Tamoxifen long-term treatment *in vitro* alters the apoptotic response of MCF-7 breast cancer cells

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In this study, we examined alterations in the apoptotic response of tamoxifen (TAM)-resistant breast cancer cells. We used an *in vitro* selection approach for TAM resistance by means of long-term culture of MCF-7 breast cancer cells with increasing concentrations of TAM. The apoptotic response to TAM was determined by means of ELISA measurement of apoptotic DNA–histone complexes in cytoplasm and by Annexin-V staining. MCF-7(LT) cells isolated after 5 months of long-term treatment with TAM exhibited a significantly reduced apoptotic response to this drug, even if administered in high concentrations up to 20 μM. This reduced apoptotic response was also observed after treatment with the topoisomerase II inhibitor etoposide, a pro-apoptotic antineoplastic drug. Microarray experiments comparing the transcriptome of MCF-7(LT) and wild-type cells revealed both the down-regulated expression of several genes coding for pro-apoptotic proteins and the up-regulation of genes coding for apoptosis inhibitors. Further experiments to determine expression changes of the receptor tyrosine kinases HER2 and epidermal growth factor receptor did not reveal any alterations in MCF-7(LT) if compared to wild-type cells. Our findings suggest that long-term treatment with TAM *in vitro* does not necessarily change the expression of receptor tyrosine kinases, but can modulate the expression of apoptotic key genes impairing the apoptotic response of MCF-7 breast cancer cells. *Anti-Cancer Drugs* 15:787–793

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Introduction

Endocrine treatment plays an important role in the management of hormone-dependent breast cancer. The non-steroidal antiestrogen tamoxifen (TAM), a selective estrogen receptor modulator (SERM), is the only drug approved for use in breast cancer chemoprevention and it remains the treatment of choice for most women with hormone receptor-positive, invasive breast carcinoma. This outstanding position of TAM in the treatment of breast cancer is caused by the combination of its good tolerability profile and its efficacy both in adjuvant and first-line settings [1,2]. However, most patients with initially responsive tumors acquire resistance to TAM.

The therapeutic efficacy of TAM in cancer therapy is thought to arise primarily from its ability to compete with estrogens for binding to the estrogen receptor (ER). It is thought that TAM-ER is unable to effectively activate transcription of genes important for the growth and development of estrogen-dependent tumors. However, several often conflicting studies show that TAM can actively induce programmed cell death of cancer cells [3,4]. The precise mechanisms by which TAM induces apoptosis are not fully understood, but recent data indicated that there are both ER-dependent and ER-independent mechanisms mediating the apoptotic effects of TAM [5,6]. Several studies demonstrated that TAM is able to induce oxidative stress, followed by mitochondrial dysfunction and activation of caspases [5,7,8]. The participation of other mechanisms like phospholipase C-protein kinase-mediated phospholipase D activation [9], upregulation of Fas ligand [10] and signaling via Jun N-terminal kinase (JNK) [11] pathways in TAM-induced apoptosis is currently discussed.

Different mechanisms for either *de novo* or acquired TAM resistance have been demonstrated so far (reviewed in [12,13]) including ER mutations, expression changes of ERs or their co-regulators [14,15] and interactions with growth factor receptor pathways like HER2 or phosphatidylinositol 3-kinase/AKT-mediated activation of ERα [16,17]. In several studies, the loss of antiestrogen responsiveness of initially responsive tumors was correlated with changes in expression of receptor tyrosine kinases [18–20]. *In vitro* studies using the method of long-time culture of breast cancer cells in TAM-containing medium to generate cells with a reduced responsiveness to TAM yielded contradictory

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results with regard to observed changes in the expression of receptor tyrosine kinases like HER2 [19,21]. Overexpression of HER2 was demonstrated to suppress the apoptotic response of MCF-7 cells by up-regulating the expression of the anti-apoptotic proteins Bcl-2 and Bcl-x [22].

However, changes in the apoptotic response triggered by long-term exposure to TAM have not been directly linked to the development of antiestrogen resistance. In this study, we generated a MCF-7 subline with a reduced responsiveness to TAM in order to analyze alterations in the apoptotic response of these cells.

