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Original Articles

Chemotherapy Cytotoxicity of Human MCF-7 and MDA-MB 231 Breast Cancer Cells Is Altered by Osteoblast-Derived Growth Factors

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Abstract

One-third of women with breast cancer will develop bone metastases and eventually die from disease progression at these sites. Therefore, we analyzed the ability of human MG-63 osteoblast-like cells (MG-63 cells), MG-63 conditioned media (MG-63 CM), insulin-like growth factor I (IGF-I), and transforming growth factor beta 1 (TGF-β1) to alter the effects of adriamycin on cell cycle and apoptosis of estrogen receptor negative (ER^-) MDA-MB-231 and positive (ER^+) MCF-7 breast cancer cells, using cell count, trypan blue exclusion, flow cytometry, detection of DNA fragmentation by simple agarose gel, and the terminal deoxynucleotidyl transferase (TdT)-mediated nick end-labeling method for apoptosis (TUNEL assay). Adriamycin arrested MCF-7 and MDA-MB-231 cells at G_2/M phase in the cell cycle and inhibited cell growth. In addition, adriamycin arrested the MCF-7 cells at G_1/G_0 phase and induced apoptosis of MDA-MB-231 cells. Exogenous IGF-I partially neutralized the adriamycin cytotoxicity/cytostasis of cancer cells. MG-63 CM and TGF-β1 partially neutralized the adriamycin cytotoxicity of MDA-MB-231 cells but enhanced adriamycin blockade of MCF-7 cells at G_1/G_0 phase. MG-63 osteoblast-like cells inhibited growth of MCF-7 cells while promoting growth and rescued MDA-MB-231 cells from adriamycin apoptosis in a collagen coculture system. These data suggest that osteoblast-derived growth factors can alter the chemotherapy response of breast cancer cells. Conceivably, host tissue (bone)–tumor cell interactions can modify the clinical response to chemotherapy in patients with advanced breast cancer.

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Introduction

Metastatic breast cancer is the second leading cause of tumor-related death in women after lung cancer (1). The biology of metastatic breast

cancer is unique in that, unlike other solid tumors that metastasize in the skeleton, estrogen receptor positive (ER^+) breast cancer patients with bone-only metastases enjoy a favorable response to chemotherapy and favorable prognosis (2–4). Unfortunately, this is not the case for patients with ER^- breast cancer and/or widespread metastatic disease beyond the skeleton (1,5).

Among the most important local modulators involved in the pathophysiology of bone metastases is the urokinase type plasminogen activator (uPA)/plasmin system, which is thought to deregulate bone matrix deposition via activation of metalloproteases and hydrolysis of insulin-like growth factor–binding proteins (IGFBPs), resulting in an increased bioavailability of IGFs and activation of latent transforming growth factor beta 1 (TGF-β1) (6–9). Therefore, the IGFs/IGFBPs/TGF-β1/uPA regulatory system appears to play an important role in mediating cell–cell interactions in bone metastasis. Recently, osteoblasts and osteoblast-derived growth factors, such as IGF-I and TGF-β1, reversed adriamycin apoptosis of metastatic PC-3 prostate cancer cells (10), and neutralization of type I IGF receptor activity, through tyrosine kinase inhibitors, was associated with a reduction in breast cancer tumor growth (11,12).

Because at the cellular level any type of cancer therapy induces either cytostasis and/or programmed cell death, apoptosis, we investigated whether local mediators of the host tissue (bone)–cancer interactions can differentially alter chemotherapy cytostasis and cytotoxicity of ER^+ and ER^- metastatic breast cancer cells in a way that could explain a favorable or unfavorable chemotherapy response in bone metastases, respectively.

Human MG-63 osteoblast-like cells, MG-63 conditioned media (MG-63 CM), and osteoblast-related growth factors, such as IGF-I and TGF-β1, were employed to test the hypothesis of whether host tissue (bone) can modify adriamycin cytotoxicity of ER^+ MCF-7 and ER^- MDA-MB-231 human breast cancer cells in vitro. Indeed, we documented that osteoblasts and osteoblast-derived growth factors, depending on the type of breast cancer cells interacting with them, can either block or enhance the chemotherapy response of breast cancer cells.

Materials and Methods

Cell Lines and Cell Culture Assays

MG-63 osteoblast-derived human osteosarcoma cells and MDA-MB-231 human breast cancer cells were obtained from the American Type Cell Culture (ATCC, Bethesda, MD). The MCF-7 cells were kindly provided by Dr. Claude Labrie, Research Center, CHUQ, Laval University. The MG-63, MCF-7, and MDA-MB-231 cells were grown in 75-cm^2 culture flasks using Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12, Gibco/BRL, Gaithersburg, MD) containing 5% calf serum (CS). The cells were plated at a cell density of 1.5×10^4 cells in 24-well plates and grown with DMEM/F-12 medium containing various concentrations (0.5 to 5%) of CS, depending on the experiment. For experiments involving MCF-7 cells, media were supplemented with 10^{-9} M estradiol (Sigma, St. Louis, MO).

We used MCF-7 (p53 wild-type) human breast cancer cells containing estrogen receptor (ER^+), thereby representing a reliable model for studying the molecular events associated with response to chemotherapy of ER^+ breast cancer cells (13–16). MDA-MB-231 (p53 mutant) breast cancer cells are ER^- cells and have proven to be a reliable model for studying the biology of ER^- breast cancer cells (15–18). MG-63 human osteosarcoma cells are well-characterized, osteoblast-like cells that express alkaline phosphatase, osteocalcin, and parathyroid hormone receptor (PTHR). They increase cAMP production in response to PTH and express vitamin D3 receptor and estrogen receptor (ER^+). In addition, MG-63 cells increase osteocalcin expression in response to estrogen and enhance expression of type I collagen when cultured on collagen gels (ref. 19 and references therein). In addition, these cell lines were successfully used in the development of a novel three-dimensional type I collagen gel system for studying bone metastases. This system enabled us to produce for the first time morphological evidence of the osteolytic and osteoblastic reaction caused by cell–cell interaction and to analyze apoptosis in vitro (10,20–22).

