Estradiol Induces Functional Inactivation of p53 by Intracellular Redistribution

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Abstract

Estradiol treatment of MCF-7 cells grown in serum-free medium induced a modification of the intracellular distribution of p53 protein. Western blot analysis and immunofluorescence staining showed that p53 was localized in the nucleus of untreated cell and that after 48 h of hormone treatment, it was mostly localized in the cytoplasm. This effect was blocked by the antiestrogen CI182,780. Intracellular redistribution of p53 was correlated to a reduced expression of the WAF1/CIP1 gene product and to the presence of degradation fragments of p53 in the cytosol. Estradiol treatment prevented the growth inhibition induced by oligonucleotide transfection, simulating DNA damage. This observation indicated that the wild-type p53 gene product present in the MCF-7 cell could be inactivated by estradiol through nuclear exclusion to permit the cyclin-dependent phosphorylation events leading to the G1-S transition. In addition, the estradiol-induced inactivation of p53 could be involved in the tumorigenesis of estrogen-dependent neoplasms.

Introduction

Different types of stress (e.g., hypoxia or DNA damage) induce a cellular response leading to growth arrest or to apoptosis through a functional activation of p53 gene product (1). DNA-dependent protein kinase (2) or the gene product mutated in ataxia telangiectasia, ATM (3, 4), are involved in alternative pathways leading to p53 activation. Activated p53 protein acts as a transcription factor, increasing the expression of genes such as p21 (5), GADD45 (6), or bax (7), whose products inhibit cell cycle progression or induce apoptosis. Activation of p53 protein is limited by a short feedback loop involving the MDM2 gene product (8). p53 stimulates transcription of the MDM2 gene (9), and MDM2 protein binds to activated p53 protein (10). This interaction inhibits p53 transcriptional activity and promotes its export to the cytoplasm for proteasome-mediated degradation (11). In many human and animal tumors, the p53 gene is functionally inactivated by deletion or point mutations, participating, by this mechanism, in the process of neoplastic transformation as an oncogene (12).

The MCF-7 breast cancer cell line responds to stimulation by a physiological concentration of estradiol with an increase in proliferation rate (13). This cell line contains a wild-type p53 gene (14) whose product is mostly localized in the nucleus during the G1 phase and moves to the cytoplasm after the G1-S transition (15). In this report, we present evidence that, in MCF-7 cells, estradiol was able to induce functional inactivation of p53 protein by intracellular redistribution.

Materials and Methods

Cell Culture. MCF-7 breast cancer cells were grown in 75-cm² flasks in DMEM, supplemented with 5% FCS, 100 units/ml penicillin G, 100 units/ml streptomycin, 50 µg/ml gentamicin, and 2 mM l-glutamine in a 5-5% CO2 atmosphere. For induction experiments, 30% confluent cells were grown in phenol red-free serum-free medium for 2 days (cells were transferred to fresh medium twice a day) and then 10 µg/ml insulin and 10 nM estradiol were added in the absence or the presence of 1 µM CI182,780. All tissue culture media, sera, and reagents were from Life Technologies, INC., Grand Island, NY.

For oligonucleotide transfection, cells were grown in 6-cm plates. Transfection was performed with 8 µg/ml of 17-mer double-stranded oligonucleotides with a 5’-end overhang (GATC) premixed with 30 µl of DOTAP1 liposomal transfection reagent (1 mg/ml; Boehringer Mannheim, Mannheim, Germany), according to manufacturer’s instructions. In mock transfections, only DOTAP liposomal transfection reagent was added. A rough estimate of the cell number per well was obtained by staining the cells with crystal violet and measuring the absorbance on a spectrophotometer (16).

Colony Formation Assay in Methylicholesterol. MCF-7 cells were cultured in 50% DMEM and 50% RPMI, supplemented with 20% FCS, and treated with dextran-coated charcoal and 0.8% methylcellulose (MethoCult H4100; Terry Fox laboratories, Vancouver BC, Canada) in 6-well plates (5 x 10⁴ cells/well). Colonies were analyzed after 7 days. Photographs were taken at ×100 magnification.