Material and methods

Material

Water-soluble 17-β-estradiol (estradiol), 4-OH tamoxifen (TAM) and etoposide were purchased from Sigma (Deisenhofen, Germany). FCS was obtained from Life Technologies (Karlsruhe, Germany). Phenol red-free DMEM medium (with 1000 mg/l glucose and with L-glutamine) and Serum Replacement 2 (SR2) were obtained from Sigma (Deisenhofen, Germany). Cell Death Detection ELISA kit and Annexin-V FLUOS staining kit were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Epidermal growth factor receptor (EGFR; 1005-G), HER2 (c-18) and ERβ (N-19) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ERα (62A3) antibodies were obtained from Cell Signaling Technology (Beverly, MA) and β-actin antibody (8226) was purchased from Abcam (Cambridge, UK). The ECL system was obtained from Amersham (Little Chalfont, UK).

Cell culture

The human breast cancer cell line MCF-7 (obtained from ATCC, Manassas, VA) was maintained in phenol red-free DMEM medium supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. In order to generate clones with a lower sensitivity towards TAM, we treated the cells with TAM and raised its concentration gradually, starting with a concentration of 0.5 μM. The cells were cultured under the condition of a humidified atmosphere with 5% CO_2 at 37°C.

Resistant cells were isolated and cloned by seeding the cells in a soft agar 96-well plate containing medium with a appropriate TAM concentration, followed by isolation of the grown colonies.

Apoptosis assays

MCF-7 cells were suspended in phenol red-free DMEM containing 5% steroid depleted charcoal-treated medium (sFCS) and seeded into 96-well plates (2.5×10^3 cells/well). After at least 3 h, we further reduced serum concentration by adding 1 volume serum-free DMEM supplemented with serum replacement SR2 (Sigma-Aldrich, Munich, Germany). After 24 h the medium was changed into 1 × SR2-DMEM, followed by a treatment with the test substances in quadruplicates. Cellular apoptosis was quantified after 18 h of treatment by counting of cells with positive Annexin-V and negative propidium iodide staining after using the Annexin-V FLUOS staining kit (Roche Applied Science) according to the manufacturer's protocol. Additionally, measurement of cytoplasmic histone-associated DNA fragments was performed using the Cell Death Detection ELISA Plus (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Apoptosis was expressed as percentage of the SR2-DMEM medium control. Statistical analysis of the data was performed by one-way ANOVA using Prism 2.0 software (Graph Pad, San Diego, CA), with statistical significance accepted at $p < 0.05$.

Western blot analysis

Cells were lysed in RIPA buffer (1% Igepal CA-630, 0.5%. sodium deoxycholate, 0.1% SDS in PBS containing aprotinin and sodium orthovanadate). Aliquots containing 10 μg protein were resolved by 10% SDS–PAGE (7.5% SDS–PAGE for PARP Western blot), followed by electrotransfer to a nitrocellulose membrane. Immunodetection was carried out using antibodies in PBS containing 5% skim milk, followed by horseradish peroxidase-conjugated secondary antibodies which were detected using the ECL system. As internal standard, β-actin expression was determined and measured using EasyWin Software (Herolab, Wiesloch, Germany). EGFR, HER2, ERα and ERβ expression were quantified as the ratio of EGFR or HER2 or ERα or ERβ to β-actin. Statistical analysis of the data was performed by one-way ANOVA using Prism 2.0 software (Graph Pad), with statistical significance accepted at $p < 0.05$.

RNA preparation and hybridization of DNA microarrays

Total RNA from MCF-7 wild-type, MCF-7 control and MCF-7(LT) cells was isolated using TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. After DNase treatment and subsequent precipitation, 20 μg total RNA was used for cDNA synthesis using a cDNA kit (Roche). After purification of the cDNA, Cy3- and Cy5-labeled cRNA was generated using the MEGAscript T7 Kit (Ambion, Dresden, Germany). After purification of the labeled cRNA using the High Pure RNA Tissue Kit (Roche) and its fragmentation, 10 μg of Cy3- and Cy5-labeled cRNA were used for the hybridization of the Human Cancer Array slides (MWG Biotech, Ebersberg, Germany) containing oligonucleotides which represent 1853 human genes associated with cancer development and progression. The measurement of the slides was performed by

the scanning service of MWG Biotech. Data are presented as relation between the MCF-7 control:MCF-7 wild-type ratios (defined as 100%) and the ratios of the MCF-7(LT) and MCF-7 wild-type arrays.

