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Growth factors and chemotherapeutic modulation of breast cancer cells

Kadriye Ciftci, Jiangfeng Su and Peter B. Trovitch

Abstract

A variety of molecules including growth factors are involved in the metastasis of breast cancer cells to bone. We have investigated the effects of osteoblast derived growth factors, such as insulin-like growth factor-1 (IGF-1) and transforming growth factor beta-1 (TGF-β1), on doxorubicin (adriamycin)-induced apoptosis and growth arrest of estrogen receptor positive (ER+) (MCF-7) and negative (ER−) (MDA-MB-435) breast cancer cell lines. Human breast normal epithelial (MCF-10A), breast cancer (MCF-7) and metastatic breast cancer (MDA-MB-435) cell lines were exposed to different doses of doxorubicin (0.1, 1 or 10 μM) at various exposure times (12, 24 or 48 h). The doxorubicin cytotoxicity was found to be higher in cancer cell lines (MDA-MB-435 and MCF-7) compared with normal breast epithelial cells (MCF-10A cells). Doxorubicin appeared to exert a blockade of MCF-7 and MDA-MB-435 cells at the G2/M phase, and induced apoptosis in MDA-MB-435 ($29 \pm 4.2\%$ vs $3.4 \pm 1.9\%$ control) as assessed by flow cytometry, DNA fragmentation and terminal deoxynucleotidyl-transferase mediated deoxyuridine 5-triphosphate and biotin nick-end labelling (TUNEL) assays. Estradiol (E2) stimulated the growth of MCF-7 cells and increased the distribution of the cells at the G2/M and S phases. Exogenous IGF-1 partially neutralized the doxorubicin cytotoxicity in both cancer cell lines (MCF-7 and MDA-MB-435). Similarly, TGF-β1 partially neutralized the doxorubicin cytotoxicity in MDA-MB-435 cells by reducing the number of cells at the <G1 phase (from 29% to 6.4%) and enhanced the doxorubicin blockade of MCF-7 (E2−) at the G0/G1 phase. Results showed that the osteoblast-derived growth factors could affect the chemotherapy response of breast cancer cells, thereby allowing for the possibility of chemotherapeutic modulation.

Introduction

Breast cancer is the most common cancer associated with osteolytic lesions. Sixty-nine percent of advanced breast cancer patients have bone metastasis, and bone is the most common site of first distant recurrence (Diel et al 1992; De Jong et al 1998). Most of these patients will require chemotherapy with the majority achieving partial responses. Complete disappearance of the disease has been reported in fewer than 20% of the patients treated (D'Andrea & Seidman 1997; Myers 1997).

The mechanisms underlying osteotropism, an extraordinary affinity to grow in bone, are complex and involve unique characteristics of both breast cancer cells and bone. A variety of molecules including cell adhesion molecules (CAMs), matrix metalloproteinases (MMP), growth factors and cytokines have been implicated in the pathophysiology of bone metastasis (Nass et al 1996; Beckman et al 1997; El-Tanani & Green 1997).

Among the growth factors, transforming growth factor beta-1 (TGF-β1) is known as a potent inhibitor of mammary epithelial cell proliferation and deregulates mammary development in-vivo (Borellini & Oka 1989; Nass et al 1996). Specifically, TGF-β1 expression has been shown to be higher in human breast tumours than in normal mammary tissue with protein levels being positively correlated with disease progression (Nass et al 1996). Aside from being a potent growth inhibitor in human breast cancer cells in-vitro, TGF-β1 has been shown to stimulate insulin-like growth factor (IGF) binding protein gene expression (Oh et al 1995).

Temple University, School of Pharmacy, Department of Pharmaceutical Sciences, 3307 N. Broad Street, Philadelphia, PA 19140, USA

Kadriye Ciftci, Jiangfeng Su, Peter B. Trovitch

Correspondence: K. Ciftci, Temple University, School of Pharmacy, Department of Pharmaceutical Sciences, 3307 N. Broad Street, Philadelphia, PA 19140, USA. E-mail: kadriye.ciftci@temple.edu

Insulin-like growth factor-1 (IGF-1) is known as a potent mitogen for breast cancer cells in-vitro (Surmacz et al 1998; Resincoff et al 1995). IGFs induce various responses in cell types including the regulation of growth, differentiation and the over-expression of IGF receptors confers protection from apoptosis (Parrizas & Leroith 1997).

