Sensitization of MCF-7 Breast Cancer Cells to the Apoptotic Effect of Estradiol

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A new substrain of hormone-resistant MCF-7/T breast cancer cells was selected after long-term culturing of estrogen-dependent MCF-7 cells in the presence of tamoxifen. These cells were resistant to the growth-stimulating and cytostatic effects of estradiol and tamoxifen, respectively. MCF-7/T cells gained paradoxical sensitivity to the apoptotic effect of estradiol. Estradiol stimulated p53 expression and decreased DNA-binding activity of NF-κB. Our findings provide indirect evidence that these proteins are involved in the regulation of estrogen-induced apoptosis. These results indicate that tamoxifen-resistant breast cancer cells can be sensitized to the apoptotic effect of estradiol. The data form a basis for the development of new methods of endocrine therapy for breast cancer patients.

Key Words: *breast cancer; MCF-7; apoptosis; hormonal resistance*

Antiestrogen drug tamoxifen was used for adjuvant therapy of receptor-positive breast cancer (BC) for more than 30 years [3,4]. It should be emphasized that hormone-independent forms of BC are resistant to antiestrogens *de novo* or gain hormonal resistance during treatment with tamoxifen [3,4]. The formation of a hormone-resistant phenotype is not necessarily accompanied by the disappearance of estrogen receptors (ER) on BC cells. Estrogen resistance and estrogen-independent growth of cells are associated with modulation of intracellular signal pathways, which includes activation of receptor tyrosine kinases, dysregulation of cell cycle proteins, and change in functional activity of ER [1,3].

Much recent attention is focused on sensitization of hormone-resistant BC cells to the apoptotic effect of estradiol [8,14,15]. Long-term culturing of BC cells with antiestrogen drugs *in vitro* (tamoxifen and raloxifene) or in a steroid hormone-free medium sometimes leads to the development of paradoxical sensitivity of these cells to the apoptotic effect of estradiol [8,15]. Previous studies showed that estrogen-induced apoptosis involves the FAS-signal pathway and activation of caspase-7 and caspase-9 [15]. However, the molecular mechanism for sensitization of tamoxifen-resistant BC cells to the apoptotic effect of estrogens remains unknown.

Here we studied sensitization of MCF-7 BC cells to the apoptotic effect of estradiol after long-term culturing with tamoxifen and possible signal pathways involved in estrogen-induced apoptosis.

MATERIALS AND METHODS

MCF-7 human BC cells were cultured in DMEM containing 5% fetal bovine serum (Gibco) and gentamicin (50 U/ml) at 37°C and 5% CO_2. The hormone-resistant substrain was obtained after long-term culturing (for more than 2 months) of parent MCF-7 cells in a standard medium with 10^{-6} M tamoxifen followed by culturing of these cells in phenol red-free DMEM containing 5% steroid-free fetal serum. Steroid-free serum was obtained from fetal

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serum by routine treatment with dextran-coated charcoal (Sigma) [13]. The cell growth rate was estimated in the MTT test. This test is based on accumulation of a fluorescent MTT reagent by viable cells [7].

ER in whole cells were studied by a modified radioligand method with ethanol extraction [10].

For immunoblotting assay the cells (80% confluence) were transferred from Petri dishes to 1 ml phosphate buffered saline. The cell extracts were obtained from these samples and layered on nitrocellulose filters (Amersham BS) [2]. Experiments were performed with antibodies against p53 (OP29, Oncogene Research Products) and ER (E-1396, Sigma-Aldrich). The filters were treated with 5% defatted milk (Nestle) and incubated with primary antibodies at room temperature for 3 h to prevent nonspecific sorption. These filters were washed and incubated with peroxidase-conjugated secondary antibodies (Amersham BS) for 1.5 h. The complexes were developed with a chemiluminescent reagent (Amersham BS).

Specific DNA-binding activity of transcription factor NF-κB was determined by electrophoretic mobility shift assay [5].

The degree of apoptosis in cells was estimated by the method of flow cytofluorometry after propidium iodide staining (Sigma). The samples were analyzed in a FACSCalibur cytometer (Becton Dickinson). Data processing involved WinMDI 2.9 software. The percentage of apoptotic cells was evaluated as the pre-G1 peak in the DNA histogram [6].

The results were analyzed by means of Statistica 6.0 and Origin 6.0 software.