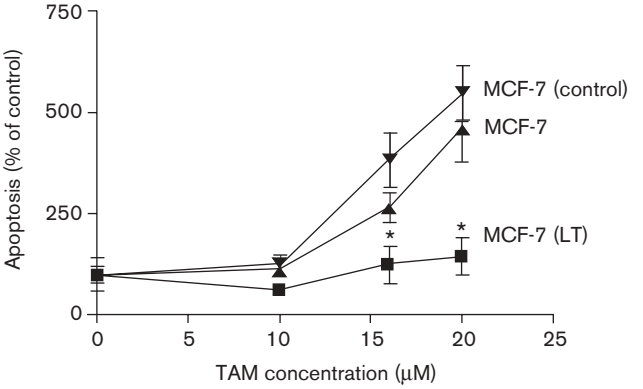
Results

Establishment of MCF-7 sublines with a reduced responsiveness to TAM

In order to generate TAM-resistant cells, we performed a long-term *in vitro* selection process culturing the ERα/β-positive cell line MCF-7 in rising concentrations of TAM. Initially cells were treated with a start concentration of 500 nM TAM, followed by an increase of 1 μM every 2 weeks until a concentration of 10 μM was reached. Since TAM was dissolved in 95% EtOH, we used EtOH as a parallel control treatment and increased its concentration corresponding to the TAM treatment. The cells generated by culture in increasing concentrations EtOH were used as negative control cells in the following experiments.

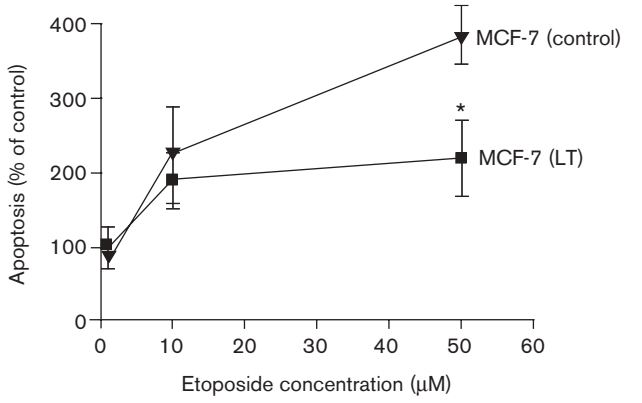
Every 4 weeks, the apoptotic effect of TAM on the long-term treated cell pool was tested and compared to its effect on control cells. After we observed significant differences, we started to isolate single clones by clonal growth assays. The reduced TAM-sensitivity of five isolated sublines was confirmed and compared in further apoptosis assays. For this purpose, we tested the ability of different TAM concentrations to induce apoptosis in the long-term treated cells in comparison with the wild-type and control cells. Apoptosis was determined by ELISA measurement of cytoplasmic histone–DNA complexes and in order to confirm the results of the ELISA exemplarily by combined staining with Annexin-V and propidium iodide. At 18 h after treatment, TAM concentrations of 16 and 20 μM were able to significantly induce apoptosis in wild-type and control MCF-7 cells, as detected by histone–DNA ELISA (Fig. 1) and Annexin-V staining (data not shown). In contrast, both concentrations of TAM were not able to induce apoptosis in the long-term treated sublines, as exemplarily shown by the MCF-7(LT) subline (Fig. 1), which was chosen for further characterization. In order to detect a delayed apoptosis in the long-term treated sublines, we performed apoptosis assays at later timepoints after TAM treatment (36 and 48 h). In these experiments, we did not observe any increase of apoptosis in the long term treated sublines, ruling out a possible delay of apoptosis in these cells (data not shown).

Fig. 1



Effects of TAM on apoptosis of MCF-7 cells isolated after long-term treatment with TAM(LT) compared with the wild-type cell lines and with clones isolated after long-term treatment with EtOH (control). MCF-7 cells were treated with the indicated concentrations of TAM. Cells were cultured in 96-well plates in serum-free DMEM-SR2 medium containing estradiol (10 nM). The induction of apoptosis in MCF-7 cells by TAM was determined by ELISA measurement of histone–DNA complexes in cytoplasm 18 h after treatment. Results were obtained from three separate experiments and expressed as means (% of untreated control) ± SD. *$p < 0.01$ versus control.

