Relationship between p21 Expression and Mutation of the *p53* Tumor Suppressor Gene in Normal and Malignant Ovarian Epithelial Cells[1]

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ABSTRACT

In many cell types, p53-mediated growth inhibition is dependent on induction of p21, which is an inhibitor of cyclin-dependent kinases that are required for cell cycle progression. Failure of mutant p53 proteins to transactivate p21 may lead to uncontrolled proliferation. Because many ovarian cancers have mutations in the *p53* gene, we examined p21 levels in normal and malignant ovarian epithelial cells to determine whether p21 expression is dependent on wild-type p53. Normal ovarian epithelial cells and two ovarian cancer cell lines with wild-type p53 expressed readily detectable levels of p21, whereas in p53 null and mutant cell lines, expression of p21 was diminished strikingly. A correlation between the status of the *p53* gene and p21 expression also was noted in 23 primary epithelial ovarian cancers. Normal levels of p21 RNA were seen in 4/7 (57%) cancers with wild-type p53, whereas 14/16 (88%) cancers with mutant p53 had reduced p21 expression ($P < 0.05$). In addition, we found that γ-irradiation of normal and malignant ovarian epithelial cells with wild-type, but not mutant, p53 resulted in induction of p21. These data are suggestive that induction of p21 is a feature of p53-mediated growth inhibition in normal ovarian epithelial cells. Conversely, mutation of the *p53* gene in ovarian cancers usually is associated with decreased p21 expression. The lack of an absolute correlation between p21 expression and the status of the *p53* gene in ovarian cancers is consistent with other studies that have suggested that p21 may also be regulated by p53-independent pathways.

INTRODUCTION

Most cancers that arise in the ovaries are found to have spread throughout the peritoneal cavity at diagnosis, and ovarian cancer is the fourth leading cause of cancer deaths in women in the United States. Although a small fraction of ovarian cancers arise because of inherited mutations in the *BRCA1* gene (1), it is thought that acquired alterations in several different oncogenes and tumor suppressor genes are responsible for more than 90% of cases. In this regard, amplification of the HER-2/*neu* and c-*myc* genes are a feature of some sporadic ovarian cancers (2). Inactivation of the *p53* tumor suppressor gene is the most common alteration described thus far in ovarian cancers, however (3, 4). Mutation and overexpression of p53, usually in concert with deletion of the other wild-type *p53* allele, occurs in 50% of advanced stage III/IV cases (5) and 15% of early stage I/II cases (6).

It is thought that wild-type p53 normally acts to restrain inappropriate cellular proliferation (7). In addition, p53 expression is increased after DNA damage due to ionizing radiation (8, 9). Growth arrest in the G_1 phase of the cell cycle allows for DNA repair prior to replication, but if repair is inadequate, p53 can trigger apoptosis. Conversely, if p53 has been inactivated, apoptosis may not occur appropriately, allowing survival of cells that have undergone DNA damage. The biological activity of p53 protein is dependent on its ability to bind to transcriptional regulatory elements in DNA. The search for critical genes regulated by p53 led to the discovery of the *p21 (CIP1/WAF1)* gene (10, 11). A consensus p53-binding site is located 2.4 kb upstream of the p21 coding sequence and confers p53-dependent transcriptional regulation on a heterologous reporter gene (10). Transcription of p21 is 50-fold lower in fibroblasts from p53-null mice compared to fibroblasts from normal mice (12). In addition, transcription of p21 is induced 10-fold after irradiation in myeloid leukemia cells with functional p53, whereas induction of this gene is not seen in those lacking functional p53 (12). The subsequent discovery that p21 acts as an inhibitor of cdks[3] provides a functional link between p53 and cell cycle control (11, 12).

This paradigm of regulation of p21 expression by p53 is well accepted based on studies performed in various immortalized cell lines. In some cases, molecular alterations observed initially in cancer cell lines have not been found when the corresponding type of primary cancer has been examined, however. Confirmatory studies in various types of primary normal and malignant cells represent a critical step toward establishing the relevance of hypotheses generated in cell lines. Because many primary human epithelial ovarian cancers have mutations in the *p53* gene, we examined p21 levels in normal and malig-

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[3] The abbreviations used are: cdk, cyclin-dependent kinase; FBS, fetal bovine serum; TGF, transforming growth factor.

nant ovarian epithelial cells to determine whether p21 expression correlates with the status of the *p53* gene.

