficient mouse or human fibroblasts underwent continued DNA replication in the presence of colcemid or nocodazole (Minn *et al.*, 1996; Di Leonardo *et al.*, 1997). Also, p53 -/- fibroblasts have been noted to become tetraploid at a high frequency, which could be indicative of a defect in the spindle checkpoint (Livingstone *et al.*, 1992).

Other groups have provided additional evidence for a role for p53 at mitosis by examining effects of p53 on the centrosome, the organizing center for the microtubules of the mitotic spindle. The p53 protein was seen to localize to the mitotic spindle using indirect immunofluorescence techniques, and a fraction of p53 protein was shown to copurify with the centrosomal protein pericentrin (Brown *et al.*, 1994). Fukusawa *et al.* reported that p53 -/- fibroblasts had multiple copies of functional centrosomes, resulting in unequal segregation of chromosomes at

mitosis (Fukasawa *et al.*, 1996). In this same study, p53 -/- cells appeared to have a defect in the regulation of centrosome duplication. Biochemical evidence can also be interpreted as indicative of p53 activity at mitosis. Wang and Prives found that cyclin-dependent kinases expressed during G2 and M were capable of phosphorylating p53 protein *in vitro* and stimulating its sequence-specific DNA binding activity (Wang and Prives 1995). Furthermore, the levels of transcriptionally active p53 protein have been shown to increase after treatment of cells with the microtubule-acting drugs paclitaxel, vinblastine, and nocodazole, suggesting that the stress of microtubule damage may result in activation of a p53-dependent response pathway (Tishler *et al.*, 1995).

Evidence against a p53-dependent checkpoint at mitosis

Despite the data discussed above, the function of p53 following microtubule disruption remains unclear. Minn *et al.* examined in detail the response of a prolymphocytic cell line to treatment with nocodazole (Minn *et al.*, 1996). Drug-treated cells were seen to arrest transiently at mitosis, then adapt into an interphase state, as is often observed with cells treated for long intervals with microtubule-acting drugs (Kung *et al.*, 1990; Rieder and Palazzo 1992). Wild-type cells remained arrested in the interphase state, while cells without functional p53 continued cell cycle progression in the absence of cytokinesis and became polyploid. As previously reported, p53 protein was found to be induced in nocodazole-treated cells following spindle damage (Tishler *et al.*, 1995; Minn *et al.*, 1996). However, levels of p53 protein did not increase immediately upon nocodazole treatment, but after the time window in which cells adapted. Thus, while p53 protein helped prevent nocodazole-treated cells from continuing DNA synthesis, it did not appear to be required until after cells had adapted from mitotic arrest. The data of Minn *et al.* would indicate that p53 does not act as a checkpoint at mitosis, but instead functions

after cells have returned to an interphase state. The third chapter of this thesis characterizes the checkpoint function of p53 after mitosis by examining the response of wild-type and p53-deficient fibroblasts to treatment with nocodazole.

The work presented in this thesis describes two distinct projects which examine how the p53 status of cells affects their response to microtubule-acting drugs. Chapter 2 will analyze how the p53 status of transformed fibroblasts determines their response to the microtubule-polymerizing agent paclitaxel, and will describe effects of paclitaxel that are independent of p53 status. Chapter 3 will address whether p53 acts as a component of a checkpoint at mitosis by examining how the p53 status of normal fibroblasts affects their ability to undergo cell cycle arrest in response to the microtubule-depolymerizing drug nocodazole.

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Chapter 2

Induction of p53-dependent and p53-independent apoptosis by the chemotherapeutic paclitaxel

The experiments described in this chapter were performed with the assistance of Scott W. Lowe (Cold Spring Harbor Laboratory, NY); Edward J. Licitra, and Jun O. Liu (Center for Cancer Research, M.I.T.).

Introduction

Most chemotherapeutic agents were identified by virtue of their cytotoxicity against tumor cell lines. The basis for their mechanism of action remains poorly understood, but has been thought to be due to inhibiting tumor cell growth. Recently, the realization that many of these agents induce apoptosis, a genetically determined form of cell death, has forced a reevaluation of the mechanisms by which cytotoxic agents inhibit tumor growth. These studies have identified correlations between drug responsiveness and tumor genotype (Lowe *et al.*, 1993a; Lowe *et al.*, 1994; rev. in Biedler 1992; Reed 1995). Consequently, a further understanding of how tumor-specific mutations affect treatment efficacy may ultimately provide a more rational basis for choice of anticancer regimen.

Mutations in the p53 tumor suppressor gene have recently been shown to have an impact on the clinical course of human tumors. Indeed, patients harboring tumors with p53 mutations often have a worse prognosis than those harboring tumors with wild-type p53 (rev. in Lowe 1995) and in several instances, p53 mutations have been associated with drug-resistant tumors (Bergh *et al.*, 1995; Goh *et al.*, 1995; Rusch *et al.*, 1995; Aas *et al.*, 1996). Consistent with these clinical findings, inactivation of p53 can promote tumorigenesis and lead to drug resistance in experimental settings. The p53 gene encodes a transcription factor that can regulate cell proliferation and survival by modulating transcription of downstream target genes, inducing either G1 arrest or apoptosis (Clarke *et al.*, 1993; El-Deiry *et al.*, 1993; Harper *et al.*, 1993; Lowe *et al.*, 1993a; Lowe *et al.*, 1993b). p53 is activated to promote G1 arrest or apoptosis by several stimuli, the most well-characterized being DNA damage. Many known anticancer agents cause DNA damage, presumably leading to p53-dependent apoptosis. Inactivation of the apoptotic response provides an attractive explanation

to account for the poor responsiveness of p53 mutant tumors to these agents. Thus, identifying chemotherapeutic agents that act independently of the p53 pathway is of fundamental importance.

Paclitaxel, a drug used for cancer therapy, is derived from the Pacific yew tree (*Taxus brevifolia*), and was initially considered promising because of its cytotoxic activity against several tumor cell lines (Wani *et al.*, 1971; Douros and Suffness 1978). Subsequent studies demonstrated that paclitaxel was effective against a variety of murine tumors and human xenografts (National Cancer Institute, 1983; Riondel *et al.*, 1986; Riondel *et al.*, 1988), as well as advanced human carcinomas refractory to traditional chemotherapy (rev. in Rowinsky and Donehower 1995). Paclitaxel promotes the assembly of microtubules *in vitro* (Schiff *et al.*, 1979). Consequently, the cytotoxic effect of the drug in tissue culture can be attributed to its ability to stabilize cellular microtubules and thus inhibit formation of the mitotic spindle (Schiff and Horwitz 1980). However, in addition to its microtubule-stabilizing activity, paclitaxel also stimulates the lipopolysaccharide (LPS) signalling pathway in murine macrophages, resulting in secretion of the cytokines interleukin-1ß (IL-1ß) and tumor necrosis factor-α (TNF-α) (Ding *et al.*, 1990; Bogdan and Ding 1992; Manthey *et al.*, 1992; Carboni *et al.*, 1993). This effect is independent of its ability to stabilize microtubules, as some derivatives of paclitaxel retain microtubule-stabilizing activity but do not stimulate cytokine secretion (Manthey *et al.*, 1993; Burkhart *et al.*, 1994). Interestingly, TNF-α itself can induce apoptosis and has well-documented anticancer activity (reviewed in Beyaert 1994; Hieber and Heim 1994). In human macrophages, paclitaxel alone has not been shown to induce TNF-α or IL-1ß secretion, but it does enhance production of these cytokines when applied in conjunction with LPS (Allen *et al.*, 1993).

Since p53 promotes apoptosis following DNA damage, it might be expected that a microtubule-stabilizing drug such as paclitaxel would have p53-independent effects. Indeed, several of the human xenografts that responded to paclitaxel in preclinical trials are documented to have p53 mutations (National Cancer Institute, 1983; Baker *et al.*, 1989; Reiss *et al.*, 1992; Rose 1993; Joschko *et al.*, 1994). However, paclitaxel-induced cell cycle arrest is compromised in murine fibroblasts lacking p53, suggesting that p53 may in fact contribute to paclitaxel's biological effects (Wahl *et al.*, 1996). Furthermore, in an ovarian teratocarcinoma cell line, paclitaxel induced apoptosis to a greater extent in cells with intact p53 function than in cells in which p53 was inactivated through expression of human papilloma virus type 16 E6 protein (Wu and El-Deiry 1996). Thus, there is an apparent discrepancy between the effects of p53 status on the response of cells to paclitaxel *in vivo* versus *in vitro*. In order to characterize the effectiveness of paclitaxel against transformed cells, we sought to determine the relationship between paclitaxel response and p53 status.

Methods

Cells and cell culture. p53 +/+ and p53 -/- mouse embryonic fibroblasts (MEFs) were derived from 13.5 day old embryos and used at early passage (Jacks *et al.*, 1994). p53 +/+ and p53 -/- MEF clones stably expressing E1A and T24 H-ras were generated by calcium phosphate coprecipitation as previously described (Lowe *et al.*, 1993a). The RAW 264.7 cell line was a gift from Dr. Gerald Wogan (M.I.T., Division of Toxicology, Cambridge, MA). All cells were maintained in Dulbeccos' modified Eagle's medium containing 10% fetal bovine serum supplemented with penicillin and streptomycin.

Dose-response assays. Exponentially growing fibroblasts were plated at a density of $8 \cdot 10^4$ cells per 34 mm well for untransformed MEFs and $1.5 \cdot 10^5$ cells per 34 mm well for transformed MEFs. Twenty-four hours after plating, growth medium was replaced with fresh medium containing the appropriate concentration of recombinant murine tumor necrosis factor-α (TNF-α) (Boehringer Mannheim), paclitaxel or cephalomannine (provided by the National Cancer Institute, Drug Synthesis and Chemistry Branch). Cell viability was determined after a 48 hour treatment by pooling adherent and nonadherent cells and measuring uptake of propidium iodide as determined by FACScan.

Conditioned medium assay. RAW 264.7 murine macrophages were plated at a density of $7.5 \cdot 10^6$ cells per 100 mm dish, allowed to adhere, and treated with 30 µM paclitaxel or cephalomannine for 24 hours. The macrophage growth medium was collected, filtered, and applied to wells of fibroblasts ($8 \cdot 10^4$ cells per 34 mm well for untransformed MEFs; $1.5 \cdot 10^5$ cells per 34 mm well for E1A-Ras expressing MEFs). Fibroblasts were grown in the conditioned medium for 24 hours and then analyzed

for cell viability by propidium iodide uptake. For anti-TNF-α experiments, macrophage growth medium was incubated with monoclonal hamster anti-mouse TNF-α (Genzyme) at concentrations of 0.1, 0.2 and 1 μg/ml of conditioned media for 2 hours at 37°C prior to addition to fibroblasts. The control antibody was monoclonal hamster anti-mouse CD3 used at a concentration of 1 μg/ml of conditioned media (Pharmingen).

Apoptosis assay. E1A-Ras expressing MEFs were plated onto poly-D-lysine coated coverslips at a density of $1.5 \cdot 10^5$ cells per 34 mm well. Twenty-four hours after plating, growth medium was replaced with fresh medium containing 10 μg/ml recombinant TNF-α. After 15 hours of treatment, cells were fixed in 2% paraformaldehyde/phosphate-buffered saline (PBS) for 15 minutes, washed with PBS, permeabilized for 15 minutes in 0.25% Triton X-100/PBS, and washed again with PBS. Cells were then stained for 5 minutes with 4,6-diamidino-2-phenylindole (DAPI) at a concentration of 1 μg/ml PBS. Coverslips were mounted on glass slides with Mowiol and analyzed via fluorescence microscopy.

Results

Induction of p53-dependent apoptosis by paclitaxel

We chose to examine paclitaxel response in a well-characterized system consisting of wild type and p53-deficient primary mouse embryonic fibroblasts (MEFs) transformed with the adenovirus-5-E1A oncogene and the T24-activated-H-ras allele (Lowe *et al.*, 1993a). These highly transformed cells differ in p53 genotype but are otherwise genetically similar, thus constituting a good model system in which to determine how p53 status affects response to chemotherapeutic agents (e.g., see Lowe *et al.*, 1993a).

The viability of E1A-Ras transformed, wild-type and p53-deficient MEFs and untransformed control MEFs treated with paclitaxel is summarized in Fig. 2.1. Cells were treated with the indicated concentration of paclitaxel for 48 hours, then analyzed for viability by propidium iodide exclusion. In the transformed fibroblast populations, paclitaxel treatment led to preferential killing of cells containing wild-type p53. p53 +/+, E1A-Ras transformed MEFs had an IC50 of 75 nM, while the IC50 of their p53 -/- counterparts was >30 μM. Death occurred by induction of the apoptotic pathway, as determined by condensed chromatin and nuclear fragmentation visible in cells stained with DAPI (data not shown). The viability of untransformed MEFs was largely unaffected by exposure to paclitaxel at the concentrations tested, although p53 -/- MEFs did show a slight decrease in viability, as has been observed previously (Hawkins *et al.*, 1996; Wahl *et al.*, 1996).

Macrophage-mediated killing of p53-deficient tumor cells

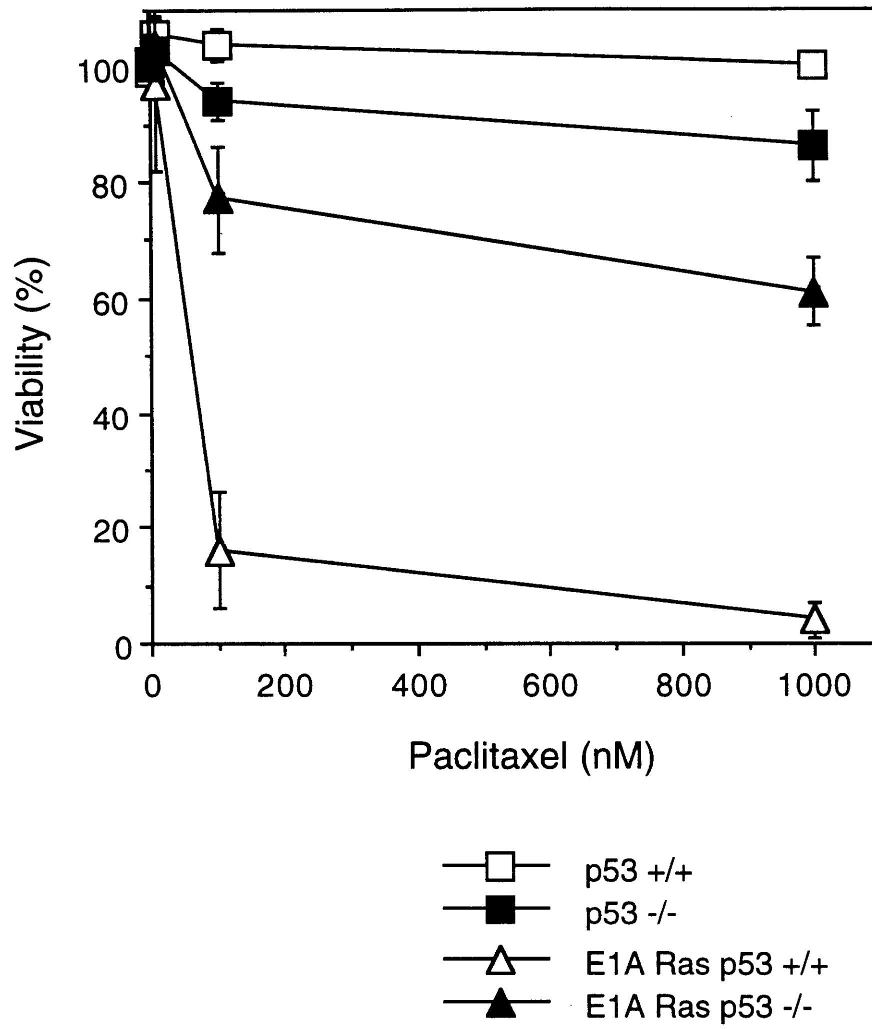
The observation that paclitaxel induced p53-dependent apoptosis in transformed cells was unexpected, since paclitaxel was effective against p53 mutant tumors in

FIG. 2.1

Induction of p53-dependent apoptosis in transformed cells by paclitaxel.

Viability of p53 +/+ untransformed fibroblasts, p53 -/- untransformed fibroblasts, p53 +/+ E1A-Ras expressing fibroblasts, and p53 -/- E1A-Ras expressing fibroblasts is shown following paclitaxel treatment. Cells were grown in paclitaxel-containing media at the indicated concentrations for 48 hours, harvested, and analyzed for viability as determined by propidium iodide exclusion using flow cytometry. Treatment of cells with equal amounts of carrier alone (ethanol) had no effect on viability (not shown). Data shown represent the analysis of 3 experiments, with error bars indicated.

Figure 2.1



preclinical studies (National Cancer Institute, 1983; Baker *et al.*, 1989; Reiss *et al.*, 1992; Rose 1993; Joschko *et al.*, 1994). This discrepancy could be resolved if, in addition to its ability to directly induce p53-dependent apoptosis, paclitaxel also acted through an indirect mechanism that required a tumor microenvironment. In this regard, the ability of paclitaxel to stimulate release of cytokines from tumor-associated macrophages provides a potential mechanism.