Cytosol and Nuclear Extract Preparation. Cells were rinsed with cold PBS containing 1 mM EDTA, and harvested with 2 ml EDTA. The cell pellet was washed twice with ice-cold PBS, once with ice-cold buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.5 mM DTT, 1.5 mM MgCl₂], resuspended in three volumes of buffer A, and homogenized in a Dounce homogenizer (10 strokes with pestle B). The homogenate was centrifuged at 3300 x g for 30 min at 4°C to obtain a nuclear pellet. The supernatant was collected and centrifuged for 1 h at 100,000 x g at 4°C; the supernatant referred to as cytosol. The nuclear pellet was resuspended with buffer B [20 mM HEPES (pH 7.9), 450 mM NaCl, 0.2 mM DTT, 1.5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 25% (v/v) glycerol], homogenized (two to five strokes in a Dounce homogenizer, pestle B), incubated for 30 min with gentle shaking, and centrifuged for 1 h at 25,000 x g at 4°C. The supernatant, referred to as nuclear extract, was clarified and aliquoted. Complete protease inhibitor cocktail TM was added to all buffers (1 tablet/50 ml; Boehringer Mannheim).

Electrophoresis and Western Blot Analysis. SDS PAGE was performed in reducing conditions in 10 or 11% acrylamide gels (acylamide/bisacylamide ratio, 40/1, w/w). Twenty-five µg of protein form cytosol or nuclear extract were applied to each lane. After Western blot analysis, proteins were electrophoretically transferred to a 0.45 µm nitrocellulose sheet. Membranes were then blocked by 5% nonfat milk for 1 h in TBST buffer [20 mM Tris-HCl (pH 7.5), 135 mM NaCl, and 0.05% Tween 20]. After repeated washes with TBST buffer, the membranes were incubated with the primary antibodies for 1 h at room temperature in the same buffer. Proteins were detected using mouse monoclonal antibodies Ab-6 (1 µg/ml; Calbiochem-Oncogene Research Products, Cambridge, MA) or BP53-12 (1 µg/ml; Sigma Immunochemicals, St. Louis, MO) to p53, or mouse monoclonal antibody to WAF1 (Calbiochem-Oncogene Research Products). At the end of the incubation, the membrane was washed once for 15 min and three times for 5 min with TBST buffer. Antibody reactions were revealed by incubation for 1 h, at room temperature with enzyme-coupled antimmunoglobulin IgG (1:10,000 dilution; Amersham, Buckinghamshire, England). This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate; TBST, Tris-buffered saline-Tween 20; EMSA, electrophoretic mobility shift assay.

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RESULTS

Estradiol produced an increase in the cell proliferation rate of MCF-7 cells, either serum-starved or grown in the presence of charcoal-stripped serum. We tested our cell line in a soft-agar colony assay (Fig. 1). A 7-day treatment of cells grown in serum-free medium with a physiological concentration of estradiol induced a 2–3-fold increase in colony number and size. Simultaneous treatment with the pure antagonist ICI182,780 prevented this effect, thus confirming that the mitogenic effect of the steroid was mediated by estrogen receptor.

To investigate whether p53 protein was affected by hormone action, Western blot analysis of cytosol or nuclear extract was performed on those cells. Analysis with antibodies to p53 protein showed that the protein was present in both compartments of hormone-deprived cells and that a 48-h estradiol treatment in the absence of serum produced a dramatic decrease in the p53 signal in the nuclear extract. This effect was complemented by a signal increase in the cytosol. The simultaneous presence of an excess of ICI182,780 also produced a minimal increase of p53 signal in the cytosol, probably as a consequence of the incomplete antagonist effect at the concentration used. However, it preserved or somewhat increased the nuclear signal (Fig. 2A). This result suggested that estradiol induced a redistribution of p53 in the MCF-7 cells, and it was confirmed by immunofluorescence staining of the cells (Fig. 3). In serum-starved cells, antibodies to p53 protein revealed a prevalent nuclear distribution, whereas the fluorescence was concentrated in the cytoplasm of cells treated with estradiol for 48 h (Fig. 3). Simultaneous treatment with the antiestrogen ICI182,780 preserved the prevalent nuclear staining in most cells. This effect was specific for estradiol, because insulin, another mitogen for MCF-7 cell, was unable to induce it.

