Oestrogen treatment enhances the sensitivity of hormone-resistant breast cancer cells to doxorubicin

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Synopsis

Recently, it was shown that the resistance of breast cancer cells to growth-stimulating oestrogen action may be accompanied by the paradoxical tumour sensitization to oestrogen apoptotic action. In the present paper, we studied the influence of oestrogens on the sensitivity of resistant breast tumours to cytostatic drugs, and to evaluate the role of NF-κB (nuclear factor κB) signalling in the regulation of the apoptotic response of the resistant cells. The experiments were carried out on the oestrogen-dependent MCF-7 breast cancer cells and resistant MCF-7/LS subline generated through long-term cultivation of the parental cells in the absence of oestrogen. The cell treatment with the combination of oestradiol and Dox (doxorubicin) was found to enhance the apoptotic action of Dox in MCF-7/LS cells but not in the parent cells. MCF-7/LS cells were characterized by the increased level of ROS (reactive oxygen species) and decreased NF-κB activity. Oestradiol in combination with Dox leads to significant NF-κB stimulation and its accumulation in the nucleus of MCF-7/LS cells. The knockdown of NF-κB with siRNA (small interfering RNA) increased the apoptotic response of the MCF-7/LS cells to both Dox and oestradiol demonstrating the important role of NF-κB in the protection of the MCF-7/LS cells against apoptosis. In general, the results obtained show that: (i) oestradiol enhances the apoptotic action of Dox in the resistant breast cancer cells; and (ii) suppression of NF-κB signalling amplifies the apoptotic response of the resistant cells to both oestrogen and Dox, demonstrating that NF-κB may serve as a potential target in the therapy of the resistant breast cancer.

Key words: apoptosis, breast cancer, MCF-7 cell, nuclear factor κB (NF-κB), oestrogen receptor (ER), oestrogen.

INTRODUCTION

Studying the mechanism of hormonal resistance of cancer cells is one of the promising directions of molecular oncology. It was shown that the acquired resistance to hormonal therapy as well as tumour ability to hormone-independent growth may be caused by the hyperactivation of hormone-independent growth signalling followed by disorders in the ER (oestrogen receptor) machinery [1,2]. Particularly, the efficiency of endocrine therapy of breast cancer is limited by the development of hormone-independent tumours that are resistant to anti-oestrogens initially or acquire resistance de novo during anti-oestrogen therapy [1–3]. Recently, the phenomenon of the paradoxical sensitization of anti-oestrogen-resistant breast tumours to oestrogen-induced apoptosis was described [4]. Oestradiol’s ability to induce apoptosis has been reported for the breast cancer cells grown in the presence of anti-oestrogens and/or in a steroid-depleted medium for a long time [4,5]. These findings correlate with the clinical and experimental data demonstrating that oestrogen treatment of resistant breast tumours can cause tumour regression in vivo [6].

Previously, we have shown that long-term growth of oestrogen-dependent MCF-7 breast cancer cells in a steroid-free medium results in the formation of the cell subline characterized by resistance to growth stimulatory oestradiol action and hypersensitivity to oestrogen-induced apoptosis [5]. We found that oestrogen treatment results in the suppression of NF-κB (nuclear factor κB) activity, and demonstrated the important role of the latter in the cells’ sensitization to oestrogen apoptosis [7].

Abbreviations used: Dox, doxorubicin; E2, 17β-oestradiol; ER, oestrogen receptor; ERE, oestrogen-responsive element; H2DCFDA, dichlorodihydrofluorescein diacetate; NF-κB, nuclear factor κB; PARP poly (ADP-ribose) polymerase; PI, propidium iodide; ROS, reactive oxygen species; siRNA, small interfering RNA.

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In the present paper, we studied the influence of oestrogens on the sensitivity of the resistant breast tumours to cytostatic drugs, and evaluated the role of NF-κB signalling in the regulation of the survival of the resistant cells.