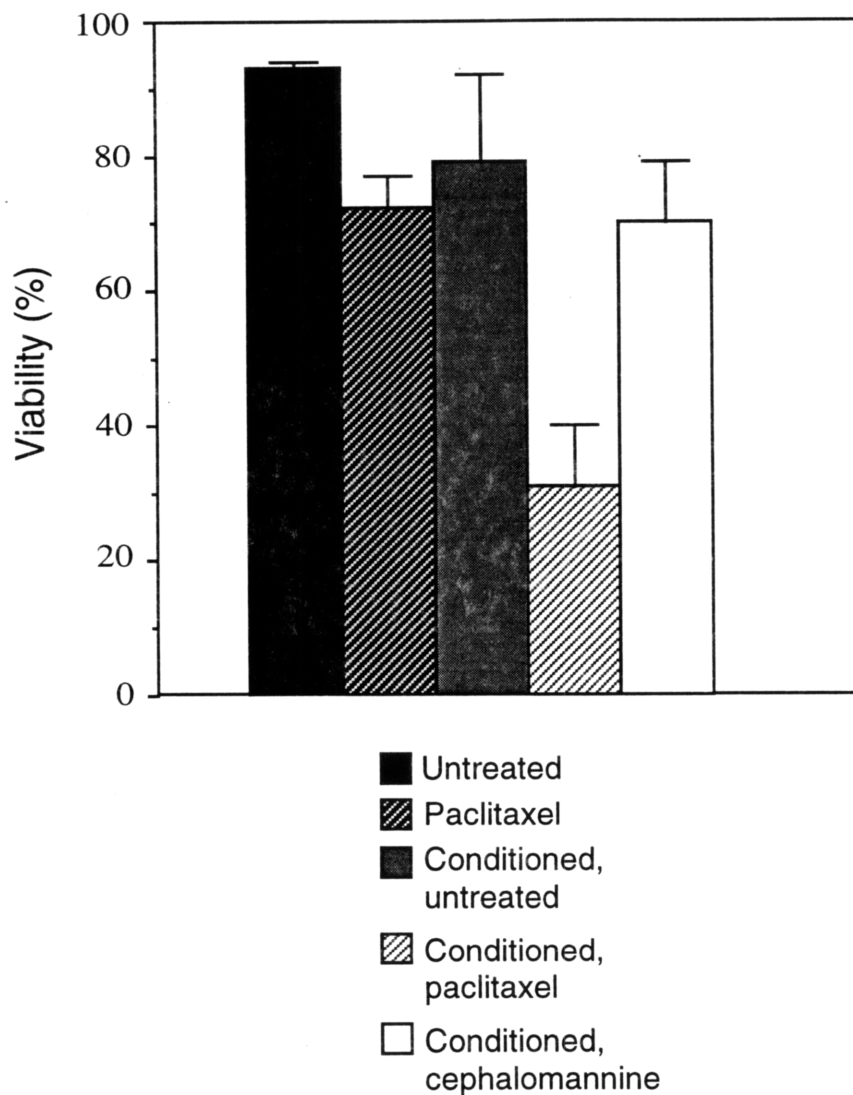
To determine whether paclitaxel could induce secretion of a cytotoxic cytokine from macrophages, we utilized an *in vitro* tissue culture assay which recapitulated events that might occur in an *in vivo* tumor setting. We included as a control the paclitaxel derivative cephalomannine, which retains the ability to stabilize microtubules but does not induce cytokine secretion (Burkhart *et al.*, 1994). Cells from the murine macrophage cell line RAW 264.7 were exposed to 30 μM paclitaxel or 30 μM cephalomannine for 24 hours. This concentration of paclitaxel is comparable to plasma levels of paclitaxel following a standard therapeutic dose (Innocenti *et al.*, 1995). The media from the treated macrophages was then collected, filtered, and applied to p53 -/- E1A-Ras expressing MEFs. After 24 hours of incubation with the macrophage-conditioned media, the transformed MEFs were assayed for viability (Fig. 2.2). As would be predicted from the results presented in Fig. 2.1, treating p53 -/- cells directly with 30 μM paclitaxel led to only a modest loss of viability. However, exposing these cells to conditioned media from macrophages treated with 30 μM paclitaxel caused a sharp decrease in viability. Importantly, incubation of the cells in media from macrophages treated with cephalomannine did not cause a decrease in viability beyond that observed following direct paclitaxel treatment. These data suggest that macrophages exposed to paclitaxel release a soluble factor into the media which induces apoptosis in p53 -/- transformed fibroblasts.

FIG. 2.2

Effects of macrophage-conditioned media on p53 -/- transformed cells.

Murine macrophages (RAW 264.7) were treated with 30 μM paclitaxel or 30 μM cephalomannine for 24 hours. Macrophage-conditioned media was collected, filtered, and applied to E1A-Ras p53 -/- fibroblasts. After 24 hours, viability of fibroblasts was determined using propidium iodide exclusion and flow cytometry. Viability of E1A-Ras p53 -/- fibroblasts was also determined following treatment with 30 μM paclitaxel directly, and following incubation in conditioned medium from untreated RAW 264.7 cells. Data shown represent the analysis of 3 experiments, with error bars indicated.

Figure 2.2



TNF-α release from macrophages

TNF-α is a likely candidate for the cytotoxic factor secreted by paclitaxel-treated macrophages, since earlier studies have shown both that paclitaxel induces secretion of TNF-α (Ding *et al.*, 1990) and that TNF-α can promote apoptosis (Beyaert 1994). To test whether TNF-α was the cytotoxic factor present in the conditioned media, RAW 264.7 macrophages were treated with paclitaxel for 24 hours, and the conditioned media was then collected and incubated for 1 hour with a monoclonal antibody to TNF-α. The MEFs were then grown in the macrophage-conditioned media for 24 hours and subsequently assayed for viability. As shown in Fig. 2.3, preincubation of the conditioned media with 0.1 μg/ml of an anti-TNF-α monoclonal antibody protected a large portion of the cell population from death. Incubation with higher concentrations of antibody restored the viability of the p53 -/- transformed MEFs to near untreated levels. However, preincubation of the conditioned media with a control monoclonal antibody against CD3 had no effect on viability. These data strongly suggest that the cytotoxic factor released by macrophages upon paclitaxel treatment is TNF-α.

Direct induction of apoptosis by TNF-α

To prove that TNF-α is capable of inducing p53-independent apoptosis, we examined the response of E1A-Ras transformed MEFs when treated directly with TNF-α (Fig. 2.4a). Untransformed and E1A-Ras expressing p53 +/+ and p53 -/- MEFs were treated with TNF-α at various concentrations for a 48 hour period and then assayed for viability. Exposure to TNF-α led to significant cytotoxicity in all E1A-Ras expressing cells, independent of their p53 status. Cells died by apoptosis, as determined morphologically and by nuclear staining with DAPI (Fig. 2.4b) The viability of untransformed MEFs was unaffected by TNF-α treatment (Fig. 2.4a). The

ability of TNF-α to induce death in E1A-Ras p53 -/- tumor cells supports the idea that paclitaxel acts via TNF-α secretion *in vivo*.

FIG. 2.3.

Protection from apoptosis by anti-TNF-α antibody.

Conditioned media from paclitaxel-treated macrophages was incubated with monoclonal anti-TNF-α or anti-CD3 antibody, then applied to E1A-Ras p53 -/- fibroblasts. Viability of fibroblasts was determined after 24 hours using propidium iodide staining and flow cytometry. Data shown represent the analysis of 3 experiments, with error bars indicated.

Figure 2.3

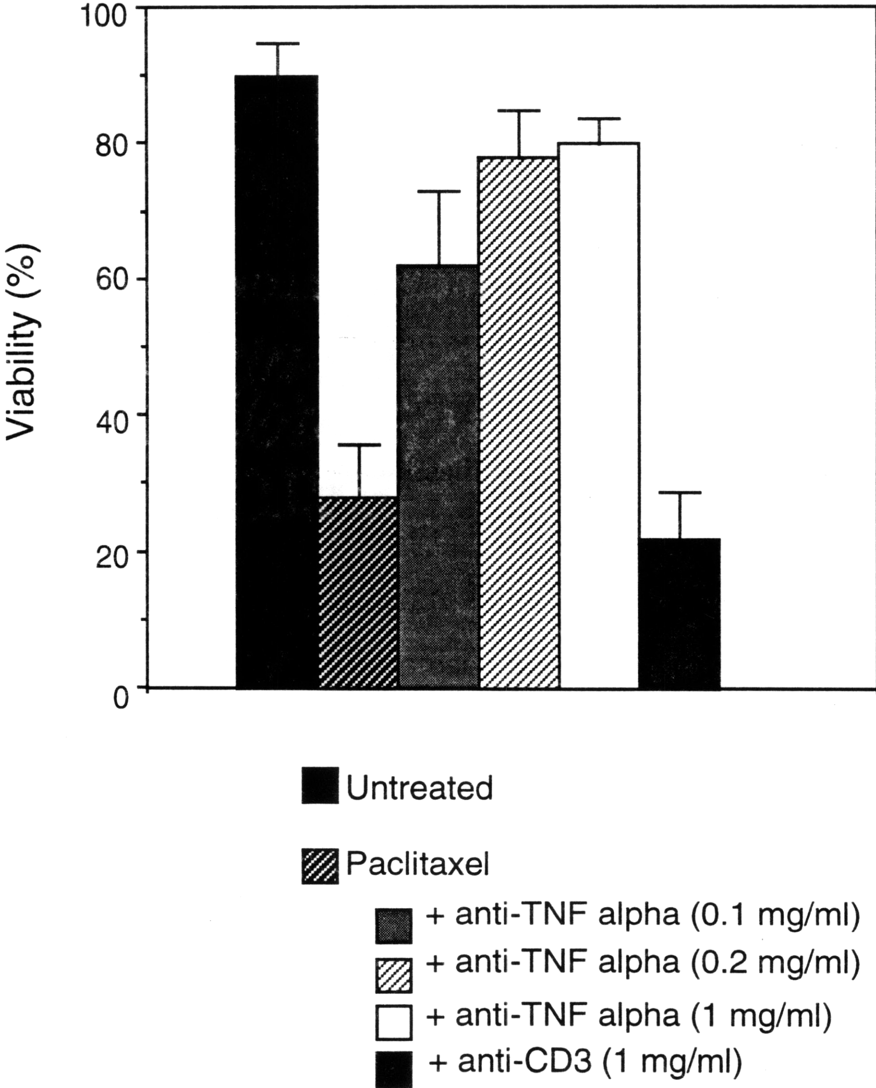


FIG. 2.4

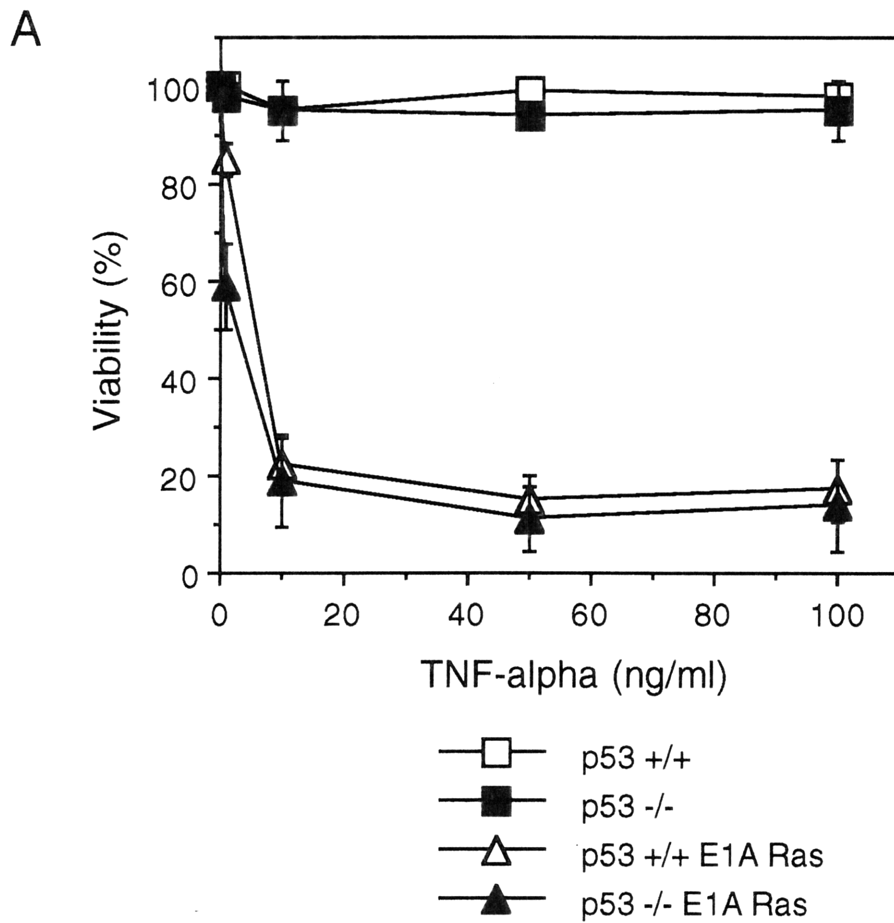
Induction of p53-independent apoptosis by TNF-α.

(A) Viability of p53 +/+ untransformed fibroblasts, p53 -/- untransformed fibroblasts, p53 +/+ E1A-Ras expressing fibroblasts , and p53 -/- E1A-Ras expressing fibroblasts following TNF-α treatment. Cells were grown in media containing TNF-α at the indicated concentrations for 48 hours, harvested, and analyzed for viability as determined by propidium iodide exclusion using flow cytometry. Data shown represent the analysis of 3 experiments, with error bars indicated.

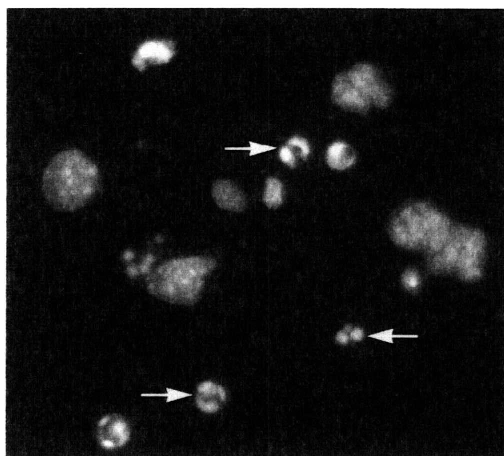
(B) Induction of apoptosis by TNF-α in E1A-Ras p53 -/- fibroblasts. Cells were treated with TNF-α (10 ng/ml) for 15 hours, at which time a significant fraction had undergone apoptosis. Cells were then fixed, stained with the nuclear stain 4,6-diamidino-2-phenylindole (DAPI), and examined using fluorescence microscopy. Arrows indicate apoptotic nuclei, with condensed, brightly staining chromatin.

Figure 2.4

A



B



Discussion

The present study describes how the chemotherapeutic agent paclitaxel may use a novel mode of action against tumors. Like traditional antineoplastic therapies such as adriamycin and gamma-irradiation (Lowe *et al.*, 1993a; Lowe *et al.*, 1994), paclitaxel can act directly on the target tumor cell and induce apoptosis via a p53-dependent pathway. However, unlike these other treatments, paclitaxel also has the potential to act indirectly against tumor cells by stimulating macrophages to secrete the cytokine TNF-α. This secondary effect could circumvent the issue of the p53 genotype of the target cell, because we have demonstrated that TNF-α induces apoptosis in transformed cells irrespective of their p53 status.

Direct induction of p53-dependent apoptosis

Given that p53's function is understood primarily from its role in the DNA damage response pathway, it is interesting that paclitaxel, a microtubule stabilizing drug, should elicit a p53-dependent response. Data from untransformed fibroblasts also suggest a p53 connection, as MEFs lacking p53 have decreased viability after paclitaxel treatment (Hawkins *et al.*, 1996; Wahl *et al.*, 1996). Also, experiments in which untransformed fibroblasts were treated with the microtubule-destabilizing agents nocodazole and colcemid indicate a possible role for p53 as a mitotic spindle checkpoint (Cross *et al.*, 1995). It is presently unclear, however, how the state of cellular microtubules influences the p53 pathway.

The extreme sensitivity of p53 +/+ E1A-Ras transformed cells to paclitaxel is striking, with apoptotic cells observed at paclitaxel concentrations as low as 10 nM. In untransformed fibroblasts, low concentrations of paclitaxel induce mitotic block not by increasing tubulin polymerization, but by suppressing the dynamic growth and shrinkage of the mitotic spindle (Jordan *et al.*, 1993). Interestingly, this

mechanism is identical to that of the microtubule destabilizing agent vinblastine, suggesting that in untransformed cells, both classes of antimitotic drugs share a common antiproliferative mechanism (Jordan *et al.*, 1991; Jordan *et al.*, 1993). A common pathway may also be functioning in transformed cells treated with antimitotic drugs, since the microtubule-destabilizing agent vincristine, which is structurally similar to vinblastine, also induces p53-dependent apoptosis in E1A-Ras transformed fibroblasts (data not shown). Thus, drugs which stabilize or destabilize microtubules both may lead to p53-dependent cell cycle arrest or apoptosis, depending on the cellular context.

TNF-α secretion and p53-independent apoptosis

Paclitaxel also has the ability to induce TNF-α release from murine macrophages (Ding *et al.*, 1990). Other studies suggest that TNF-α-induced apoptosis proceeds through a complex of molecules that link the trimeric TNF-α receptor to several downstream effectors (Baker and Reddy 1996). In this study, we observe that p53 is dispensable for TNF-α-induced apoptosis, since TNF-α treatment triggered apoptosis in E1A-Ras transformed fibroblasts in a p53-independent manner. Therefore, our data suggest that the p53 protein is not a component of the TNF-α apoptotic pathway.

The observation that TNF-α can induce p53-independent apoptosis in transformed cells would suggest that TNF-α itself could be an effective antineoplastic treatment, particularly against tumors which have sustained p53 mutations. TNF-α has long been recognized as an agent capable of inducing marked tumor regression, and has undergone considerable testing against tumors in patients and animal models. Systemic administration of TNF-α in humans produced severe side effects and was ineffective against tumors (reviewed in Spriggs and Yates 1992). More recently,

however, local administration of TNF-α alone, or in combination with other drugs and cytokines, was effective at inhibiting tumor growth with minimal side effects in both tumor models and human patients (Baisch *et al.*, 1990; Asher *et al.*, 1991; Lejeune *et al.*, 1994; Lasek *et al.*, 1995; Lasek *et al.*, 1996). These results lend support to further testing of TNF-α as an anticancer agent.