Estradiol treatment of cells also modified the electrophoretic pattern of p53 immunoreactive peptides. As evident in Fig. 2B, Lane b, in a number of experiments, the p53 immunoreactivity in the cytosol of estradiol-treated cells was shared by more than one band of variable mass, but smaller than 53 kDa. Although this result was not constant, it was repeated several times in conditions of strict proteolysis control during cell disruption and fractionation (see “Materials and Methods”). This evidence suggested that the observed increase in p53 protein in the cytosol after estradiol treatment was associated with its proteolytic deg-
Fig. 2. Western blot analysis of MCF-7 cell extracts. A, Western blot analysis with monoclonal antibody Ab-6 to p53 protein (53) in cytosol or nuclear extract, as indicated at the bottom of the panels (25 μg protein/lane), from MCF-7 cells treated with vehicle alone (Lane a), 10 nM estradiol (Lane b), or 10 nM estradiol and 1 μM ICI182,780 (Lane c) for 48 h, as indicated in “Materials and Methods.” SDS-PAGE was performed on an 11% polyacrylamide gel. B, Western blot analysis with monoclonal antibody Ab-6 to p53 protein (53, Lanes a and b) or with mouse IgG (Lanes c and d) of cytosol (25 μg protein/lane) from MCF-7 cells treated with vehicle alone (Lanes a and c) or 10 nM estradiol (Lanes b and d) for 48 h, as indicated in “Materials and Methods.” SDS-PAGE was performed on a 10% polyacrylamide gel. C, Western blot analysis with monoclonal antibody Ab-6 to p21-WAF-1 protein (21) of nuclear extract (25 μg protein/lane), from MCF-7 cells treated with vehicle alone (Lane a); 10 nM estradiol (Lane b), or 10 nM estradiol and 1 μM ICI182,780 (Lane c) for 48 h, as indicated in “Materials and Methods.” SDS-PAGE was performed on an 11% polyacrylamide gel.

Discussion

MCF-7 is a breast cancer cell line extensively studied for the expression of a functional estrogen receptor (13). Growth of these cells is stimulated by estradiol and by other factors such as insulin. The mechanism underlying the mitogenic effect of estradiol has not been elucidated definitively. The hypothesis that the mitogenic effect was mediated solely by hormone-regulated autocrine growth factor secretion has been contradicted by the evidence that estrogens can directly promote G1 progression through direct activation of cell cycle genes or through the ras-mitogen-activated protein kinase pathway. This evidence suggests that, as the result of hormone-receptor interaction, more than one intracellular pathway becomes activated or inactivated for the proliferating response. Inactivation of p53 negative control of the G1-S transition by estradiol could greatly contribute to the hormonal control of cell proliferation. Expression of the mdm2 gene correlates with the estrogen receptor status of many human breast carcinoma cell lines, and mdm2 mRNA is accumulated after estradiol treatment of MCF-7 cell line (18). The mdm2 gene product inhibits p53 function by direct binding, hindering the transactivation domain and targeting p53 protein for degradation. mdm2 protein contains the nuclear export signal for the complex that is eventually degraded by the proteasome. It is conceivable that the mdm2 gene product mediated the observed effect, although p53 protein has a leucine-rich nuclear export signal necessary and sufficient for nuclear export (21). The biochemical and morphological evidences presented in this report suggest that estradiol was able to induce a redistribution of p53 from the nucleus to the cytoplasm. This was correlated to a reduction of p53 activity, as evident from the reduced expression of p21 in the estrogen-treated cells and the presence of immunoreactive fragments of p53 protein in the cytosol. The estradiol effect was produced in cells grown in serum-free medium, and it was mediated...
by estrogen receptor, because the estrogen antagonistICI182,780 prevented it.

Nuclear exclusion is one of the mechanisms of p53 inactivation in breast cancer, and cytoplasm staining for p53 of neoplastic cells strongly correlated with the presence of a wild-type, intact p53 protein by sequence analysis (22). In addition, cytoplasm sequestration of p53 is visible in normal lactating breast tissue (22). Our observations, therefore, strengthen the hypothesis that p53 inactivation by nuclear exclusion is a necessary step for estrogen-induced cell proliferation in the presence of an active, wild-type p53 gene. This p53 inactivation has a permissive role for the observed induction of cyclin-dependent kinase activity and the consequent phosphorylation of retinoblastoma protein induced by estradiol and inhibited by coadministration of ICI182,780 in the MCF-7 cell line (23). Apparently contradictory evidence that in the T47D breast cancer cell line estradiol is able to increase the expression of p53 protein (24) further confirms our hypothesis that nuclear exclusion is a nonmutational mechanism for abrogating the inhibitory function of wild-type p53 protein. This cell line, in fact, expresses an inactive p53 protein harboring a point mutation at codon 194 (14) and responds to estradiol treatment with increased proliferation and retinoblastoma protein phosphorylation despite the induced expression of p53 protein (25).

These results, indicating that estradiol treatment prevented the p53-mediated growth arrest induced by the presence of intracellular DNA fragments, have allowed further speculation. Through inactivation of the tumor suppressor function of p53, estradiol could play an additional role in the multistep process of tumorogenesis of target tissues in which the p53 gene is not altered by genetic mutations. Absence of an active p53 protein by nuclear exclusion could, in fact, contribute to the genetic instability of target cells in which estradiol is able to induce a proliferative response. A hormone-independent phenotype usually emerges during the process of tumor progression, both in human breast cancer and in mouse mammary tumor models, despite the presence of a hormonal environment conferring a growth advantage to the hormone-dependent cells.

References


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