**MATERIALS AND METHODS**

**Cell culture**

The resistant breast cancer cell subline MCF-7/LS was developed by long-term (60 days) cultivation of the parental MCF-7 cells in Phenol Red-free DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 5% (v/v) steroid-free fetal serum at 37°C and 5% CO₂. The steroid-free serum was prepared by the treatment of fetal serum with dextran-coated charcoal (Sigma–Aldrich), according to the routine method described in [8]. The cell growth was evaluated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide]-test based on the accumulation by living cells of an MTT reagent [9].

**Transient transfection and measurement of reporter gene activity**

To determine the transcriptional activity of NF-κB and the ER, the cells were transfected with the plasmids containing the luciferase reporter gene controlled by the promoter with NF-κB-responsive elements or EREs (oestrogen-responsive elements) respectively. The plasmids used in this work were kindly provided by Dr George Reid (European Molecular Biology Laboratory, Heidelberg, Germany) [10] and Dr Alexander Gasparian (Cleveland Biolabs, Buffalo, NY, U.S.A.) [11]. The transfection was carried out for 4 h at 37°C using Lipofectamine™ Reagent (Life Technologies BRL). To control the efficiency and potential toxicity of the transfection, the cells were transfected with the β-galactosidase plasmid. All subsequent experiments were performed during 48 h after the transfection. The luciferase activity was measured according to a standard protocol (Promega) using a Turner Biosystems 20/20n luminometer. The luciferase activity was calculated in arbitrary units evaluated as the ratio of the luciferase activity to the galactosidase activity.

**siRNA (small interfering RNA) oligonucleotides**

Scrambled non-specific siRNA (sense 5′-CAGUCCGCUU-UGCGACUGGdTdT-3’) and p65 NF-κB-specific siRNA (sense 5′-GCCCUAUCCCUCUUGCAdTdT-3’) along with their corresponding anti-sense RNA oligonucleotides were purchased from Syntol. These RNAs were dissolved in annealing buffer [10 mM Tris/Cl (pH 7.5), 50 mM NaCl and 1 mM EDTA] as 10 μM solutions and annealed at room temperature (25°C) following heating to 95°C.

Transfection of the RNA oligonucleotides was performed using Lipofectamine™ Reagent to result in a final RNA
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Figure 2 NF-κB signalling and ROS level in MCF-7 and MCF-7/LS cells

(a) ROS level in MCF-7 and MCF-7/LS cells. The cells were treated with $5 \times 10^{-7}$ M Dox and/or $10^{-9}$ M E2 for 3 days, the ROS level was assessed using the standard protocol as described in the Materials and methods section. Data represent means±S.D. of at least three independent experiments. *$P < 0.05$ compared with MCF-7 cells; #$P < 0.05$ compared with control (Co). (b) The transcriptional activity of NF-κB. The MCF-7 and MCF-7/LS cells were co-transfected with the plasmid containing the luciferase reporter gene under the NF-κB-sensitive promoter and β-galactosidase plasmid. At 24 h after transfection the cells were treated with or without $10^{-9}$ M E2 and/or $5 \times 10^{-7}$ M Dox for 24 h with subsequent determination of the reporter luciferase activity. Data represent means±S.D. of at least three independent experiments. *$P < 0.05$ compared with control (Co); #$P < 0.05$ compared with Dox+E2-treated MCF-7 cells. (c) p65 NF-κB DNA binding. The cells were treated with $10^{-9}$ M E2 and/or $5 \times 10^{-7}$ M Dox for 2 days, and subjected to p65 NF-κB DNA-binding assay. p65 NF-κB DNA-binding was determined using a standard enzyme immunoassay kit (NF-κB, p65 ELISA). Data represent means±S.D. of at least three independent experiments. *$P < 0.05$ compared with control (Co); #$P < 0.05$ compared with Dox+E2-treated MCF-7 cells.

concentration of 50–100 nM. The cells were treated with either Dox (doxorubicin; $5 \times 10^{-7}$ M) or E2 (17β-oestradiol; $10^{-9}$ M) and lysed in the buffer for Western-blot analysis.