MATERIALS AND METHODS

Cell Culture. Ovarian cancer cell lines OVCA 420, 429, 432, and 433 were established and maintained in modified Eagle's medium with 10% heat-inactivated FBS as described previously (13). The SKOV3 ovarian cancer line was obtained from the American Type Culture Collection and maintained in RPMI supplemented with 10% FBS. Primary monolayer cultures of ovarian epithelial cells were established from surgical specimens of normal ovaries as described previously (14, 15). Briefly, the surface of the ovary was scraped gently, and the epithelial cells were then plated in a 1:1 mixture of MCDB 105/M199 medium supplemented with 15% heat-inactivated FBS and epidermal growth factor (10 ng/ml). Cells were cultured at 37°C in 5% CO_2 and 95% humidified air.

Primary Ovarian Cancers. Twenty-three snap-frozen samples of epithelial ovarian cancer were obtained from patients who underwent primary surgery at Duke University Medical Center from 1985 to 1993. The disease was staged in accordance with the International Federation of Gynecologists and Obstetricians staging system for ovarian carcinoma. The tissues were frozen in liquid nitrogen immediately after extirpation, and the presence of viable cancer was confirmed by histological analysis. In the tissues selected for this study greater than 90% of the sample comprised malignant cells. Sixteen of the 23 cases had been shown previously to overexpress mutant p53 protein by immunohistochemical analysis (5, 6). In 8 of 23 cases, the precise mutation in the *p53* gene also had been identified by DNA sequencing (4). Complete clinical information including survival was abstracted from the hospital chart.

Cloning CIP1/WAF1. Whole-cell RNA was extracted from tissue culture cells and intact tissue by the method of Chomczynski and Sacci (16). Forward (ACTCAGAGGAGGCGCCATGT) and reverse (TTCCTGTGGGCGGATTAGGG) primers were designed based on the published cDNA sequence of p21 to include the entire coding sequence (12). Whole-cell RNA from normal mammary epithelial cells was reverse transcribed using random hexamers, and p21 cDNA was amplified by the PCR. A product of the anticipated size was obtained and cloned into the *Eco*RV site of Bluescript SK (Stratagene, La Jolla, CA). Sequencing of the cloned product confirmed the identity of the p21 cDNA.

Northern Analysis. Ten μg of total RNA from each source was separated electrophoretically on a 1% formaldehyde/agarose gel and transferred to a nylon membrane. Membranes were hybridized overnight at 65°C with 2×10^7 cpm of a labeled probe to the human p21 gene. Washing was performed at 65°C in 0.2× SSC (1× SSC, 0.15 M NaCl–0.015 M sodium citrate) and 0.1% SDS. The intensity of the p21 bands present in the resulting autoradiographs was quantitated using laser densitometry. Expression of p21 was corrected for relative loading of each lane as determined by densitometric scanning of the 28S RNA.

Radiation Treatment and Western Analysis. Subconfluent cells were exposed to γ-irradiation (0, 3, and 7 Gy). Six h after irradiation, the cells were lysed and protein was extracted according to the method of Li *et al.* (17). Aliquots of protein (100 μg) were electrophoresed on a 12.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. After incubating with blocking buffer (PBS, 5% nonfat dry milk, and 0.1% Tween 20), the membrane was probed with a mouse antihuman p53 monoclonal antibody (Ab-2; Oncogene Science, Manhassett, NY) and detected using goat antimouse IgG-horse radish peroxidase conjugate. The reaction was visualized with a chemiluminescent substrate solution using the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) and exposed to Kodak X-OMAT film for 3 min at room temperature. The membrane was probed and assayed in the same manner for p21 and B-actin using rabbit antihuman polyclonal antibody for p21 (PharMingen, San Diego, CA) and mouse antihuman monoclonal antibody for B-actin (Oncogene Science, Manhassett, NY).