The mitogenic action of IGF-1 is synergistic with estrogen, in part because estrogen upregulates expression of IGF-1 receptor (IGFR-1). Many breast cancer cells express IGFR-1 and interference with its expression leads to inhibition of cell growth (Nass et al 1996). In addition high levels of IGFR-1 are positively correlated with estrogen receptor (ER) expression (Oh et al 1995).

Estrogens are thought to play an important role in the development, progression and treatment of human breast cancer (Nass et al 1996). Although the molecular mechanism of breast cancer cell growth stimulation by estradiol remains unclear, it has been reported that growth factors may be an integral part of the mechanism. Estrogens can also function as a survival factor for estrogen receptor positive (ER+) breast cancer cells. In other words, tumours that express estrogen receptor tend to grow more slowly and are associated with longer disease-free survival than estrogen receptor negative (ER−) tumours. Therefore, ER+ breast cancer patients with bone-only metastases benefit from a favourable response to chemotherapy and a favourable prognosis. However, patients with ER− breast cancer and/or widespread metastatic disease beyond the skeleton do not benefit from such a response (Borellini & Oka 1989; Choki et al 1998; Rasmussen & Cullen 1998; Surmacz et al 1998).

Based on these observations, we have investigated the effects of osteoblast-derived growth factors (IGF-1 and TGF-β1) on doxorubicin (adriamycin)-induced apoptosis and growth arrest in G1 and G2/M in MDA-MB-435 (ER−) and MCF-7 (ER+) cell lines. Since both cell lines produce local modulators and MCF-7 cells express estrogen receptor, they represent a reliable in-vitro model for this study. Among the other anthracene derivatives, doxorubicin was chosen as a model drug due to its broad spectrum of activity and known mechanisms of action (Myers 1997). The effect of estradiol on MCF-7 (ER+) breast cancer cells undergoing doxorubicin-induced apoptosis and the modulatory effect of the osteoblast derived growth factors were investigated also.

Materials and Methods

Materials

All human breast cancer cell lines (MCF-7, MDA-MB-435) and the normal breast epithelial cell line (MCF-10A) were obtained from American Type Culture Collection (ATCC, Rockville, MD). Minimum essential medium (MEM), Leibovitz's L-15 medium, Ham's F12 medium, Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), foetal calf serum (FCS), horse serum and RNase-A were purchased from Gibco BRL Laboratories (Grand Island, NY). IGF-1 and TGF-β1 were purchased from R&D Systems (Minneapolis, MN). Doxorubicin, Proteinase-K and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma (St Louis, MO). Tdt-dUTP (terminal deoxynucleotidyltransferase-mediated deoxyuridine 5-triphosphate) and biotin nick-end labelling (TUNEL) assay kit was obtained from Boehringer Mannheim (Indianapolis, IN). All other chemicals were reagent or molecular grade, as appropriate.

Cell cultures

The normal breast epithelial (MCF-10A) and cancer cell lines (MCF-7 (ER+) and MDA-MB-435 (ER−)) were maintained in DMEM, MEM and Leibovitz's medium without phenol red, respectively. MCF-7 and MDA-MB-435 cell culture medium were supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.01 mg mL^{-1} bovine insulin and 10% charcoal-treated FBS. The cells were plated in tissue culture flasks and incubated in a humidified 5% CO_2 incubator.

Treatment of the cells

The cells were seeded at a density of 3×10^5 in six-well plates and incubated until the cells reached 60% confluency. All cells were initially exposed to 10^{-5}, 10^{-6} and 10^{-7} M doxorubicin solution in cell medium for 12, 24 or 48 h. A range of cytotoxic effects was analysed and the optimum dose of doxorubicin was selected. To determine the influence of recombinant IGF-1 and TGF-β1 on protecting MCF-7 and MDA-MB-435 cells from doxorubicin cytotoxicity, the cells were treated with doxorubicin at optimum dose (0.1 μM) for 12, 24 or 48 h in the presence of 50 ng mL^{-1} IGF-1 and 25 ng mL^{-1} TGF-β1. The medium for MCF-7 cells was supplemented with 0.1 μM 17β-estradiol (E2) unless otherwise indicated.