**Western-blot analysis, p65 NF-κB assay and measurement of ROS (reactive oxygen species) levels**

The cells were removed from the dishes with 1.2 ml of phosphate buffer, washed twice, and incubated for 10 min on ice in the modified lysis buffer containing 50 mM Tris/HCl (pH 7.4), 1 % SDS, 1 % Igepal CA-630, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and 1 mM PMSF; 1 μg/ml each aprotinin, leupeptin, pepstatin; 1 mM sodium orthovanadate and 1 mM NaF. Samples were sonicated four times for 5 s each at 30 % output and centrifuged for 5 min at 15000 g, and supernatants were then used as total cell extracts. Protein content was determined by the Bradford method. Cell lysates (70 μg protein) were separated in SDS/10 % PAGE under reducing conditions, transferred to a nitrocellulose membrane (Hybond-C extra; GE Healthcare) and processed according to the standard protocol. To prevent non-specific absorption, the filters were treated with 5 % (v/v) non-fat milk solution in TBS buffer [20 mM Tris/HCl (pH 7.5) and 500 mM NaCl] and then incubated with primary antibodies overnight at +4°C.

Primary antibodies to PARP [poly (ADP-ribose) polymerase] (BD Pharmingen™), ER (Sigma–Aldrich) and p65 NF-κB (Cell Signaling Technology) were used; antibody against β-actin (Cell Signaling Technology) was used to standardize loading. Appropriate IgGs (GE Healthcare) conjugated to horseradish peroxidase were used as secondary antibodies. Signals were detected using ECL® (enhanced chemiluminescence) reagent (GE Healthcare). p65 NF-κB DNA-binding was quantitatively determined using a standard NF-κB p65 ELISA (EKS-446; Stressgen Assay Designs) according to the manufacturer’s instructions, and expressed in relative units/mg of protein.

**ROS measurement**

Intracellular ROS was measured by flow cytometry following staining with H2DCFDA (dichlorodihydrofluorescein diacetate) according to the standard protocol [12]. Briefly, the cells were
stained with 5 μM H2DCFDA for 30 min at 37°C. Then the cells were harvested, washed thrice with PBS and resuspended in PBS. Fluorescence was then analysed using a FACSCalibur™ cytometer. In some experiments, cells were pre-treated with Dox or oestradiol before analysis of ROS generation.

Assessment of apoptosis

Apoptosis was determined by flow cytometry using staining with PI (propidium iodide) (Sigma–Aldrich). Cells were fixed in 70% cold ethanol, centrifuged and resuspended in 1 ml of solution containing PI (5 μg/ml), 0.1% sodium citrate, 0.1% Triton X-100, and then incubated for 15 min in the dark. Then the samples were analysed in the FACSCalibur™ cytometer. The further processing of the data was performed with the WinMDI 2.9 software program (Joseph Trotter, La Jolla, San Diego, CA, U.S.A.). The percentage of apoptotic cells was determined as a pre-G1 peak in the DNA histogram.

Statistical analysis was performed using the Origin 5 software program. The criterion for statistical significance was *P < 0.05.

RESULTS

The experiments were performed on the oestrogen-dependent MCF-7 breast cancer cells and oestrogen-independent MCF-7/LS subline generated through a 2-month cultivation of the parental cells in a steroid-free medium. We have shown that MCF-7/LS cells were characterized with the active growth in a steroid-free medium, low sensitivity to growth-stimulating oestrogen action, and at the same time the hypersensitivity to oestrogen-induced apoptosis [13]. The apoptotic response of MCF-7/LS cells to oestradiol was found to be developed after at least 8 days of oestrogen treatment [13].

The combined effect of oestradiol and Dox on the apoptosis in MCF-7 and MCF-7/LS cells

The efficiency of the apoptotic action of low dose of Dox, given alone or in combination with oestradiol was analysed. The data presented show no significant changes in the apoptosis level in MCF-7 cells treated with a low dose of Dox or oestradiol. At the same time, oestradiol in combination with Dox significantly enhanced the apoptosis in the resistant MCF-7/LS cells within 3 days after addition. Importantly, oestradiol alone did not induce the apoptosis in this time (Figure 1a).