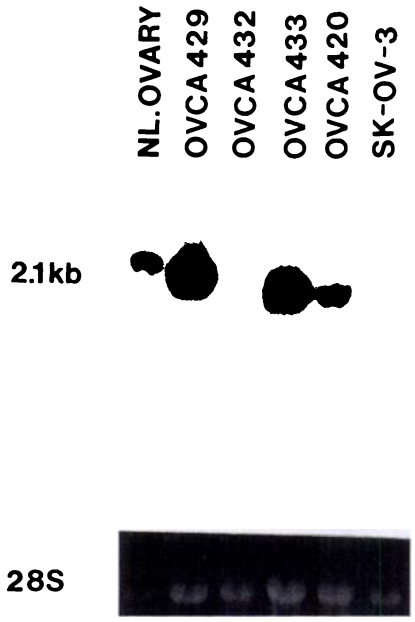

Fig. 1 Northern analysis of the 2.1-kb p21 mRNA transcript in normal and malignant ovarian epithelial cells that were hybridized with a p21 cDNA probe. Decreased p21 expression is seen in the OVCA 432 cell line, which contains a mutant *p53* gene, and in the p53-null SKOV3 cell line.

RESULTS

The coding sequence of the *p21* gene was isolated by reverse transcription and subsequent PCR amplification of RNA derived from normal ovarian epithelial cells. An amplification product of the expected size was obtained and cloned. Sequencing of the cloned product confirmed that this was the *p21* gene. All hybridizations were performed using this cloned cDNA as probe. Total cellular RNA was prepared from immortalized and primary epithelial ovarian cancers. Northern blot hybridization of these RNAs with the p21 probe revealed a single band of 2.1 kb (Figs. 1 and 2). The level of p21 transcript varied widely. Detectable p21 message was present in primary monolayer cultures of normal ovarian epithelial cells. In three immortalized

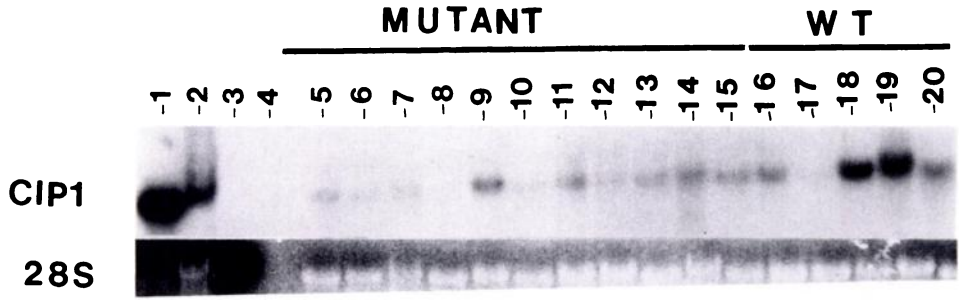

Fig. 2 Northern analysis of p21 (*CIP1*) in primary ovarian cancers with mutant or wild-type (*WT*) *p53* genes. *Lanes 1* and *2* (positive control), OVCA 429 and OVCA 433, which have normal *p53* genes. *Lanes 3* and *4* are blank. *Lanes 5–15*, primary ovarian cancers with mutant *p53* genes; in all cases, p21 expression is decreased by greater than 75% compared to OVCA 433. *Lanes 16–20*, ovarian cancers with wild-type *p53* genes. Although p21 expression is reduced in *Lanes 16*, *17*, and *20*, p21 expression in *Lanes 18* and *19* is normal.

ovarian cancer cell lines with wild-type *p53* genes (OVCA 420, 429, and 433) the level of p21 transcript was equal to or greater than that seen in normal ovarian epithelial cells. In contrast, the p53-null SKOV3 cell line, in which both copies of the *p53* gene have been deleted, had barely detectable amounts of p21 mRNA. In the OVCA 432 cell line, which has a missense mutation in codon 277 of the *p53* gene, p21 message was only detectable upon reverse transcription and PCR amplification of the RNA (data not shown).

