**Advances in Brief**

**Alteration of p53 Damage Response by Tamoxifen Treatment**

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**Abstract**

Hormone therapy is often used in association with chemotherapy in the treatment of estrogen-responsive breast cancers. By using breast adenocarcinoma cell lines, we show that antiestrogen treatment leads to a dramatic decrease of p53 protein levels. This effect leads to a loss of wild-type p53 response to genotoxic treatment. This inhibition is assessed by the lack of p53 protein accumulation and the loss of the p53-dependent induction of p21^{WAF1/CIP1} expression. Given that the effects of several anticancer agents are mediated through DNA damage, these observations suggest that antiestrogen treatment could modulate cellular response to chemotherapeutic agents.

**Introduction**

Breast cancer is an increasingly prevalent cause of death among women. The use of adjuvant therapy, either chemotherapeutic or hormonal, is recommended by many clinicians after surgery. Recently, cytotoxic chemotherapy has been combined with antiestrogen treatment (tamoxifen) in patients with hormone-responsive human breast cancer with the hope of achieving additional antitumor activity (1). Despite extensive research, there is a paucity of information regarding the effects of steroids on tumor suppression function, and the exact mechanisms by which antiestrogens induce tumor regression are still not clear. Antiestrogen treatment is attributed generally to its ability to bind to and translocate ER to the nucleus, where presumably it inhibits estrogen-mediated events leading to cell growth. The recent demonstration that antiestrogens modulate cdk activity provided a functional link between antiestrogen and cell cycle arrest (2).

The p53 tumor suppressor gene product is a participant in the cellular response to DNA damage resulting in either G1 cell cycle arrest (3) or cell death by apoptosis (4, 5). Transcriptional regulation appears to be essential for its antiproliferative function. p53 activates transcription of a set of genes containing p53-responsive elements. These genes include the cdk inhibitor p21^{WAF1/CIP1} (6) that inhibits kinases responsible for G_{1}-S transition. Because of the crucial role that p53 plays in tumor suppression, and its relation with the activity of the cdk, we investigated p53 response to genotoxic agents in the presence of estrogen and/or antiestrogen in breast cancer cells.

**Materials and Methods**

**Cell Cultures and Treatments.** Human breast adenocarcinoma cell lines MCF-7, BT20, and T47D and hepatoblastoma cell line HepG2 were obtained from the American Type Culture Collection. They were maintained routinely in DMEM supplemented with phenol red and 5% FCS. For experiments involving estrogen or tamoxifen, cells were passaged in phenol red-free DMEM containing 5% of serum stripped of steroids by absorption to dextran-coated charcoal.

4-Hydroxytamoxifen was a gift from ICI Laboratories (Macclesfield, UK) and 17β-estradiol was purchased from Roussel-Uclaf (Romainville, France). Concentrated stock solutions (10^{-2} M) of these compounds were prepared and stored at 4°C. 4-Hydroxytamoxifen and 17β-estradiol were added to the culture medium in ethanolic solution at the appropriate concentration. The amount of ethanol represented less than 0.1% of the final volume, and the same volume of ethanol was added to control samples.

Cells were treated with the chemotherapeutic agent hydroxyl-doxorubicin (Adriamycin) or with CDDP (cisplatin) for 18 h at 37°C at a concentration of 0.2 or 2 μg/ml, respectively, or exposed to X-ray irradiation at 6 Gy.

**Western Blot and Immunoprecipitation.** For Western blot analysis, cell lysates adjusted to equal amount of proteins (100 μg) were harvested in sample-loading buffer, separated by SDS-PAGE and transferred to Immobilon-P polyvinyl-divinyl fluoride transfer membrane (Millipore Corp., Bedford, MA). Analysis of p53, p21, and RB proteins was performed using p53 (Ab-2) and WAF1 (Ab-1) monoclonal antibodies (Oncogene Science Inc., Cambridge, MA) and RB (IF8) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Detection was performed using a peroxidase-conjugated antibody and enhanced chemiluminescence detection reagent.