Therapeutic effectiveness of paclitaxel

This study supports prior observations that paclitaxel can promote the release of TNF-α from murine macrophages, and additionally shows that the secreted cytokine can efficiently induce apoptosis in p53 -/- E1A-Ras transformed MEFs. We suggest that this mode of action contributes to the activity of paclitaxel in murine tumor model systems, as solid tumors are often infiltrated by macrophages (Mantovani *et al.*, 1992). Accordingly, our study predicts that paclitaxel should be effective against tumors with inactivated p53. We have attempted to demonstrate this by treating tumors generated in nude mice from E1A-Ras transformed cells; however, we found that paclitaxel was not measurably active against such tumors, regardless of their p53 genotype (data not shown). However, data obtained from testing of paclitaxel in other tumor models support our prediction: paclitaxel was effective in preclinical testing against the human tumors CX-1 (colon), A431 (vulva), and FaDu (hypopharynx) when xenografted into nude mice (National Cancer Institute, 1983; Rose 1993; Joschko *et al.*, 1994), all of which are documented to contain p53 mutations (Baker *et al.*, 1989; Reiss *et al.*, 1992). Additionally, paclitaxel was effective against numerous other tumor xenografts (Nicoletti *et al.*, 1993; Rose 1993; Joschko *et al.*, 1994; Milross *et al.*, 1995), few of which have been screened for their p53 status but at least some of which are likely to contain mutated p53.

It will be important to establish whether in human patients, paclitaxel activity is contingent upon macrophage stimulation and subsequent cytokine release. The activation of the LPS pathway by paclitaxel alone has only been documented in mouse macrophages, and could represent a species difference (Allen *et al.*, 1993; Ding *et al.*, 1993). The observation that paclitaxel can enhance TNF-α and IL-1B gene expression in the presence of LPS might suggest that paclitaxel does activate the LPS pathway in human macrophages, but only in conjunction with a second signal (Allen *et al.*, 1993). How the p53 status of human tumors determines their responsiveness to paclitaxel is unclear, but it is noteworthy that paclitaxel is effective in treating breast cancer, a tumor type with a high frequency of p53 mutations (Ozbun and Butel 1995). Left unexplained is the apparent selectivity of paclitaxel, which is effectively used to treat ovarian and breast cancer, among others. One might expect that neither the microtubule-based nor the cytokine-based effects would be specific to tumor tissue type. Based on the present work, one possible explanation for this apparent selectivity would be that different tumor types have different degrees of macrophage infiltration.

If paclitaxel acts through a cellular intermediate, then it represents a new mode of action for chemotherapeutic agents. Moreover, these data suggest that the critical feature of paclitaxel as an anti-tumor agent may not be its microtubule-stabilizing activity, but its ability to activate components of the immune system. Therefore, this aspect of the drug's action should be a focus of efforts to improve its effectiveness through modification.

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Chapter 3

Characterization of the p53-dependent postmitotic checkpoint following spindle disruption

Introduction

The proper execution of events in the eukaryotic cell cycle is regulated by a number of different checkpoints. For example, to ensure stable maintenance of the genome, cells arrest in G1 or G2 upon detection of DNA damage, providing time for DNA repair before the initiation of DNA synthesis or entry into mitosis (Elledge 1996). Other checkpoints function during mitosis to monitor successful assembly of the spindle and control the initiation of metaphase, thus protecting the cell from chromosome missegregation (Rudner and Murray 1996). Genes that are required for cellular arrest at different checkpoints have been identified, demonstrating that each checkpoint is a genetic pathway activated by specific signals. The inactivation of checkpoint genes leads to increased mutation rate, chromosome loss, or changes in ploidy, depending on the genes affected (Paulovich *et al.*, 1997). Interestingly, some genes that are mutated in human cancers are involved in checkpoint functions, suggesting that without such controls in place, the resulting genetic damage predisposes cells to malignancy (Sherr 1996).

The p53 tumor suppressor gene is mutated in over half of all sporadic human cancers. p53 has an essential role in the G1 checkpoint in response to DNA-damaging agents such as radiation (Kastan *et al.*, 1991; Kastan *et al.*, 1992; Kuerbitz *et al.*, 1992). Functional analysis of the p53 protein has shown that it is a transcription factor with sequence-specific DNA binding activity (Farmer *et al.*, 1992; Kern *et al.*, 1992; Zambetti *et al.*, 1992). After DNA damage, p53 activates the transcription of several downstream target genes, including p21, an inhibitor of cyclin-dependent kinases (CDKs) (El-Deiry *et al.*, 1993). The induction of p21 causes subsequent arrest in the G1 phase of the cell cycle by binding of cyclin-CDK complexes (Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993). Additional p53 target genes are likely to

cooperate with p21 in implementing G1 arrest, as p21-deficient mouse cells are only partially defective in their DNA damage arrest response, while p53-deficient cells are completely defective (Brugarolas *et al.*, 1995; Deng *et al.*, 1995).

Recently, p53 has been proposed to have an additional, novel function as a checkpoint at mitosis. Wild-type fibroblasts arrest when the mitotic spindle assembly is disrupted by the addition of drugs which bind microtubules. However, studies have shown that p53-deficient fibroblasts fail to arrest under such conditions but instead undergo a new round of DNA synthesis in the absence of cell division, becoming polyploid (Cross *et al.*, 1995; Minn *et al.*, 1996; Di Leonardo *et al.*, 1997). This phenotype is very similar to that observed in yeast strains that have inactivated spindle assembly checkpoints. *S. cerevisiae* strains with mutations in the MAD or BUB genes, which monitor spindle assembly, become polyploid when treated with spindle inhibitors (Hoyt *et al.*, 1991; Li and Murray 1991). Based on this similarity, one might conclude that, like the MAD and BUB gene products, p53 monitors spindle integrity and can thus be termed a mitotic checkpoint. Also, additional data have suggested that the spindle components themselves may be under p53 regulation, as p53 has been shown to localize to the centrosome (Brown *et al.*, 1994), and p53 −/− MEFs contain abnormal numbers of centrosomes (Fukasawa *et al.*, 1996). However, later work has shown that in cells treated with spindle inhibitors, p53 is neither expressed during mitosis nor required for mitotic arrest (Minn *et al.*, 1996). Instead, expression of p53 protein occurs only after cells exit mitotic arrest and progress to an interphase state while still retaining 4N DNA content. These data argue that p53 does not function as part of a mitotic checkpoint but acts at some subsequent point in the cell cycle to induce arrest following a defect in M phase.

In this study, we have characterized in greater detail the p53-dependent checkpoint following disruption of the mitotic spindle with microtubule-destabilizing drugs. Upon examining the response of wild-type and p53-deficient mouse embryo fibroblasts to spindle inhibitors, we observed that cells of either genotype underwent a transient arrest at mitosis and subsequently progressed into a G1-like state without ever completing cell division. Wild-type MEFs remained arrested in this state, while p53 –/– MEFs initiated another round of DNA synthesis. Using time-lapse videomicroscopy, we were able to define the length of mitotic arrest and establish precisely the timing of S phase reentry in p53 –/– MEFs after exit from mitotic arrest. We also demonstrated a requirement for the p53 target gene product, p21, in executing cell-cycle arrest after spindle disruption. Upon further characterization of arrested cells, we determined that cells expressed molecular markers associated with the G1 phase of the cell cycle despite having 4N DNA content. These data demonstrate that the p53-dependent checkpoint in cells with disrupted mitotic spindles has strong similarity to the p53-dependent checkpoint in G1 following DNA damage. Our data suggest that rather than having a novel role as part of a spindle checkpoint, p53 functions in the G1 phase of the cell cycle and via the same downstream pathway in murine cells which have sustained either DNA damage or microtubule disruption.

Methods

Cells and cell culture. p53 +/+ and p53 –/– mouse embryonic fibroblasts (MEFs) were derived from 13.5 day old embryos and used between passages 3 and 8 (Jacks *et al.*, 1994). MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum supplemented with penicillin and streptomycin. For flow cytometry and immunoblot experiments, MEFs were plated at a density of 5 x 10^5 - 1 x 10^6 cells per 100 mm dish, 24 h in advance of experiments. Nocodazole (Sigma) was kept as a 1 mg/ml stock in DMSO. Nocodazole treatment of MEFs was performed at a concentration of 0.125 μg nocodazole/ml media.

NIH 3T3 cells were obtained from the American Type Culture Collection, and were grown in DMEM containing 10% calf serum supplemented with penicillin and streptomycin. For flow cytometry and immunoblot experiments, NIH 3T3 cells were plated at a density of 5 x 10^5 cells per 100 mm dish. 24 h after plating, cells were serum synchronized by washing twice with DMEM, then placing in DMEM plus 0.5% calf serum for 48 h. Nocodazole treatment of NIH 3T3 cells was performed at a concentration of 0.5 μg nocodazole/ml media.

Flow cytometry. Approximately 1 x 10^6 cells per 100 mm dish were detached in 0.25% trypsin and washed in phosphate-buffered saline (PBS). Following centrifugation, cells were resuspended in 0.5 ml cold PBS. Cells were fixed by adding 4.5 ml cold 100% ethanol dropwise to the sample while vortexing gently, and then placing at -20°C for at least 60 min. After fixation, cells were spun out of ethanol, washed once with PBS, and resuspended in 20 μg propidium iodide (Sigma) + 200 μg RNAse (Sigma)/ml PBS. Cells were incubated at 37°C for 30 min and allowed to

stain for at least 8 h at 4°C. Samples were analyzed for DNA content on a Becton Dickinson FACScan.

Immunofluorescence. 1 x 10^5 MEFs were plated onto glass coverslips in a 34 mm tissue culture well. MEFs were allowed to adhere to coverslips for 24 h, then were changed into medium with or without nocodazole (0.125 μg/ml) and incubated for 24 h. Bromodeoxyuridine (BrdU) and fluorodeoxyuridine (FdU) (Sigma) were added to the medium from a 1000x stock solution in H_20, for a final concentration of 3 μg BrdU + 0.3 μg FdU/ml medium; cells were incubated for an additional 4 h. Coverslips were then fixed in 4% paraformaldehyde/PBS for 20 min at room temp, washed in PBS, permeabilized for 15 min in 0.25% Triton X-100/PBS, and washed again with PBS. For BrdU detection, slips were denatured in 1.5 N HCl for 10 min and washed several times with PBS. Slips were incubated with a murine monoclonal antibody to BrdU (Becton Dickinson, 1:50 dilution) in 10% goat serum/PBS for 30 min at 37°C. After 2 PBS washes, fluorescein-conjugated anti-mouse antibody (Jackson Immunoresearch Laboratories, 1:200 dilution) was added in 10% goat serum/PBS, and slips were incubated for 30 min at 37°C. Slips were washed with PBS and stained for 5 min in 0.2 μg 4,6-diamidino-2-phenylindole (DAPI; Sigma)/ml PBS. Coverslips were mounted on glass slides with Mowiol and analyzed via fluorescence microscopy.

Time-lapse videomicroscopy. MEFs were grown on 12 mm glass coverslips as above. A single coverslip was placed in a dish containing 2 ml HEPES-buffered DMEM + 10% fetal bovine serum, with or without nocodazole (0.125 μg/ml). Each coverslip was observed for up to 24 h under a Nikon microscope. Recording was done by taking one picture frame every 8 seconds using a GYYR TLC2100 time-lapse VCR. During recording, MEFs were maintained at 37°C using a Delta temperature

controller and kept in an environment of 5% CO_2 by bubbling gas across the surface of the media. Pictures were printed out using a Sony color video printer. All equipment was obtained from Micro Video Instruments, Inc.

For time-lapse videomicroscopy followed by immunofluorescence, MEFs were grown on gridded cover slips (Bellco) placed in 34 mm wells, with 1×10^5 MEFs plated per well. A single square of the grid was followed by time-lapse videomicroscopy for 18 to 24 h. For BrdU incorporation assays, 2 μl BrdU/FdU 1000x stock solution was added directly to the dish 4 h before the end of the experiment, for a final concentration of 3 μg BrdU + 0.3 μg FdU/ml medium. After recording was complete, the gridded slip was fixed in 4% paraformaldehyde/PBS and immunofluorescence was performed. The cells which had been recorded on video were identified during immunofluorescent analysis by locating the same square of the grid.

Immunoblotting. Whole cell extracts were made by scraping approximately 1×10^6 cells in 200 μl boiling lysis buffer (100 mM NaCl, 10 mM Tris pH 8.0, 1 % SDS). Lysates were heated at 100°C for 10 min, quick frozen in a dry ice/ethanol bath, and stored at -80°C. Protein concentration of lysates was determined using a bicinchoninic acid (BCA) kit (Pierce). For cyclin E, cyclin B1, and p21 immunoblots, 100 μg of protein was loaded per lane on a 10% (29:1) SDS-PAGE gel. Gels were electrophoresed at 150 V for 4 h, then transferred to to Immobilon-P membrane (Millipore) in low MW transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) for overnight at 25 V at 4°C. Blots were pre-blocked in 0.01% Tween-20/ PBS (PBS-T) + 5% dried milk for 1 h at room temp, then probed with primary antibody diluted in block solution for 1 h at room temp. Antibodies and dilutions were as follows: cyclin E, Santa Cruz M-20, 1:200 dilution; cyclin B1, Pharmingen GNS-1, 1:200

dilution; p21, Santa Cruz C19-G, 1:100 dilution. Blots were then washed with PBS-T 3 times for 10 min. For p21 detection, blots were put through an additional incubation step in mouse anti-goat antibody (Jackson Immunoresearch Laboratories, 1:12000) diluted in block solution for 1 h at room temp, followed by 3 washes in PBS-T. All blots were then incubated in peroxidase-linked secondary antibody (Amersham, 1:5000 dilution) for 1 h at room temp and washed 3 times in PBS-T. Blots were developed with ECL reagents and exposed to Kodak X-OMAT 5 film for 1-20 minutes.

For pRB immunoblots, 350 μg of protein was loaded per lane on a 6% (29:1) SDS-PAGE gel. Gels were electrophoresed at 20 mAmp overnight, then transferred to Immobilon-P membrane in high MW transfer buffer (50 mM Tris, 380 mM glycine, 0.1% SDS, 20% methanol) for 14 h at 25 V at 4°C, and for an additional hour at 40 V. Blots were pre-blocked in 0.03% Tween-20 in 10 mM Tris pH 7.5, 150 mM NaCl (TBS-T) + 5% dried milk for 1 h at room temp, then probed with primary antibody diluted in block solution for 1 h at room temp (Pharmingen G3-245, 1:100 dilution). After 3 10 min washes in TBS-T, blots were incubated in rabbit anti-mouse antibody (Jackson Immunoresearch Laboratories, 1:14,000) for 1 h at room temp. Blots were washed 3 times in TBS-T, incubated in peroxidase-linked tertiary antibody (Amersham, 1:5000 dilution) for 1 h at room temp, and washed 3 times in TBS-T. Blots were developed as above.

Results

Inappropriate DNA synthesis in nocodazole-treated p53 -/- fibroblasts

Because previous results regarding p53 function at mitosis were contradictory, we chose to characterize in greater detail the effect of p53 deficiency on cells treated with spindle inhibitors. To confirm a requirement for p53 following spindle disruption, wild-type and p53-deficient MEFs were grown in the presence of the microtubule-destabilizing drug nocodazole. After 24 h of treatment, cells were fixed and stained with propidium iodide for analysis by flow cytometry (Fig. 3.1A). Wild-type MEFs treated with nocodazole arrested with primarily a 4N DNA content. A small subpopulation of wild-type cells that had 8N DNA content was observed, perhaps reflecting the presence of cycling tetraploid cells in the normal population, which would be predicted to arrest with 8N DNA content upon spindle disruption. As described previously, p53 –/– fibroblasts did not arrest with a 4N DNA content upon drug treatment, but continued through an additional round of S phase to become 8N (Fig. 3.1A; Cross *et al.*, 1995; Minn *et al.*, 1996; Di Leonardo *et al.*, 1997).

In order to examine specifically the fraction of cells capable of S-phase reentry following nocodazole treatment, cells were treated with nocodazole for 24 h, pulsed with BrdU for an additional 4 h while still in the presence of drug, and analyzed by immunofluorescence for anti-BrdU staining (Fig. 3.1B). Untreated wild-type and p53 –/– MEFs had similar proportions of cells in S phase. After 28 h of nocodazole treatment, very few BrdU-positive cells were observed in the wild-type MEF population, consistent with their ability to induce cell cycle arrest after nocodazole treatment. However, many p53 –/– MEFs incorporated BrdU while in the presence of nocodazole, indicating that they were able to reenter the cell cycle and initiate DNA synthesis, despite having failed to undergo proper mitosis. Both wild-type and p53 –/– cells were observed to contain micronuclei following nocodazole

FIG. 3.1

p53-dependent cell cycle arrest following nocodazole treatment.

(A) FACS profiles of wild-type and p53 –/– MEFs untreated or treated with nocodazole for 24 h. X-axis is DNA content; y-axis is number of cells counted. Data shown are representative of multiple experiments performed on 3 different wild-type and p53 –/– clones.

(B) Immunofluorescent staining of wild-type and p53 –/– MEFs. MEFs were treated with nocodazole for 24 h, pulsed with BrdU in the presence of nocodazole for an additional 4 h, and fixed. Immunofluorescence was performed to detect BrdU incorporation (α-BrdU; green staining) and nuclear staining (DAPI; blue staining). Immunofluorescence was performed on 3 different wild-type and p53 –/– clones.