Fig. 2



Effects of etoposide on apoptosis of MCF-7(LT) and MCF-7 control cells. Cells were cultured in 96-well plates in SR2-DMEM medium. At 18 h after treatment with etoposide in the indicated concentrations, cellular apoptosis was determined by ELISA measurement using a Cell Death Detection Assay. Results were obtained from three separate experiments and expressed as means (% of control) ± SD. *$p < 0.05$ versus control.

Effects of etoposide on apoptosis in MCF-7(LT) cells

In order to address the question, whether the reduced apoptotic effects of TAM on MCF-7(LT) cells resulted from general changes in the apoptotic signaling complex triggered by the long-term treatment with TAM, we additionally examined the effect of treatment with the pro-apoptotic drug etoposide. Etoposide is an agent which complexes with topoisomerase II and DNA to enhance double-strand and single-strand cleavage of DNA and reversibly inhibits religation. It blocks the cell cycle in the S and G_2 phase of the cell cycle and induces apoptosis in normal and tumor cell lines [23]. Under serum-free DMEM-SR2 culture conditions, the apoptotic effects of etoposide concentrations of 1, 10 and 50 μM were tested.

Only concentrations of 10 and 50 μM led to a significant increase of cellular apoptosis in MCF-7 cells. In MCF-7(LT) cells, the apoptotic effect observed after treatment with 50 μM etoposide was significantly lower than in control cells (Fig. 2).

Expression of EGFR, HER2, ERα and ERβ in MCF-7(LT) cells

In order to examine the expression of key molecules of classical ER and receptor tyrosine kinase signal transduction pathways in MCF-7(LT) cells, we performed a Western blot analysis of EGFR, HER2, ERα and ERβ. We intended to examine changes in receptor expression, which could underlie the reduced efficacy of TAM in these cells.

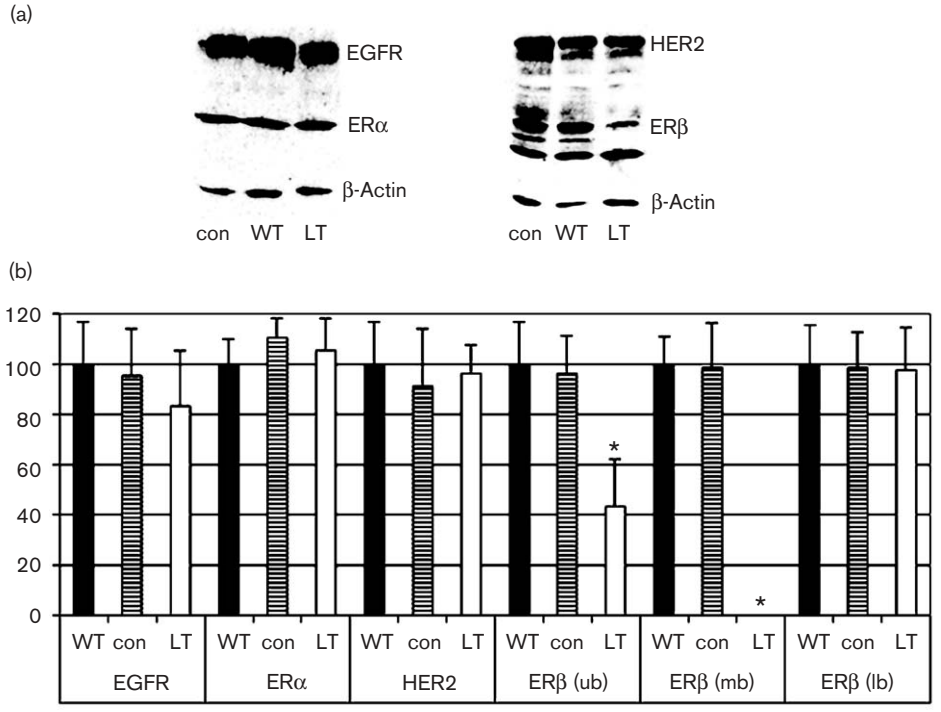
The receptor tyrosine kinases EGFR (170 kDa) and HER2 (185 kDa) could be detected in all tested cell lines (Fig. 3), with an only moderate expression level if compared to overexpressing cells like the ovarian cancer cell line SK-OV-3 or breast cancer cell line BT-474 (data not shown). When we compared the expression level of MCF-7(LT) cells with the one in control and wild-type cells, no significant difference could be observed.