For immunoprecipitation, tamoxifen or estradiol-treated cells were pulse-labeled for 2 h with [35S]methionine. Cell extracts adjusted to equal amounts of proteins were precleared with protein G-sepharose beads (Amersham International plc, Buckinghamshire, UK) for 1 h at 4°C, and p53 was immunoprecipitated with p53 (Ab-2) monoclonal antibody (Oncogene Science). The immunoprecipitates were separated by SDS-PAGE, and p53 was detected by autoradiography.

**Northern Blot Analysis.** Total cellular RNA was extracted using the guanidium isothiocyanate-phenol-chloroform extraction procedure as described previously (7). RNA samples (20 μg) were separated by electrophoresis through agarose gel and transferred to nylon membranes (Hybond-N; Amersham). Membranes were hybridized with a 32P-labeled p53 cDNA.
Fig. 1  A. effects of tamoxifen or 17β-estradiol on the growth of MCF-7 cells. Cells were passaged at time 0 with phenol red-free medium supplemented with charcoal-stripped serum in the presence of tamoxifen (OHT) or 17β-estradiol (E2) in the indicated molar concentration. Data points are the means of three dishes. B. effects of tamoxifen or 17β-estradiol on phosphorylation of the RB protein in MCF-7 cells. OHT, phenol red-free medium supplemented with charcoal-stripped serum. with 10^{-8} M tamoxifen. E2, phenol red-free medium supplemented with charcoal-stripped serum. with 10^{-8} M 17β-estradiol. Upper band, ppRB, hyperphosphorylated form of RB; lower band, pRB, hypophosphorylated form of RB.

Plasmids and Transfections. The reporter plasmid pRGΔFosLacZ contains two copies of the RGC-p53 binding fragment in head-to-head orientation upstream of a minimal Δfos promoter that controls the expression of bacterial LacZ gene to produce β-galactosidase protein (provided kindly by Thierry Frebourg; Ref. 8). Cells were plated at 50% confluence and transfected with 10 μg plasmid DNA using lipofectin reagent (Life Technologies, Inc., Gaithersburg, MD). The β-galactosidase activity of transfected cells was analyzed 48 and 72 h after DNA transfection, as described previously (8).

Results and Discussion

Effects of Antiestrogen Treatment on p53 Levels. Basal levels of endogenous p53 were studied in MCF-7 cells in the absence or presence of antiestrogen. The breast adenocarcinoma cell line MCF-7 is ER-positive and expresses a wild-type p53 (9). Cells were maintained routinely in DMEM supplemented with phenol red and 5% FCS. Culture in phenol red-free medium supplemented with stripped serum and tamoxifen (10^{-6} M) causes a growth arrest (Fig. 1A), with a resulting decrease in the relative proportion of cells synthesizing DNA (10, 11). This cell cycle arrest is associated with a decrease in the degree of RB phosphorylation (Fig. 1B), which could be an essential event for the inhibition of entry into S phase (2). As shown in Fig. 2A, tamoxifen treatment leads to a gradual decrease of p53 levels detectable after 24–48 h to reach a plateau corresponding to a 10–15-fold decrease after 96 h as assessed by densitometry. This effect is reversed by the addition of estrogen in the medium (data not shown), suggesting that the down-regulation of p53 levels is due to the inhibition of estrogen activity. According to this hypothesis, culture of MCF-7 cells in an estrogen-free medium and in the absence of antiestrogen leads to a similar decrease of p53 protein levels (Fig. 2B, Lane 2). Tamoxifen can compete with estrogen for the nuclear receptor, but it displays 100-fold less affinity (12). Consequently, we studied p53 levels in the presence of both 17β-estradiol and tamoxifen. Treatment with a 100-fold excess of tamoxifen produces a significant decrease of p53 levels (Fig. 2B, Lane 6). However, in equimolar concentrations of 17β-estradiol and tamoxifen, p53 protein levels remain unchanged (Fig. 2B, Lane 7). Conventionally used doses of tamoxifen (10–20 mg daily) result in steady-state levels of approximately 0.4–1.0 μM. Thus, at clinically relevant concentrations, tamoxifen provokes a decrease of p53 protein levels. The competition effect of estrogen and tamoxifen suggests strongly a mechanism mediated via the ER.