Figure 3.1

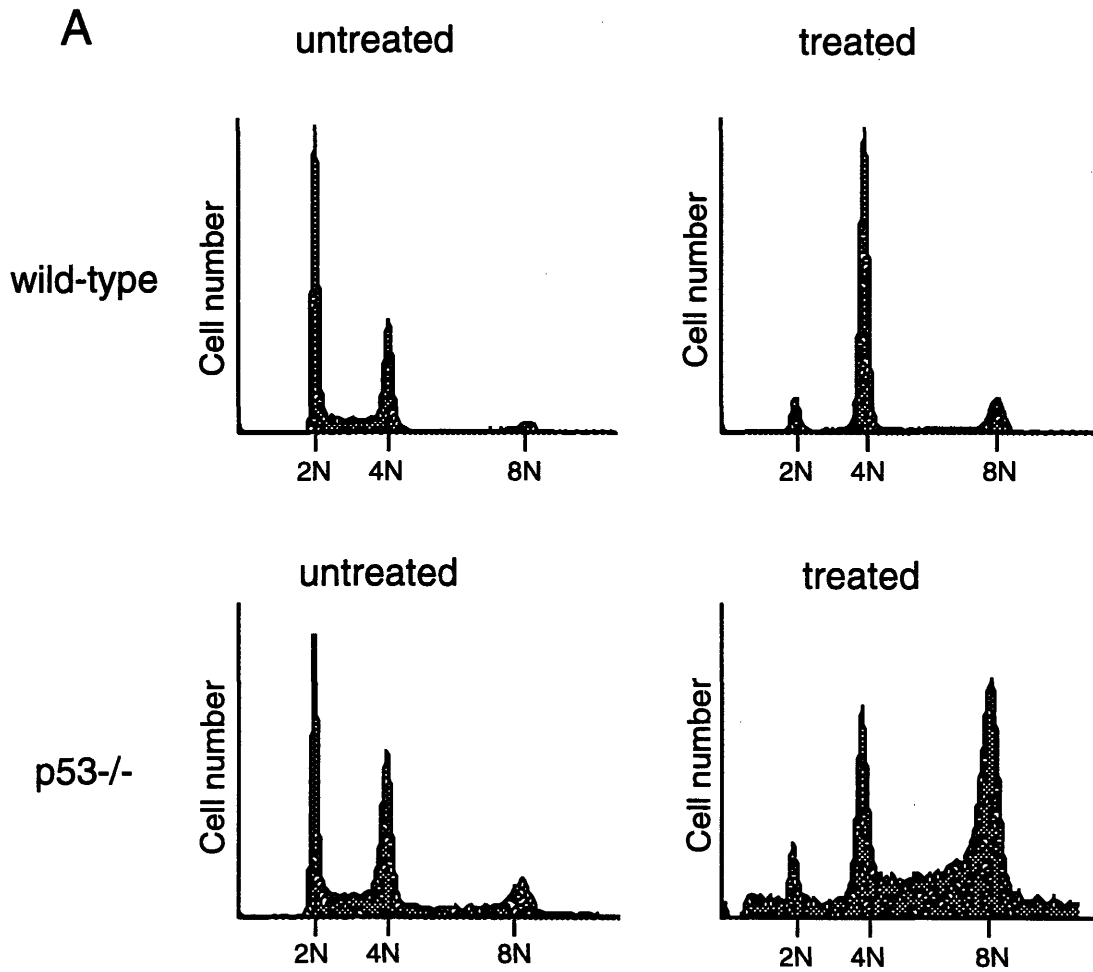
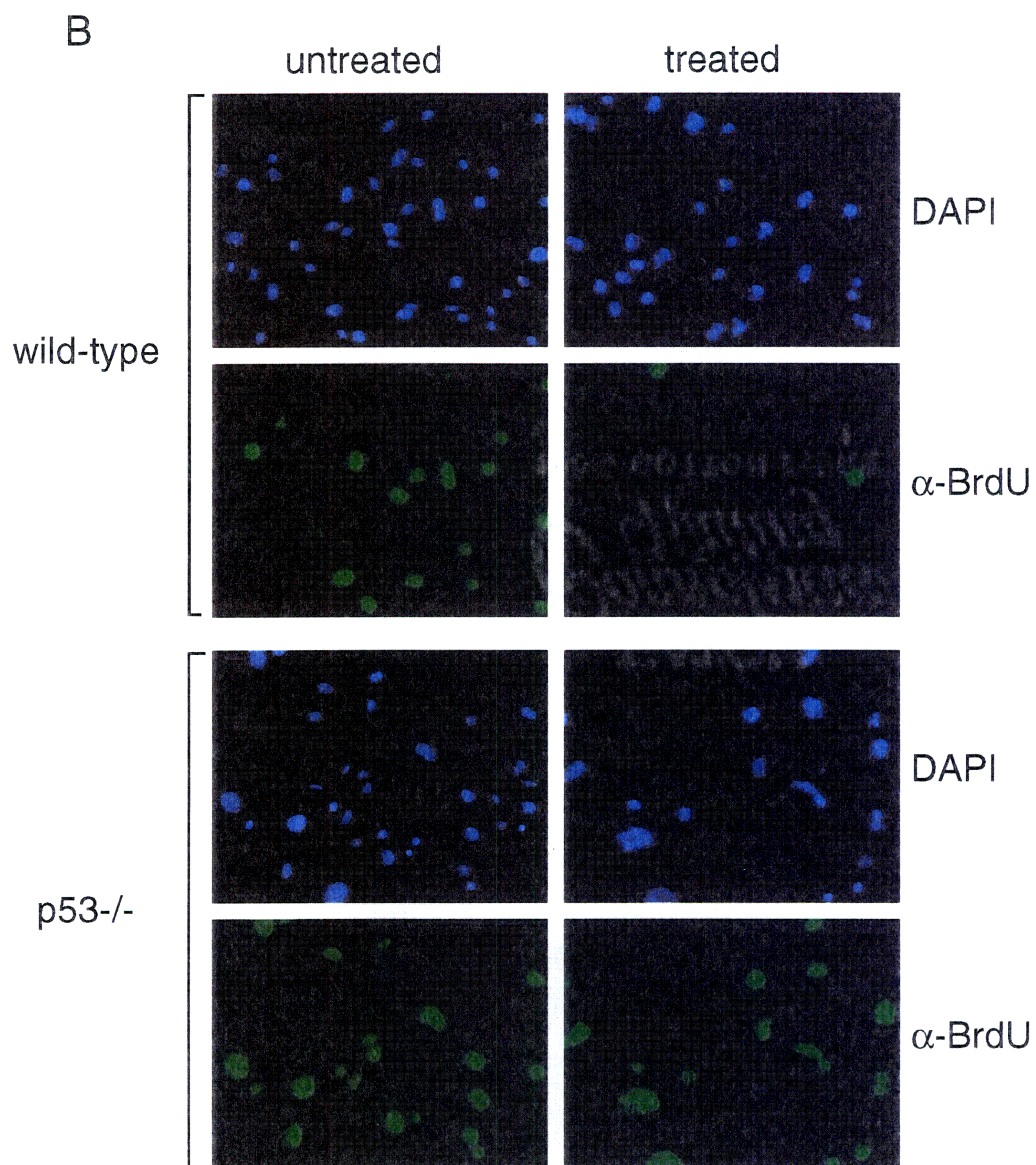


Figure 3.1



treatment, resulting from chromosome decondensation and subsequent reformation of the nuclear membrane (Kung *et al.*, 1990). These results confirm previous observations describing a p53-dependent arrest following destabilization of the mitotic spindle (Cross *et al.*, 1995; Minn *et al.*, 1996; Di Leonardo *et al.*, 1997).

Time-lapse videomicroscopy of mitotic arrest

One interpretation of the results described in Fig. 3.1 is that p53 functions in a checkpoint at mitosis, based on the fact that p53 −/− MEFs behave abnormally when the mitotic spindle is disrupted. To test this hypothesis directly, we used time-lapse videomicroscopy to observe p53 +/+ and p53 −/− MEFs during mitosis. If p53 were part of a mitotic checkpoint, then we would expect to see a difference in the behavior of nocodazole-treated p53 +/+ and p53 −/− MEFs at mitosis. Figure 3.2A shows a representative untreated p53 −/− fibroblast during normal cell division. The cell rounded up from the coverslip (Fig. 3.2A, time 5:21 PM), underwent cytokinesis (Fig. 3.2A, time 5:32 PM), and completed division into two daughter cells (Fig. 3.2A, time 5:40 PM). Thus, the length of mitosis, as determined visually by rounding of the parent cell and separation into two daughter cells, was approximately 20 min. Normal mitosis lasted 26 ± 8 min (mean ± standard deviation) in wild-type MEFs and 27 ± 7 min in p53 −/− MEFs, based on observations made on at least 15 cells of each genotype (data not shown). For our next experiments, we used time-lapse techniques to determine the fate of fibroblasts that initiated mitosis while in the presence of the spindle inhibitor nocodazole. Figure 3.2B shows two nocodazole-treated p53 +/+ fibroblasts that entered mitosis within the same 30 min window. Each cell rounded up from the coverslip as was observed for normal mitosis (Fig. 3.2B, time 1:14 PM and 1:43 PM). However, the cells then changed shape rapidly in apparent attempts to complete cell division over a period of approximately 4 h (Fig. 3.2B, time 3:33 PM and 4:31 PM). Strikingly, after

FIG. 3.2

Time-lapse videomicroscopy of wild-type and p53 –/– nocodazole-treated fibroblasts.

(A) Control p53 –/– MEF that underwent mitosis in normal media. Each photograph lists the time during videorecording when the picture was taken. The cell entered mitosis at 5:21 PM (arrowhead), cytokinesed at 5:32 PM (two arrowheads), and completed division by 5:40 PM. The cell shown is representative of over 30 cells (wild-type and p53 –/–) observed undergoing normal mitosis.
(B) Two wild-type MEFs that initiated mitosis in the presence of nocodazole. Each cell entered mitosis (time 1:14 PM and time 1:43 PM), remained arrested at mitosis for several hrs (time 3:33 PM and time 4:31 PM), then adapted (time 5:33 PM and time 6:02 PM). Black and white arrowheads indicate the two different cells.
(C) p53 –/– MEF that entered mitosis in the presence of nocodazole (time 10:08 AM; arrowhead). It arrested at mitosis for several hours (time 11:00 AM and time 12:17 PM) and eventually adapted (time 1:47 PM and time 2:13 PM).
(D) Quantitation of length of time that individual wild-type and p53 –/– MEFs spent at mitotic arrest. Length of mitotic arrest was determined morphologically, beginning when a cell first became rounded and refractile, and ending when it flattened back onto the coverslip. Wild-type MEFs spent an average of 4.4 ± 2.4 hrs at mitotic arrest, while p53 –/– MEFs spent an average of 4.6 ± 2.2 hrs at mitotic arrest. At least 60 cells were observed for each genotype.

Figure 3.2

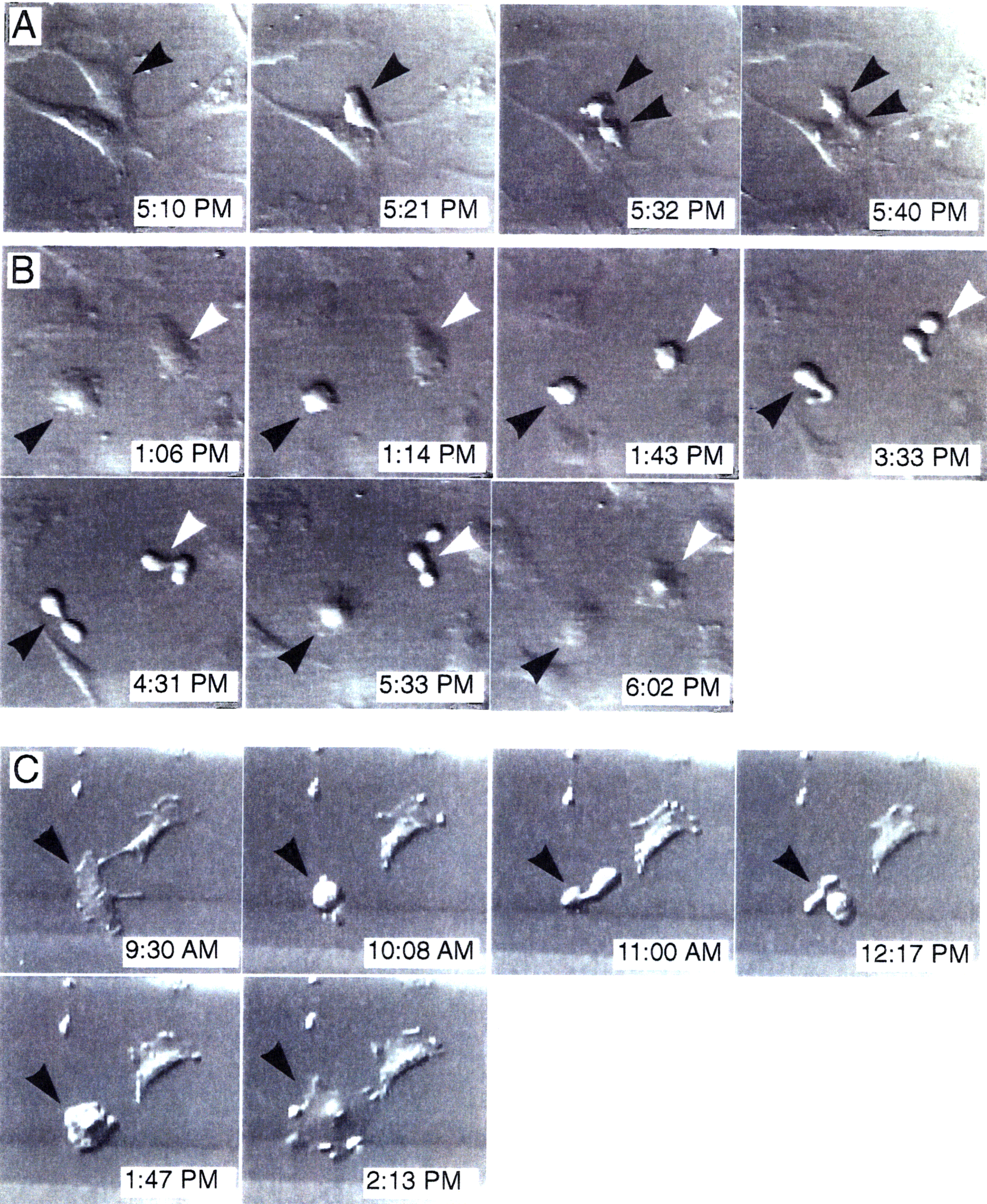
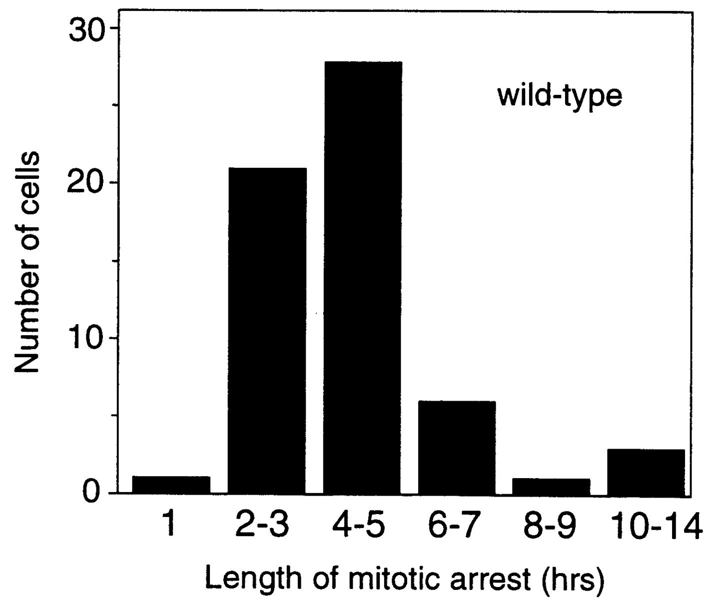
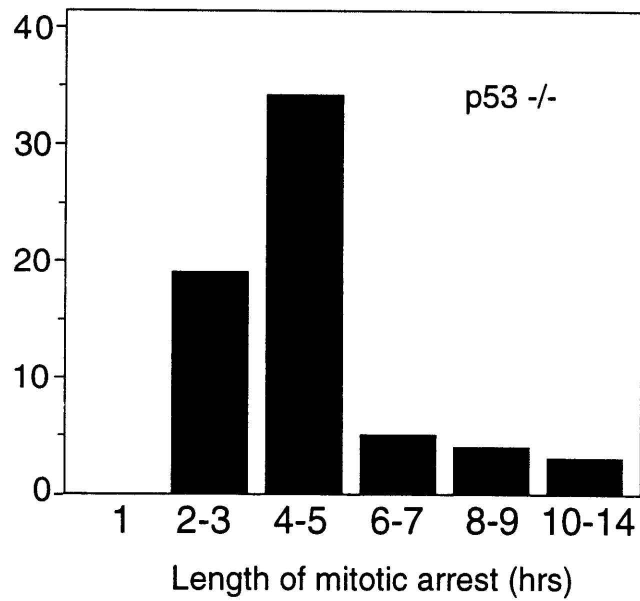


Figure 3.2

D





this time, the cells returned to a flattened morphology without completing mitosis (Fig. 3.2B, time 5:33 PM and 6:02 PM). This behavior of exiting from mitotic arrest into an apparent interphase state without actually completing mitosis has been previously described (Kung *et al.*, 1990; Rieder and Palazzo 1992) ; we will refer to it as "adaptation". We also examined the behavior of p53 –/– fibroblasts that entered mitosis while in the presence of nocodazole. A representative p53 –/– fibroblast that initiated mitosis while growing in nocodazole is shown in Figure 3.2C. Like the p53 +/+ cells, the p53 –/– fibroblast rounded up (Fig. 3.2C, time 10:08 AM), changed morphology over a 4 h period (Fig. 3.2C, time 11:00 AM and 12:17 PM), and then flattened back out again onto the coverslip (Fig. 3.2C, time 1:47 PM and 2:13 PM).

Figure 3.2D shows quantitation of the time spent at mitotic arrest for individual p53 +/+ and p53 –/– MEFs that were followed by time-lapse videomicroscopy. The length of arrest was determined for at least 60 cells of each genotype. Both p53 +/+ and p53 –/– MEFs spent a much longer period of time in a rounded mitotic state when they were grown in nocodazole-containing medium than when they were grown in normal medium. The average time spent arrested at mitosis under these conditions was 4.4 hr for p53 +/+ MEFs and 4.6 hr for p53 –/– MEFs compared to 26-27 min normally spent at mitosis in untreated cells. Cells of each genotype subsequently exited from mitosis and flattened onto the coverslip without completing cytokinesis. Interestingly, several cells of each genotype were observed to undergo prolonged mitotic arrest of 8-14 h prior to adaptation (Fig. 3.2D). These data demonstrate that p53 does not act as a mitotic checkpoint per se, because p53 +/+ and p53 –/– MEFs behave identically at mitosis in the presence of nocodazole.