RESULTS

The substrain of MCF-7/T cells exhibited a higher growth rate in steroid-free medium compared to the parent MCF-7 cells (Fig. 1, *a*). When studying the effects of 17β-estradiol (E2) and tamoxifen on the cell growth rate we found that MCF-7/T cells had a lower sensitivity to the growth-stimulating and cytostatic effects of E2 (10^{-9} M) and tamoxifen (10^{-6} M),

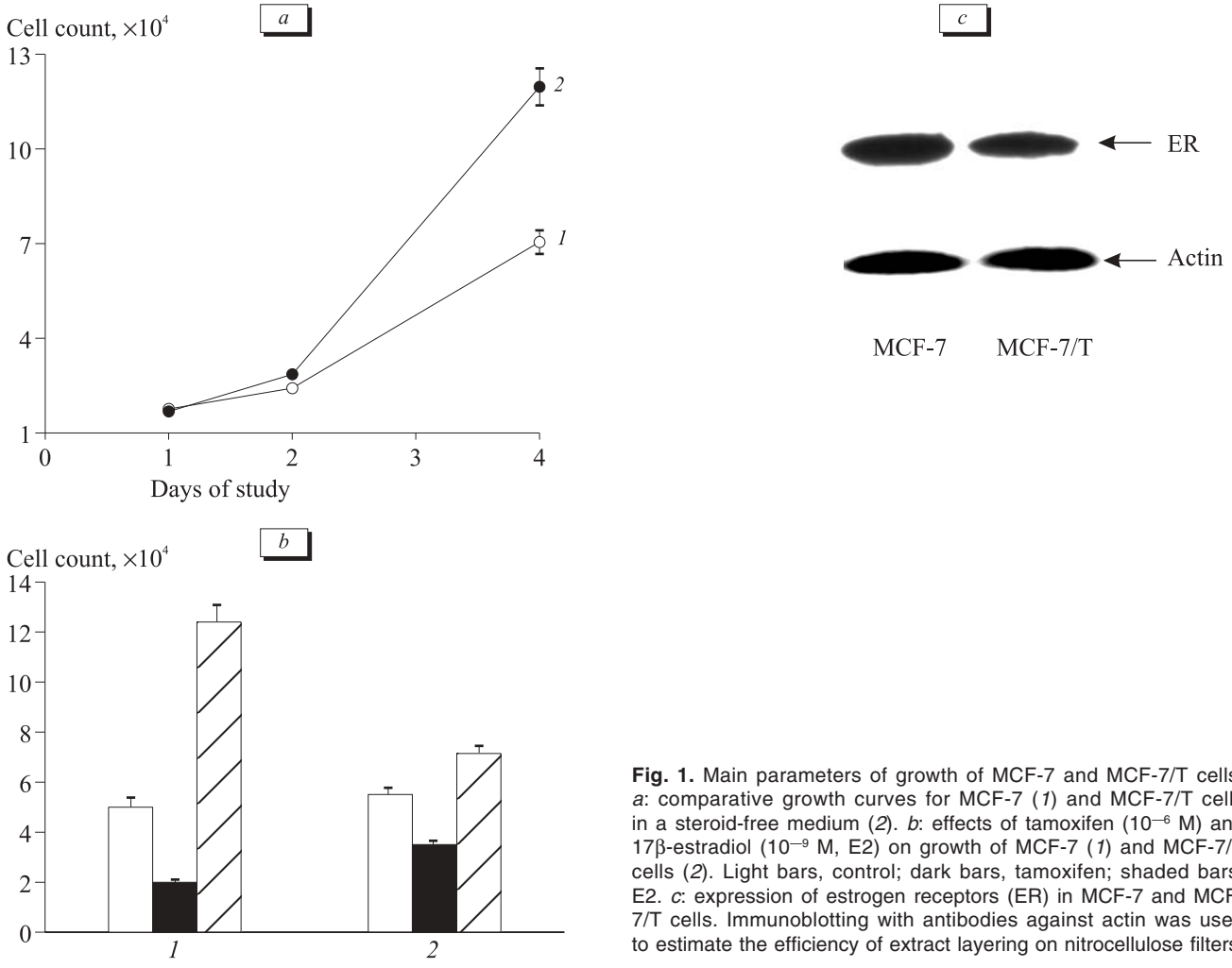

Fig. 1. Main parameters of growth of MCF-7 and MCF-7/T cells. *a*: comparative growth curves for MCF-7 (*1*) and MCF-7/T cells in a steroid-free medium (*2*). *b*: effects of tamoxifen (10^{-6} M) and 17β-estradiol (10^{-9} M, E2) on growth of MCF-7 (*1*) and MCF-7/T cells (*2*). Light bars, control; dark bars, tamoxifen; shaded bars, E2. *c*: expression of estrogen receptors (ER) in MCF-7 and MCF-7/T cells. Immunoblotting with antibodies against actin was used to estimate the efficiency of extract layering on nitrocellulose filters.