Cell growth and viability

Cytotoxicity studies of IGF-1, TGF-β1, doxorubicin, doxorubicin + IGF-1 and doxorubicin + TGF-β1 were performed using the trypan blue dye exclusion and MTT assays. Briefly, cells were grown in six-well plates (40–50% confluent) and then treated with the chemotherapeutic agent as outlined above. For trypan blue assay, trypsinized cells were stained with 0.4% trypan blue dye and trypan blue positive and negative cells were counted using a haemocytometer under a phase contrast microscope (Nikon Instrument INC, NY). The MTT assay is based on the observation that the mitochondria in living cells can catalyze MTT molecules to a colorimetrically detectable dye (Mosmann 1983). For the assay, trypsinized cells were seeded in a 96-well plate at 10^5 cells/well. MTT was dissolved in phosphate-buffered saline (PBS) at a concentration of 5 mg mL^{-1} and filtered through a 0.2-μm membrane filter. MTT solution (10 μL) was then added to each well and the cells were incubated at 37 °C for 4 h. Thereafter, acid-isopropanol solution (100 μL 0.04 M HCl

in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue precipitate and incubated for 10 min at room temperature. Absorbance of samples was determined at a wavelength of 580 nm and a reference wavelength of 630 nm using a Bio-Tek (EL 300) Microplate Reader. The extent of cytotoxicity was defined as the relative reduction of the optical density (OD), which correlated to the amount of viable cells in relation to cell control (=100%).

Detection of apoptosis

Apoptotic DNA was assessed by detecting DNA fragmentation on agarose gel electrophoresis (Hermann et al 1994), flow cytometry and TUNEL methods (Gavrieli et al 1992).

DNA fragmentation
Cells were lysed with 0.1 mL DNA lysis buffer (Nonidet p-40, 10 mM Tris-HCl and 25 mM EDTA, pH 7.4) and centrifuged at 15 000 rev min^{-1}. The lysate was treated with 500 g mL^{-1} Proteinase-K and incubated at 37 °C for 2 h. The samples were then centrifuged using a high-speed centrifuge at 4 °C and the supernatant was treated with Ribonuclease-A (0.1 mg mL^{-1}) at 37 °C for 18 h. DNA in the final extract was resolved by agarose gel electrophoresis. Agarose gel (1.2%) contained 0.1 mg mL^{-1} ethidium bromide.

TUNEL assay
Apoptosis was detected also using a TUNEL assay. This in-situ cell death detection method was performed according to the manufacturer's protocol. Briefly, the cells were treated with doxorubicin in the presence or absence of growth factors as explained in the Methods section. The cells were fixed using paraformaldehyde solution (4% in PBS, pH 7.4) for 1 h at 37 °C and then rinsed with PBS. The samples were incubated in 2% H_2O_2 in methanol for 10 min at 37 °C to block endogenous peroxidase and the cells were permeabilized by rinsing the cells with PBS and incubating the samples in 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4 °C. Cells were then labelled with 50 μL TUNEL reaction mixture and incubated at 37 °C for 60 min. Signal conversion was achieved by adding 50 μL of converter-POD (anti-fluorescein antibody, Fab fragment from sheep, conjugated with horse-radish peroxidase) followed by incubation in a humidified chamber for 60 min at 37 °C. To complete the substrate reaction, 50–100 μL diaminobenzidine (DAB) solution was added and rinsed with PBS. The samples were analysed under a light microscope.

Cell cycle analysis
Flow cytometry was utilized to determine the indices of the cell cycle and apoptosis. Cells were treated with doxorubicin (0.1 or 10 μM) as explained above. The cells were then trypsinized and washed with PBS followed by fixing with ice-cold methanol. After incubation on ice for 30 min, cells were centrifuged and resuspended in PBS followed by treatment with RNaseA (500 U mL^{-1}) to digest the residual RNAs and stained with propidium iodide (50 μg mL^{-1}). Samples were incubated for 30 min at room temperature and cell cycle analysis was performed with a Becton-Dickinson Fluorescence-Activated Cell Sorter (FACS). The data were analysed to obtain cell cycle distribution using the MODFIT program. Results are illustrated as histograms based on distinction of cells with diploid (G0/G1 phases), hyperdiploid (S phase) and tetraploid (G2/M phase).

Statistical analysis

Cytotoxicity experiments were performed three times and data were analysed by the non-parametric comparator, the Kruskal-Wallis test. Nemenyi's test was performed as post hoc analysis with accepted alpha level ($P < 0.05$). Cell cycle experiments were repeated six times, data were analysed by analysis of variance and Bonferroni correction was applied to the groups after analysis of variance.