Requirement for p53 after adaptation

Given that p53 does not affect mitotic arrest kinetics, we wished to identify the stage at which p53 is required for cell cycle arrest following nocodazole treatment. To address this question, individual p53 –/– MEFs treated with nocodazole were followed through mitotic entry and arrest, mitotic exit/adaptation, and S phase entry. Cells were plated onto gridded cover slips and followed by time-lapse videomicroscopy as above. After 18-22 h of treatment, cells were pulsed with BrdU for an additional 4 h in the presence of nocodazole and then analyzed by immunofluorescence. Figure 3.3A shows a representative p53 –/– MEF that attempted mitosis, adapted, and then incorporated BrdU. The cell entered mitosis at 12 AM, remained in mitotic arrest for several hours, and then exited mitosis at 5:30 AM. BrdU label was added at 11 AM, and the cell was fixed 4 h later. Immunofluorescent detection for BrdU uptake revealed that the cell had been in S phase during the time the BrdU label was present, which was 5.5 to 9.5 hours after adaptation had occurred. Ten individual p53 –/– fibroblasts were tracked with this method to determine the number of hours which elapsed between morphological adaptation and S phase entry (Fig. 3.3B). Importantly, cells did not enter S phase immediately after exiting mitosis, as cells labelled with BrdU 0 to 3.5 h post-adaptation failed to stain positively. The p53 –/– MEFs began to incorporate BrdU in a time window approximately 4 to 6 h post-adaptation. One p53 –/– MEF incubated with BrdU for 9 to 13 h after it had undergone adaptation failed to incorporate label. The behavior of this fibroblast does not necessarily indicate the time limit for when p53 –/– MEFs can reenter S phase, however, because the fibroblast may either already have completed S phase at the time of the BrdU label or may not yet have initiated it. These data show that p53 is required to prevent entry into S phase beginning in a specific time interval after nocodazole-treated MEFs have attempted mitosis and subsequently adapted into an interphase state.

FIG. 3.3

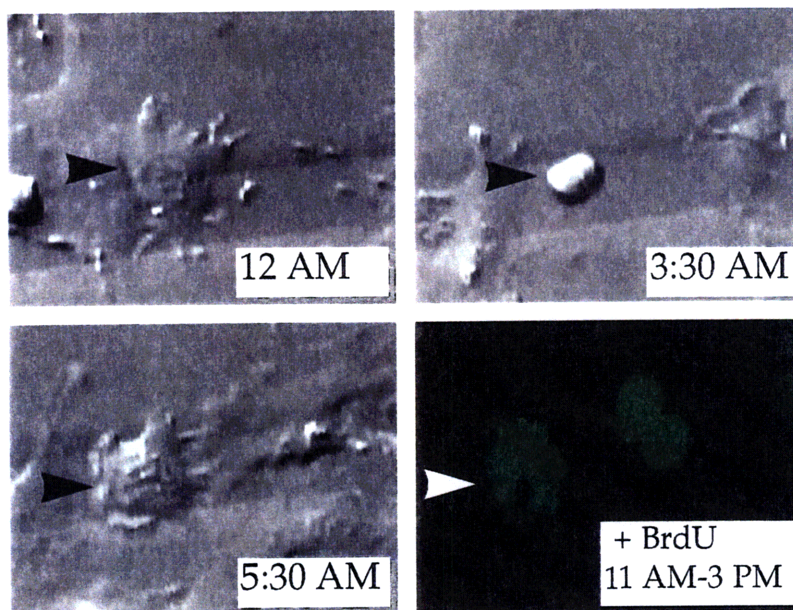
Timing of S phase entry in p53 –/– MEFs treated with nocodazole.

(A) Time-lapse videomicroscopy and subsequent immunofluorescence of representative p53 –/– MEF that arrested at mitosis in the presence of nocodazole. The cell was at mitotic arrest starting shortly after 12 AM, then adapted at 5:30 AM (top panels and lower left panel). At 11 AM, BrdU label was added to the media; the cell was then recorded for an additional 4 h and fixed. Immunofluorescence was performed to detect BrdU incorporation (lower right panel; green staining). As indicated by arrowheads, the same cell was identified during videorecording and immunofluorescence by its position on a gridded coverslip.

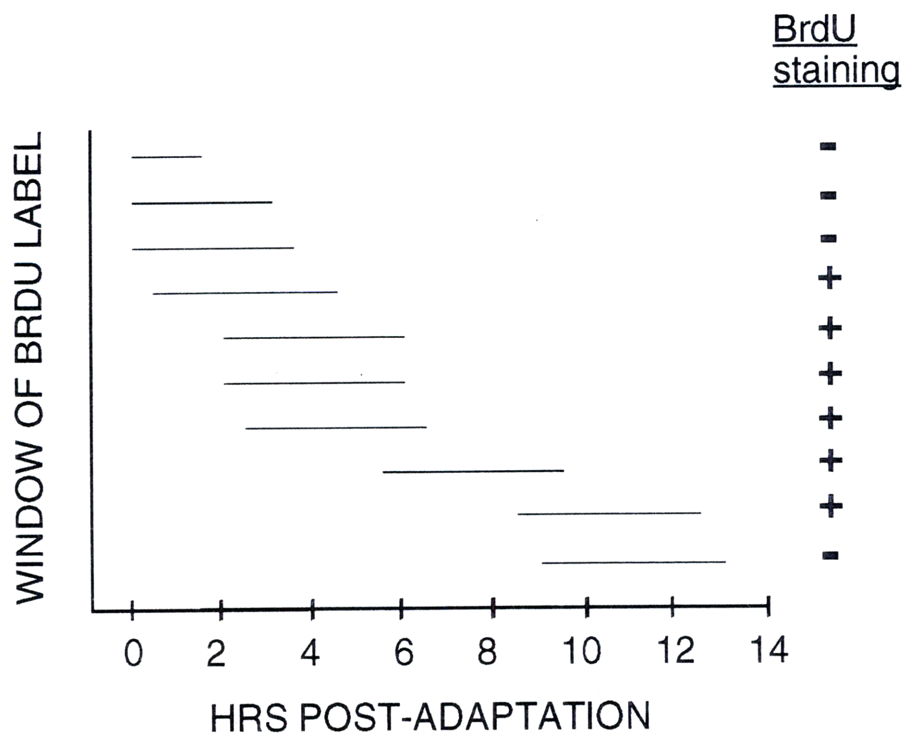
(B) Measurement of time of S phase entry relative to time of adaptation from mitotic arrest. 10 p53 –/– MEFs on gridded cover slips were treated with nocodazole, monitored by time-lapse videomicroscopy, and pulsed with BrdU for 4 h at various times following adaptation. For each cell, the horizontal line indicates the period during which BrdU was present, measured in hrs elapsed since the cell underwent adaptation. The + or – indicates whether the cell stained positive for BrdU incorporation by immunofluorescence.

Figure 3.3

A



B



Cells lacking p21 fail to maintain postmitotic arrest

p53 has been shown to prevent cells in G1 from entering S phase following DNA damage (Kastan *et al.*, 1991; Kastan *et al.*, 1992; Kuerbitz *et al.*, 1992). This arrest is mediated in large part by p21, a transcriptional target of the p53 protein which inhibits CDKs and prevents phosphorylation of the Rb protein, a requirement for S phase entry (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). To test whether a similar mechanism might be functioning to prevent S phase entry in nocodazole-treated adapted cells, we performed immunoblot analysis for p21 protein on wild-type and p53 –/– MEFs treated with nocodazole (Fig. 3.4A). In wild-type cells, levels of p21 protein increased after 16 h of nocodazole treatment and remained elevated over a 48 h period. No p21 protein was detectable in nocodazole-treated p53 –/– MEFs. These observations suggested a possible role for p21 in maintaining arrest following nocodazole treatment. To establish whether p21 was required for the arrest following spindle disruption, wild-type, p53 –/–, and p21 –/– MEFs were treated with nocodazole for 24 h and analyzed by flow cytometry (Fig. 3.4B). As was observed in Figure 3.1, wild-type MEFs arrested with 4N DNA content, while p53 –/– MEFs continued to increase in ploidy. p21 –/– MEFs were observed to have a phenotype similar to that of p53 –/– MEFs, with a significant fraction of cells undergoing an additional round of S phase to become 8N in DNA content.

To demonstrate further that p21 –/– cells were capable of reentry into S-phase following nocodazole treatment, p21 –/– MEFs were treated with nocodazole for 24 h, pulsed with BrdU while still in the presence of nocodazole for another 4 h, and analyzed by immunofluorescence (Fig. 3.4C). A high percentage of nocodazole-treated p21 –/– MEFs were found to incorporate BrdU under these conditions, indicating inappropriate S-phase entry. Similar results were observed when wild-type, p53 –/–, and p21 –/– MEFs were treated with the microtubule-destabilizing

FIG. 3.4

Requirement for p21 in cell cycle arrest following nocodazole treatment.

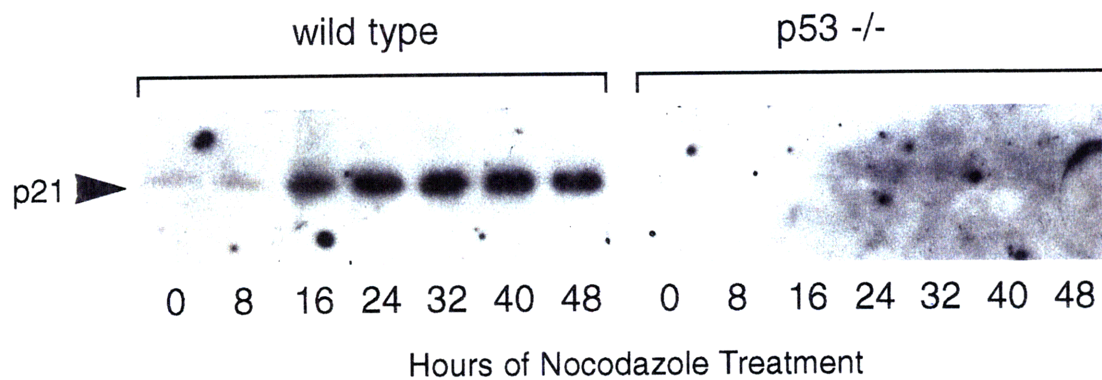
(A) Immunoblot analysis for p21 protein on extracts from wild-type and p53 –/– MEFs treated with nocodazole. Cells were treated with nocodazole for times indicated.

(B) FACS profiles of p21 –/– MEFs untreated or treated with nocodazole for 24 h. X-axis is DNA content; y-axis is number of cells counted. Data shown are representative of 3 experiments performed on 2 different p21 –/–clones.

(C) Immunofluorescent staining of p21 –/– MEFs. Cells were treated with nocodazole for 24 h, pulsed with BrdU in the presence of nocodazole for an additional 4 h, and fixed. Immunofluorescence was performed to detect BrdU incorporation (α-BrdU; green staining) and nuclear staining (DAPI; blue staining). Immunofluorescence was performed on 2 different p21 –/–clones.

(D) Quantitation of number of cells in S phase in wild-type, p21 –/–, and p53 –/– MEFs following nocodazole treatment. Cells were treated with nocodazole and immunofluorescence was performed as described in Fig. 3.4C. Data shown are the averages from 3 different experiments, with standard deviations as indicated. In each experiment, 100 cells of each genotype were examined and the number of BrdU-positive nuclei was counted to determine the percent of S phase cells. Two different clones were tested for each genotype.

Figure 3.4



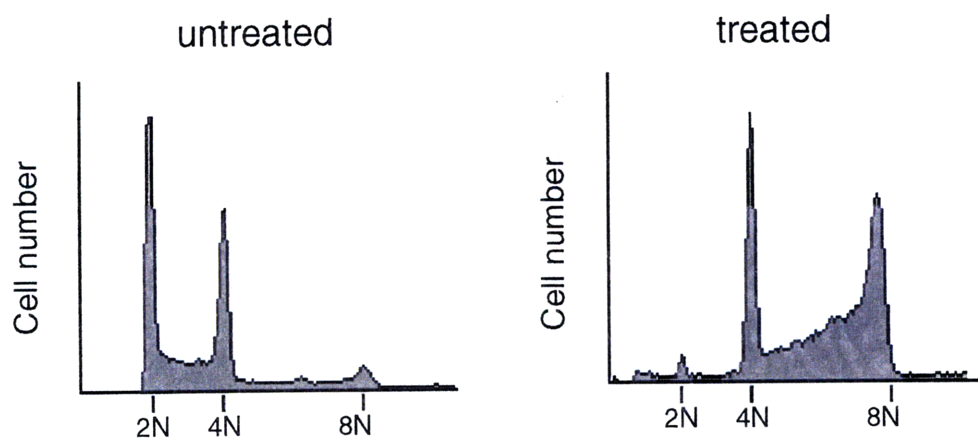
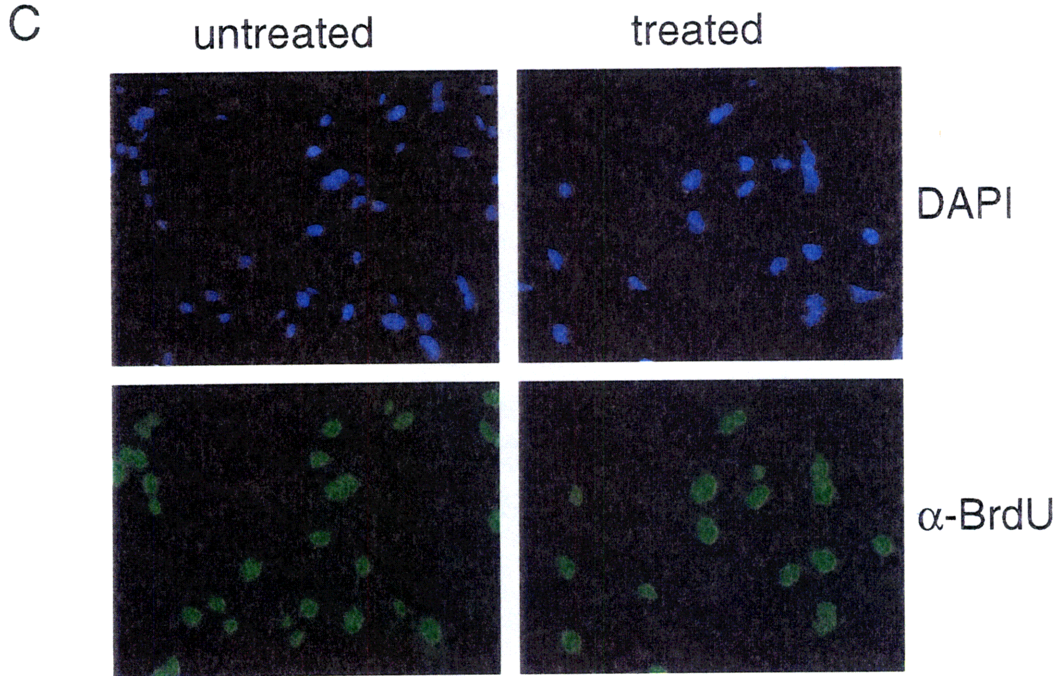
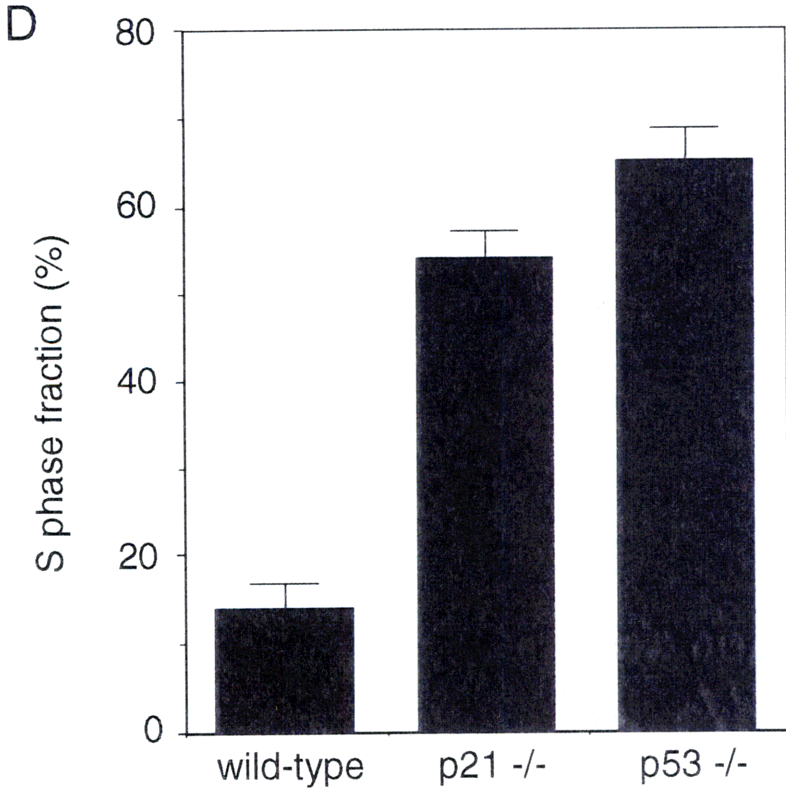


Figure 3.4





drug colcemid, demonstrating the generality of this response (data not shown). Quantitation of the percentage of wild-type, p53 –/–, and p21 –/– MEFs that stained positively for BrdU incorporation after 28 h of nocodazole treatment is presented in Figure 3.4D. Approximately 54% of p21 –/– MEFs incorporated BrdU during the labelling period, as compared to 65% of p53 –/– MEFs and 14% of wild-type MEFs. Untreated MEFs of all three genotypes had similar percentages of cells in S phase (approximately 50-60%; data not shown). Thus, the p21 –/– MEFs had an abnormal arrest phenotype which was similar to that of p53 –/– MEFs, although not as severe. Given these results, we concluded that p21 is required to fully prevent cells treated with spindle drugs from reentering S phase.