Additionally, we detected the expression of ERα (65.5 kDa), and a triplet of ERβ bands previously described [24] in MCF-7 wild-type and control cells. In MCF-7(LT) cells, the intensity of the upper and the middle ERβ band was significantly diminished when compared to the wild-type and control cells (Fig. 3).

Expression of apoptotic response genes in MCF-7(LT) cells

In order to examine molecular mechanisms underlying the observed reduced apoptotic response of MCF-7(LT) cells, we performed an expression analysis by means of DNA microarrays (MWG Biotech). MWG Human Cancer Arrays containing 1853 human genes associated with cancer development and progression were hybridized with Cy3- or Cy5-labeled cRNA representing the transcriptome of MCF-7, MCF-7 control or MCF-7 (LT) cells. In these experiments, we compared the transcriptome of MCF-7 wild-type cells with the one of MCF-7(LT) or MCF-7 control cells, respectively. The procedure from RNA isolation to microarray hybridization was repeated once including a dye swap in order to confirm the generated data. In a second step, the ratios MCF-7 control:MCF-7 wild-type and MCF-7(LT):MCF-7

Fig. 3



Western blot analysis of receptor tyrosine kinase and estrogen receptor (ER) expression in long-term treated MCF-7(LT), wild-type (WT) and control (con) cells. 10 ig protein of cell lysates prepared from cells cultured in 10% FCS were loaded and resolved by 10% SDS–PAGE. Detection of β-actin expression was used as a loading control. (a) Representative Western blot experiment, (b) quantification of Western blot band intensity, normalized to the β-actin signal, in percent of the intensity in wild-type cells (defined as 100%). (ERβ: ub = upper band, mb = middle band, lb = lower band). Results were obtained from three separate experiments and expressed as means (% of control) ± SD. *$p < 0.01$ versus control.

were compared in order to reveal expression changes, which were not triggered by long-term culture with the solution agent EtOH only, but specifically by long-term culture in TAM-containing culture medium.

The microarray experiments revealed clear expression alterations of genes involved in the apoptotic response. If compared to the gene expression of control cells, our data demonstrates more than 2-fold elevated transcript levels of the anti-apoptotic genes *bag4* [25], *tnfrsf5* (CD40) [26], *tnfrsf17* (BCMA) [27], *tnfsf18* (GIRTL) [28] and *fksg2* in MCF-7(LT) cells (Fig. 4a). Furthermore, we were able to detect more than 2-fold decreased mRNA levels of the pro-apoptotic genes *casp1* [29], *casp10* [30], *tnfrsf6* (FAS) [31], *tnfsf7* (CD70) [32] and *bad* [33] in MCF-7(LT) cells if compared to the expression in MCF-7 control cells (Fig. 4b). In contrast, the microarray experiments did not show any significant alteration in the expression of receptor tyrosine kinases like EGFR or HER2 in MCF-7(LT) cells, confirming the results of our Western blot experiments (Fig. 4c). In MCF-7(LT) cells, the microarray experiments also did not show any significant expression changes of the genes *bcar1* and *bcar3* (breast cancer anti-estrogen resistance 1 and 3) (data not shown), which were previously reported to confer antiestrogen resistance on breast cancer cells [36,37].

Discussion

In order to analyze the effects of long-term treatment with TAM on the apoptotic response of MCF-7 breast cancer cells, we used an *in vitro* selection approach by long-term cell culture in TAM-containing medium. After treatment with increasing concentrations of TAM for 5 months, the ability of this antiestrogen to induce apoptosis in the isolated sublines was significantly diminished if compared to wild-type cells or control cells being isolated after long-term treatment with the solvent EtOH. In the following experiments, we further analyzed the MCF-7(LT) subline to elucidate molecular mechanisms which can underlie the observed impaired apoptotic response.

