Organ-selective chemoresistance in metastasis from human breast cancer cells: inhibition of apoptosis, genetic variability and microenvironment at the metastatic focus

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We hypothesized that the development of the most resistant cells during metastasis is favored by anti-apoptotic proteins, leading to the acquisition of an adaptive phenotype crucial to drug resistance at the metastatic foci. In order to test it, we induced metastasis in nude mice, injecting orthotopically 435/Bcl-xL or 435/Neo cells, transfected previously with the luciferase gene to use it as a tumor marker, and treated them with a therapeutic dose of docetaxel. We monitored metastasis in mice by calculating tumor cell equivalents (TCEs) present in tissues. Between docetaxel-treated and non-treated 435/Bcl-xL, luc mice significant differences in the metastatic burden of lymph nodes (P = 0.02) and viscera (P = 0.02) were observed. However, treatment did not significantly decrease metastatic burden in bones (P = 0.19). Additionally, we analyzed the clonality of metastasis from lung, bone and lymph node by genomic DNA fingerprinting. Bcl-xL enhanced cell genetic instability in terms of gain and loss fractions (GF = 0.18 and LF = -0.21) when compared with the control 435/Neo (GF = 0.15 and LF = -0.14). Thus, genetic instability might be a molecular mechanism favored by Bcl-xL, evolved in the selection process of breast cancer progression, which results in different genetic changes among metastases from lung, bone or lymph node, favoring organ-selective chemoresistance.

Introduction

There is a need for markers that can predict the efficacy of adjuvant chemotherapy in patients with breast carcinomas. In fact the main efforts are being concentrated on the accurate prediction of the outcome of primary treatment among patients with early stage disease (1). Inhibition of apoptosis can influence the response of tumor cells to anticancer therapy (2). It is reasonable to suggest that one cause why recurrent or metastatic disease is less responsive to chemotherapeutic regimens, when compared with the response rate obtained for the same disease at initial presentation or before dissemination, is because of the tumor’s acquisition of an anti-apoptotic gene. Indeed, highly metastatic cells tend to exhibit a greater survival ability and resistance to apoptosis than poorly metastatic ones (3–6).

The proteins encoded by the Bcl-2 family genes play a central role in apoptosis regulation, functioning as either inducers or blockers of cell death (7,8). Most Bcl-2 family proteins reside in mitochondrial membranes, and anti-apoptotic members such as Bcl-2 and Bcl-xL can prevent cell death induced by reactive oxygen species, ceramide and anticancer drugs (9–11).

Bcl-2, like Bcl-xL, is phosphorylated by agents that disrupt microtubule architecture, including taxanes (paclitaxel and docetaxel), vincristine, vinblastine or colchicine. The loss of microtubule integrity and cytotoxicity are accompanied by Raf-1 phosphorylation (12). The anticancer drug taxol induces serine phosphorylation of the loop domain of Bcl-2 or Bcl-xL leading to loss of their anti-apoptotic function by affecting membrane channel function, which may regulate the pre-apoptotic mitochondrial release of cytochrome c into cytosol or mitochondrial permeability transition, followed by apoptosis (13–15).

Recently we have analyzed the cytotoxicity of taxanes in several breast cancer cells showing that apoptosis significantly decreased in vitro when cells over-express Bcl-2 or Bcl-xL (16). The survival advantage to metastatic cells over-expressing anti-apoptotic proteins was induced by the activation of the Stat3 pathway conferring their chemoresistance (17).

Since anti-apoptotic proteins might promote displacement in the death/proliferation equilibrium in breast cancer cells, in the direction of enhanced survival and adaptation to a new microenvironment, we hypothesized that the development of the most resistant cells during metastasis are favored by anti-apoptotic proteins, leading to the acquisition of an adaptive phenotype in breast cancer cells crucial to drug resistance at the metastatic foci.

In an attempt to answer this question, we monitored the metastatic activity of MDA-MB 435 tumors in Nude Balb/c mice treated with or without docetaxel. The human breast cancer cell line 435 transfected previously with the Bcl-xL gene and labeled with the luciferase gene, as a tumor cell marker, was inoculated into the mammary fat pad (i.m.f.p.) to obtain tumors, and metastasis. Docetaxel was administrated after removing the tumor, as conventional clinical strategy. Metastatic cells lodged in bones were more resistant than cells lodged in lymph nodes or in viscera. Genetic instability, which resulted in different genetic changes among metastases from lung, bone or lymph node foci might allow the selection of the organ-specifically most adaptive phenotype, contributing to the fact that excellent responses after treatment with docetaxel are not necessarily translated into cures.

Materials and methods

Human breast carcinoma cell cultures and transfections

MDA-MB 435 cell cultures (435) were maintained in 1:1 (v/v) mixture of DMEM and Ham F12 medium (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), and 2 mM l-glutamine in 5% CO2–95% air at 37°C in a
humidified incubator. Transfections were carried out with Lipofectin (Life Technologies, Gibco BRL, Gaithersburg, MD). Selection of 435/Bcl-xL and 435/Neo cells started 48 h after transfection of pSFFV-Neo-Bcl-xL or pSFFV-Neo-Bcl-2, and pSFFV-Neo using 500 µg/ml of neomycin G418 (Life Technologies, Gibco BRL). The pool of transfectants was used to avoid the possibility of clonal variations.