Cytotoxicity Assays

We analyzed the number of live (L) and dead (D) cells, as assessed by trypan blue exclusion assay, in cell cultures exposed to 100 nM of adriamycin (Sigma) for 48 hr. The MG-63, MCF-7, and MDA-MB-231 cells were plated at 15,000 cells/well and cultured with DMEM/F-12 containing 5% CS for 24 hr. The cells were then washed with serum-free medium and cultured with DMEM/F-12 containing either 0.5% or 5% CS plus 100 nM of adriamycin, depending on the experiment. For the experiments involving

MCF-7 cells, media were supplemented with 10^{-9} M estradiol (Sigma). The ratio of live (L) to dead (D) cells (L/D) was computed and compared with that of cell cultures grown without adriamycin under identical experimental conditions (10,13,14).

Detection of Apoptosis in Simple Agarose Gel

Apoptosis was assessed by DNA fragmentation (DNA ladders with multiples of 180 base pairs) detected through simple agarose gel electrophoresis of total cellular DNA as described previously (13).

Phase Distribution in the Cell Cycle and Detection of Apoptosis by Flow Cytometry

The indices of cell cycle and apoptosis were assessed by flow cytometry; DNA content in propidium iodide (PI) and Hoechst 33342–stained cells was analyzed with a counter EPICS 753 pulse cytometer (Coulter Cytochemistry, Hialeah, FL). This technique permits the calculation of apoptotic cells excluding necrotic cells as reported previously (23). The MG-63, MCF-7, and MDA-MB 231 cells were exposed to 100 nM of adriamycin for 6, 24, and 48 hr cultured with DMEM/F-12 medium supplemented with 0.5% or 5% CS, depending on the experiment. We analyzed apoptosis (hypodiploid cells; $<G_1$) after having excluded necrotic cells and computed the percentage of apoptotic cells and percent phase distribution of all cell types into the cell cycle (23).

Effects of IGF-I, TGF-β1, and MG-63 CM on Cell Growth

We analyzed the ability of recombinant human IGF-I and TGF-β1 (R&D Systems, Minneapolis, MN) to alter the growth of MCF-7 and MDA-MB 231 cells and to protect these cells from adriamycin cytotoxicity; this was assessed by counting the number of live (L) and dead (D) cells and computing the L/D ratio in monolayer cultures. Similarly, MG-63 conditioned media (CM) was prepared using confluent MG-63 cell cultures grown in 225-cm^2 culture flasks using DMEM/F-12 without CS. CM was collected every 48 hr, pooled together, and centrifuged at 3000 rpm for 3 min. The supernatant was then filtered over protein filters with a cut-off molecular weight (MW) of 15,000 daltons (D) (Amicon, Danvers, MA) (10,20). Increasing doses of CM containing from 1 ng/ml to 100 μg/ml (final concentration in culture well) were tested in MCF-7 and MDA-MB cells to assess cell growth and cytotoxicity. Then 50 μg/ml (optimum concentration) of MG-63 CM was analyzed for its putative protective action against adriamycin cytotoxicity in breast cancer cells.

Three-Dimensional Type I Collagen Gel System (3-D System)

Collagen was prepared in our laboratory using native type I collagen extracted from rat tail tendons (21). We used this type I collagen in an aqueous solution containing 0.002 M acetic acid in a final concentration of 1.5 mg/ml. Three-dimensional gels of native collagen type I were prepared in 24-well plates by rapidly mixing MG-63, MCF-7, and MDA-MB 231 cells, or MG-63 and breast cancer cell types with type I collagen in a final concentration of 10^6 cells/ml of collagen gel. The collagen gels were left for 30 min at 37°C and then incubated in a humidified CO_2 incubator using 1 ml of DMEM/F-12 containing 5% CS (20,21). For the experiments involving MCF-7 cells, media were supplemented with 10^{-9} M estradiol (Sigma). In addition, we prepared collagen gels containing mixed cell populations of 0.5×10^6 of MG-63 and 0.5×10^6 of MCF-7 or MDA-MB 231 cells (final concentration 10^6 cells/ml of gel). Both the MG-63 and breast cancer cells were homogeneously dispersed into the type I collagen gel (21,22). After 24 hr, we changed culture media and added 100 nM of adriamycin for 48 hr. For the experiments involving MCF-7 cells, media were supplemented with 10^{-9} M estradiol (Sigma). Control collagen gels were cultured without adriamycin under identical experimental conditions. Controls containing only MG-63 cells, only MCF-7 cells, only MDA-MB 231, and mixed MCF-7 or MDA-MB 231 cells and MG-63 cells were used. Because the morphology of MG-63 and breast cancer cells (MCF-7 and MDA-MB 231 cells) is quite different from that of MG-63 cells (21,22), each cell type in the co-culture 3-D system was easily assessed. Results of the analysis were extracted from 10 different sections (number of cells) and were presented as percent above or below the appropriate controls.

