p53 Mutation and Tamoxifen Resistance in Breast Cancer

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ABSTRACT

A substantial portion of patients with estrogen receptor-positive breast cancer fail to respond to estrogen depletion or to the antiestrogen tamoxifen. The molecular changes that lead to tamoxifen resistance and estrogen-independent growth are unknown. To test the hypothesis that a p53 mutation could result in tamoxifen resistance and estrogen-independent growth, the MCF-7 cell line was transfected with p53 cDNA which was mutated at codon 179 (histidine to glutamine). MCF-7 is an estrogen receptor-positive, estrogen-dependent, tamoxifen-sensitive cell line with only wild-type p53. The presence of transfected mutant p53 cDNA was verified by the PCR, and overexpression of mutant p53 protein was assessed by Western blotting. Five separate mutant-transfected clones were selected and tested in subsequent growth experiments. In monolayer culture, there was no consistent evidence of estrogen-independent growth or tamoxifen resistance in the mutant transfectants compared with vector-only controls or the parental cell line. In soft agar growth experiments, four of five mutant transfectants remained sensitive to tamoxifen in a dose-dependent manner. In the presence of wild-type p53, mutant 179 p53 protein does not result in estrogen-independent growth or tamoxifen resistance. These results do not exclude the possibility that other p53 mutational types could result in tamoxifen resistance, or that loss of the remaining wild-type allele may be necessary to result in this phenotype.

INTRODUCTION

The growth of some breast cancers has long been known to be regulated by estrogen. Conversely, antiestrogens such as tamoxifen can inhibit breast cancer growth in laboratory models, and treatment with tamoxifen can result in a prolonged survival of breast cancer patients in the adjuvant setting (1) or for metastatic disease (2). Unfortunately, however, only a portion of breast cancers are sensitive to the effects of tamoxifen and estrogen. This unresponsiveness is only partially explained by lack of ER, since 50% of patients who have ER-positive tumors still do not respond to tamoxifen therapy, and even those who do eventually become resistant. The cause of this unresponsiveness remains unclear.

p53 plays a central role in the negative growth regulation of a wide variety of cells (3, 4). Mutations in the p53 gene result in the loss of this negative growth regulation, an acquired ability to transform cells (5), and promotion of malignant cell growth (6). p53 alterations are common in breast cancer and are associated with a worse prognosis, which is an indirect manifestation of a more aggressive cell growth (7-9).

By a number of speculative mechanisms, p53 status could alter a breast tumor’s responsiveness to hormonal therapy and result in estrogen-independent growth. One mechanism that might explain tamoxifen resistance in p53-mutated breast cancer cells relates to the ability of tamoxifen to induce apoptosis, as seen in a mouse mammary carcinoma cell line (10). Studies suggest that p53 plays an important role in apoptosis induced by anticancer agents (11). If p53 became mutated, it is possible that tamoxifen-induced apoptosis might also become blocked. Also, p53 mutations can alter growth factor interactions potentially important in the therapeutic response to tamoxifen. Evidence suggests that tamoxifen can produce an inhibitory effect on breast cancer by increasing TGF-β, a growth factor that slows breast cancer cell growth (12). In human bronchial epithelium, mutant p53 causes loss of the inhibitory response usually seen with TGF-β (13). Thus, breast cancers with a p53 mutation may no longer be responsive to the normal growth-retarding effects of TGF-β. Finally, p53 negatively regulates cell proliferation and entry of cells into the S-phase. ER also influences cell proliferation and transit through the cell cycle. In the presence of mutated p53, the influence of the ER on cell cycle control might be lost, so that the cell would no longer be responsive to tamoxifen or estrogen.

By any of these mechanisms, loss of the function of p53 would disrupt the functional integrity of the ER pathway because of a shared or common component. This would in turn lead to loss of response to agents exerting their effect through ER. Using MCF-7, a human breast cancer cell line that is estrogen dependent, tamoxifen sensitive, and ER positive, we tested the hypothesis that a p53 mutation could result in tamoxifen resistance and estrogen-independent growth.

MATERIALS AND METHODS

Vector. A full-length p53 cDNA was cloned into a 5.5-kb pRC/CMV plasmid (Invitrogen, San Diego, CA). The cDNA was derived from a lung cancer cell line and contained a

1 The abbreviations used are: ER, estrogen receptor; TGF-β, transforming growth factor β; FBS, fetal bovine serum; CMV, cytomegalovirus.
mutation that resulted in a histidine to glutamine substitution at codon 179 (14). This is a frequent site of mutation in a number of different cancers (15). The construct also contained a CMV promoter for p53 expression and a neomycin gene allowing G418 selection. A pRC/CMV plasmid without p53 cDNA served as a control.