Experimental model to test tumorigenic and metastatic activity of cells

Seven-week-old athymic Nude Balb/c female mice were used to generate experimental model to test tumorigenic and metastatic activity of cells elsewhere (20). Briefly, 1

The MTT tetrazolium assay was used to measure cell viability, as described elsewhere (20). Different tissues and organs were examined by luciferase assay.

Metastatic variants were obtained by primary culture from bone, lung and lymph nodes metastatic 435/Bcl-xL tumors, and lung metastasis from 435/Neo tumors induced in mice. Monolayers of metastatic cells were obtained from trypsin-treated histocultures (tumor fragments of 1 mm3) maintained until growth in medium supplemented with 20% fetal bovine serum, in the presence of 500 µg/ml of neomycin G418.

Seven-week-old athymic Nude Balb/c female mice were used to generate orthotopic primary tumors (19) by inoculation of 1 x 105 cells in 0.05 ml of medium without serum in the right inguinal mammary gland (i.m.f.p.). Tumors were obtained at day 45, and at day 110 animals were killed and organs removed, weighed and examined for metastasis. Metastatic involvement was determined by microscopic examination of hematoxylin–eosin stained paraffin sections.

Tumor volume was calculated using the formula: Volume (mm3) = L x W x 0.5, where L and W are the major and minor diameters in millimeters, respectively.

Drugs used

For in vitro experiments we used stock solutions (1 mg/ml) of docetaxel (Aventis Pharma, France), or paclitaxel (Sigma, St Louis, MO) prepared with 100% ethanol and stored at –80°C; doxorubicin (Fedic-Mejji Farma S.A., Acalá de Henares); and vincristine (Pharmacia & Upjohn, S.p.A., Nerviano, Italy).

For in vivo treatments, treatment solutions were obtained by mixing 1 vol of docetaxel stock solution at 50 mg/ml and 1 vol of Tween 80. The final dilution was prepared by adding 5% glucose in water to required concentrations. Chemotherapy was begun after 2 days of removing tumors. Docetaxel at 15 mg/kg was administered intraperitoneally every 4 days by three injection treatments. Chemotherapy was begun after 2 days of removing tumors. Docetaxel at 15 mg/kg was administered intraperitoneally every 4 days by three injections in the treated group. The control group was treated with physiological saline.

Cell viability assay

The MTT tetrazolium assay was used to measure cell viability, as described elsewhere (20). Briefly, 1 x 105 cells/well were seeded in 96-well microtiter plates in complete medium. After 48 h incubation, the cells were rinsed twice and incubated with serum-free media supplemented with the drugs for 24 h. Drug dilution was prepared by adding 5% glucose in water to required concentrations. Five microliter of MTT solution (5 mg/ml) was added to the cultures and the incubation continued for a further 4 h period after which 100 ml of DMSO were added. Forming formazan crystals were allowed to dissolve for 16 h before measuring optical density at 540 nm on a microplate reader.

Clonogenic survival

Colony formation of 435 cells over-expressing Bcl-xL and Neo control was assessed as described previously (21). Briefly, cells were seeded at 100 cells/well in 6-well plates, and incubated overnight. After a 24-h exposure to the drug solutions, the wells were washed in drug-free medium, followed by a 10-day incubation in drug-free medium to allow colony formation. Then the colonies were fixed and stained by the addition of 0.5% Löffler’s Methyline Blue (Merck KgA, Darmstadt, Germany) in 50% ethanol for 45 min at room temperature. Visible colonies were counted to determine the percent colony formation of plated cells for each drug treatment with regard to colony-forming values of untreated controls. Values were expressed as the mean ± SD from triplicate experiments.

Conditioned medium (CM) obtainment

CM was collected from histocultures, 50 ml/100 mg tissue fragments. Medium supplemented with HEPES, 1% of FBS and 50 µg/ml gentamicine was used to rock cells for 6 h. Then supernatant was removed and supplied by culture medium supplemented with 0.26 mg/ml seroalbumina followed by 24 h placed in 5% CO2-95% air at 37°C in a humidified incubator. CM was harvested, filtered in 0.22 µm filter and stored at –80°C until used.

Before drug treatment, cells were incubated for 4 days with CM supplemented with 1% FBS (22).