Detection of Apoptosis in the 3-D System

After 48 hr of exposure to 100 nM of adriamycin, collagen gels underwent fixation with phosphate-buffered formalin 10% for 4 hr and were

then routinely processed and embedded in paraffin blocks. Serial sections of 5 μm were cut and stained with the classic trichrome-Masson techniques described previously (21,22) or were processed by the terminal deoxynucleotidyl transferase (TdT)-mediated nick end-labeling method for detection of apoptosis (TUNEL assay) (10). The sections consecutive to those stained with the TUNEL assay (Oncor, Gaithersburg, MD) were stained with standard hematoxylin/eosin to evaluate histological features of apoptosis, including cell shrinkage, chromatin condensation, and apoptotic body formation. Results from 10 different sections were presented as percent above or below controls (10).

Statistical Analysis

Our results were analyzed by Dunnett's test and the paired *t*-test, depending on the experiment. The analysis was done on triplicate determination. Statistical analysis is reported here as either statistically significant ($p < 0.05$) or statistically insignificant ($p > 0.05$; NS).

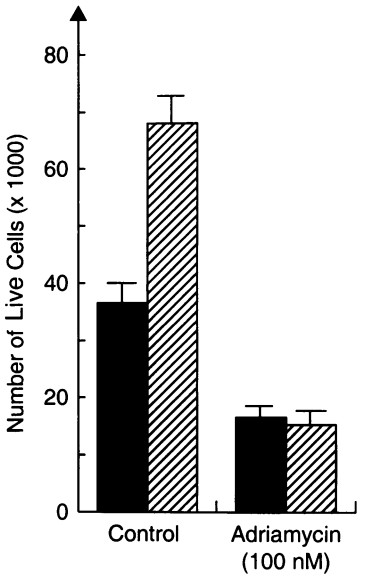

Fig. 1. Effects of adriamycin (100 nM) on number of ER$^+$ MCF-7 cells (■) and ER$^-$ MDA-MB 231 cells (▨) in monolayer cultures. Cells were cultured using DMEM/F-12 media supplemented with 5% calf serum (CS) and exposed to adriamycin for 48 hr. Adriamycin significantly decreased the number of both MCF-7 and MDA-MB 231 cells.

Results

Adriamycin Cytotoxicity of MCF-7 and MDA-MB 231 Cells in Monolayer Cultures

A 48-hr exposure to 100 nM of adriamycin remarkably decreased the number of live cells in both the MCF-7 and MDA-MB 231 monolayer cultures as documented by cell count using trypan blue exclusion (Fig. 1). In addition, the number of dead cells was significantly increased ($p < 0.05$) after 48-hr exposure to adriamycin in the MDA-MB 231 cell cultures. The same treatment did not significantly alter the number of dead cells in the MCF-7 cultures [MCF-7 cultures: control = 1084 ± 203; adriamycin = 981 ± 325 ($x \pm$ SE; p = NS); MDA-MB 231 cells: control = 2615 ± 145; adriamycin = 3503 ± 165; number of dead cells ($x \pm$ SE; $p < 0.05$)]. Because adriamycin cytotoxicity cannot be assessed without considering the number of live and dead cells in each culture (cytostasis can decrease cytotoxicity), we analyzed the ratio of live (L) to dead (D) cells in these cell cultures. The L/D ratio was decreased from 32.8 (controls) to 20.5 (adriamycin) in MCF-7 cells. Similarly, the L/D ratio decreased from 28.5 (controls) to 5 (adriamycin) in the MDA-MB 231 cells. From these results, along with the data obtained by trypan blue exclusion, we concluded that adriamycin possibly exerted both a cytostatic and cytotoxic effect on MBA-MB 231 cells whereas it exerted only a cytostatic effect on MCF-7 cells.

To confirm the cytostatic and/or cytotoxic effect of adriamycin on these cells, we used flow cytometry with the double staining method (PI and Hoechst 33342) as described in Materials and Methods (22). A few apoptotic MCF-7 and MDA-MB 231 cells were detected by flow cytometry in control cultures (apoptosis = $<G_1$; hypodiploid cells). Apparently, the MDA-MB 231 cells progressed more rapidly than MCF-7 cells in the cell cycle under these experimental conditions (Tables 1 and 2). This was in concert with data obtained by trypan blue exclusion where, although both these cell lines were plated at an equal cell concentration (15,000 cells/well), the number of MDA-MB 231 cells was significantly higher than that of MCF-7 cells [74,525 ± 1839 vs. 35,560 ± 1114 ($x \pm$ SE; $p < 0.05$)] after 24 hr of post-plating incubation and 48 hr incubation under the experimental conditions described in Materials and Methods (Fig. 1).

In addition, a 6-hr exposure to 100 nM of adriamycin produced little change in the phase distribution of MCF-7 and MDA-MB 231 cells and no evidence of apoptosis in both cell cultures

Table 1. Apoptosis and phase distribution of ER^+ MCF-7 human breast cancer cells in cell cycle ($x \pm SE$)[a]

	Controls	ADR	ADR + MG-63 CM	ADR + IGF-I	ADR + TFG-β1
Apoptosis (% at $<G_1$)					
$<G_1$	1.9 ± 1	2.4 ± 2	3.0 ± 2	1.8 ± 1	2.5 ± 1
Phases in cell cycle (% at G_1, S, G_2/M)					
G_1/G_0	64.5 ± 2	80.0 ± 4	89.0 ± 3	69.5 ± 3	87.5 ± 4
S	26.5 ± 1	4.5 ± 2	5.5 ± 1	21.5 ± 3	8.0 ± 1
G_2/M	9.0 ± 1	15.5 ± 2	5.5 ± 1	9.5 ± 2	4.5 ± 2

[a]Cells were cultured in DMEM/F-12 medium supplemented with 10^{-9} M estradiol and 5% CS. Exposure to adriamycin (ADR: 100 nM), MG-63 CM (50 μg/ml), IGF-I (50 ng/ml), and TGF-β1 (25 ng/ml) was for 48 hr.