Results and Discussion

Cytotoxicity studies

Varying doses of doxorubicin have been known to exhibit a wide range of effect. Low doses of doxorubicin interfere with DNA unwinding and inhibit topoisomerase II expression while suprapharmacological concentrations produce DNA strand breaks that suggest free radical-mediated apoptosis (Myers 1997). These pathways may mediate growth arrest and cell death depending on the biochemical properties of the target tissues and experimental conditions. Here, the effects of the experimental conditions such as dose and exposure time on doxorubicin cytotoxicity in human breast cancer (MCF-7 and MDA-MB-435) and normal breast epithelial (MCF-10A) cell lines were assessed by trypan blue and MTT assays as outlined in the Methods section. The MTT results revealed that the cell viability was significantly higher in normal breast epithelial cell line (MCF-10A) compared with both breast cancer cell lines following exposure to doxorubicin at all doses and exposure times tested (Figure 1). This can be explained by the difference in the physiological stage of the normal and malignant cells. The viability of MCF-7 cells as a function of exposure time and doxorubicin dose showed similar trend as MDA-MB-435 cell line (Figure 1). The number of live cells declined progressively with increasing doses of doxorubicin and exposure times in both cancer cell lines tested. For example, after 48-h exposure to 1 or 10 μM doxorubicin, the viability of MCF-7 (E2−) was approximately 68% and 41%, respectively ($P < 0.05$). The difference in cytotoxicity between the two exposure times (12 h and 24 h) at low doses of doxorubicin (0.1 or 1 μM) was found to be statistically insignificant ($P > 0.05$) in both cancer cell lines tested (Figure 1).

These results were compared with the trypan blue exclusion assay (TBE) (data not shown) and the antiproliferative effect of doxorubicin was found to be higher in