Protein expression

Cells from exponential cultures or frozen breast tumor tissue were lysed in 200 µl buffer (50 mM Tris, 150 mM NaCl, 0.01% SDS, 1% NP-40, 0.5% sodium deoxycholate) for western-blot analysis. Sample volumes were adjusted to contain 50 µg of protein as determined by the BCA, Protein Assay Reagent (Pierce, Rockford, IL). Following polyacrylamide gel electrophoresis the immunodetected proteins were transferred onto nitrocellulose membrane, stained with ECL (Amersham, Life Science, Cleveland, OH) and non-specific protein-binding sites blocked by a 5% solution of non-fat dried milk in PBS. Membranes were first incubated with polyclonal rabbit antibodies specific for human Bcl-x at a 1:1000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) or Bcl-2 monoclonal antibody 124 (Cambridge Research Biochemicals, Cheshire, UK) followed by a peroxidase-conjugated goat anti-rabbit (second antibody) (Amersham). Immunoreactive bands were visualized on Hyperfilm MP (Amersham) using the ECL system chemiluminescent reaction (Amersham). Molecular weights (MW) were established using pre-stained MW markers (Bio-Rad Laboratories S.A., Madrid, Spain).

An antihuman actin monoclonal antibody (Sigma) was also used as an internal standard for densitometric analysis of X-ray film, ‘Molecular Analyst Software’ (Bio-Rad, Richmond, CA).

Luciferase assay

Luciferase activity in cell or tissue extracts was measured by chemiluminescence. The presence of substrate luciferin using the standard luciferase assay kit (Promega, Madison, WI). Production of light was measured using a Turner Designs luminometer model TD 20/20, after addition of 100 µl of luciferase assay reagent (Promega) to 20 µl of cell lysate or tissue homogenate.

Cell and tissue lysates were prepared by performing a freeze-thaw cycle in reporter lysis buffer (RLB). Whole organ and tissue extracts were prepared by mechanical homogenization of tissue in RLB at a 1:1 ratio (weight/volume), using an Ultra-Turrax T-25, tissue homogenizer (Janke and Kunkel, Staufen, Germany) followed by centrifugation at 25 000 g for 45 min at 4°C, to remove insoluble particles. In the case of lymph node samples, tissue homogenates were made in the presence of 10% bovine serum albumin, as a protection against proteases.

Light detection standard curves. Light detector measurements are expressed in relative light units (RLU). Standard curves to evaluate light detection linearity and sensitivity were generated by measuring light production in a mixture consisting of lysate from a predetermined number of 435 luc cells, ranging from 0 to 5000 in RLB and a 20 µl aliquot of clear tissue homogenate, prepared as described above, from mice lacking 435 luc cells. A standard control reference curve was also generated with the only difference that tissue homogenate was substituted by RLB (18).

Counting of tumor cell equivalents and detection sensitivity. To calculate the number of 435/Neo.luc or 435/Bcl-xL.luc tumor cell equivalents (TCEs) present in mice tissues, the corresponding background light was subtracted from the RLU produced by a 20 µl aliquot of tissue homogenate and the result divided by the slope of the corresponding tissue homogenate standard curve. Tissue homogenates in which the luciferase activity of a 20 µl aliquot varied in range from 0 to 5000 luc cells, were diluted with RLB and assayed as described above. Sixteen to seventeen weeks later mice were killed by carbon dioxide and metastasis in the right inguinal mammary gland (i.m.f.p.). Metastatic involvement was determined by microscopic examination of hematoxylin–eosin stained paraffin sections.

DNA extraction

Genomic DNA was extracted from cells by the phenol–chloroform method. DNA was diluted to a concentration of 20 µg/ml and 1 µl transferred. Sample was analyzed in 1% agarose gel electrophoresis, and stained with ethidium bromide to verify its quality and concentration. When necessary, DNA concentration was adjusted according to the ethidium bromide signal.

AP-PCR fingerprinting

Four primers were used to generate four different fingerprints by AP-PCR (arbitrarily primed polymerase chain reaction) for the detection of genomic variability on cell variants, as described (23). Primers were selected based on reproducibility and pattern readability. Three AP-PCR experiments were performed with a single arbitrary primer per reaction, for the fourth experiment,
two primers were added simultaneously to the reaction mix (D12S77). Primer sequences were as follows: Ocean, 5'-CAGAATTTCTCAGACATCGC-3'; pU6, 5'-GCTCTGACTTATTTGCTCTTAG-3'; Blue, 5'-CCGAATTCGCAAAGCTCTGA-3'; D12S77, U: 5'-GAAGGCGAAACAGTGAA-3'; D, 5'-CTTTCATTCCTCCCCTCCTTC-3'.

AP-PCR with primers Blue and Ocean were carried out with 50 ng of genomic DNA, PCR buffer (supplied by Boehringer Mannheim, Mannheim, Germany), 108 mM of arbitrary primer, 2.16 mM MgCl₂, 1.5 mCi [α-32P]dATP (Amersham, Buckinghamshire, UK) and 0.75 or 1.1 U Ocean and Blue primer, respectively. Taq DNA polymerase (Boehringer Mannheim) was added in a final volume of 25 μl. AP-PCR conditions with pU6 primer was performed using 40 ng of genomic DNA, PCR buffer, 100 mM each dNTP, and 1.25 U Taq DNA polymerase in a final volume of 25 μl. AP-PCR with D12S77 primers was performed using a higher dNTP concentration (200 μM each dNTP) and Blue primer, respectively.