(data not shown). The phase distribution of MCF-7 and MDA-MB 231 cells in the cell cycle was significantly altered in a time-dependent manner by the exposure to adriamycin. After 24 hr exposure and, more evidently, after 48 hr exposure to 100 nM of adriamycin, the distribution of MCF-7 cells at G_1/G_0 phase increased ($p < 0.05$) and that at S phase decreased ($p < 0.05$) in the cell cycle without producing apoptosis (Table 1). Moreover, a 24-hr exposure to 100 nM of adriamycin produced a blockade of MDA-MB 231 at G_2/M phase ($p < 0.05$) and apoptosis ($p < 0.05$). This finding was even more evident after 48 hr exposure. The G_1/G_0 and S phases of surviving MDA-MB 231 cells contained a few cells after 48 hr treatment with 100 nM of adriamycin, suggesting that MDA-MB 231 cells overcoming G_2/M blockade in the cell cycle had undergone apoptosis (Table 2). Therefore, exposure to adriamycin produced G_2/M blockade and apoptosis in a time-dependent manner in (p53 mutant/ER^-) MDA-MB 231 cells but not in (p53 wild-type/ER^+) MCF-7 cells, which were apparently blocked at G_1/G_0 phase (Tables 1 and 2).

Adriamycin apoptosis of MDA-MB 231 cells, detected by flow cytometry and trypan blue exclusion, was also confirmed by analysis of DNA fragmentation on a simple agarose gel, a classical method of detecting the DNA ladders that accompany programmed cell death, apoptosis, in vitro. The adriamycin apoptosis of (p53 mutant) MDA-MB 231 cells apparently took place via a p53-independent mechanism. The failure of adriamycin (100 nM) to induce apoptosis of (p53 wild-type) MCF-7 cells, as noted by flow cytometry and trypan blue exclusion, was also confirmed by analysis of DNA fragmentation on a simple agarose gel (Fig. 2). Consequently, adria-

Table 2. Apoptosis and phase distribution of ER^- MDA-MB 231 human breast cancer cells in cell cycle ($x \pm SE$)[a]

	Controls	ADR	ADR + MG-63 CM	ADR + IGF-I	ADR + TFG-β1
Apoptosis (% at $<G_1$)					
$<G_1$	2.6 ± 1	29.4 ± 2	9.0 ± 2	10.8 ± 1	7.5 ± 1
Phases in cell cycle (% at G_1, S, G_2/M)					
G_1/G_0	57.0 ± 2	9.0 ± 4	31.0 ± 3	32.0 ± 3	49.5 ± 4
S	34.0 ± 2	18.5 ± 1	25.0 ± 1	33.5 ± 3	15.0 ± 1
G_2/M	9.0 ± 1	72.5 ± 2	35.0 ± 1	34.5 ± 2	35.5 ± 2

[a]Cells were cultured in DMEM/F-12 medium supplemented with 5% CS. Exposure to adriamycin (ADR: 100 nM), MG-63 CM (50 μg/ml), IGF I (50 ng/ml), and TGF-β1 (25 ng/ml) was for 48 hr.

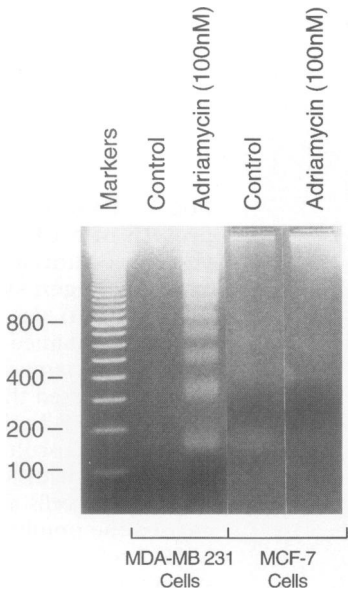

Fig. 2. Adriamycin apoptosis as assessed by analysis of DNA fragmentation on a simple agarose gel. MCF-7 (p53 wild-type) and MDA-MB 231 (p53 mutant) cells were maintained in DMEM/F-12 media supplemented with 5% DCC-CS and exposed to 100 nM of adriamycin for 48 hr. DNA was then extracted and analyzed as described in Materials and Methods. The presence of ~180 bp DNA ladders (DNA fragmentation) marks apoptotic death in vitro. Notably, only MDA-MB 231 cells extracts presented with evidence of DNA fragmentation. The adriamycin apoptosis in MDA-MB 231 cells was apparently p53-independent. Obviously, MCF-7 cells were resistant to adriamycin (100 nM) apoptosis in vitro.

mycin cytostasis, not adriamycin apoptosis, mediated the reduction in the number of live cells and the L/D ratio of MCF-7 cells. These data suggest that a pharmacological dose of 100 nM adriamycin for 48 hr will produce an arrest of MCF-7 cells at G_1/G_0 phase (cytostastic effect) and of MDA-MB 231 cells at G_2/M phase (cytostatic effect) and apoptosis (cytotoxic effect).