There also was a correlation between the status of the *p53* gene and p21 expression in primary ovarian cancers (Fig. 2). We examined p21 expression in 23 ovarian cancers, of which overexpression of mutant p53 had been demonstrated previously in 16 cases (4). Easily detectable levels of p21 were seen in four of seven cancers (57%) containing wild-type *p53* genes. In contrast, in primary cancers with p53 mutations, in 14 of 16 cases (88%) p21 was either absent or reduced by greater than 75% compared to OVCA 433 cells, which have wild-type p53. In addition, after densitometric scanning and correcting for relative loading of each lane, the mean p21 expression in the 16 cases with p53 alteration was significantly lower than that seen in the 7 cases with normal p53 (Student's t test, $P < 0.05$). There was no relationship between p21 expression and other clinical features of primary ovarian cancers (Table 1).

Table 1 Relationship between p21 expression and other features of primary ovarian cancers

	Reduced p21 expression no./total (%)	P value[b]
FIGO[a] stage		
I/II	2/5 (40%)	
III/IV	14/18 (78%)	NS[c]
Histological grade		
Well differentiated		
Moderately differentiated	5/7 (71%)	
Poorly differentiated	10/14 (71%)	NS
Histological type		
Serous	7/8 (88%)	
Other	11/15 (73%)	NS
Disease status		
No evidence disease	3/6 (50%)	
Alive with disease	10/12 (80%)	
Dead of disease	4/5 (80%)	NS
p53		
Wild-type	3/7 (43%)	
Mutant	14/16 (88%)	0.05

[a] FIGO, Fédération Internationale des Gynaecologistes et Obstetristes.
[b] χ^2 test.
[c] NS, not significant.

We examined expression of the *p21* gene in normal and malignant ovarian epithelial cells after γ-irradiation using Western analysis. In normal ovarian epithelial cells (NOSE1 and -2) and the OVCA 429 ovarian cancer cell line, which have wild-type *p53* genes, expression of both p53 and p21 mRNA was increased 6 h after radiation (Fig. 3). In contrast, in the SKOV3 ovarian cancer cell line, which lacks wild-type p53, induction of p53 and p21 was not seen after irradiation.

DISCUSSION

Disruption of cell cycle control is central to the process of malignant transformation. The decision to begin DNA synthesis (S phase) or, alternatively, to arrest in G_1 appears to be one of the main checkpoints in the cell cycle (18). G_1 arrest is dependent on a family of cdk inhibitors including p21, p16, p15, and p25 that interact with cdks, various cyclins, and proliferating cell nuclear antigen (19, 20). Cell cycle progression is characterized by decreased expression of p21, which allows cdks to phosphorylate the Rb (retinoblastoma) protein and other substrates, leading to initiation of DNA synthesis. The hypothesis that p21 expression is requisite for G_1 arrest is also supported by the finding that cells transformed by DNA tumor viruses such as SV40 do not contain the p21 in the cyclin-cdk complex (21) and that transfection of p21 into cancer cells suppresses growth (10). Numerous studies have shown that p53 is an important regulator of p21 transcription (10, 22–24), and it is thought that p21 is the primary effector of the growth-inhibitory effect of p53.

Although the *p53* tumor suppressor gene is inactivated because of mutation and/or deletion in approximately half of epithelial ovarian cancers, the other half of these cancers appears to retain functional p53. In cancers with wild-type p53, it is possible that alterations in p21 and other downstream effectors might have the same effect as loss of p53 function, namely, loss of the ability to arrest in G_1. Inactivating mutations in the *p21* gene do not appear to be a feature of human cancers, however (25–27).