Electrophoretic analysis of the AP-PCR products. Radioactive PCR products were diluted 1:3 in 95% deionized formamide denaturing buffer, heated at 100°C for 1 min, and then cooled on ice. The samples were loaded onto a 6% polyacrylamide 8 M urea denaturing sequencing gel at room temperature without intensifier screen for 1–3 days.

Densitometric analysis of the AP-PCR bands. Films were scanned (EPSON GT-8500 with transparency unit, Seiko Epson, Nagano, Japan) and the peak intensity of those bands, clearly distinguishable from the background, were densitometrically measured using Phoretix ID software (Phoretix International, Newcastle upon Tyne, UK).

For these experiments we used cells transfected with anti-apoptotic genes, Bcl-2 and Bcl-xL or the control 435/Neo cells. The pool of transfectants was used to avoid the possibility of clonal variations. The parental cells used in this study expressed low levels of Bcl-2 and Bcl-xL protein, and the transgene protein expression was 2-fold higher than in the control cells. Comparative analyses of Bcl-2 and Bcl-xL protein expression by western blot showed stable over-expression of these genes in 435 cells (Figure 1A and B).

We treated cells with different drugs (10–50 ng/ml). As shown in Figure 2, colony formation was more efficient (40–80%) in cells treated with paclitaxel than in cells treated with docetaxel (10–30%), without significant differences between 435/Bcl-2, 435/Bcl-xL and 435/Neo control cells. All transfectants showed resistance to doxorubicin.

Previously, we have reported how standard plots of light production versus the number of 435/Neo.luc and 435/Bcl-xL.luc cells were linear over a range of 0–5000 cells (18). The slopes of the regression plots, which are a measure of the amount of light produced during 1 min by a single cell, corresponded to 0.023 RLU/cell for 435/Neo.luc and 0.021 RLU/cell for 435/Bcl-xL.luc cells.

The amount of light produced by known numbers of 435/Neo.luc and 435/Bcl-xL.luc cells added to tissue homogenates was measured to determine the effect of tissue-specific components in light detection sensitivity. The slopes of the resulting standard curves measured the amount of light/(cell·minute) that can be effectively detected under the experimental conditions and were used to calculate the number of TCEs in the different target organs (18).

Assay sensitivity was defined as the number of TCEs required to generate a quantity of light equivalent to 2 SD of the background signal. Only TCE values above the assay sensitivity limit were included as part of the data.

Since the luciferase gene was introduced by permanent transfection in the 435/Bcl-xL and 435/Neo clones, we used it as a tumor marker to measure the number of tumor cells lodged in metastatic foci in Nude Balb/c mice that had the primary tumors excised at day 45. Orthotopical implantation of 435/Neo.luc and 435/Bcl-xL.luc transfectants in Nude Balb/c mice resulted in the development of primary tumors with a 100% incidence. Tumor weight was 178.8 ± 19.6 mg in 435/Neo.luc tumors and 281.4 ± 13.2 mg in 435/Bcl-xL.luc tumors (P = 0.002). We measured the stability of light production capacity during tumor growth assessing the number of 435/Neo.luc and 435/Bcl-xL.luc cells in tumors using the

Results

Colonization as a predictor of chemoresistance in breast cancer cells that over-express anti-apoptotic genes

We tested in vitro the sensitivity of 435 cells to docetaxel before the experimental metastasis treatment.

For these experiments we used cells transfected with anti-apoptotic genes, Bcl-2 and Bcl-xL or the control 435/Neo cells. The pool of transfectants was used to avoid the possibility of clonal variations. The parental cells used in this study expressed low levels of Bcl-2 and Bcl-xL protein, and the transgene protein expression was 2-fold higher than in the control cells. Comparative analyses of Bcl-2 and Bcl-xL protein expression by western blot showed stable over-expression of these genes in 435 cells (Figure 1A and B).

We treated cells with different drugs (10–50 ng/ml). As shown in Figure 2, colony formation was more efficient (40–80%) in cells treated with paclitaxel than in cells treated with docetaxel (10–30%), without significant differences between 435/Bcl-2, 435/Bcl-xL and 435/Neo control cells. All transfectants showed resistance to doxorubicin.

In the light of these results, docetaxel was the most specific treatment to kill in vitro 435 breast cancer cells, even when they over-expressed anti-apoptotic proteins.

Distribution of metastatic 435/Neo.luc and 435/Bcl-xL.luc cells in vivo, and response to docetaxel treatment in metastatic foci

In order to assess the efficiency of docetaxel in the treatment of in vivo breast cancer metastasis, we induced metastasis in nude mice in which breast tumors were obtained injecting orthotopically 435/Bcl-xL or 435/Neo cells, transplanted previously with the luciferase gene to use it as a tumor marker.
The ratio remained similar in both types of tumors, 1.2 \times 10^5 \pm 1.6 \times 10^4 in 435/Neo.luc and 1.2 \times 10^5 \pm 5.7 \times 10^3 in 435/Bcl-xL.luc tumors (P = 0.508). These results indicated that the light generated in tissues is proportional to tumor mass and comparable between them, in consequence TCEs detected in 435/Neo cells is similar to that generated by 435/Bcl-xL cells.