BT20 and T47D cells display a missense p53 mutation at codons 132 and 194, respectively (13). Consequently, p53 levels are not affected by genotoxic treatment in these cells (Fig. 3). However, although to a lesser degree than in MCF-7 cells, culture in an estrogen-free medium for 96 h leads to a significant decrease (3-fold) of endogenous p53 protein levels in the ER-positive T47D cells (Fig. 3). A similar observation was reported recently by Hurd et al. (14). In contrast, p53 levels are not affected by the absence of estrogen in the ER-negative BT20 cells. Similar results are obtained in the ER-negative HepG2 hepatoblastoma cell line that expresses wild-type p53. These
Fig. 2 A. effects of tamoxifen on p53 protein levels in MCF-7 cells. MCF-7 cells were treated with 10^{-8} M tamoxifen in a culture medium depleted of estrogen. Cell lysis was performed at the indicated times, and Western blot was processed using equal amounts of proteins. B. p53 protein levels in MCF-7 cells grown in different culture media as assessed by Western blot. MCF-7 cells were grown for 96 h in the indicated culture medium, and cell extracts were prepared as indicated in “Materials and Methods.” Lanes: C. DMEM supplemented with 5% FCS. Csx, phenol red-free DMEM supplemented with charcoal-stripped serum; OHT-6. Csx with 10^{-8} M tamoxifen; OHT-8. Csx with 10^{-8} M 17β-estradiol; E2-8 + OHT-6. Csx with 10^{-7} M 17β-estradiol and 10^{-8} M tamoxifen; E2-8 + OHT-8. Csx with 10^{-7} M 17β-estradiol and 10^{-6} M tamoxifen.

Fig. 3 p53 levels in two adenocarcinoma cell lines. T47D (ER{+}), mutant p53 and BT20 (ER{−}, mutant p53), and the hepatoblastoma cell line HepG2 (ER{+}, wild-type p53), as measured by Western blot. Cells were grown for 96 h in the indicated culture medium, as follows. C. DMEM supplemented with 5% FCS. Csx, phenol red-free DMEM supplemented with charcoal-stripped serum; OHT. Csx with 10^{-8} M tamoxifen; E2. Csx with 10^{-8} M 17β-estradiol; Ad. cells were cultured in DMEM supplemented with FCS and treated with 0.2 μg/ml Adriamycin for 18 h.

observations support the hypothesis that p53 protein levels are modulated through a mechanism mediated by the ER.

To study the mechanisms involved in the modulation of p53 levels, we analyzed p53 mRNA from antiestrogen- and estrogen-treated MCF-7 cells. No fluctuation in p53 mRNA levels was observed (Fig. 4A). This suggests that p53 levels are regulated by a posttranscriptional mechanism. Thus, we measured the rate of [^{35}S]methionine incorporation, and we estimated the half-life of the labeled protein. The rate of p53 synthesis in tamoxifen-treated MCF-7 cells was much lower compared with estrogen-treated cells (Fig. 4B), but no dramatic change of the p53 half-life was observed (data not shown). These preliminary data suggest that estrogen can modulate p53 levels by a translational mechanism.

As described previously, p53 functions are activated in response to DNA damage. Different p53 target genes transcriptionally activated by wild-type p53 have been identified, including the inhibitor of cdks p21^{WAF1/CIP1} (6). To investigate biological consequences of the down-regulation of p53 levels in the presence of tamoxifen, we studied p53 and p21^{WAF1/CIP1} response after treatment with chemical and physical genotoxics used in breast cancer therapy.