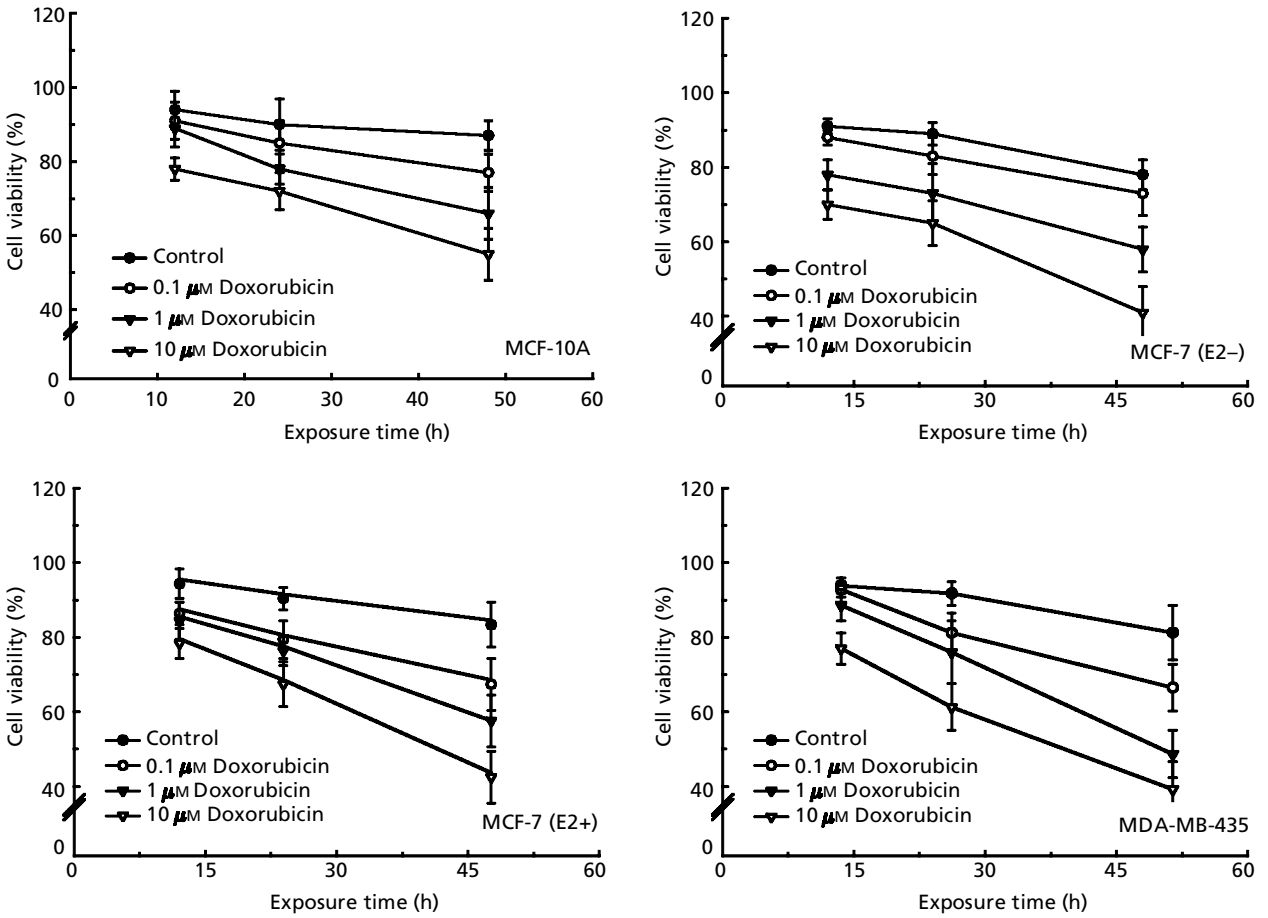

Figure 1 In-vitro cytotoxic effect of doxorubicin determined by MTT assay in breast cancer cells (MCF-7, MDA-MB-435) and normal breast epithelial cells (MCF-10A). The cells were exposed to 0.1, 1 or 10 μM doxorubicin for 12, 24 or 48 h in the presence or absence of 17β-estradiol as detailed in Materials and Methods. Results are presented as mean $\pm$ s.d. of three experiments.

both cancer cell lines. This could be due to the action of doxorubicin to increase mitochondrial density and hence the ability of MTT to measure total succinate dehyrogenase activity in mitochondria. Based on these results 0.1 μM doxorubicin was selected as an optimum dose for further studies. This dose was known to be a common pharmacological dose for doxorubicin in clinical practice as well as a suitable dose to characterize its action on breast cancer cells (Choki et al 1998).

Effects of estradiol and growth factors on cell growth and doxorubicin cytotoxicity

Due to the ability of estradiol (E2) to prevent the regression of many ER+ mammary tumours by a process involving apoptosis, we have studied the influence of E2 on doxorubicin cytotoxicity in ER+ breast cancer cells (MCF-7). MCF-7 cells were treated with different concentrations of doxorubicin in the presence or absence of E2 as described previously. The results indicated a more pronounced depression of cell viability against the doxorubicin cytotoxicity in MCF-7 (E2−) cells compared with MCF-7 (E2+) cells. It has been reported that estrogen can promote resistance of ER+ human breast cancer cells to chemotherapeutic drugs through a mechanism that involves regulation of the proto-oncogene (Bcl-2) expression (Texixeira et al 1995).

The use of IGF-1 in cell medium increased the growth of MCF-7 and MDA-MB-435 cells by 25 and 30%, respectively (data not shown). IGF-1 partially reversed the doxorubicin (0.1 μM) cytotoxicity in MCF-7 (E2−) and MDA-MB-435 cells (Figure 2), whereas it did not affect significantly the viability of MCF-7 (E2+) cells (Figure 2). Since estradiol is known to increase IGF type 1 receptor level in breast cancer cells grown in culture, it might sensitize the cells to this mitogenic growth factor (Oh et al 1995; El-Tanani & Green 1997).

TGF-β1 inhibits the growth of normal mammary cells in-vivo (Daniel et al 1989) and breast cancer cells in-vitro (Valverius et al 1989), therefore it was originally hypothesized that TGF-β1 could be used to inhibit tumour growth in mammary tissue. In this study, exogenous TGF-β1 (25 ng mL^{-1}) decreased the growth of breast cancer cells tested by approximately 20–30% (data not shown). The presence of TGF-β1 in cell medium did not significantly effect the viability of doxorubicin treated MCF-7 cells

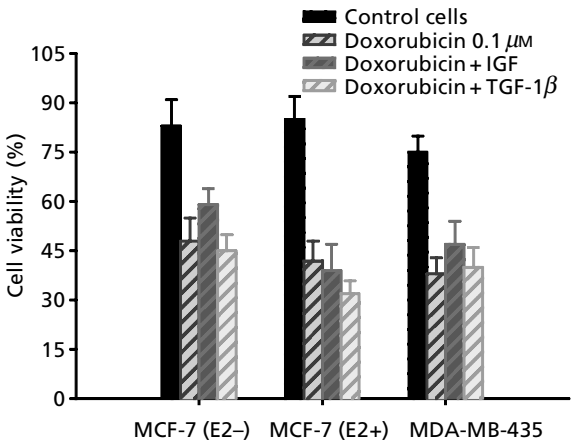

Figure 2 The effect of IGF-1 and TGFβ-1 on doxorubicin cytotoxicity in breast cancer cell lines. The cells were exposed to different concentrations of doxorubicin in the presence of osteoblast-derived growth factors as explained in the text. Results are shown as mean ± s.d. of three experiments.

regardless of the presence of E2 but it partially neutralized the doxorubicin (0.1 μM) cytotoxicity in MDA-MB-435 (Figure 2) and the number of cells undergoing apoptosis in MDA-MB-435 (Table 1). This difference is presumably a consequence of the ER function in MCF-7 cells by perhaps decreasing the expression of TGF-β1 or increasing the expression of TGF receptor (Koutsilieris et al 1999).