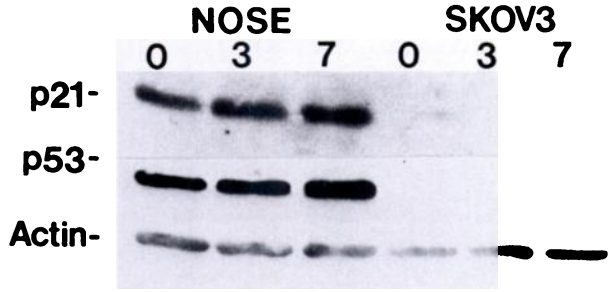

Fig. 3 Western analysis of the effect of γ-irradiation on p53 and p21 expression in normal ovarian epithelial cells (*NOSE*) with wild-type p53 and malignant ovarian epithelial cells (*SKOV3*) in which p53 is absent.

Consistent with the notion that p21 is transactivated by p53, we found that monolayer cultures of normal and malignant ovarian epithelial cells with wild-type *p53* genes expressed readily detectable levels of p21 RNA and protein. Conversely, an ovarian cancer cell line with a missense mutation in the *p53* gene and an ovarian cancer cell line in which both copies of the *p53* gene have been deleted did not express detectable p21. We were able to detect p21 transcript using PCR in the former cell line, suggesting that the *p21* gene is not deleted. Thus, in epithelial ovarian cancer cell lines, there was a close correlation between the status of the *p53* gene and expression of p21. These data are consistent with reports that have examined expression of p21 in other types of cancers (28–30).

Prior studies have shown that irradiation-induced cell cycle arrest is associated with a p53-dependent increase in p21 expression in most types of cells (23, 24, 31, 32). Exceptions to this model have been noted, however. For example, in KG human myeloblastic leukemia cells, which lack functional p53, irradiation leads to increased p21 expression by way of mechanisms that appear to be independent of p53 (33). Similar to most other types of cells, however, we found that irradiation increased p21 expression in normal and malignant ovarian epithelial cells with wild-type *p53* genes but not in those with mutant or absent *p53* genes.

Although convenient to maintain in culture, immortalized ovarian cancer cell lines are not necessarily representative of primary ovarian cancers. In this regard, we have shown previously that although most immortalized epithelial ovarian cancer cell lines are insensitive to the growth-inhibitory effect of TGF-β (13), like normal ovarian epithelial cells, more than 90% of primary ovarian cancers are growth inhibited by TGF-β (34). These studies and others underscore the importance of recapitulating findings obtained in immortalized cell lines in primary cancers obtained directly from patients. In this study, we noted a correlation between p53 mutation and reduced p21 expression in 23 primary epithelial ovarian cancers. Among the 16 cancers with mutant p53, 14 had either absent or significantly reduced levels of p21, consistent with the hypothesis that p53 is an important regulator of p21 expression. We did not find a relationship between p21 expression and clinical features such as stage, grade, and histological type or survival, but larger studies with greater statistical power are needed to address this issue.

Regulation of p21 expression in ovarian epithelial cells does not appear to be solely under the control of p53. Two primary ovarian cancers known to contain p53 mutations were found to express normal levels of p21, suggesting the presence of other regulatory pathways. In this regard, we have shown previously that p21 expression is induced by TGF-β in ovarian cancers with either wild-type or mutant p53 (35), and other groups also have demonstrated induction of p21 via a p53-independent pathway (28, 33, 36–38). Our finding that three of seven primary ovarian cancers with wild-type *p53* gene had low levels of p21 also suggests that p53 is not the sole regulator of p21 expression. Inactivation of this pathway may occur downstream from p53 and p21 and may involve cyclins and cdks (39).

It also appears that proliferation of some ovarian cancers may proceed despite expression of p21. In this study, we found that two of the immortalized ovarian cancer cell lines with relatively high levels of p21 continue to proliferate in monolayer culture. This suggests the possibility that inhibitors of p21 may exist or that other molecular signals may be generated in G_1 that allow cells to overcome p21-induced arrest. Recently, it has been reported that p21 can exist in both active and inactive cdk complexes depending on the stoichiometry of the complex components (40). We had previously assayed the downstream targets of p21 specifically, cdk activity and Rb phosphorylation, as an indirect measurement of p21 function in these cell lines (35). Our results indicate that p21 and the cdks in these cell lines are biologically active. Thus, although p21 may be a key regulator of cell cycle progression, it does appear that it can be circumvented.

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