Cell Line. MCF-7 is a well-characterized ER-positive and progesterone receptor-positive, hormone-dependent, tamoxifen-sensitive human breast cancer cell line. It has no detectable mutations in the conserved region (exons 4–9) of the p53 gene (16). Recently, the entire coding region of the gene was sequenced and no mutation was found. 4 When used as a control, MCF-7 cells were not clonally derived from a single cell.

Transfection. Twenty μg CsCl-purified control or mutant-containing plasmid was transfected into MCF-7 cells with 50 μg Lipofectin (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer’s instructions. Cells were incubated overnight, then transferred to MEM (GIBCO, Grand Island, NY) supplemented with 10% FBS and 10–7 M insulin. The cells were initially grown in 800 μg/ml G418 (GIBCO-BRL) for 3 weeks, after which individual resistant colonies were harvested and screened for the presence of transfected plasmid by amplifying regions unique to the vector or vector-p53 construct using the PCR.

Western Blotting. Cells were harvested at 50–75% confluency, cytosols were prepared in 5% SDS, and protein determination was made with the bicinchoninic acid method. Samples were run on 8.5% polyacrylamide gels. The primary antibody for p53 was D01 (Oncogene Science, Uniondale, NY) at a concentration of 10 μg/ml. The antibody for proliferating cell nuclear antigen was PC10 (DAKO, Carpentry, CA) at a 1:100 dilution. The secondary antibody was an antimouse IgG antibody linked to horseradish peroxidase, NA931 (Amersham, Arlington Heights, IL). Blots were developed using a chemiluminescence reagent (Dupont New England Nuclear, Boston, MA) and exposed to film for 1 to 2 min.

Growth Studies. For 17β-estradiol-induced monolayer growth experiments, 1 × 105 cells were plated and grown for 24 h in MEM plus 10% FBS plus 10–7 M insulin plus 25 μg/ml gentamicin. The cells were then washed, and the medium was changed to phenol red-free MEM plus 10% charcoal-stripped FBS plus 10–7 M insulin and 25 μg/ml gentamicin. For 4-hydroxytamoxifen growth experiments, 2 × 104 cells were plated, and 10–10 M estradiol was added to the phenol red containing 10% MEM and 10% stripped FBS. Cells were grown to approximately 50% confluency over a period of 5–7 days. All experiments were done in triplicate. Cells were harvested and counted with a hemocytometer. For anchorage-independent soft agar cloning, a base plate composed of 0.8% agarose in MEM phenol red-free medium, 10% stripped FBS, 10–10 M estradiol, 10–7 M insulin, and 25 μg/ml gentamicin was used. An upper layer of 0.4% agarose contained the above constituents, 30,000 cells/well, and the indicated concentration of 4-hydroxytamoxifen. Colonies were counted at 7 days. All experiments were performed in triplicate. Cloning efficacy was expressed as a percentage of colonies formed by wild-type cells.

RESULTS

Protein Expression. MCF-7 cells were transfected with a 5.5-kb plasmid that contained a full-length p53 cDNA mutated at codon 179 (see “Materials and Methods”). Approximately 70 clones were harvested after 3 weeks of G418 selection. Of these, five contained plasmid with mutant p53 cDNA as verified by DNA PCR. Five separate clones were studied to avoid artificial results due to individual clone variation. From separate transfection experiments, several different clones were selected which contained the plasmid vector without p53. An untransfected MCF-7 parental cell line was used as a negative control and MDA-231, an ER-negative human breast cancer cell line with a single mutant p53 allele (codon 280, arg → lys), was used as a positive control. Western blot analysis (Fig. 1) showed that mutant-transfected clones all overexpressed p53 relative to the MCF-7 parental line or plasmid-only controls, although this expression was variable. VM4K and VM10L had the highest protein expression, although this expression was less than MDA-231, the positive control. MCF-7 had a trace amount of p53 detectable as determined by this method. Protein expression was verified both before (Fig. 1) and after all growth experiments (data not shown), and there was no significant change in the level of expression.

Monolayer Growth. After verifying overexpression of p53, clones were next grown in the absence or presence of increasing concentrations of 17β-estradiol (Fig. 2). There was little, if any, growth in the absence of estrogen for any of the clones or parental line. As expected, with the addition of estradiol, growth dramatically increased, usually in a dose-dependent manner. Thus, there was no evidence of estrogen-independent growth induced by mutant p53. Next, similar experiments were performed with increasing concentrations of 4-hydroxytamoxifen.
**Fig. 2** Growth of clones in various concentrations of estrogen. Cells ($1 \times 10^5$) were plated and exposed to phenol red-free MEM with 10% stripped FBS and the concentrations of 17β-estradiol (E2) shown. Cells were grown until 50% confluency, which occurred at approximately 7 days. All clones were grown in triplicate. Bars, SEM.