Cell cycle studies

Chemotherapeutic agents are known to damage the DNA and are associated with cell cycle arrest at G2/M phase in tumour cells (Myers 1997). To investigate the effect of doxorubicin on cell cycle progression and apoptosis in MCF-7 and MDA-MB-435, the cells were treated with doxorubicin as described in the Methods section and analysed by flow cytometry. The control cells (no treatment) maintained a uniform phase distribution throughout the experiments, whereas exposure to doxorubicin changed the phase distribution of both cancer cell lines in a dose (data not shown) and time dependent manner (Table 1).

Twelve-hour exposure to optimum dose (0.1 μM) or high dose (10 μM) of doxorubicin did not alter significantly the cell cycle distribution of MCF-7 and MDA-MB-435 cells compared with control cells (no treatment) ($P > 0.05$) (data not shown). The number of apoptotic cells was minimum in both cancer cell lines suggesting that doxorubicin toxicity was not due to apoptosis during the 12-h period. In addition, the presence of E2 had minimum effect on the cell cycle distribution of MCF-7 cells during this period.

In contrast, 24-h and more evidently 48-h exposure to doxorubicin significantly changed the cell cycle distribution of both cancer cell lines. The distribution of MCF-7 cells increased at G0/G1 phase and decreased at S phase following exposure to doxorubicin (0.1 or 10 μM) for 24 h and few apoptotic cells were detected in the presence or absence of E2 (data not shown). After 48-h exposure to doxorubicin at optimum dose (0.1 μM), MCF-7 (E2−)

Table 1 The cell cycle progression of MCF-7 and MDA-MB-435 cells after 48-h exposure to doxorubicin (0.1 or 10 μM).

	< G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
MCF-7				
Control (E2−)	2.0 ± 1.3	79.1 ± 1.0	14.0 ± 1.7	5.0 ± 2.1
Control (E2+)	1.5 ± 1.1	64.0 ± 3.2	28.2 ± 2.2	5.7 ± 1.4
(E2−) + IGF-1	2.8 ± 1.5	60.9 ± 4.2	26.2 ± 3.5	10.1 ± 2.1
(E2+) + IGF-1	1.0 ± 0.6	61.3 ± 3.9	30.6 ± 3.5	7.0 ± 1.4
(E2−) + TGF-β1	2.4 ± 1.4	78.3 ± 2.8	13.2 ± 2.7	6.1 ± 1.1
(E2+) + TGF-β1	2.7 ± 0.9	75.2 ± 1.2	17.8 ± 3.0	4.3 ± 2.0
Doxorubicin 0.1 μM (E2−)	4.1 ± 1.2	65.2 ± 0.9	5.2 ± 0.6	24.5 ± 2.3
Doxorubicin 0.1 μM (E2+)	3.4 ± 1.5	78.1 ± 0.8	3.4 ± 1.8	11.2 ± 2.1
Doxorubicin 10 μM (E2−)	4.8 ± 2.2	62.0 ± 2.5	18.2 ± 1.9	15.1 ± 2.3
Doxorubicin 10 μM (E2+)	4.0 ± 1.7	45.1 ± 1.9	36.0 ± 2.6	12.1 ± 1.8
Doxorubicin* + IGF-1 (E2−)	3.5 ± 1.0	69.1 ± 2.8	10.2 ± 1.3	16.8 ± 0.9
Doxorubicin* + IGF-1 (E2+)	3.2 ± 1.7	76.4 ± 3.1	16.7 ± 4.1	5.5 ± 2.6
Doxorubicin* + TGF-β1 (E2−)	2.5 ± 1.1	82.1 ± 2.7	7.1 ± 1.3	8.5 ± 2.1
Doxorubicin* + TGF-β1 (E2+)	2.8 ± 0.9	74.0 ± 5.2	11.1 ± 4.0	12.1 ± 1.4
MDA-MB-435				
Control	3.4 ± 1.9	55.0 ± 4.1	33.0 ± 1.9	8.6 ± 1.1
Control + IGF-1	4.4 ± 1.5	46.1 ± 3.8	40.0 ± 3.0	11.2 ± 1.3
Control + TGF-β1	4.1 ± 1.9	66.2 ± 2.8	21.5 ± 2.4	9.8 ± 2.4
Doxorubicin 0.1 μM	29 ± 4.2	12.0 ± 4.9	17.0 ± 3.0	46.1 ± 2.0
Doxorubicin 10 μM	27 ± 5.4	7.3 ± 2.1	39.4 ± 2.8	23.0 ± 2.4
Doxorubicin* + IGF-1	9.2 ± 3.2	31.3 ± 2.7	34.5 ± 3.0	25.7 ± 1.4
Doxorubicin* + TGF-β1	6.4 ± 2.1	52 ± 2.7	11.0 ± 4.2	30.6 ± 1.9