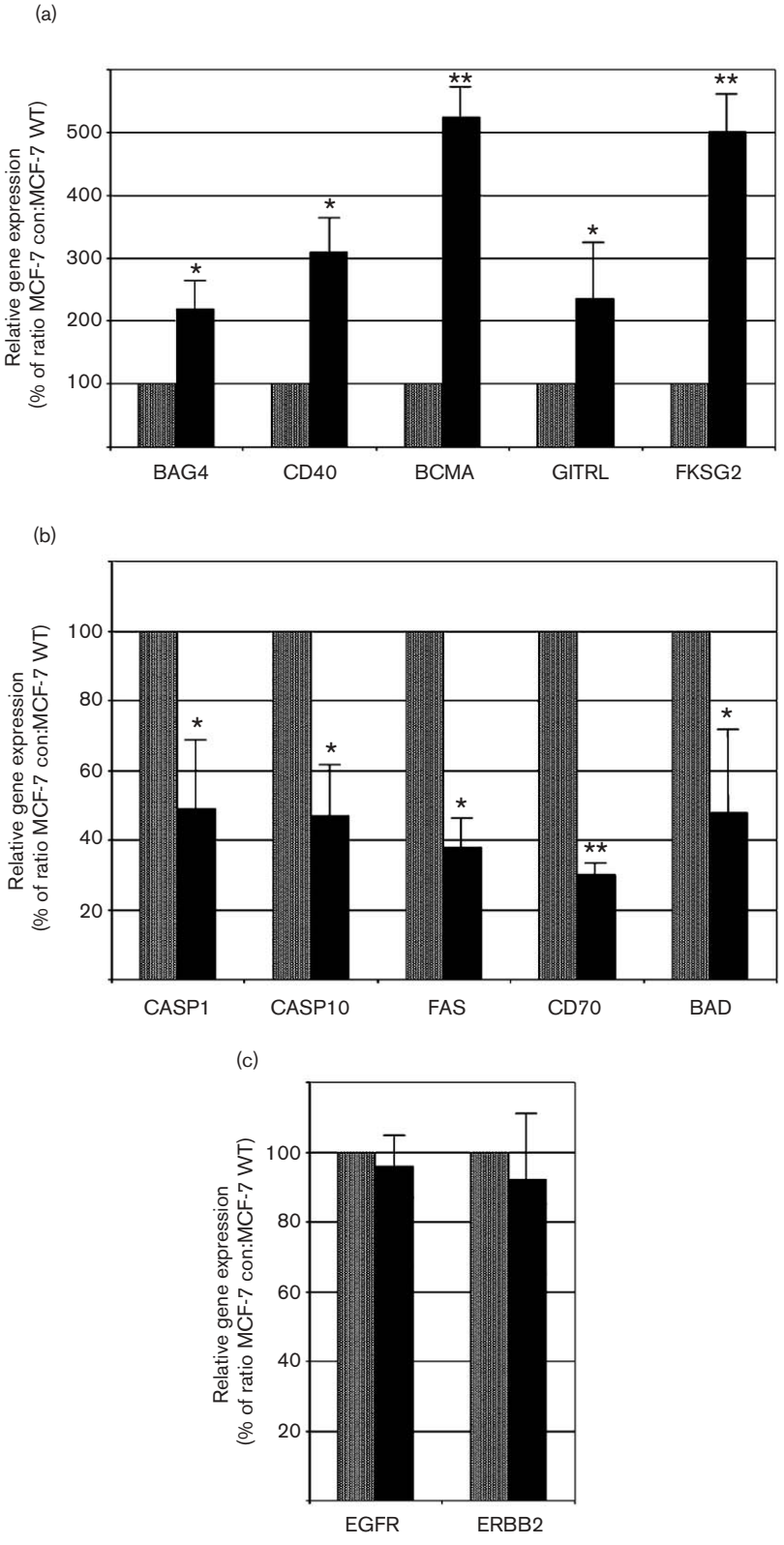
In order to examine whether the apoptotic machinery of MCF-7(LT) cells was affected in general, we tested the effects of the pro-apoptotic drug etoposide on these cells. Etoposide is known to induce both apoptotic morphological changes and DNA degradation into oligonucleosomal fragments in MCF-7 breast cancer cells [34]. Our data demonstrated that the ability of etoposide to induce apoptosis was diminished in MCF-7(LT) cells when compared to control cells. These results suggested that a long-term treatment with TAM is not only able to impair the apoptotic response to this agent, but is able to affect the apoptotic response of MCF-7 cells in general.