Mitotic arrest and adaptation in NIH 3T3 cells

Thus far, our data demonstrated that p53 functions to prevent S phase entry following mitotic arrest and adaptation. However, while inappropriate S phase entry had been observed to occur in thousands of cells using FACS analysis, mitotic arrest and adaptation had only been observed in approximately 100 cells in these experiments. To demonstrate that mitotic arrest and adaptation also occurred on the level of entire populations of cells, we turned to methods other than time-lapse videomicroscopy. NIH 3T3 cells were used in these experiments, for ease of synchronization and to show that our previous observations did not reflect a phenotype specific to our MEF clones. The NIH 3T3 cell line has been shown to contain wild-type p53 (Rittling and Denhardt 1992; Tishler *et al.*, 1993; Hermeking and Eick 1994; Michieli *et al.*, 1994; Lu *et al.*, 1996). NIH 3T3 cells were synchronized for 48 h in low serum medium, then released into high serum medium with or without nocodazole. At 6 h time points, cells were photographed and then fixed for flow cytometric analysis of DNA content (Fig. 3.5). At the time of release into high serum, cells were synchronized with a 2N DNA content (Fig. 3.5A, untreated, and B,

FIG. 3.5

Mitotic arrest and adaptation occur in synchronized, nocodazole-treated NIH 3T3 cells.

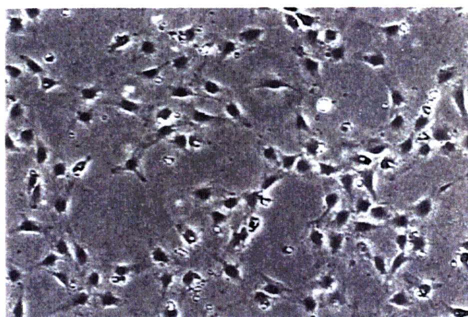
(A) Untreated NIH 3T3 cells. Cells were synchronized in low serum for 48 h, then released into high serum and followed for 32 h. At each time point, cells were photographed (left panels), then prepared for FACs analysis for DNA content (right panels). Time points are measured in hrs post-release from serum starvation. Cells were initially in G0 (0 h), then entered S phase at 14 h, and completed cell division by 26 h.

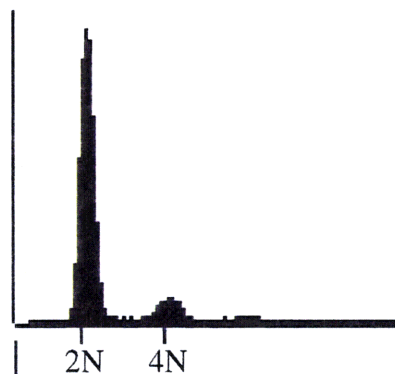
(B) Nocodazole-treated NIH 3T3 cells. Cells were synchronized in low serum for 48 h, then released into high serum + nocodazole and followed for 32 h. Progression of cells through cell cycle was monitored as above. Cells exited G0 and entered S phase by 14 h, remained arrested at mitosis through 26 h, and adapted by 32 h.

Figure 3.5

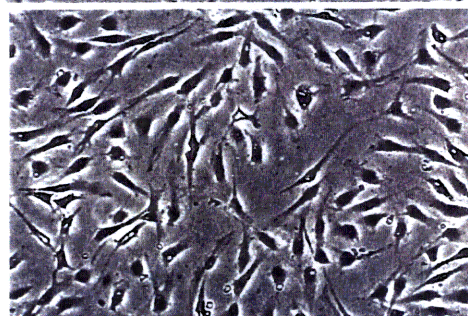
A

0 hr

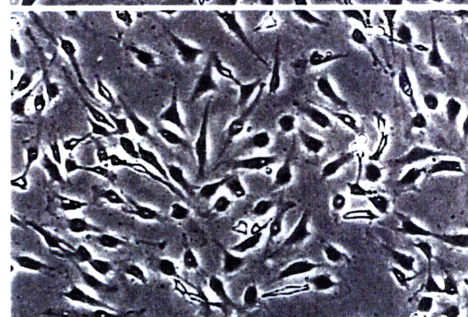


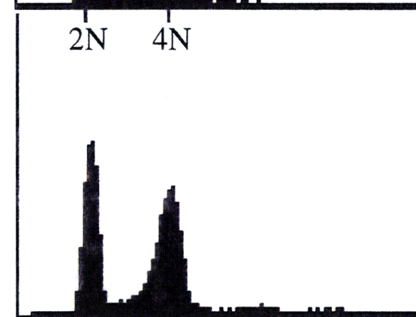


14 hr

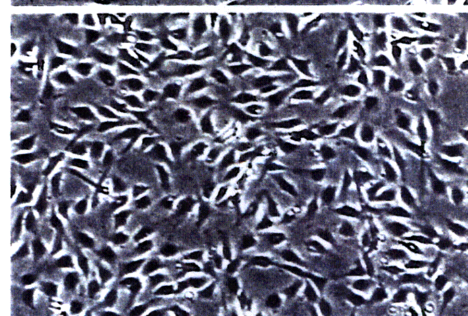


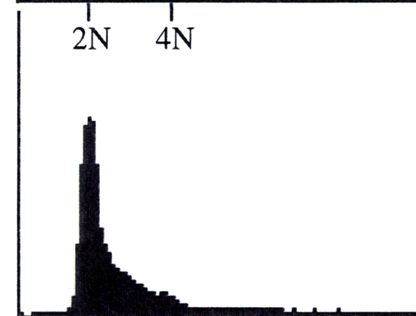
20 hr



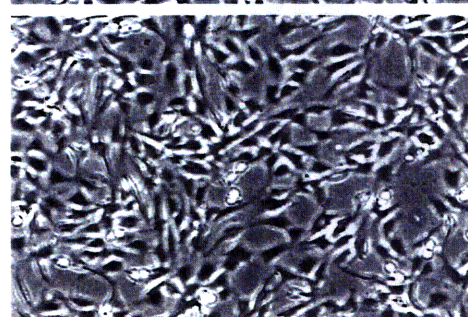


26 hr





32 hr



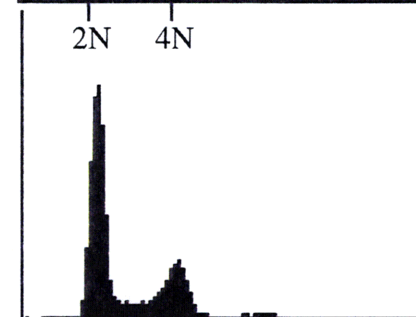
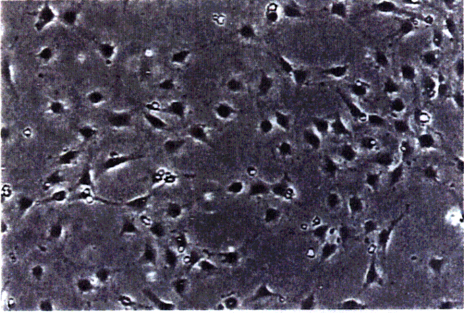
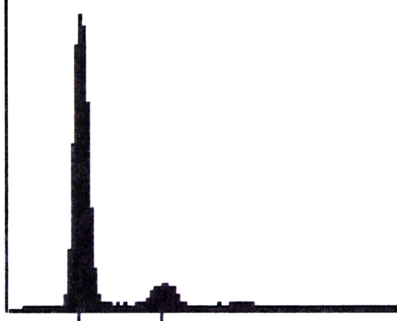


Figure 3.5

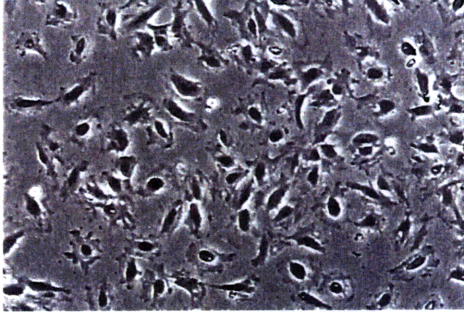
B

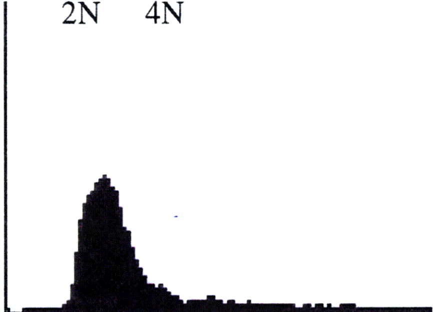
0 hr



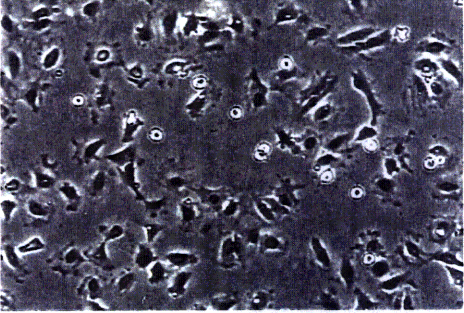


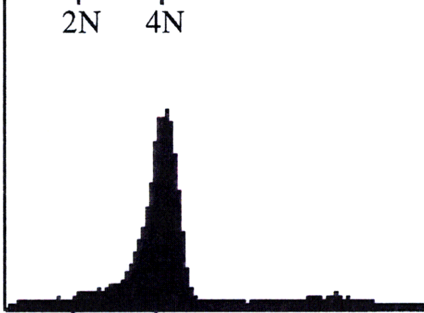
14 hr





20 hr





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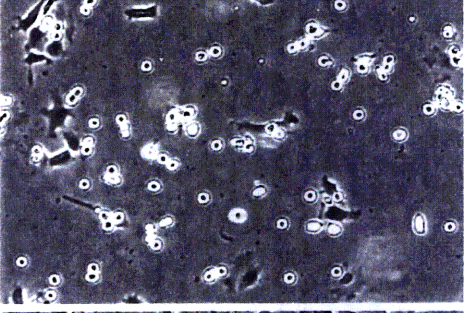
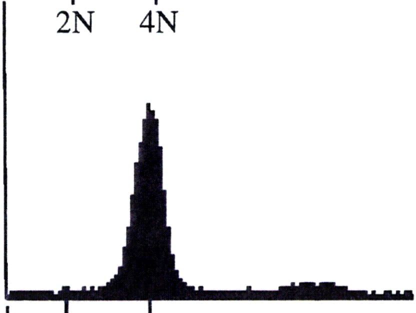
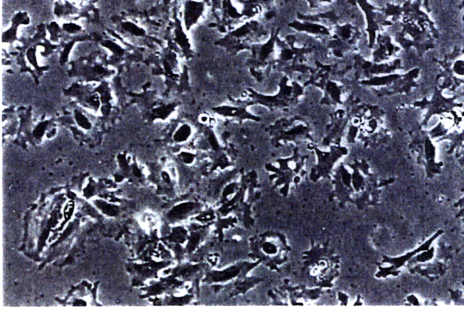
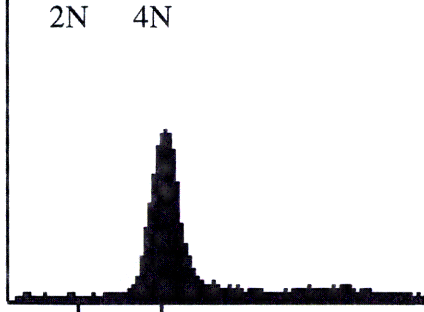




32 hr





nocodazole-treated; 0 h). Cells entered S phase synchronously (Fig. 3.5A and B, 14 h) and completed DNA synthesis by 20 h after release into high-serum containing medium (Fig. 3.5A and B, 20 h). The presence of nocodazole did not noticeably delay progression into or completion of S phase, as judged by DNA content. The first mitoses were visible at the same time point in photographs of untreated and nocodazole-treated cells as rounded refractile cells (Fig. 3.5A and B, 20 h). Within 6 h, all of the cells in the untreated population had completed mitosis, as the 4N population had been replaced by cells with 2N and S phase DNA content (Fig. 3.5A, 26 h). In contrast, all the cells in the nocodazole-treated population had a 4N DNA content at 26 h, and most appeared to be rounded up and arrested at mitosis (Fig. 3.5B, 26 h). By the next time point, however, these cells had flattened out again but still had a 4N DNA content, indicating their failure to complete mitosis (Fig. 3.5B, 32 h). In the adapted cells, microtubules were depolymerized and chromatin was decondensed, as determined by indirect immunofluorescence for tubulin and nuclear staining with DAPI, respectively (data not shown). Thus, as observed with wild-type MEFs, NIH 3T3 cells did not undergo an additional round of DNA synthesis during nocodazole treatment. These results indicate that the great majority of murine fibroblasts in a synchronized population undergo mitotic arrest and adaptation when treated with nocodazole.

Expression of cyclins B and E following nocodazole treatment

We observed some similarities between the p53-dependent checkpoint following nocodazole treatment and the p53-dependent G1 checkpoint following irradiation. Our data showed that a primary effector of the G1 checkpoint response, p21, was also required for arrest after spindle disruption. Also, adapted cells had the flattened morphology and decondensed chromatin chararacteristic of cells in G1. To determine whether adapted cells were similar to G1 cells at the molecular level, we

FIG. 3.6

Expression of cyclin E protein is upregulated in nocodazole-treated, adapted cells.

Immunoblot analysis of cyclin B1 and cyclin E expression. NIH 3T3 cells were synchronized in low serum for 48 h and released into high serum + nocodazole. Extracts were collected at 8 h time points for the next 48 h and analyzed by immunoblotting for cyclin B1 and cyclin E expression. Cyclin E expression was highest in cells entering S phase (16 h) and in cells which had adapted (40 and 48 h), while cyclin B1 expression was highest in mitotic cells (24 h). Asterisk (*) indicates constant band detected by cyclin E antibody that serves as internal loading control.

Figure 3.6

cyclin E

cyclin B1

*

0 8 16 24 32 40 48

Hours of Nocodazole Treatment

measured expression of cyclin B1, a mitotic cyclin, and cyclin E, a G1 cyclin, in nocodazole-treated cells. Cyclin B1 is expressed at high levels in mitotic cells and declines when cells enter G1, while cyclin E expression is highest in cells in the late G1 phase of the cell cycle (Dunphy *et al.*, 1988; Lew *et al.*, 1991). NIH 3T3 cells were synchronized in low serum, released into medium with high serum plus nocodazole, and collected at time points for immunoblotting. The blot was probed with antibodies to cyclin B1 and cyclin E (Fig. 3.6). Cyclin B1 levels peaked at 24 h and decreased afterward. The appearance of this peak corresponded with the presence of mitotic cells (data not shown). Cyclin E levels were elevated at 16 h as cells progressed from G1 into S phase and then decreased with the onset of mitosis. At 32 h, mitotic-arrested cells had just adapted into a flattened state (data not shown) and cyclin E levels remained low (Fig. 3.6). Cyclin E levels then increased again in the adapted cells at 40 h (Fig. 3.6). Thus, adapted cells express high levels of cyclin E, a G1 marker, despite having a DNA content characteristic of cells in the G2 or M phase of the cell cycle. The delayed expression of cyclin E relative to adaptation may reflect the fact that cyclin E is normally expressed in late G1 and that adapted cells take several hours to progress to this state.

Presence of hypophosphorylated pRB in adapted cells

To characterize further the nocodazole-induced adapted state, we examined adapted cells for another marker of G1, hypophosphorylated Rb protein. In the G1 phase of the cell cycle, the Rb protein (pRB) is in a hypophosphorylated state (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Mihara *et al.*, 1989). As part of the G1-S phase transition, pRB becomes hyperphosphorylated on multiple sites, and remains so until anaphase of mitosis (Ludlow *et al.*, 1993). We analyzed serum-synchronized, nocodazole-treated NIH 3T3 cells at various time points for their pRB phosphorylation status (Fig. 3.7A) and DNA content (Fig. 3.7B). At time 0, cells had

FIG. 3.7

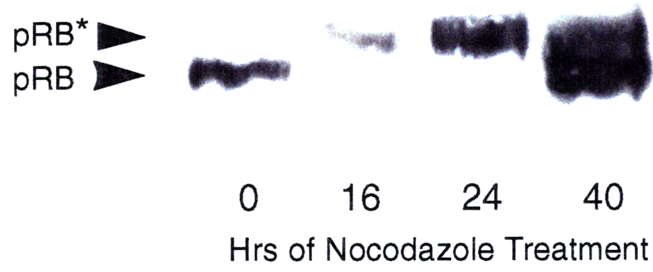
RB protein is hypophosphorylated in nocodazole-treated, adapted cells.

(A) Immunoblot analysis of pRB. NIH 3T3 cells were synchronized in low serum for 48 h and released into high serum + nocodazole. Extracts were collected at time points for the next 48 h and analyzed by immunoblotting for pRB. Hypophosphorylated pRB (pRB) and hyperphosphorylated pRB (pRB*) are indicated by arrows.