*Cells were treated with 0.1 μM doxorubicin. n = 6.

cells were arrested at the G2/M phase (24.5% vs 5% control) and the distribution of the cells at S (5.2% vs 14% control) and G0/G1 phases (65.2% vs 79.1% control) decreased (Table 1). Exogenous estradiol also changed the phase distribution in the cell cycle of doxorubicin-treated MCF-7 cells (Table 1). The number of MCF-7 cells at the G0/G1 phase was increased (from 65.2% to 78.1%) but it was decreased in the G2/M phase (from 24.5% to 11.2%) of the cycle ($P < 0.05$). Doxorubicin did not produce apoptosis in MCF cells either in E2− (4.1%) or E2+ (3.4%) ($P > 0.05$) (Table 1). Although it is clear that E2 has an important role on the doxorubicin action of ER+ cells, our data cannot conclude whether E2 function was the only reason for the differential action of doxorubicin. It has been reported that simultaneous exposure of the cells in-vitro to an optimal concentration of estradiol (10^{-8} M) was found to stimulate the cell cycle progression in ER+ breast cancer cells but this action was limited to early G1 phase (Fornari et al 1994).

We have tested the high dose of doxorubicin (10 μM) and the results showed that 10 μM doxorubicin did not induce apoptosis in MCF-7 cells but enhanced the cell cycle distribution at the G2/M and S phases ($P < 0.05$) (Table 1). The results are in agreement with previous findings in the literature (Fornari et al 1994; Choki et al 1998).

The effect of doxorubicin on cell cycle progression was also determined in MDA-MB-435 cells and similar results were obtained in both cancer cell lines tested. MDA-MB-435 cells were arrested at the G2/M phase (46.1% vs 8.6% control) ($P < 0.05$) following 48-h exposure to 0.1 μM doxorubicin and the cells showed a higher susceptibility towards the cytotoxic effect of doxorubicin based on the number of apoptotic cells ($29 \pm 4.2\%$) (Table 1). Likewise, the high dose of doxorubicin (10 μM) produced apoptosis in MDA-MB-435 cells ($27 \pm 5.4\%$ vs $3.4 \pm 1.9\%$ control) and increased the number of cells at the G2/M phase.

Doxorubicin-induced apoptosis of MDA-MB-435 (p53 mutant) was also confirmed by analysis of DNA fragmentation in agarose gel and TUNEL assay. As shown in Figure 3, continuous smear of low molecular weight DNA (~180 bp) was noted in MDA-MB-435 cells following 48-h exposure to doxorubicin (0.1 μM). Clearly, MCF-7 cells were relatively resistant to doxorubicin apoptosis at the same doxorubicin dose and exposure time, whereas the cytotoxic effect present in the MDA-MB-435 was induced by apoptosis. Apparently, the doxorubicin apoptosis in MDA-MB-435 (p53 mutant) cells was p53 independent. The results were confirmed by TUNEL assay. As shown in Figure 4, there was evidence of apoptotic cell death in MDA-MB-435 cells. Results suggested that doxorubicin induced apoptosis in p53 mutant/ER− cells but not in p53 wild type/ER+ cells. These results correlated with the results of cytotoxicity studies.