Clinical and laboratory data have suggested that growth factor signaling may play a role in antiestrogen resistance of breast cancer. Our data do not support studies reporting increased expression of HER2 and EGFR in TAM-resistant breast cancer cells generated by *in vitro* selection [19,35]. In the MCF-7(LT) subline, we did not observe changes in the expression of both receptor tyrosine kinases in long-term treated MCF-7 cells, supporting a previous study with similar observations [21].

Several studies have demonstrated that TAM can actively induce apoptosis of cancer cells [3,4]. Recently it has been demonstrated that there are both ER-dependent and ER-independent mechanisms mediating the apoptotic effects of TAM [5,6]. Cellular effects of TAM concentrations higher than 15 μM are considered to be not reversible by adding back estrogen, suggesting that in our experiments, the apoptotic response observed in MCF-7 wild-type and control cells was ER independent. However, our data demonstrate the weaker expression of ERβ-isoforms in the MCF-7(LT) subline. Previous studies on the expression of ERβ in TAM-resistant tumors yielded contradictory results. A small clinical study demonstrated that ERβ mRNA was significantly up-regulated in the TAM-resistant group when compared to the TAM-sensitive group of patients [14]. In contrast, a second study demonstrated that total ERβ protein expression was more frequently observed in TAM-sensitive tumors than in resistant tumors [15]. Though we were not yet able to identify the specific ERβ subtype exhibiting a weaker expression in MCF-7(LT) cells, our data support findings demonstrating a decreased ERβ expression in TAM-resistant cells.

In order to reveal further molecular mechanisms which could underlie the reduced apoptotic response of the MCF-7(LT) subline, we analyzed the expression of apoptotic genes in these cells. If compared to MCF-7 wild-type or control cells, our data revealed significant changes in the expression of genes involved in the apoptotic machinery. In the MCF-7(LT) subline, the transcript level of genes coding for the BCL-2 related proteins BAD and BAG4, tumor necrosis factor (TNF)-related cell surface ligands CD70 and GIRTL, TNF-related cell surface receptors CD40, BCMA and FAS, and apoptosis effector molecules caspase 1 and 10 was changed. Whereas a participation of BAD, BAG4, FAS, CD40, and caspases 1 and 10 in the apoptotic response of breast cancer cells was reported previously, the function of the other genes in the apoptotic machinery of this cell type remains unclear. However, our data suggest that up-regulation of anti-apoptotic genes and down-regulation of pro-apoptotic genes is a possible mechanism underlying the observed diminished apoptotic response of MCF-7(LT) cells.

Fig. 4



Gene expression in MCF-7(LT) cells (black bars) in relation to control cells (grey bars). (a) Expression of anti-apoptotic genes, (b) expression of pro-apoptotic genes and (c) expression of receptor tyrosine kinases. Data was generated in DNA microarray experiments ($n=2$, including dye swap) as described in Methods. Microarrays were hybridized with MCF-7 control plus MCF-7 wild-type cRNA or with MCF-7(LT) plus MCF-7 wild-type cRNA, respectively. The ratio MCF-7 control:MCF-7 wild-type was defined as 100% for every gene and compared to the ratio of the corresponding gene observed in the MCF-7(LT) plus MCF-7 wild-type array. $^{**}p<0.01$ versus control, $^{*}p<0.05$ versus control.

In conclusion, our findings indicate that long-term treatment with TAM *in vitro* is able to impair the apoptotic response of MCF-7 breast cancer cells. The modulated expression of apoptotic genes observed in the MCF-7(LT) subline might reveal an alternative mechanism leading to development of acquired resistance to this drug. Whether this mechanism of TAM resistance is also present in the *in vivo* situation, has to be determined in further studies.

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