Effects of MG-63 CM, IGF-I, and TGF-β1 on Cell Growth and Adriamycin Cytotoxicity

Increasing doses of MG-63 CM stimulated the growth of MDA-MB 231 cells in a dose-dependent manner. A dose of 50 μg/ml (final protein concentration) of MG-64 CM produced maximum stimulation of MDA-MB 231 cell growth (25% above controls; $p < 0.05$), whereas MG-63 CM exerted a dose-dependent inhibitory effect on MCF-7 cells. A dose of 25 μg/ml of MG-63 CM produced maximum inhibition (30% below controls; $p < 0.05$) of MCF-7 cell growth. In addition, 50 ng/ml of IGF-I increased by 30–35% ($p < 0.05$) and 25 ng/ml of TGF-β1 decreased by 50% and 65% ($p < 0.05$) the growth of MCF-7 and MDA-MB 231 cells, respectively. These data are in concert with previous studies assessing the role of osteoblast-related growth factors and osteoblast CM in breast cancer cells (23,24).

A dose of 50 μg/ml of MG-63 CM and 25 ng/ml of TGF-β1 increased the percent distribution of MCF-7 cells at G_1/G_0 phase [from 64.5 ± 2 to 70.5 ± 4 and 64.5 ± 2 to 71.5 ± 1, respectively ($x \pm SE$; $p < 0.05$)] and decreased it at S phase [from 26.5 ± 1 to 20 ± 3 and 26.5 ± 1 to 20.5 ± 2, respectively ($x \pm SE$; $p < 0.05$)]. In addition, these doses enhanced adriamycin (100 nM) cytostasis of MCF-7 cells, increasing further the percent distribution of MCF-7 cells at G_1/G_0 phase (Table 1). Exogenous IGF-I (50 ng/ml) decreased the percent distribution of MCF-7 cells in G_1/G_0 phase [from 64.5 ± 2 to 55.0 ± 3 ($x \pm SE$; $p < 0.05$)] and increased it at S phase [from 26.5 ± 1 to 36.0 ± 2 ($x \pm SE$; $p < 0.05$)]. Moreover, IGF-I significantly reversed adriamycin (100 nM) cytostasis of MCF-7 cells as assessed by flow cytometry (Table 1).

Furthermore, the L/D ratio was decreased from 32.8 (controls) to 20.5 by adriamycin (100 nM) in MCF-7 cultures. This effect of adriamycin on MCF-7 cells was further enhanced by the addition of MG-63 CM (50 μg/ml; L/D ratio decreased from 20.2 to 15.5) and of TGF-β1 (25 ng/ml; L/D ratio decreased from 20.5 to 14.0); the effect of adriamycin was partially reversed by IGF-I (50 ng/ml; L/D ratio increased from 20.5 to 23.5). Concomitant treatment with adriamycin, MG-63 CM, and TGF-β1 further decreased the L/D ratio to 10.0, which suggests that MG-63 CM and TGF-β1 additively enhanced adriamycin cytostasis of MCF-7 cells.

Doses of 50 ng/ml of IGF-I and 50 μg/ml of MG-63 CM increased the distribution of MDA-MB 231 cells at S phase [from 34.0 ± 2 to 41.0 ± 2 and from 34.0 + 2 to 40.5 ± 3, respectively ($x \pm SE$; $p < 0.05$)], but decreased this distribution at G_1/G_0 phase [from 57.0 ± 2 to 50.0 ± 4 and 57.0 ± 2 to 48.5 ± 4, respectively ($x \pm SE$; $p < 0.05$)]. TGF-β1 (25 ng/ml) increased distribution of MDA-MB cells in G_1/G_0 phase [from 57 ± 2 to 63 ± 3 ($x \pm SE$; $p < 0.5$)]. Doses of 50 ng/ml of IGF-I, 50 μg/ml of MG-63 CM, and 25 ng/ml of TGF-β1 partially reversed the adriamycin cytotoxicity of MDA-MB 231 cells as noted by the decreasing number of cells undergoing apoptosis and decreased distribution of