A dose of 50 ng mL^{-1} IGF-1 decreased the percent distribution of MCF-7 cells at G0/G1 phase and increased it at S phase regardless of the presence of estradiol (Table 1). IGF-1 partially reversed the doxorubicin (0.1 μM) cytotoxicity in the MCF-7 (E2−) cells as shown by a decreasing number of cells (3.5%) undergoing apoptosis as compared with that of control cells (4.1%) (treated with doxorubicin alone) (Table 1). In addition, the distribution of MCF-7 (E2+) cells was increased at S phase (from $3.4 \pm 1.8\%$ to $16.7 \pm 4.1\%$) but it did not change significantly at the G0/G1 phase ($78.1 \pm 0.9\%$ to $76.4 \pm 3.1\%$) in the presence of IGF-1.

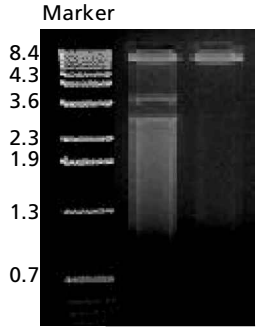

Figure 3 Analysis of DNA fragmentation on agarose gel. The breast cancer cells were exposed to 100 nM doxorubicin for 48 h then DNA was extracted as explained in Materials and Methods (n = 3). Left to right: lane 1, marker; lane 2, MDA-MB-435; lane 3, MCF-7.

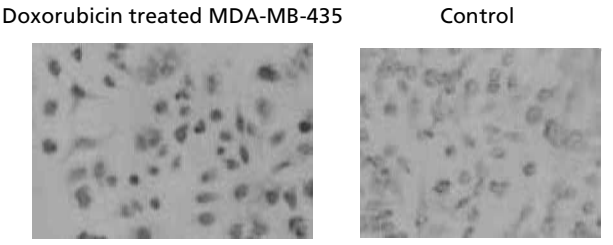

Figure 4 Example of doxorubicin-induced apoptosis detected by TUNEL assay in MDA-MB-435 cells. The detail of the experiment was described in Materials and Methods. Arrows show apoptotic cells.

Exogenous IGF-1 increased the distribution of MDA-MB-435 (ER−) cells at S phase (from $33 \pm 1.9\%$ and $40 \pm 3.0\%$) and decreased it at the G0/G1 phase (from $55 \pm 4.1\%$ to $46 \pm 3.8\%$). The doxorubicin cytotoxicity in MDA-MB-435 cells was partially reversed by IGF-1 as noted by the decreasing number of cells undergoing apoptosis (9.2%) and decreased distribution of the cells at the G2/M phase (from 11.2% to 25.7%) in the cell cycle (Table 1).

On the other hand, exogenous TGF-β1 did not effect significantly the cell cycle distribution of MCF-7 cells (E2−) but arrested (E2+) cells at the G0/G1 phase (Table 1). The percent distribution of MCF-7 (E2+) cells was increased at the S phase (11.1%) compared with MCF-7 (E2−) cells (7.1%) ($P < 0.05$) (Table 1). Therefore it is possible that ER function either decreased the expression of TGF-β1 or increased the expression of TGF-β1 receptor. In addition, TGF-β1 exhibited nearly a 5-fold increase in G0/G1 phase (52%) in doxorubicin-treated MDA-MB-435 cells compared with the cells treated with doxorubicin alone (~12%) and the number of apoptotic cells as well as the cells at G2/M phases decreased. In other words, TGF-β1 partially reversed the

doxorubicin cytotoxicity in MDA-MB-435 cells. Previously, it was demonstrated that TGFs could induce apoptosis as well as cell cycle arrest and several studies reported increased expression in breast cancer cells, which had been stimulated to undergo apoptosis by cytotoxic drugs (Fornari et al 1994; Choki et al 1998).

Conclusion

Overall results revealed that IGF produced E2-like changes in ER+ cells in regards to the cell cycle distribution, whereas TGF-β1 reversed these changes in the same cells. TGF-β1 action in human breast cancer cells might be mediated by IGF binding protein (IGFBP-3), which controls the IGF bioavailability, through an IGF independent and p53 independent mechanism (Kyprianou et al 1991). In addition, TGF-β1/TGF-β1R and IGF-1/IGFR1 can regulate growth factor bioavailability thereby controlling the growth of breast cancer cells and their responses to chemotherapy. Thus, depending on the type of manifestation, the tumour can acquire either cytotoxic drug-resistant growth in the presence of osteoblast-related growth factors IGF-1 and TGF-β1 as exemplified by (ER−) MDA-MB-435 cells, or a favourable response to chemotherapy, as in the (ER+) MCF-7 cells. Conceivably, these facts can optimize efficiency and efficacy of cytotoxic drug chemotherapy in patients with advanced breast cancer via further study into these growth factors and their potential in modulating cell cycle regulation.

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