The animals were treated with docetaxel (15 mg/kg) and maintained until symptoms of clinical disease were evident, day 110 post-i.m.f.p., at which time they were killed and analyzed for metastasis in the different organs. We assessed the incidence and distribution of metastasis, taking into account lungs; viscera as well as liver, kidneys and brain; lymph nodes, including peripheral nodes, the majority of which were localized in cervical and axillary lymph nodes, mediastenial nodes; and bones that include vertebra, ribs and femur.

The over-expression of Bcl-xL increased the metastatic potential of MDA-MB-435 breast cancer cells when implanted i.m.f.p.. The total number of TCEs detected in organs from mice with 435/Neo tumors was 7.8 \times 10^4 \pm 4.2 \times 10^3 and in mice carrying 435/Bcl-xL.luc tumor was 5.7 \times 10^5 \pm 5.2 \times 10^4.

The metastatic burden significantly decreased in treated 435/Bcl-xL.luc mice, 1.7 \times 10^5 \pm 1.2 \times 10^6 (P = 0.02), but not in 435/Neo.luc mice, 1.4 \times 10^4 \pm 1.3 \times 10^4 (P = 0.44).

Table II describes the distribution of TCEs detected in several organs from treated and non-treated mice. The affected organs in 435/Bcl-xL.luc mice were lungs, 5.1 \times 10^5 \pm 4.9 \times 10^4 TCEs; lymph nodes, 6.1 \times 10^6 \pm 2.5 \times 10^5 TCEs; bones, 0.8 \times 10^5 \pm 0.4 \times 10^5 TCEs; and viscera, 8.9 \times 10^2 \pm 5.4 \times 10^2 TCEs. Between treated and non-treated 435/Bcl-xL.luc mice significant differences in the metastatic burden of lymph nodes (P = 0.02) and viscera (P = 0.02) were observed.

Table 1. Stability of light production during 435/Neo.luc and 435/Bcl-xL.luc tumor growth

<table>
<thead>
<tr>
<th>435/Neo.luc</th>
<th>435/Bcl-xL.luc</th>
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<tbody>
<tr>
<td>Mean ± SE (n = 9)</td>
<td>Mean ± SE (n = 9)</td>
</tr>
<tr>
<td>Weight (mg)a</td>
<td>178.8 ± 19.6</td>
</tr>
<tr>
<td>TCEs/mgb</td>
<td>1.2 \times 10^5 \pm 1.6 \times 10^4</td>
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</table>

aTumor weight at day 45 from injecting cells i.m.f.p.

bNumber of TCEs present in 1 mg of tumor tissue.

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However, treatment did not significantly decrease metastatic burden in bones (P = 0.19) or in lungs (P = 0.30).

In contrast, 435/Neo.luc tumor-bearing mice preferentially metastasized in lungs, in which TCEs were 7.6 × 10⁴ ± 4.1 × 10⁴ (2/3), and less effectively in lymph nodes, 1.9 × 10³ ± 1.5 × 10³ TCEs; bones, 0.8 × 10² ± 0.7 × 10² TCEs; and viscera, 0.3 × 10² ± 0.3 × 10² TCEs. Between treated and non-treated mice, TCEs found in lungs (P = 0.23), bones (P = 0.26), lymph nodes (P = 0.19) and viscera (P = 0.72) were not significant statistically.

Since lymph node metastasis are more abundant in mice with 435/Bcl-xL luc tumors than in mice with 435/Neo.luc tumors, the global differences between the two groups could be attributable to the specificity of the treatment to lymph node metastasis. Indeed, no significant therapeutic effect was observed in lung or bone metastasis in both groups.

Sensitivity to challenge by chemotherapy and epigenetic factors from target organs of metastatic variants

Tumor microenvironment may play a key role as a determinant of drug response (24–27). To test the influence of microenvironmental factors on the cytotoxicity induced by docetaxel, we obtained metastatic variants by primary cultures from lung, lymph node and bone metastases. Monolayer cultures were established by trypsination of tissue cultures and maintained under genetinase selection.

The cytotoxic assay was carried out in metastatic variants treated with different doses of docetaxel (Figure 3A).

The most sensitive variant was 435/NeoL3; the viability in the presence of 5 ng/ml of docetaxel was 50%. In contrast, 80% of 435/Bcl-xL L1 cells were viable in the same conditions. These differences were significant statistically (P = 0.03), and point to the fact that Bcl-xL might induce docetaxel resistance in lung metastasis.

Furthermore, 435/Bcl-xL lung metastasis exerted the highest resistance against docetaxel. At 50 ng/ml the survival of 435/Bcl-xL lung cells was 40%. In contrast, only 20% of 435/Bcl-xL lymph node cells survived at this condition, validating in vivo experiments in which the metastatic burden significantly decreased in lymph nodes but not in bones or lungs.