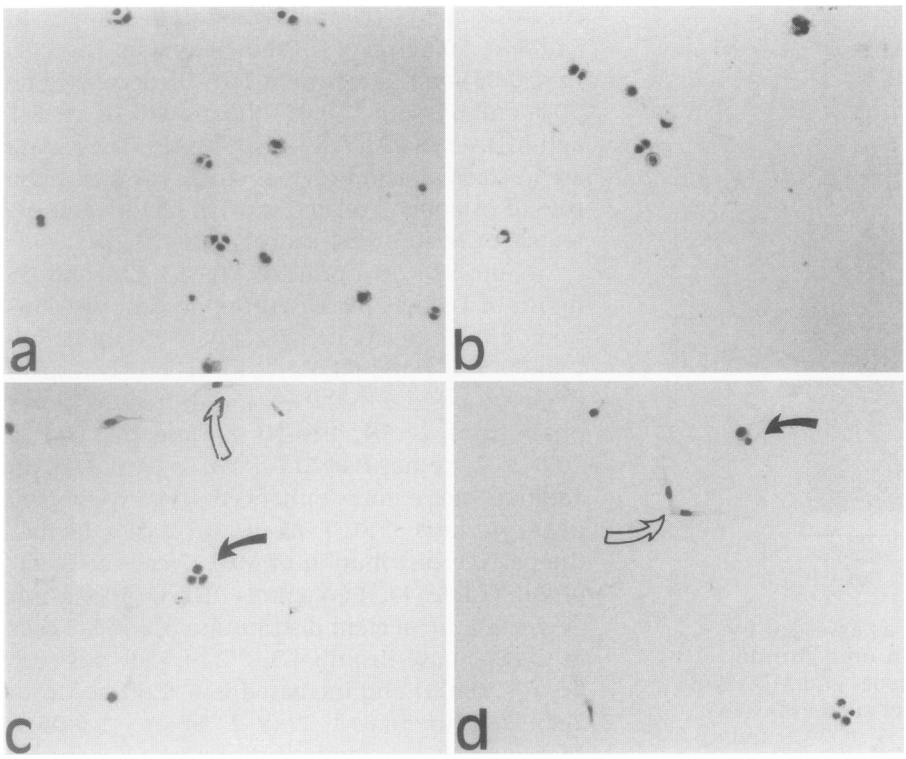

Fig. 3. Example of growth of MCF-7 cells in the three-dimensional type I collagen system (3-D system). (a) The 3-D system maintained MCF-7 cells. (b) Adriamycin significantly decreased the number of MCF-7 cells in the 3-D system. (c) Co-culture of MCF-7 cells with MG-63 osteoblast-like cells also decreased the number of MCF-7 cells grown in this system. (d) Adriamycin exposure further decreased the number of MCF-7 cells under these co-culture conditions. White arrows, MG-63 cells; black arrows, MCF-7 cells.

MDA-MB 231 cells at G_2/M phase in the cell cycle (Table 2).

In addition, the L/D ratio was accordingly changed in MDA-MB 231 cells [controls: L/D ratio = 28.5; adriamycin (100 nM): L/D ratio = 5; adriamycin + MG-63 CM: L/D ratio = 10.9; adriamycin + TGF-β1: L/D ratio = 9.2; adriamycin + IGF-I: L/D ratio = 15.0; adriamycin + MG-63 CM + TGF-β1: L/D ratio = 19.8]. The combination of MG-63 CM with IGF-I and TGF-β1 was maximally effective in protecting MDA-MB 231 cells from adriamycin cytotoxicity, which suggests that MG-63 CM, IGF-I, and TGF-β1 may act via distinct molecular pathways to protect MDA-MB 231 cells.

MG-63 Osteoblast-Mediated Protection of MDA-MB 231 Cells from Adriamycin Apoptosis in the 3-D System

The 3-D type I collagen system maintained the growth of MCF-7 cells well (Fig. 3a). The MCF-7 cells were resistant to adriamycin apoptosis in this system (Fig. 4c). The number of MCF-7 cells decreased by 30% ± 5 ($p < 0.05$) after 48 hr exposure to adriamycin compared to controls (Fig. 3b). Co-culture of MG-63 with MCF-7 cells inhibited growth of both cell types by 40% ± 8 ($p < 0.05$) compared to control cultures in the 3-D system, which suggests that cell–cell interactions inhibit the growth of both MCF-7 and MG-63 cells (Fig. 3c). This result is in agreement with a recent report documenting the existence of MCF-7, breast cancer–derived, specific inhibitors of a protein nature (MW = 700 to 4000 D) for osteoblasts, including MG-63 cells (24,25). Adriamycin (100 nM) further decreased (55% ± 7; $p < 0.05$) the number of MCF-7 cells in this 3-D system containing MG-63 osteoblast-like cells compared with that of control cultures using adriamycin-free media.

The 3-D system also maintained the growth of MDA-MB 231 cells (Fig. 5a). After 48 hr exposure to adriamycin, the number of MDA-MB 231 cells decreased by 45% ± 6 ($p < 0.05$) in the 3-D system (Fig. 5b). It is noteworthy that the number of MDA-MB 231 cells in the 3-D systems containing MG-63 cells did not decrease with either the presence of MG-63 cells using adriamycin-free media (Fig. 5c) or the 48-hr exposure to adriamycin, compared to controls (Fig. 5d). These data suggest that the early establishment of local cell–cell interactions between MG-63 and MDA-MB 231 cells in the 3-D system protects MDA-MB 231 cells from adriamycin cytostasis/apoptosis, thereby promoting the adriamycin-resistant growth of MDA-MB 231 cells in vitro.