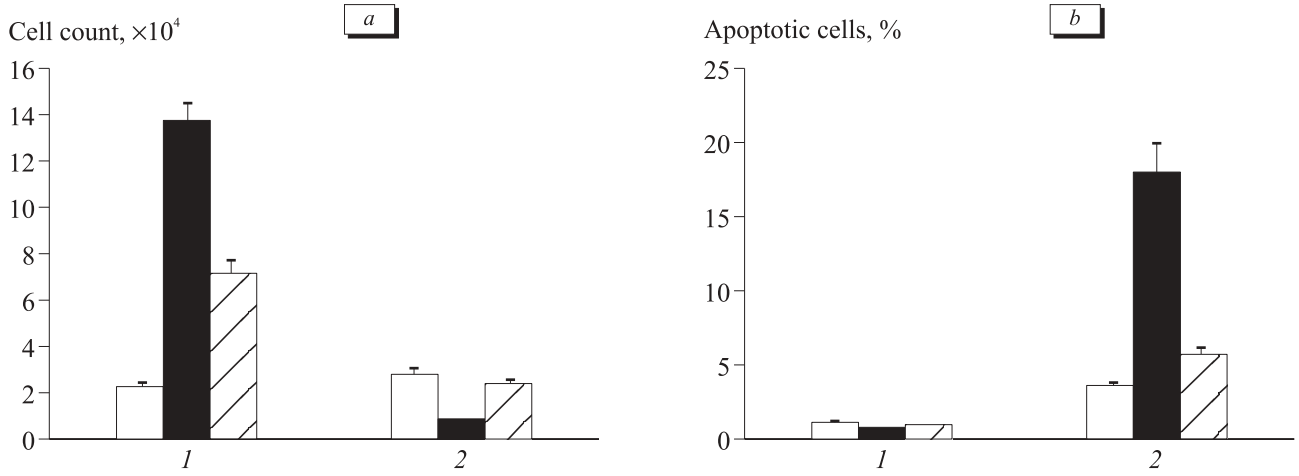

Fig. 2. Growth rats (*a*) and apoptosis (*b*) in MCF-7 (*1*) and MCF-7/T cells (*2*) after culturing with E2 and/or tamoxifen for 8 days. Light bars, control; dark bars, E2; shaded bars, tamoxifen and E2.

respectively, than parent MCF-7 cells (Fig. 1, *b*). Immunoblotting assay revealed a slight decrease in ER expression on hormone-resistant MCF-7/T cells compared to parent cells (Fig. 1, *c*). Similar changes were observed in the study of binding activity of ER by the standard radioligand method [10]. E2 binding to MCF-7/T cells was lower compared to MCF-7 cells. The mean number of binding sites was $(3.7\pm 0.4)\times10^5$ and $(6.1\pm0.5)\times10^5$, respectively.

The count of parent MCF-7 cells sharply increased after 8-day culturing with 10^{-9} M E2 (by 6 times). The stimulatory effect of E2 decreased after addition of 10^{-6} M tamoxifen to the medium (Fig. 2, *a*). Long-term culturing of hormone-resistant MCF-7/T cells with E2 was followed by a sharp decrease in the cell growth rate. Addition of tamoxifen partially prevented these changes (Fig. 2, *a*). Flow cytofluorometry showed that the inhibition of MCF-7/T cell growth under the influence of E2 is associated with activation of apoptosis, while parent MCF-7 cells were practically insensitive to the apoptotic effect of E2 (Fig. 2, *b*).