Since we did not find in vivo differences between 435/Bcl-xL lung and 435/Neo lung metastasis in response to docetaxel treatment, we hypothesize that microenvironmental factors at the metastatic foci could contribute to drug resistance. To assess their influence, we tested docetaxel cytotoxicity in the presence of CM obtained from histocultures.

Docetaxel was less effective at inducing cytotoxicity in cells cultured in the presence of CM from each respective tissue (Figure 3B). The survival increased from 30 to 50% in lung metastatic cells from both systems, 435/Bcl-xL and 435/Neo, and in bone metastatic cells. In contrast, lymph node metastatic cells did not have a survival advantage in the presence of CM at high doses of docetaxel.

To corroborate these results we tested several 435/Bcl-xL metastatic variants from bone and lymph node metastasis. As shown in Figure 3C, the cytotoxicity of docetaxel significantly decreased in bone metastatic variants in the presence of CM from bone tissue (P < 0.001). In contrast, the CM from lymph nodes did not modify significantly the cytotoxicity of the drug against lymph node metastatic cells.

We concluded that microenvironmental factors in the target organ of metastasis reduce the sensitivity of breast cancer cells to docetaxel, with more effective prevention at low doses of the drug. Moreover, since the lymph node metastatic foci seem to be the most sensitive to docetaxel treatment, we hypothesized that some intrinsic differences exist among metastatic variants that might influence cell response to therapy.

Genetic variability from tumoral cells to metastatic variants

In attempt to understand why cells that belong to the same tumor showed different responses to therapy, we analyzed, using genomic DNA fingerprinting, the clonality of metastasis from lung, bone and lymph node using AP-PCR, which permits the detection of phylogenetic and ontogenetic DNA sequence variation.

We carried out genomic DNA fingerprinting of the 435/Bcl-xL tumor cells and metastatic cells. We analyzed by AP-PCR a total of 175 DNA bands amplified by primers Ocean (45), D12S77 (45), pU6 (39) and Blue (46). The pattern of bands was analyzed by densitometry capturing the images by the Adobe Photoshop program and analyzing them by the Phoretix program. To quantify genetic changes between specimens, we used Microsoft Excel. The intensity of each band was calculated as a relative intensity, taking into account the intensity of the experiment, the absolute intensity of each ladder and the number of analyzed bands, as has been described previously (23).

<table>
<thead>
<tr>
<th>Organ</th>
<th>110 days</th>
<th>110 days (TXT)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>TCEs mean ± SE (incidence)</td>
<td>TCEs mean ± SE (incidence)</td>
<td></td>
</tr>
<tr>
<td>Lungs</td>
<td>7.8 × 10⁴ ± 4.2 × 10⁴ (3/3)</td>
<td>1.4 × 10⁴ ± 1.3 × 10⁴ (6/6)</td>
<td>0.44</td>
</tr>
<tr>
<td>Viscera (liver/brain)</td>
<td>1.9 × 10³ ± 1.5 × 10³ (3/3)</td>
<td>0.2 × 10³ ± 0.8 × 10³ (6/6)</td>
<td>0.19</td>
</tr>
<tr>
<td>Lymph nodes (peripheral/mediastinal)</td>
<td>8.0 × 10² ± 0.7 × 10² (2/3)</td>
<td>4.8 × 10¹ ± 3.3 × 10¹ (2/6)</td>
<td>0.26</td>
</tr>
<tr>
<td>Bones (vertebra/femur)</td>
<td>7.8 × 10³ ± 4.2 × 10³ (3/3)</td>
<td>1.4 × 10⁴ ± 1.3 × 10⁴ (6/6)</td>
<td>0.44</td>
</tr>
<tr>
<td>Total</td>
<td>5.7 × 10³ ± 5.1 × 10³ (3/3)</td>
<td>1.7 × 10⁴ ± 1.2 × 10⁴ (6/6)</td>
<td>0.02</td>
</tr>
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</table>

The mean and SD of TCEs in different organs at day 110 from injecting cells i.m.f.p., with or without docetaxel treatment.

*Number of mice with metastasis from total mice in each group.

**The mean and SD of TCEs in different organs at day 110 from injecting cells i.m.f.p., with or without docetaxel treatment.

Table II. Metastatic activity of 435/Neo.luc and 435/Bcl-xL luc breast tumors, and response to docetaxel (TXT) treatment at metastasis foci.
for each band from independent experiments using the parental cell line was measured, and a regression model curve was calculated to normalize results taking into account these changes. We considered the limit of change to be a curve below which 99% of changes detected for the primers used fell.

As a result of this procedure, the intensity of bands was defined by $1/C_0$ and 0; for GF, LF or no intensity changes, respectively. The gain or loss of band intensity was stipulated in regard to the referent control parental MDA-MB 435 cells, to evaluate genetic alterations that occur in transfec-
tants. In the case of metastasis from 435/Bcl-xL, the GF or LF of bands was stipulated with regard to the tumor.