The analysis of degradation of PARP, one of the commonly used apoptotic markers, showed a similar tendency: the combination of oestradiol and Dox induced the weak degradation in the parent MCF-7 cells and increased PARP degradation in the resistant MCF-7/LS cells (Figure 1b).

The measurement of ROS revealed the marked increase in ROS production in the resistant MCF-7/LS cells in comparison with MCF-7 cells. Dox treatment stimulated ROS production in both the cell lines, whereas oestradiol did not affect the level of ROS (Figure 2a), demonstrating that oestrogen action occurs independently of ROS generation.

Taken together, the results presented show the increased apoptotic potency in the resistant MCF-7/LS cells as well as the oestradiol’s ability to potentiate the apoptotic action of Dox in these cells, whereas the parent MCF-7 cells are insensitive to pro-apoptotic oestrogen action.

Influence of Dox and oestradiol on NF-κB transcriptional activity

Previously, we have described the decrease in the NF-κB activity in the MCF-7/LS subline in comparison with MCF-7 cells [7]. In present study, analysis of the influence of Dox and oestradiol on NF-κB activity showed that, given alone, Dox resulted in a weak increase in NF-κB activity, whereas oestradiol inhibited NF-κB in both the cell lines. Furthermore, in MCF-7 cells, the effect of the combination of Dox and oestradiol on NF-κB activity was not different from that induced by oestradiol alone. At the same time, in MCF-7/LS cells, Dox in combination with oestradiol led to significant NF-κB stimulation when compared with the cells treated with oestradiol alone (Figure 2b).

These data were confirmed in the following experiments based on the analysis of DNA-bound NF-κB. As shown in Figure 2(c), in contrast with parent MCF-7 cells, the treatment of MCF-7/LS cells with a combination of oestradiol and Dox results in the marked accumulation of the DNA-bound NF-κB in the nucleus. Interestingly, we did not find a significant difference in NF-κB...
levels with the combination of oestradiol and Dox; when compared with Dox alone, which seems to be caused by oestradiol’s ability to inhibit NF-κB [14].

To compare the efficiency of ER machinery in the MCF-7 and MCF-7/LS cells after Dox treatment, the transcriptional activity and expression of ER were analysed. For this purpose, the cells were transfected with the luciferase reporter plasmid containing the ERE, with subsequent oestradiol and Dox treatment. The results show that Dox did not significantly decrease the oestradiol-induced ER transcriptional activity or expression demonstrating the retention of ER machinery in the cells treated with Dox (Figures 3a and 3b). We conclude that NF-κB stimulation by the combination of Dox/oestradiol in MCF-7/LS cells is not connected with the alteration in ER machinery, but may reflect the substantial activation of an apoptotic response in these cells.

To further elaborate the role of NF-κB in the regulation of cell response to oestradiol and/or Dox, we suppressed intracellular NF-κB by the siRNA to the p65 subunit of NF-κB. As observed, siRNA in a final concentration of 100 nM was sufficient to cause approx. 2-fold decrease in NF-κB activity (Figure 4a) that correlated with the reduction of p65 expression (Figure 4b). The comparative analysis of the apoptotic response of the non-transfected cells and cells transfected with scrambled or NF-κB siRNAs showed a slight increase in spontaneous apoptosis after scrambled siRNA and a marked increase in Dox/oestradiol-induced apoptosis in the cells transfected with siRNA NF-κB (Figure 4c). Taken together, these data give evidence of NF-κB involvement in the protection of the MCF-7/LS cells against apoptotic action of oestradiol or Dox, and demonstrate the ability of NF-κB inhibitors to sensitize the resistant cells to apoptotic action of these drugs.

**DISCUSSION**

Paradoxical induction of apoptosis by oestrogens has been reported for oestrogen-deprived breast cancer cells and/or breast tumours after anti-oestrogen treatment. Several observations revealed that the long-term oestrogen deprivation as well as the retained high level of ERs may play a causal role in the cell sensitization to oestradiol-induced apoptosis [5,15,16].