The molecular mechanism of estrogen-induced apoptosis was determined by studying the effect of E2 on tumor suppressor p53 and transcription factor NF-κB. Tumor suppressor p53 is one of the main transmitters of the apoptotic signal. Transcription factor NF-κB possesses high proliferative and anti-apoptotic activity [12]. E2 significantly increased p53 expression in cells (Fig. 3, *a*) and inhibited

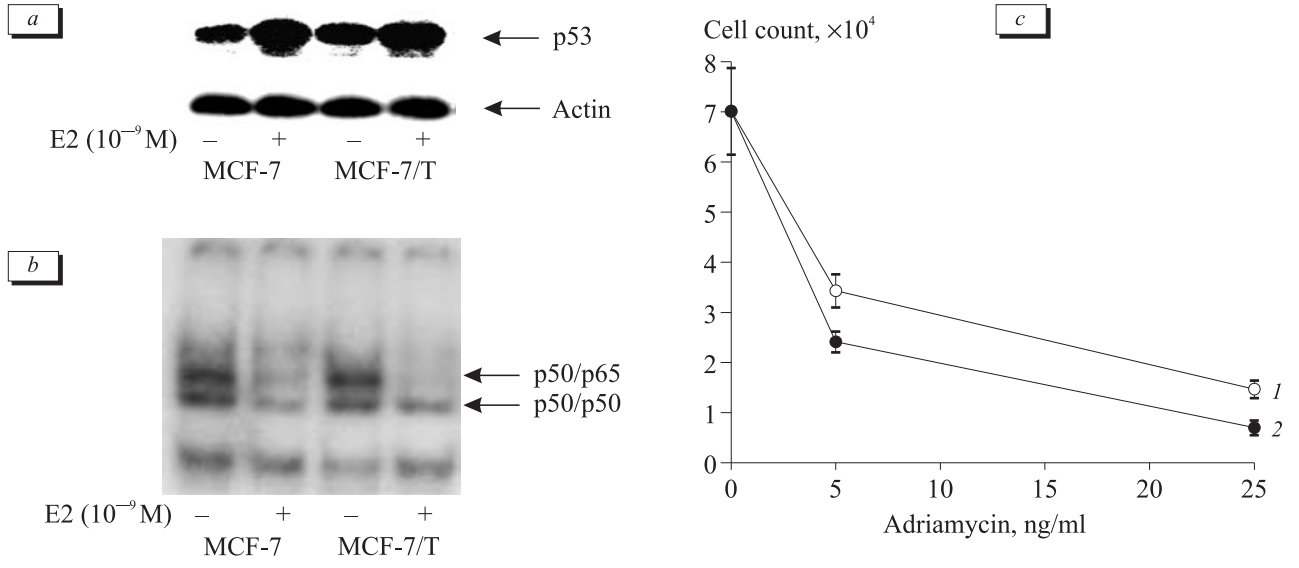

Fig. 3. Effect of E2 on expression of tumor suppressor p53 (*a*) and DNA-binding activity of homodimers (p50/p50) and heterodimers (p50/p65) of NF-κB (*b*) in MCF-7 (*1*) and MCF-7/T cells (*2*). Influence of adriamycin on cell growth (*c*).

binding of p50/p50 NF-κB homodimer to specific DNA sites (Fig. 3, *b*). These changes were observed in parent cells and resistant MCF-7/T cells.

Only MCF-7/T cells were sensitive to E2-induced apoptosis, therefore we hypothesized that resistant cells are characterized by an imbalance between prooxidant and antioxidant signal pathways, which determines high sensitivity of these cells to apoptosis. To test this hypothesis, we compared the sensitivity of MCF-7 and MCF-7/T cells to a classic antiapoptotic agent adriamycin. Resistant cells were highly sensitive to adriamycin (Fig. 3, *c*). These data support the hypothesis on higher apoptotic activity of MCF-7/T cells compared to parent cells. The type of signal pathways involved in E2-induced apoptosis, as well as their role in sensitization of BC cells to the apoptotic effect of E2, should be evaluated in further experiments.

A new hormone-resistant substrain of BC cells (MCF-7/T cells) was selected and characterized in the present work. These cells are resistant to the growth-stimulating and cytostatic effects of estradiol and tamoxifen, respectively. We revealed sensitization of hormone-resistant MCF-7/T cells to estrogen-induced apoptosis. E2 regulates p53 expression and DNA-binding activity of NF-κB in parent and resistant cells. Our findings provide indirect evidence that these molecules are involved in the regulation of estrogen-induced apoptosis. These results indicate that tamoxifen-resistant BC cells can be sensitized to the apoptotic effect of estradiol (at least, under *in vitro* conditions). Some clinical studies demonstrated a positive effect of additive therapy with estrogens in patients with hormone-resistant BC tumors [9,11]. Our results and published data form a basis to develop new methods of endocrine therapy for BC patients.

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