As shown in Table III, cells with Bcl-xL over-expression had more genetic instability (GF = 0.17 and LF = $1/C_0$ 0.17) than the control 435/Neo (GF = 0.15 and LF = $1/C_0$ 0.14), which was maintained in a clone 435/Bcl-xL C1 (GF = 0.18 and LF = $1/C_0$ 0.21), derived from 435/Bcl-xL pool. Among transfec-
tants 435/Bcl-2 had the most genetic instability (GF = 0.27 and LF = $1/C_0$ 0.28). These differences were not significant statistically.

Moreover, genetic variability increased in metastatic variants with regard to its respective 435/Bcl-xL tumor, in metastasis from lungs (435/Bcl-xL Lung GF = 0.13 and LF = $1/C_0$ 0.09), bones (435/Bcl-xL Bone GF = 0.09 and LF = $1/C_0$ 0.10), and lymph nodes (435/Bcl-xL Node GF = 0.07 and LF = $1/C_0$ 0.08). To avoid clonal artifacts comparing genetic changes between metastasis, we tested lymph node and lung metastasis from different 435/Bcl-xL tumors. As shown in Table III, genetic

Table III. Genetic variability in cells transfected with anti-apoptotic genes and in metastatic variants

<table>
<thead>
<tr>
<th>Transfectant cells with regard to parental cells</th>
<th>Gain fraction</th>
<th>Loss fraction</th>
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<tbody>
<tr>
<td>435/Neo</td>
<td>0.15</td>
<td>0.14</td>
</tr>
<tr>
<td>435/Bcl-2</td>
<td>0.27</td>
<td>0.28</td>
</tr>
<tr>
<td>435/Bcl-xL</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>435/Bcl-xL C1</td>
<td>0.18</td>
<td>0.21</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metastatic variants with regard to tumors</th>
<th>Gain fraction</th>
<th>Loss fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>435/Bcl-xL Lung</td>
<td>0.13</td>
<td>0.09</td>
</tr>
<tr>
<td>435/Bcl-xL Bone</td>
<td>0.09</td>
<td>0.10</td>
</tr>
<tr>
<td>435/Bcl-xL Lymph Node</td>
<td>0.07</td>
<td>0.08</td>
</tr>
<tr>
<td>435/Bcl-xL T2L1</td>
<td>0.13</td>
<td>0.09</td>
</tr>
<tr>
<td>435/Bcl-xL T2N1</td>
<td>0.06</td>
<td>0.09</td>
</tr>
<tr>
<td>435/Bcl-xL T3L1</td>
<td>0.25</td>
<td>0.23</td>
</tr>
<tr>
<td>435/Bcl-xL T3N1</td>
<td>0.18</td>
<td>0.18</td>
</tr>
</tbody>
</table>

As a result of this procedure, the intensity of bands was defined by $1/C_0$ and 0; for GF, LF or no intensity changes, respectively. The gain or loss of band intensity was stipulated in regard to the referent control parental MDA-MB 435 cells, to evaluate genetic alterations that occur in transfec-
tants. In the case of metastasis from 435/Bcl-xL, the GF or LF of bands was stipulated with regard to the tumor.

As shown in Table III, cells with Bcl-xL over-expression had more genetic instability (GF = 0.17 and LF = $1/C_0$ 0.17) than the control 435/Neo (GF = 0.15 and LF = $1/C_0$ 0.14), which was maintained in a clone 435/Bcl-xL C1 (GF = 0.18 and LF = $1/C_0$ 0.21), derived from 435/Bcl-xL pool. Among transfec-
tants 435/Bcl-2 had the most genetic instability (GF = 0.27 and LF = $1/C_0$ 0.28). These differences were not significant statistically.

Moreover, genetic variability increased in metastatic variants with regard to its respective 435/Bcl-xL tumor, in metastasis from lungs (435/Bcl-xL Lung GF = 0.13 and LF = $1/C_0$ 0.09), bones (435/Bcl-xL Bone GF = 0.09 and LF = $1/C_0$ 0.10), and lymph nodes (435/Bcl-xL Node GF = 0.07 and LF = $1/C_0$ 0.08). To avoid clonal artifacts comparing genetic changes between metastasis, we tested lymph node and lung metastasis from different 435/Bcl-xL tumors. As shown in Table III, genetic
variability increased in metastatic variants with regard to its respective tumor, 435/Bcl-xL LT2 and 435/Bcl-xLT3. Lung metastasis had more genetic changes (435/Bcl-xLT2L1: GF = 0.13, LF = −0.09; and 435/Bcl-xLT2L3: GF = 0.25, LF = −0.23) than lymph node metastasis (435/Bcl-xLT2N1: GF = 0.06, LF = −0.09; and 435/Bcl-xLT3N1: GF = 0.18, LF = 0.18) from the same tumor.

Discussion
Disruption of the intrinsic apoptotic pathway is extremely common in cancer cells. It is currently believed that the metastatic cascade involves a series of interrelated events within which are some that tumor cells use to withstand severe pro-apoptotic pressures from host-cell cytokines and growth factors (28,29). Because the mitochondrial pathway is thought to be the principal target of survival signaling pathways, which act by stabilizing mitochondrial function and integrity and suppressing release of cytochrome c, we hypothesized that anti-apoptotic oncoproteins, which exert their principal effects through stabilization of the mitochondrion, could promote enhanced survival of breast cancer cells and adaptation to a new microenvironment, leading to resistance to chemotherapy.