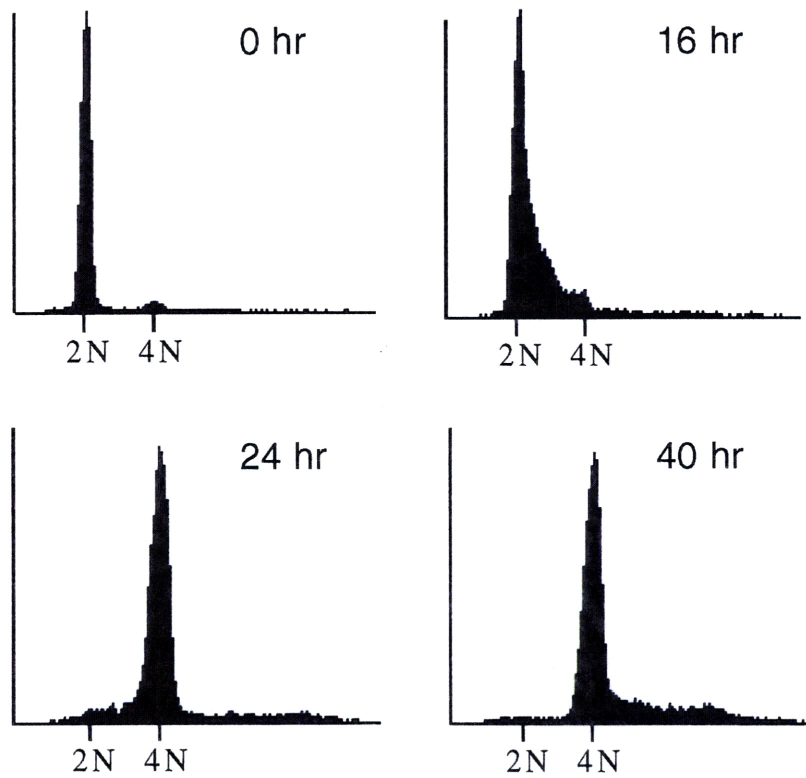
(B) DNA content of NIH 3T3 cells. Cells were treated as described in Fig. 3.7A. At each time point, a duplicate plate was collected and prepared for FACS analysis of DNA content. Cells were initially synchronized in G0 (0 h), then progressed to S phase (16 h), and arrested at mitosis (24 h and 40 h).

Figure 3.7

A



B



been serum-starved for 48 h and were 2N in DNA content. All pRB was hypophosphorylated, as would be expected for cells in G0. Sixteen hours after release into medium containing high serum plus nocodazole, pRB had become hyperphosphorylated, corresponding with the entry of cells into S phase. At 24 h post-release, cells had completed DNA synthesis and were entering mitotic arrest, from which they later adapted (Fig. 3.7B and data not shown). By 40 h of treatment, cells had adapted and pRB was predominantly in the hypophosphorylated state. Therefore, like cyclin E expression, pRB phosphorylation status in adapted cells resembles the pattern seen in G1 cells. Because pRB is present in adapted cells in the active, or hypophosphorylated, state, it is possible that like p53 and p21, pRB functions to prevent S-phase reentry during nocodazole treatment. Such a mechanism seems likely, given that p21 is required for arrest in nocodazole-treated cells and that p21 induces cell cycle arrest in part by inhibiting pRB phosphorylation.

Discussion

p53 is a postmitotic checkpoint

Many functions have been attributed to the p53 protein, which is clearly a critical cell cycle regulator in mammalian cells (Levine 1997). In this study, we have addressed the question of whether the ability of cells to initiate and maintain the mitotic spindle checkpoint is affected by their p53 status. We have attempted to clarify the role of p53 at mitosis by monitoring the response of individual p53 +/+ and p53 −/− cells to spindle inhibitors. In order for p53 to qualify as part of a true mitotic checkpoint, p53 +/+ and p53 −/− MEFs would have to behave differently at mitosis. However, we observed that both wild-type and p53-deficient MEFs arrested at mitosis following disruption of the spindle and that cells of either genotype arrested for the same length of time and behaved identically during the mitotic arrest. This behavior is opposite to that of mammalian cells in which the MAD2 or BUB1 mitotic checkpoint gene products, which monitor the integrity of the mitotic spindle, have been inhibited. Recent studies have identified a human homolog for the *S. cerevisiae* MAD2 checkpoint gene, hsMAD2, as well as a murine homolog of the *S. cerevisiae* BUB1 checkpoint gene. As would be predicted from experiments with *S. cerevisiae mad* strains, mammalian cells electroporated with an anti-hsMAD2 antibody did not undergo arrest at mitosis when the cells were treated with nocodazole (Li and Benezra 1996). Similarly, when treated with nocodazole, mammalian cells which expressed a dominant negative version of the murine Bub1 gene arrested at mitosis much less frequently than their normal counterparts. (Taylor and McKeon 1997). Thus, when contrasted with genes which are known to act at mitotic checkpoints in mammalian cells, p53 acts in a different manner. We can conclude that, unlike the hsMAD2 and murine Bub1 genes, the presence or absence of p53 has no impact on the ability of cells to detect microtubule disruption

and initiate metaphase arrest. Thus, we have shown definitively that p53 does not act to implement a checkpoint at mitosis in mouse embryonic fibroblasts.

p21 is required for postmitotic arrest

We observed that p53 does have a checkpoint function in MEFs following nocodazole treatment, specifically, to prevent S phase reentry following adaptation from mitotic arrest. Consistent with this finding is the observation that levels of p53 protein and its downstream target gene product p21 both increase following treatment with nocodazole or other microtubule drugs (Fig. 3.4A; Minn *et al.*, 1996; Tishler *et al.*, 1995). Importantly, we have found that the downstream arrest mechanism activated by p53 requires the CDK inhibitor p21, as p21 –/– MEFs fail to arrest in response to nocodazole treatment and enter another round of S phase, in contrast to the results of an earlier study (Deng *et al.*, 1995). Although our data were obtained with cells treated with nocodazole, we obtained similar results with the spindle inhibitor colcemid (data not shown), which was the microtubule-destabilizing drug used in the earlier study. We eliminated the possibility that the p21 protein itself was acting at a mitotic checkpoint, because like wild-type and p53 –/– MEFs, p21 –/– MEFs treated with nocodazole arrested at mitosis for 4 to 5 h and then adapted (data not shown). The simplest explanation for our observation is that in adapted cells, activation of p53 leads to increases in p21 protein, which then initiates cell cycle arrest by inhibiting cyclin-CDK activity. Interestingly, the contribution of p21 to the arrest response following nocodazole treatment and adaptation is proportionately similar to the contribution of p21 to the G1 arrest response following DNA damage. In nocodazole-treated, adapted MEFs, p21 is responsible for about 80% of the p53-mediated arrest response, while in irradiated MEFs, p21 accounts for approximately 70-80% of the p53-dependent arrest in G1 (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). These data demonstrate not only that p21

is required for the arrest following nocodazole treatment but also that the requirement for p21 is quantitatively similar to the requirement for p21 in the G1 arrest following DNA damage.

Postmitotic arrest resembles G1 arrest

Taken together, our results suggest that p53 could be acting as a component of a G1 checkpoint in nocodazole-treated cells. First, p53 function is required to prevent reentry into S phase following checkpoint activation, as occurs in the G1 checkpoint following DNA damage. Second, p21 is required to execute this arrest, and to an extent similar to its role in the G1 arrest checkpoint. Finally, cells which have exited mitotic arrest have the flattened appearance and decondensed chromatin of G1 cells and express the G1-specific markers cyclin E and hypophosphorylated pRB. Thus, p53 could be implementing a G1-like arrest in 4N, adapted cells.

Potential signalling pathways

An interesting and as-yet-unanswered question is the nature of the upstream signal which triggers p53-dependent arrest following nocodazole treatment. One possibility is that as during the normal G1 checkpoint, p53 detects DNA damage. It is readily conceivable that a cell which attempted to undergo chromosome separation at metaphase when its spindle was destabilized by nocodazole could incur DNA damage, which would then be detected following exit from mitotic arrest. In this case, both the upstream and downstream signals for arrest following nocodazole treatment would be identical to those for G1 arrest following irradiation. Alternatively, p53 could be activated by other types of signals, such as the presence of two centrosomes in a G1-like cell. Another possible signal could be the presence of excess chromosomes in a G1-like cell, which would trigger a p53-dependent checkpoint to prevent endoreduplication. Our data do exclude one possibility,

however, which is that p53 is activated in response to a loss of microtubule stability induced by nocodazole treatment. Our data in NIH 3T3 cells, which contain functional p53, show that like other cell types, they progress unhindered from G1 to mitosis while in the constant presence of nocodazole (Fig. 3.5; Jordan *et al.*, 1992). Were p53 detecting a loss of microtubule stability in G1 or G1-like cells, these cells would never have progressed through S phase, but instead would have arrested shortly after entry into G1.

While it has been speculated to act at multiple different cell cycle checkpoints, the p53 protein appears to have overlapping checkpoint functions following irradiation and spindle disruption. After irradiation, cells undergo p53-dependent arrest in G1 (Kastan *et al.*, 1991; Kastan *et al.*, 1992; Kuerbitz *et al.*, 1992); after nocodazole-induced disruption of the mitotic spindle, cells arrest transiently at mitosis, adapt into a G1-like state, and then undergo p53-dependent arrest (Minn *et al.*, 1996). In both instances, the p53-mediated arrest requires the CDK inhibitor p21 (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). Thus, both the stage of the cell cycle at which p53 induces arrest and the downstream mechanism by which it implements arrest are similar after either irradiation or nocodazole treatment. In cells which lack functional p53 protein, failure to arrest following irradiation or nocodazole treatment would be predicted to lead to two distinctly different outcomes of chromosomal damage and polyploidy, respectively. Thus, the loss of p53 checkpoint function during tumorigenesis could lead to decreased genomic stability by multiple mechanisms.

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Chapter 4

Discussion and Conclusions

In this thesis, I have described research relating the effects of p53 status on the response of cells to microtubule disruption. We have found that in our model system, the p53 genotype of transformed cells determines their ability to undergo apoptosis in response to treatment with the microtubule-stabilizing drug paclitaxel. Additionally, we have identified a potential mechanism by which paclitaxel may exert p53-independent effects, via induction of TNF-α secretion from macrophages. In a separate project also described in this thesis, we examined whether p53 acts as part of a mitotic checkpoint in response to the microtubule-destabilizing drug nocodazole. We have found that p53 does help implement cell cycle arrest after nocodazole treatment, but only after cells have adapted from mitotic arrest into a G1-like state. The implications of these findings are discussed below, as well as future experiments to address unanswered questions.

Paclitaxel and p53-dependent apoptosis

We found that p53 +/+ fibroblasts transformed with the oncogenes E1A and activated-H-Ras underwent apoptosis when treated with paclitaxel. However, similarly transformed p53 -/- fibroblasts were resistant to treatment. This finding may be significant for several reasons. First, it suggests that paclitaxel could act *in vivo* by inducing apoptosis in transformed cells. Such a mechanism has been suggested for other anticancer agents, such as adriamycin and ionizing radiation, which also induce apoptosis in E1A-Ras transformed cells *in vitro*. (Lowe *et al.*, 1993). Both adriamycin and ionizing radiation were subsequently shown to cause massive apoptosis *in vivo*, by treating tumors grown in nude mice from the E1A-Ras transformed cell lines (Lowe *et al.*, 1994). Based on our observations, one logical next step would be to determine whether paclitaxel inhibits tumor progression by inducing apoptosis in tumors *in vivo*. We have attempted to do so using the same

model system of E1A-Ras tumors in nude mice, but found that paclitaxel treatment did not consistently inhibit tumor progression in either p53 +/+ or p53 -/- tumors in this system. A possible explanation for this is that the E1A-Ras tumors are fibrosarcomas, which are not a tumor type against which paclitaxel has been shown to be effective (Rowinsky and Donehower 1995). It would be of interest to test paclitaxel in a group of murine tumor models in which it has been proven to be effective, and determine whether apoptosis was taking place. Paclitaxel has already been shown to induce apoptosis in a murine tumor model in at least one instance, in a mouse model of lung adenocarcinoma (Kalechman *et al.*, 1996).

Another interesting feature of the ability of paclitaxel to induce apoptosis in E1A-Ras transformed cells is that it was dependent on the presence of wild-type p53 protein. This observation was unexpected, as no functional connection has been made between the microtubule cytoskeleton and p53. Interestingly, we also observed that the chemotherapeutic drug vincristine, which destabilizes microtubules, induced p53-dependent apoptosis in transformed cells (J.S. Lanni and T. Jacks, unpublished data). These data suggest that any disruption of microtubule stability can result in the activation of p53-dependent apoptosis in transformed cells. It is possible that as has been proposed, p53 is a general detector of cellular stresses, such as DNA damage, poor nutrient supply, or in this instance, changes in microtubule stability (Levine 1997). Alternatively, cells which have been treated with microtubule-acting drugs may incur DNA damage when they attempt to undergo mitosis with a defective spindle, leading to activation of the p53-dependent DNA damage response pathway. It would be valuable to determine whether treatment with paclitaxel or vincristine did result in DNA damage, which would lend support to the latter model.

Tumor necrosis factor-α and p53-independent apoptosis

As part of investigating a possible secondary mechanism of paclitaxel, we tested the response of transformed cells to treatment with tumor necrosis factor-α (TNF-α), which is released from murine macrophages upon paclitaxel stimulation. We found that TNF-α induced apoptosis equally effectively in p53 +/+ and p53 -/- E1A-Ras transformed fibroblasts. This observation is significant in that it suggests a mechanism by which paclitaxel could inhibit the growth of tumors with mutated p53. Paclitaxel has been shown to be effective against tumors with documented p53 mutations in several xenograft models (National Cancer Institute 1983; Baker *et al.*, 1989; Reiss *et al.*, 1992; Rose 1993; Joschko *et al.*, 1994). These data are difficult to explain given that in our system, paclitaxel was seen to induce apoptosis only in p53 -/- transformed cells. However, if paclitaxel were able to induce TNF-α secretion from macrophages infiltrating the tumor stroma, it could act by an indirect mechanism to inhibit growth of both p53 +/+ and p53 -/- tumors *in vivo*. It is important to note that if TNF-α release is relevant to the activity of paclitaxel in the whole organism, it may be affected by species-specific differences. Treatment with paclitaxel alone has been found to stimulate secretion of TNF-α from murine macrophages, but a second stimulus appears to be required to activate TNF-α release from human macrophages (Ding *et al.*, 1990; Allen *et al.*, 1993).

Regardless of the underlying mechanism of paclitaxel activity, the observation that TNF-α causes p53-independent apoptosis has potential clinical relevance. Many anticancer treatments which induce apoptosis appear to do so via p53-dependent pathways, rendering them of limited use against tumors containing p53 mutations (Lowe *et al.*, 1994; Lowe 1995). The identification of an agent capable of inducing p53-independent apoptosis could be a valuable addition to the repertoire of anticancer treatments, particularly considering the high frequency of tumors with

p53 mutations and the strong correlation between p53 mutation and poor response to therapy (Hollstein *et al.*, 1994; Lowe 1995). It should be noted that treatment with TNF-α is far from an established therapy; in point of fact, many of the early clinical trials with TNF-α found it to be of limited efficacy against tumors and with toxic side effects in patients (Spriggs and Yates 1992). Recent trials have yielded more encouraging results, however, in which localized treatment with TNF-α in combination with other drugs has effectively inhibited tumor progression in several systems (Baisch *et al.*, 1990; Asher *et al.*, 1991; Lejeune *et al.*, 1994; Lasek *et al.*, 1995). Our data would suggest that further development of TNF-α as an anticancer agent is worthwhile, given its unique ability to activate the cell death program in p53-deficient tumor cells.

<u>Recent clinical data on paclitaxel and p53 status</u>

The research presented in Chapter 2 implies that paclitaxel may act by both p53-dependent and p53-independent mechanisms in mouse tumor models, and possibly in cancer patients as well. In order to determine the mechanism underlying paclitaxel activity in humans, it is important to establish whether paclitaxel is effective against tumors with p53 mutations. Since the time our experiments were performed, more data have accumulated on the efficacy of paclitaxel against tumors with mutated p53. One study directly addressing this question was carried out on patients with non-small cell lung carcinoma (NSCLC) who were treated with paclitaxel and radiation. Safran *et al.* determined the p53 status of 30 NSCLC tumors using a single-strand conformation polymorphism assay and then confirmed the presence of p53 mutations by sequence analysis (Safran *et al.*, 1996). Of the 12 patients who had p53 mutations, 75% responded to treatment, as compared to 83% of the 18 patients without p53 mutations. Thus, combination therapy with both paclitaxel and radiation was found to be equally effective against NSCLC tumors

with wild-type and mutated p53. As p53-mutant tumors have been documented to be resistant to radiation therapy (Lowe 1995), this finding suggests that the paclitaxel component of the therapeutic regimen is acting independently of p53 status.

In another recent study, Dunphy *et al.* examined a panel of 36 head and neck squamous cell carcinomas for p53 overexpression prior to treatment with paclitaxel and carboplatin (Dunphy *et al.*, 1997). High levels of p53 expression are often correlated with the presence of p53 mutations, providing an approximate indicator of the p53 status of the tumors (Donehower and Bradley 1993). Twenty-four patients in the study had tumors with detectable p53 expression (as seen by immunohistochemical staining); of these, 6 underwent complete remission following chemotherapy. The remaining 12 patients had tumors which did not express p53; two of them showed complete remission after treatment. Therefore, the expression of high levels of p53 protein did not appear to predict the responsiveness of head and neck carcinomas to chemotherapy with paclitaxel and carboplatin. Finally, Sikov and Safran have investigated the efficacy of combination therapy with paclitaxel and radiation against advanced gastric and pancreatic cancers (Sikov and Safran 1997). These tumor types are often resistant to chemotherapy with DNA-damaging agents and radiation, and have a high frequency of p53 mutations. The researchers found that the combination of paclitaxel and radiation therapy had substantial activity in patients. Although the p53 status of individual tumors was not determined in this study, this finding could be indicative of paclitaxel activity against tumors with p53 mutations.

The reports summarized above all suggest that paclitaxel is equally effective against tumors with wild-type and mutated p53. This finding is in agreement with earlier studies on the efficacy of paclitaxel *in vivo* discussed in Chapter 1. The observation

that paclitaxel may act independently of p53 status in humans is supportive of our model in which paclitaxel may inhibit tumor growth by both direct and indirect mechanisms. Our data predict that paclitaxel treatment could cause TNF-α secretion leading to p53-independent apoptosis. If TNF-α release is a component of paclitaxel's mode of action in humans, this could explain in part the similarity in the drug's activity against tumors with wild-type and mutant p53 protein. Alternatively, paclitaxel could be exerting p53-independent effects by another unidentified mechanism.