Inhibition of p53 Response to DNA Damage by Tamoxifen. To compare the effects of estrogen and antiestrogen on p53 response, MCF-7 cells were cultured 4 days in either a 17β-estradiol- or a tamoxifen-containing medium prior to the
genotoxic treatment. In the presence of estrogen, CDDP (cisplatin) treatment, Adriamycin treatment, or ionizing radiation lead to an accumulation of p53 (Fig. 5, A and B). Similar results are obtained with MCF-7 cells cultured in DMEM supplemented with phenol red and 5% FCS. The increase of p53 levels parallels an intranuclear accumulation of the protein and an increase of p53-transcriptional activity (see Fig. 5C). After tamoxifen treatment, the p53 response to DNA damage is strongly inhibited. This inhibition occurs as soon as 48 h after tamoxifen treatment and is visualized by an absence of p53 accumulation and a loss of the p53-dependent induction of p21WAF1/CIP1 (Fig. 5B). To confirm the inactivation of the p53 transcriptional activity in the absence of estrogen, we transfected the plasmid pRGCΔFosLacZ in MCF-7 cells. The reporter plasmid pRGCΔFosLacZ contains two copies of the RGC-p53 binding fragment upstream of a minimal fos promoter that controls the ß-galactosidase gene expression. In the presence of wild-type p53, there is a specific stimulation of ß-galactosidase activity that can be revealed by an enzymatic reaction. After transfection, cells were cultured 48 h in either a 17ß-estradiol- or a tamoxifen-containing medium. Then, Adriamycin was added to the medium for 18 h. As expected, Adriamycin treatment leads to an increase of p53 transcriptional activity in a 17ß-estradiol-containing medium. This increase is inhibited in the presence of tamoxifen (Fig. 5C). Furthermore, in the absence of genotoxic stress, basal p53-transcriptional activity decreases when cells are cultured for 72 h in a tamoxifen-containing medium (data not shown).

We show in this study that, at clinically relevant concentrations, antiestrogen treatment provokes a decrease of p53 protein levels by a posttranscriptional mechanism and a loss of wild-type p53 response to DNA damage. Similar results are obtained when cells are cultured in charcoal-treated serum. Estrogen is sufficient to restore normal p53 levels and its biological response to DNA-damaging agents. This estrogen “rescue” effect is observed even in the presence of tamoxifen, suggesting strongly that estrogen modulates p53 via the ER pathway. This hypothesis is further supported by the absence of modulation of p53 levels in ER(-) epithelial cells.

The biological mechanisms responsible for this effect remain unknown. The p53 modulation occurs after alterations in the degree of RB phosphorylation. Thus, p53 does not appear to be involved in the cell cycle arrest induced by tamoxifen, and its down-regulation is probably a secondary effect of the absence of estrogenic activity. Nevertheless, the absence of p53 modulation after the G1 arrest induced by transforming growth factor ß1 or dexamethasone (data not shown) suggests that the down-regu-
expression of genes involved in the apoptotic pathway, such as p53- and bcl-2-related genes, may modulate the sensitivity of cells to cancer therapy. It was first reported that the inactivation of p53 function in cells programmed for apoptosis, especially hematopoietic and lymphoid cells, results in a decrease of sensitivity to a wide variety of DNA-damaging agents (15–18). In contrast, in cells not programmed inherently for apoptosis, it was shown recently that p53 disruption sensitizes cells to multiple chemotherapeutic agents either by preventing the G1 arrest or by inhibiting the nucleotide excision repair pathway (19). These data were confirmed in MCF-7 cells in which disruption of p53 by constitutive expression of the human papillomavirus type 16 E6 gene or a dominant negative mutant p53 sensitized cells to cisplatin and pentoxifylline (20). On the basis of these observations, the modulation of both bcl-2 (Refs. 21 and 22) and p53 activity could provide a mechanistic explanation for the difference in sensitivity to chemotherapeutic or pre- and postmenopausal women (23, 24) and to the beneficial effect of chemoendocrine therapy in breast cancers (1). Future confirmation of this hypothesis and advances in our knowledge of molecular mechanisms involved in drug resistance of breast cancers may allow us to better select breast cancer therapy (chemotherapy and/or hormonotherapy) by studying specific genetic disorders of an individual tumor.

At last, evidence has emerged that women treated with tamoxifen have an increased risk of developing endometrial cancer, with relative risk ranging as high as 7.5 (25). The recent observation that tamoxifen displays no genotoxicity in human endometrium (26) suggests an alternative and non-genotoxic mechanism of initiation of human cancer by tamoxifen. On the basis of our data, long-term treatment with tamoxifen could inactivate the tumor suppression function of p53 in endometrial cells. Loss of the p53 response to DNA damage may render cells more susceptible to accumulation of multiple genetic lesions associated with carcinogenesis and tumor progression. This hypothesis is currently under investigation.

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References


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