**Fig. 3** Growth of clones and parental cell line in various concentrations of 4-hydroxytamoxifen (TAM). Cells ($2 \times 10^5$) were plated and grown in MEM medium with 10% stripped FBS, $10^{-11}$ M estradiol, and the concentrations of 4-hydroxytamoxifen shown. Cells were grown for 6 days and counted at 50–75% confluency. All clones were grown in triplicate. Bars, SEM.
ifé, the more active metabolite of tamoxifen. Clones transfected with plasmid alone or plasmid containing mutant p53 both showed similar inhibition by $10^{-6}$ and $10^{-8}$ 4-hydroxytamoxifen, and there was little evidence of tamoxifen resistance (Fig. 3). The parental MCF-7 cell line had a more rapid basal growth rate compared to all transfectants. It is possible that the transfected cells grew more slowly because they were maintained in 400 $\mu$g/ml G418 until 1 day before these growth experiments. The MCF-7 cells were grown in the absence of G418.

**Soft Agar Cloning.** Because soft agar cloning can sometimes reveal differences in growth rate or characteristics not seen under monolayer conditions, experiments were performed in soft agar looking for tamoxifen resistance (Fig. 4). Four of five mutant-transfected clones showed no evidence of tamoxifen resistance compared with parental MCF-7 cells. The one exception to this was clone VM20G, which appeared to be partially resistant to tamoxifen in these experiments. This finding may be explained by some unknown factor related to clonal variability. By Western blotting, mutant protein expression was relatively low in this clone. Overall, these data suggest that mutant p53 does not cause tamoxifen resistance.

**DISCUSSION**

Both estrogen and tamoxifen are thought to exert their growth regulatory effects through the estrogen receptor. Loss of response to estrogen or tamoxifen could occur through a number of different general mechanisms, including loss or alteration of ER (17, 18), postreceptor defects, and changes in autocrine or paracrine growth factor interactions (12, 13), and for tamoxifen, pharmacological alteration (19). Preliminary data from the San Antonio tumor bank indicated that node-negative ER-positive breast tumors with an accumulation of p53 protein were relatively resistant to tamoxifen. Accumulation of protein can be an indicator of a p53 mutation. As a result of this observation, in a series of experiments, we tested the possibility that p53 mutation could result in estrogen-independent growth and tamoxifen resistance in human breast cancer. From these experiments, there was no evidence that a mutant p53 gene could produce either of these two states in human breast cancer cells *in vitro*.

These results could be explained in two ways. First, p53 pathways simply may not influence or be connected with those that govern estrogen growth regulation or tamoxifen effects on the cell. There is no direct experimental evidence documenting such a connection. Second, these observations could be due to the experimental model that was used. By definition, both alleles of a classical tumor suppressor gene must be inactivated, either through loss or mutation, before loss of growth regulation and other deleterious effects occur. Initially, it was thought that the p53 gene did not function as a classical tumor suppressor gene, and that protein from a single mutated allele inactivated the remaining wild-type protein in a “dominant negative” manner (20–22). More recently, evidence has suggested that the wild type may actually be dominant to mutant, especially if mutant and wild-type protein are present in approximately equal amounts (23–25). In untransfected MCF-7 cells, only wild-type p53 is present. The efficiency of the transfections may not have been high enough to deliver a sufficient number of copies of the
mutant p53 gene for the cell to acquire a mutant phenotype. Furthermore, if mutant p53 actually can result in tamoxifen resistance and estrogen-independent growth, this may occur only in the absence of wild-type protein.

Different mutations of p53 result in different biological effects (26–29). Different regions of the molecule perform distinct functions, and mutations in these areas may result in the loss of specific functions depending on where they occur (30, 31). The p53 cDNA used in this experiment was mutated at codon 179 and was derived from a transformed lung cancer cell line. It is also commonly found in other malignant tumor types (15). Both of these observations indicate that this particular mutation is involved in malignant transformation. It is possible, however, that this type of mutation does not result in tamoxifen resistance, but that other types of mutations may still produce this effect. Only testing with other mutational types would clarify this possibility.

p53 mutation plays an important role in the molecular pathogenesis of breast cancer and could influence its hormone sensitivity. However, in the presence of wild-type p53, mutant 179 p53 protein does not result in estrogen-independent growth or tamoxifen resistance. These results do not exclude the possibility that other p53 mutational types could result in tamoxifen resistance or that the loss of the remaining wild-type allele may be necessary to result in this phenotype in vivo.

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