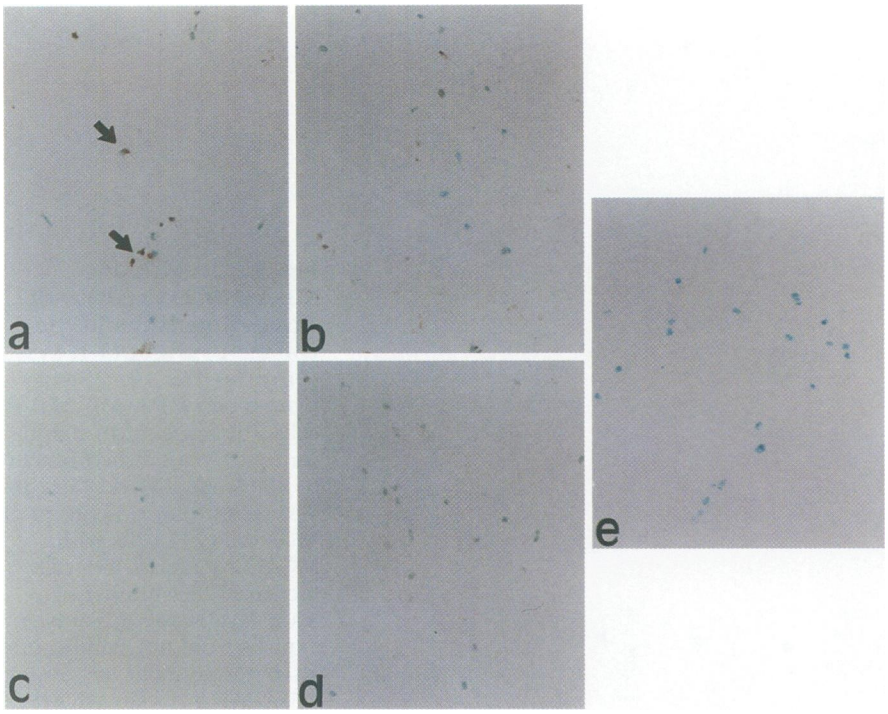

Fig. 4. Example of adriamycin (100 nM) apoptosis in MCF-7 and MDA-MB 231 cells grown in type I collagen gel system documented by terminal deoxynucleotidyl transferase (dTd)-mediated nick-end labeling method (TUNEL assay). (a) Adriamycin apoptosis of MDA-MB 231 cells (arrows); postive TUNEL assay. (b) Inhibition of adriamycin apoptosis in MDA-MB 231 cells by co-culturing with MG-63 osteoblast-like cells under identical experimental conditions. (c) MCF-7 cells were resistant to adriamycin apoptosis. (d) Adriamycin did not induce apoptosis in the 3-D system containing MCF-7 cells and MG-63 cells. (e) Negative control of TUNEL assay.

In addition, homogeneous dispersion of MG-63 and MDA-MB 231 cells partially rescued MDA-MB 231 cells from adriamycin apoptosis, decreasing the number of apoptotic cells by 55% $\pm$ 4 ($p < 0.05$) as detected by TUNEL assay in the 3-D system (compare Fig. 4a with 4b). The MG-63 cells did not undergo apoptosis (p = NS) after 48 hr exposure to adriamycin (100 nM). Conceivably, MG-63 osteoblast-like cells secrete "survival factors" that can optimize their own defense and that of MDA-MB 231 cells to adriamycin apoptosis in vitro.

Discussion

The ability of various neoplasms to metastasize selectively into specific organs depends on metastatic properties of tumor subclones, stochastic elements that interfere with the metastatic process, and local interactions with the host tissue (9,26,27). Because breast cancer patients with bone-only ER^+ tumor metastases have been reported to have a favorable response to chemotherapy and favorable prognosis (2–4), we assessed the ability of human osteoblast-like cells and osteoblast-derived growth factors to differentially influence chemotherapy cytotoxicity of ER^+ MCF-7 and ER^- MDA-MB 231 cells.

It is known that relatively low concentrations (50–500 nM) of adriamycin interfere with DNA unwinding (28), 1 μM of adriamycin inhibits topoisomerase II expression (29), and suprapharmacological concentrations (5–10 μM) of adriamycin produce non-protein-associated DNA strand breaks, suggesting free radical–mediated apoptosis (30). Therefore, we have chosen to use the concentration of 100 nM of adriamycin in our experiments because this represents a common pharmacological dose in clinical practice and it is a well-characterized dose with respect to its action on ER^+ MCF-7 cells. It is noteworthy that chronic exposure to relatively low concentrations (50–100 nM) of adriamycin, typically sustained in the peripheral blood for up to 12 hr following i.v. administration of adriamycin in breast cancer patients, appears to engage a unique growth/cell arrest/death pathway involving damage to nascent DNA, endoreduplication of DNA, and differentiation induction of proteins. This phenomenon is related to the increased phase distribution at G_1/G_0 phase and is associated with a gradual reduction in expression of the c-*myc* oncogene in ER^+ MCF-7 breast cancer cells (31).

Our data are in agreement with the previous report of adriamycin cytostasis (100 nM) occurring with blockade of ER^+ MCF-7 cells at G_1/G_0 phase (31). Unlike adriamycin cytostasis of MCF-7 cells, here adriamycin exerted a blockade

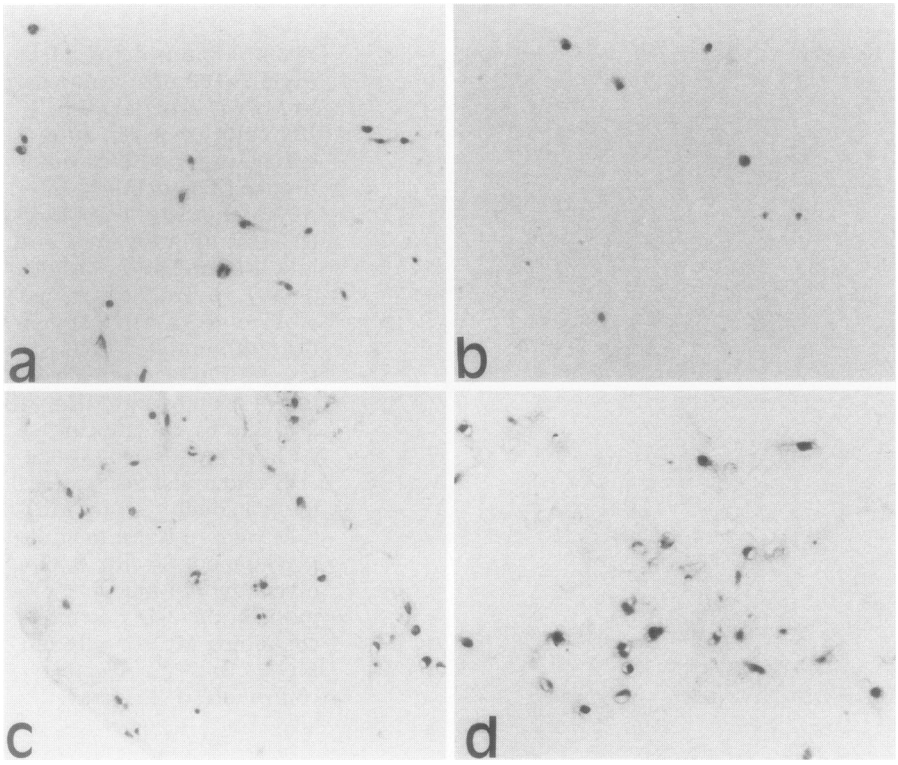

Fig. 5. Growth of MDA-MB 231 cells in the three-dimensional type I collagen system (3-D system). (a) The 3-D system maintained MDA-MB 231 cells. (b) Adriamycin significantly decreased the number of MDA-MB 231 cells in the 3-D system. (c) Co-culture of MDA-MB 231 cells with MG-63 osteoblast-like cells increased the number of MCF-7 cells. (d) Adriamycin exposure did not significantly affect the number of MDA-MB 231 cells when co-cultured with MG-63 cells.