Functional significance of TNF-α secretion

One question raised by our experiments is whether TNF-α secretion is relevant to the effectiveness of paclitaxel in preclinical and clinical settings. The fact that paclitaxel has been seen to be equally active against p53 +/+ and p53 -/- tumors in a number of different clinical settings supports a role for induction of p53-independent apoptosis caused by TNF-α release. Ideally, this hypothesis would be tested directly in a mouse tumor model setting in which both p53 +/+ and p53 -/- tumors are available. Our data would predict that if TNF-α release is occurring *in vivo*, then paclitaxel would inhibit tumor growth in tumors with and without p53. Furthermore, we would expect that histology on tumors following paclitaxel treatment would reveal an increased number of apoptotic cells, as TNF-α can induce apoptosis in transformed cells *in vitro*. As an important control, one could also test the activity of cephalomannine, a paclitaxel-related compound, in the same model system. This analog stabilizes microtubules but does not induce TNF-α release (Burkhart *et al.*, 1994). It would therefore be predicted to be effective only against p53 +/+ tumors, a prediction which could be tested in the same tumor model system.

Other possible experiments to test the TNF-α secretion model would require performing histology on paclitaxel-treated tumors and analyzing them for the presence of infiltrating macrophages. If TNF-α secretion occurs *in vivo*, then macrophages should be detectable near or in the tumor mass, as local release of TNF-α is most effective at inhibiting tumor growth (Malik 1992). Furthermore, these experiments would allow one to examine individual paclitaxel-treated tumors to determine whether any correlation exists between therapeutic success and the presence or absence of macrophages in the tumor. It would also be worthwhile to try to establish a more general correlation between the tumor types against which paclitaxel has been found to be effective and the degree of macrophage infiltration in these tissues. Tumors of different tissue types and at different stages of progression are known to exhibit varying degrees of macrophage infiltration (Mantovani *et al.*, 1992). If paclitaxel were found to be most effective against tumor types with a significant macrophage presence, that would support a possible role for TNF-α release from macrophages *in vivo*.

Another means of determining the relevance of TNF-α secretion is by comparing the activity of paclitaxel analogs against tumors in cancer patients. Docetaxel (trade name Taxotere) is a compound closely related to paclitaxel from a structural standpoint but with significant functional differences (Burkhart *et al.*, 1994). Docetaxel binds and stabilizes microtubules more effectively than paclitaxel, and can induce mitotic arrest in cells at one third the concentration of paclitaxel required to do so. However, docetaxel does not induce any release of TNF-α from macrophages. If TNF-α release is a component of the therapeutic activity of paclitaxel, then docetaxel, which lacks the ability to induce TNF-α secretion, should be less effective as a chemotherapeutic agent. Data obtained from cancer patients treated with docetaxel would suggest that this is not the case. In fact, docetaxel has proven widely

effective against a number of different tumor types, including advanced breast and ovarian cancer, head and neck cancer, and non-small cell lung cancer (Goldspiel 1997; Rowinsky 1997). All of these tumors are types against which paclitaxel has also demonstrated activity, suggesting that the mechanism of these drugs in human patients may depend primarily on their ability to stabilize microtubules rather than on their ability to induce TNF-α secretion. Of course, other characteristics of docetaxel could affect its activity *in vivo*, such as its increased cellular uptake and intracellular retention time relative to paclitaxel (Aapro 1996). These additional features of docetaxel may explain in part why docetaxel and paclitaxel do not show complete cross-resistance (Von Hoff 1997). However, the proven efficacy of docetaxel in anticancer treatments would seem to indicate that induction of TNF-α release is not essential to the therapeutic success of paclitaxel.

Possible model for paclitaxel activity

Given the data presented above, it is clear that no simple relationship exists between paclitaxel efficacy and p53 status. Our experiments on the response of transformed cells to paclitaxel treatment found that microtubule disruption resulted in p53-dependent apoptosis. However, this observation does not explain why paclitaxel has been found to be effective against p53 -/- tumors in the clinic. One possible way to reconcile our data could be by considering the concentration of paclitaxel present following a standard therapeutic dose. When present at high concentrations, many chemotherapeutic agents, including paclitaxel, can induce death in some p53 -/- tumor cells (J.S. Lanni and T. Jacks, unpublished data). These drugs can still be considered to function by p53-dependent mechanisms, as they are cytotoxic against p53 +/+ tumor cells at far lower concentrations. Thus, paclitaxel may be noticeably more effective against p53 +/+ tumor cells at the concentrations tested *in vitro*, but

may show activity against both p53 +/+ and p53 -/- tumor cells at the concentrations present *in vivo*.

Another important consideration is the presence of tissue and cell-type specific factors which may affect cellular responses to microtubule damage. In our model system of oncogenically transformed fibroblasts, paclitaxel induced p53-dependent cell death. However, in tumor cell lines derived from other tissue types, paclitaxel has been found to be cytotoxic against cells regardless of their p53 status. Delia *et al* examined a group of p53 +/+ and p53 +/- immortalized lymphoblastoid cell lines, and found that while p53 genotype affected their response to radiation, it did not determine the cytotoxicity of paclitaxel treatment (Delia *et al.*, 1996). Similarly, Debernardis *et al.* observed that in a panel of nine different ovarian carcinoma cell lines with and without p53 mutations, paclitaxel demonstated cytotoxic effects independently of p53 status (Debernardis *et al.*, 1997). These data suggest that the p53-dependence of paclitaxel may be affected by differences between tumors of fibroblast origin and those from lymphoblastoid or ovarian tissues. Even within a group of tumors derived from the same tissue type, there are likely to be additional genetic and cellular characteristics that develop during tumor formation which affect paclitaxel sensitivity. Gibb and colleagues observed that of four different ovarian carcinoma cell lines, all containing p53 mutations, each underwent apoptosis to a differing extent following treatment with paclitaxel (Gibb *et al.*, 1997). From these data, it seems probable that paclitaxel may have p53-dependent and p53-independent effects depending on the particular tumor context.

Additional factors such as induction of TNF-α secretion may also contribute to the activity of paclitaxel *in vivo*. We have proposed that one component of paclitaxel activity may be induction of TNF-α release, but were unable to determine the

clinical relevance of TNF-α secretion in the scope of this work. Although the data from docetaxel treatment in cancer patients suggest that TNF-α release is not a requirement for the antitumor activity of paclitaxel, it remains possible that TNF-α release is still a factor in paclitaxel activity in some tumors. Depending on the degree of macrophage infiltration in a particular tumor, release of TNF-α could augment the cytotoxic effects mediated directly by paclitaxel.

p53 is a post-mitotic checkpoint

We examined the response of wild-type and p53 -/- fibroblasts to treatment with the microtubule-depolymerizing agent nocodazole, and found that p53 functions as part of a post-mitotic checkpoint following spindle disruption. Earlier studies had proposed that p53 acted as a component of a cell cycle checkpoint at mitosis, because p53 -/- cells failed to maintain cell cycle arrest after treatment with microtubule-acting drugs (Cross *et al.*, 1995). We characterized in detail the response of wild-type and p53-deficient fibroblasts to nocodazole treatment, and found that in fact, p53 activity was required only after cells exited from a temporary, p53-independent mitotic arrest. Using time-lapse videomicroscopy techniques, we were able to observe nocodazole-treated wild-type and p53 -/- fibroblasts as they entered mitosis and determined that they remained in mitotic arrest for several hours. Nocodazole-treated cells of both genotypes then exited from mitotic arrest and assumed a flattened morphology, in a process known as adaptation (Rudner and Murray 1996). The duration of time spent at mitotic arrest in the presence of nocodazole (4.5 hours) was considerably longer than the time required for normal mitosis in this cell type, which lasts approximately 25 minutes.

From our data, we concluded that p53 did not act as part of a mitotic checkpoint. If p53 function were required at mitosis, then p53 -/- fibroblasts would not have been

predicted to undergo mitotic arrest in the presence of nocodazole. However, both wild-type and p53 -/- fibroblasts remained in mitotic arrest for several hours in the presence of drug. This behavior is in contrast to that of mammalian cells in which components of the mitotic checkpoint have been inhibited from carrying out their normal functions. The MAD and BUB genes were found in *S. cerevisiae* screens designed to isolate mitotic checkpoint mutants (Hoyt *et al.*, 1991; Li and Murray 1991). The human homolog of the *S. cerevisiae* MAD2 checkpoint gene has been identified, hsMAD2. When an anti-hsMAD2 antibody was electroporated into human cells, they exhibited a decrease in the number of cells at mitotic arrest in the presence of nocodazole (Li and Benezra 1996). A murine homolog of the yeast BUB1 gene has also been identified. Expression of dominant negative mouse Bub1 in cells was seen to inhibit the ability of cells to implement mitotic arrest following nocodazole treatment (Taylor and McKeon 1997). Unlike the MAD2 and Bub1 proteins, the p53 gene product does not function at a mitotic checkpoint, because its absence does not affect the ability of cells to arrest at mitosis in the presence of spindle inhibitors. Our finding is agreement with an earlier study by Minn *et al*, who found that in prolymphocytic cells treated with nocodazole, cells expressing a dominant negative p53 gene still underwent mitotic arrest in the presence of the drug (Minn *et al.*, 1996).

We found that p53 did act as part of a checkpoint to prevent S phase reentry following the sequence of spindle disruption, mitotic arrest, and adaptation described above. Beginning 2 hours after adaptation, p53 -/- fibroblasts began to initiate S phase, despite never having successfully completed mitosis. This behavior resulted in the accumulation of p53 -/- cells with 8N DNA content, an observation that has been reported by other groups (Cross *et al.*, 1995; Minn *et al.*, 1996; Di Leonardo *et al.*, 1997). Wild-type adapted fibroblasts did not initiate an

additional round of DNA synthesis, but instead remained in a quiescent interphase state. Therefore, after spindle disruption, p53 acts to prevent adapted cells from progressing into S phase, in a checkpoint function analogous to its well-characterized checkpoint role in G1 following DNA damage. Our result supports an earlier conclusion by Minn and colleagues, who observed that expression of increased levels of p53 protein did not occur until after cells exited from mitotic arrest, indicating a post-mitotic checkpoint function for p53 (Minn *et al.*, 1996).

Other genes implicated in the postmitotic checkpoint

In addition to the p53 gene product, a number of other proteins appear to function as part of the postmitotic checkpoint. All of these proteins have previously been identified as components of the G1 cell cycle checkpoint, indicating that there is a large degree of functional overlap between these two checkpoints. The p53 target gene p21 is a CDK inhibitor which acts as a primary downstream mediator of the p53-dependent G1 cell cycle arrest following radiation (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). We found that induction of p21 was required to maintain the arrest state in cells treated with the spindle inhibitors nocodazole and colcemid, in contrast to previously published data (Deng *et al.*, 1995). We observed that like p53 -/- cells, p21 -/- cells demonstrated inappropriate DNA synthesis in the presence of nocodazole, although to a lesser degree. Notably, the extent of the requirement for p21 was similar in G1-arrested cells and postmitotically-arrested cells, approximately 70-80% in each instance (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). Thus, expression of p21 protein is required to the same degree in both the p53-dependent arrest in G1 following DNA damage and the p53-dependent arrest in adapted cells following spindle damage. Interestingly, the CDK inhibitor p16 also helps prevent cell cycle reentry following microtubule disruption, as p16 -/- mouse fibroblasts also become 8N upon treatment with spindle inhibitors (Khan and Wahl 1998).

The retinoblastoma protein (pRB) also appears to be required for maintaining postmitotic arrest. pRB is a key component in G1 regulation whose activity is regulated by cell-cycle specific phosphorylation. The hypophosphorylated, active form of the protein is present in G1, and becomes phosphorylated upon entry into S phase, coincident with a loss of activity (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Ludlow *et al.*, 1993). We found that in adapted, nocodazole-treated cells, the predominant form of pRB was hypophosphorylated, indicating a potential role for the Rb protein as part of the postmitotic checkpoint. Experiments using fibroblasts which lack functional pRB have also determined that the Rb protein is necessary for maintaining cell cycle arrest following nocodazole treatment. Di Leonardo *et al.* observed that human fibroblast cells expressing the human papillomavirus type 16 E7 protein, which binds to pRB, exhibited a large fraction of 8N cells after nocodazole treatment (Di Leonardo *et al.*, 1997). Rb -/- murine fibroblasts obtained from knockout mice also appear to initiate DNA synthesis inappropriately in the presence of spindle inhibitors, although with a phenotype less severe than HPV-E7 expressing cells (Di Leonardo *et al.*, 1997; Khan and Wahl 1998). Taken as a whole, these data indicate that the absence of functional Rb protein in adapted cells appears to permit inappropriate entry into S phase.

<u>Linking microtubule disruption to p53: possible upstream signals</u>

Loss of microtubule stability

An interesting subject which was not in the scope of our experiments is the nature of the signal leading to activation of p53 following microtubule damage. A number of possibilities exist for how this signal may be transmitted. One reasonable model is that changes in microtubule stability lead to p53 activation. If such a mechanism existed, it would define a novel signalling pathway linking the p53 protein directly

to the cytoskeleton. However, our data suggest that changes in microtubule stability do not directly lead to activation of p53. In our experiments using synchronized NIH 3T3 cells, we saw that cells progressed unhindered from G0 through S phase while grown in the constant presence of nocodazole. This result is similar to earlier findings reported by Jordan *et al.* ,who observed that low concentrations of microtubule-depolymerizing agents did not affect interphase microtubules or inhibit cell-cycle progression at times other than mitosis (Jordan *et al.*, 1992). In our experiments, if the loss of microtubule stability induced by nocodazole treatment were sufficient to cause activation of p53, we would have expected to see the NIH 3T3 cells arrest soon after their initial entry into G1, since they contain functional p53 protein (Rittling and Denhardt 1992; Tishler *et al.*, 1993; Hermeking and Eick 1994). However, since the cells did not arrest until much later in the cell cycle, we concluded that microtubule destabilization alone was not a stimulus monitored by p53.

In contrast to our findings, other research suggests that loss of microtubule stability may be able to activate p53, perhaps in a cell type- or species-specific manner. Khan and Wahl performed experiments using serum-synchronized human fibroblasts treated with nocodazole (Khan and Wahl 1998). They reported that when a synchronized population of wild-type cells progressed from G0 into G1 in nocodazole-containing medium, the cells arrested in G1. However, cells expressing the human papillomavirus type 16 E6 protein, which binds p53, did not arrest in G1 but instead went on to enter S phase. One possible explanation for these data is that the destabilization of cellular microtubules caused by nocodazole treatment is detected by p53 in G1, resulting in p53-dependent cell cycle arrest. This mechanism could differ between human and murine cells, which could reconcile our apparently disparate results. If microtubule destabilization can lead to p53 activation, then

elucidation of the molecules in the signalling pathway will be an important next step. Blagosklonny *et al.* have demonstrated that treatment of cells with microtubule-acting drugs leads to activation of the kinase Raf-1 and phosphorylation of the bcl-2 gene product (Blagosklonny *et al.*, 1997). It would be interesting to determine whether the p53 protein has any apparent functional connection to the molecules present in this pathway in cells which have experienced microtubule disruption.

Centrosomal number/ hyperploidy

Another model for p53 activation following microtubule disruption is based on the premise that p53 could become activated by the presence of abnormal elements in a G1-like cell. We and others have shown that cells treated for an extended interval with microtubule inhibitors will adapt from mitotic arrest into a G1-like interphase state (Kung *et al.*, 1990; Rieder and Palazzo 1992). These adapted cells have some normal characteristics of G1 cells, in that they contain predominantly hypophosphorylated pRB, express cyclin E, and have decondensed chromatin. However, because these cells never successfully completed mitosis, they also have a 4N DNA content and two centrosomes, neither of which are typically present in a G1 cell. It is possible that the presence of one or both of these characteristics triggers a p53-dependent arrest in the adapted cell. In support of this model, p53 -/- cells have been shown to contain abnormal numbers of centrosomes, suggesting a role for p53 in regulating centrosome duplication (Fukasawa *et al.*, 1996). Also, it has been reported that the p53 protein can be localized to the centrosome using immunodetection techniques, and that a subfraction of cellular p53 copurifies with centrosomal proteins (Brown *et al.*, 1994). While p53 has not been shown to detect hyperploidy in cells, absence of p53 has been associated with amplification of the number of copies of the genome. Over 50% of p53 -/- fibroblasts spontaneously

became tetraploid after 7 passages *in vitro* (Cross *et al.*, 1995). Additionally, cells derived from the pancreas of mice expressing SV40-T-antigen, which inactivates the p53 protein, also developed significant tetraploid populations *in vivo* (Cross *et al.*, 1995). These data may provide preliminary evidence for a role for p53 in detecting hyperploidy or centrosomal number, both of which are eventually present in cells following microtubule disruption, mitotic arrest, and subsequent adaptation.

DNA damage

A third possible model for how p53 becomes activated after spindle disruption is that treatment with microtubule inhibitors results in DNA damage, which is then detected by p53 upon progression of cells into the adapted state. In this model, p53 would carry out a function identical to its checkpoint role in G1 following other kinds of DNA damage such as radiation. This model could be addressed in part by determining whether the ATM gene product is necessary for the checkpoint in adapted cells following spindle damage. The ATM protein is known to function upstream of p53 in response to DNA damage, in a signalling pathway leading to G1 arrest (Kastan *et al.*, 1992). If absence of this protein were found to reduce the ability of cells to arrest after nocodazole treatment, it would indicate that detection of DNA damage via the ATM signalling pathway was a necessary step in checkpoint activation. Such an experiment would be readily performable using ATM-deficient fibroblasts obtained from knockout mice. It would also be useful to test whether DNA damage was physically detectable in cells following nocodazole treatment, using assays designed to detect DNA strand breaks. Using a standard terminal-deoxynucleotide transferase assay, we did not detect any broken ends of DNA in nocodazole-treated fibroblasts (J.S. Lanni and T. Jacks, unpublished data); however, a more sensitive assay might reveal the presence of damaged DNA. The presence of DNA strand breaks would not prove conclusively that they constitute part of the

upstream signalling pathway, but it would lend support to a mechanism whereby p53 was activated following detection of DNA damage. In the future, it would be valuable to address the mechanism of p53 activation following treatment with spindle drugs via these and other experiments.

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