It should be noted that the ability of sex steroid hormones to stimulate apoptotic signalling proteins [p53 and Fas/FasL (Fas ligand)] has been known for a long time [17–19], but in the
hormone-dependent cells this effect is fully compensated by the proliferative action of these hormones. Earlier, using long-term cultivation of the hormone-dependent MCF-7 cells in steroid-free medium, we developed and described the resistant subline, MCF-7/LS, characterized with the low sensitivity to growth-stimulating oestrogen action, but the hypersensitivity to oestrogen-induced apoptosis. We have shown that oestrogen treatment results in the suppression of NF-κB activity in the MCF-7 breast cancer cells and its resistant derivatives [7]. Furthermore, oestradiol was found to suppress NF-κB in the MCF-7/LS cells, demonstrating the possible NF-κB involvement in the oestrogen apoptotic action [7]. Importantly, the ability of oestradiol to suppress the transcriptional activity and expression of NF-κB has been described for various experimental models [14,20].

Several reports have demonstrated the existence of the reciprocal antagonism between NF-κB and ERs based on the formation of inactive complexes between these proteins and/or the competition for binding with common activator proteins, such as CBP [CREB (cAMP-response-element-binding protein)-binding protein]/p300 [14,21].

In this study, we found that oestradiol increases the sensitivity of the resistant MCF-7/LS subline but not of the parent MCF-7 cells to the apoptotic action of the anti-tumour drug Dox. The study of NF-κB signalling showed that oestradiol alone suppresses NF-κB transcriptional activity without marked alterations in its DNA-binding properties in both cell lines, that is, in accordance with well-documented oestrogen ability to interact with and inactivate DNA-bound NF-κB complexes [21,22]. At the same time, the cell treatment in combination with oestradiol/Dox results in the increased transcriptional and DNA-binding activity of NF-κB in the resistant MCF-7/LS cells in comparison with the parent cells, that is, correlated with the highest apoptotic response in the MCF-7/LS cells. We suggest that the effect of NF-κB activation by the combination with Dox/oestradiol reflects the compensatory cell reaction to increased apoptosis, showing the important role of NF-κB signalling in the regulation of the cell apoptotic response. Furthermore, knockdown of NF-κB by siRNA results in the increase in the sensitivity of the resistant MCF-7/LS cells to both oestradiol and Dox, demonstrating that NF-κB may be considered as a potential target in the therapy of the resistant breast cancer. Based on these observations, we conclude that: (i) oestradiol enhances the apoptotic action of Dox in the resistant breast cancer cells; (ii) suppression of NF-κB signalling amplifies the apoptotic response of the resistant breast cancer cells to both oestrogen and Dox.

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**ACKNOWLEDGEMENTS**

We thank Olga Burova (Russian N.N. Blokhin Cancer Research Centre, Moscow, Russia) for assistance with the FACS analysis. We thank Dr Elena Gershtein (Russian N.N. Blokhin Cancer Research Centre, Moscow, Russia) for providing the p65 NF-κB ELISA assay reagents.

**FUNDING**

This work was supported in part by Russian Foundation for Basic Research (www.rfbr.ru) [grant numbers 07-04-00573, 09-04-00189 and 10-04-00788] and the CV Protek Fund.

**AUTHOR CONTRIBUTION**

Alexander Scherbakov performed the Western-blot analysis and p65 NF-κB assays, data analysis, statistical analysis, prepared the Figures and wrote the manuscript. Yulia Lobanova performed the transient transfection, measurement of reporter gene activity and assessment of apoptosis. Olga Andreeva performed the siRNA oligonucleotide experiments and measurement of reporter gene activity. Valentina Shatskaya performed the MTT test and measurement of ROS levels. Mikhail Krasil’nikov performed the data analysis, managed and consulted on the project, wrote the manuscript and prepared the Figures.
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Received 7 May 2010/22 June 2010; accepted 27 July 2010
Published as Immediate Publication 27 July 2010, doi 10.1042/BSR20100052