at the G_2/M phase and apoptosis of MDA-MB 231 cells. Apparently, the MDA-MB 231 cells withstanding DNA damage were arrested first at the G_2/M blockade and those overcoming the G_2/M blockade underwent apoptosis. Because the MDA-MB 231 cells are p53 mutants, adriamycin apoptosis of MDA-MB 231 cells is obviously p53-independent.

It is interesting to note that ER^+ MCF-7 cells in control cultures presented with an increased cell distribution at G_1/G_0 phase. The latter were further enhanced with exposure to increasing concentrations of estradiol (10^{-7} M; unpublished data, Koutsilieris et al.). Therefore, ER function is strongly associated with an increased distribution of MCF-7 cells at G_1/G_0 phase. However, our data cannot conclude whether ER function is the only reason for such differential action of adriamycin on ER^+ MCF-7 cells (cytostasis; blockade at G_1/G_0 phase) and ER^- MDA-MB 231 cells (cytostasis; blockade at G_2/M, followed by apoptosis).

In addition, exogenous TGF-β1 and MG-63 CM inhibited the growth of ER^+ MCF-7 cells, increasing the distribution of MCF-7 cells at G_1/G_0 phase in the cell cycle. In our study, adriamycin (100 nM) cytostasis of (ER^+) MCF-7 cells was further enhanced by exogenous MG-63 CM and TGF-β1, but was partially reversed by exogenous IGF-I. Because it is known that MG-63 CM contains TGF-β1 (19), it is conceivable that TGF-β1 mediated, at least in part, MG-63 CM cytostasis (blockade at G_1/G_0 phase) of ER^+ MCF-7 cells. Recently, it was reported that TGF-β1 action of human breast cancer cells may be mediated by IGFBP-3 through an IGF-independent and p53-independent mechanism (24). It is noteworthy that IGFBPs can control the IGF's bioavailability, in vitro and in vivo (6,7,20). Therefore, it is conceivable that TGF-β1/TGF-β receptor, type I IGF receptor/IGF-I/IGFBP-3, and proteases that can regulate growth factor bioavailability, are modulating the biology of breast cancer cells, thereby controling the growth, apoptosis, and possibly, tumor response to chemotherapy (14,24).

Unlike growth factor actions on MCF-7 cells, exogenous IGF-I and MG-63 CM stimulated whereas TGF-β1 inhibited the growth of ER^- MDA-MB 231 cells. MG-63 osteoblast-like cells, MG-63 CM, IGF-I, and TGF-β1 protected ER^- MDA-MB-231 breast cancer cells from adriamycin cytotoxicity. This protection from adriamycin apoptosis of MDA-MB-231 cells resulted in a cytotoxic drug-resistant growth of MDA-MB-231 cells in our 3-D co-culture system containing MG-63 osteoblasts. These data are similar to

those documented using human PC-3 prostate cancer cells in this system (10).

The importance of host tissue–tumor interactions in the pathophysiology of bone metastases was recently recognized by analyzing the osteoblastic reaction produced by PC-3 prostate cancer cells (21), developing morphological evidence of "osteolysis" in response to local growth of MCF-7 and ZR-75 breast cancer cells (22), and documenting evidence for the angiogenic potential of prostate cancer cells in only the presence of stromal fibroblasts (32). In addition, interleukin 6 was found to be a resistance factor for etoposide-mediated cytotoxicity of PC-3 and DU145 prostate cancer cells (32) and IGF-I was named as the survival factor of PC12 cell apoptosis (33). Furthermore, IGF-I inhibition of the apoptosis of PC12 cells has been associated with an increased expression of the *bcl-xL* gene product, suggesting that growth factors can regulate apoptosis-related genes, which in turn modulate apoptosis and cell survival (34). It is noteworthy that a correlation exists between apoptosis, tumorogenicity, and expression of IGF-I/type I IGF receptor (35–37). Recently, it has been reported that particular checkpoints in the molecular pathways of apoptosis can meet with growth factor–signal transduction pathways (38,39). These checkpoints implicate serine phosphorylation-mediated elimination of a death agonist *Bad* gene product as a response to survival factors (40).

In this context, our data suggest that osteoblast-derived growth factors can exert differential influence on ER^- MDA-MB-231 and ER^+ MCF-7 breast cancer cells, thereby modifying their response to chemotherapy. Conceivably, depending on the type of breast cancer clones, the tumor can acquire either cytotoxic drug-resistant growth in the presence of osteoblast-related growth factors, as exemplified by ER^- MDA-MD 231 cells, or a favorable response to chemotherapy, as exemplified by ER^+ MCF-7 cells. Therefore, it is possible that chemotherapy cytotoxicity is favorably enhanced by bone-derived modifiers in this selective group of patients with advanced ER^+ breast cancer having bone-only metastases, resulting in favorable prognosis.

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