Anti-apoptotic proteins are over-expressed in tissue samples from patients with residual breast tumor after the completion of treatment (30). Moreover, it has been reported that increased activation of the Stat3–Bcl-2 pathway contributes to chemoresistance of estrogen-receptor negative metastatic breast cancer cell lines (17). We have shown that 435/Bcl-xL metastatic cells lodged in different organs might have a different sensitivity to docetaxel, because modulation of cancer cell response to chemotherapy is done by the microenvironment.

The anti-apoptotic Bcl-xL protein increases in vitro docetaxel chemoresistance of breast cancer cells that metastasize in lungs, with regard to lung metastatic cells from the control 435/Neo tumor. Moreover, the sensitivity of metastatic cells against docetaxel is organ-dependent, between treated and non-treated 435/Bcl-xL Luc mice a significant difference in the metastatic burden of lymph nodes and viscera was observed, whereas treatment did not significantly decrease the metastatic burden in bones. Since breast cancer tumors that over-express Bcl-xL show a significant increase of metastasis in peripheral lymph nodes after the treatment, we found metastatic burden significantly decreased with regard to non-treated mice.

These results suggest that microenvironmental factors at the metastatic foci seem to be more important in increasing drug resistance than the expression of Bcl-2 or Bcl-xL in metastatic cells.

The data are validated in vitro, and reinforced by the fact that the presence of CM resulted in attenuating docetaxel cytotoxicity to lung and bone but not to lymph node metastatic cells.

Taxanes are plant alkaloids that promote tubulin assembly and induce apoptosis of tumor cells by a p53-independent G2/M cell-cycle checkpoint (31). Among these drugs docetaxel, a semi-synthetic member of the taxoid class of anti-neoplastic agents, is effective in the treatment of patients with advanced (locally advanced or metastatic) breast cancer. Despite the trend of results suggesting that these agents have the potential for significant improvements in advanced and adjuvant therapy of breast cancer (32–35), the impact of taxanes on the natural history of breast cancer is yet to be defined.

Docetaxel probably does not completely eliminate cells in bones and lungs, as occurs in patients under other treatment strategies currently used for chemotherapy in high-risk breast. We suggest that some organ-specific microenvironmental factors might inhibit its deleterious effect in breast cancer cells. Indeed, epigenetic mechanisms such as fibroblast growth factors have been implicated in the failure of paclitaxel treatment in prostate cells (22). Moreover, breast cancer cells have an autocrine production of some cytokines that determine the susceptibility or resistance of tumors to drug treatment (36).

The docetaxel concentrations required to reduce murine and human cell survival by 50% ranged from 4 to 35 ng/ml (37). We used a standard dose to treat mice, at which the drug disposition has been described as similar in spleen, lung, kidney and liver (37–39). Furthermore, docetaxel had no market schedule dependence and was found active by the i.v. and the i.p. routes (40).

Pharmacokinetic evaluation of docetaxel has been widely reported. Antitumor activity and pharmacokinetics in mice revealed linear pharmacokinetics, the increase in area under the therapeutic curve was proportional to the increase in dose, with a good tumor retention compared with normal tissues. Therefore, this evidence in pre-clinical pharmacokinetics and metabolism, suggests that the different response among metastases are secondary to intrinsic properties of the tumor cells and the protection that the microenvironment of the metastatic lesion exerts, these two probably acting together in the selection process.

By selecting cells for resistance to growth-limiting conditions and lack of apoptosis (41), selecting the organ specifically with the most adaptive phenotype and overcoming dormancy (18), the anti-apoptotic protein Bcl-xL might be a hallmark of organ-specific lodging of metastasis, contributing to therapy resistance. These results point to the value of anti-apoptotic genes as targets for adjuvant therapy to prevent metastasis.

The selective nature of the metastatic process and the rapid evolution and phenotypic diversification of clonal tumor growth, results from the inherent genetic and phenotypic instability of many clonal populations of tumor cells (42). Bcl-xL over-expression increased genetic instability in cells that result in biological diversification and the generation of tumor heterogeneity, indeed metastasis from Bcl-xL tumors is more ubiquitous than metastasis from control tumors. Its effect is translated in different genetic changes among metastasis from lung, bone or lymph node foci with regard to the primary tumor. The heterogeneity and diversity seen might be also adding to the genetic changes from the dynamic and stochastic evolutionary force that varies with differing somatic environments. Thus, the ubiquity of metastatic cells might be a result of genetic alterations and variability that metastatic cells evolve in different somatic environments, each of which imposes its constraints (43). Therefore, cell sensitivity to chemotherapeutic drugs is dependent on host cellular and tissue response and not solely on the quality or quantity of changes induced by the drug in tumor cells.

Identification of therapeutic agents with demonstrable clinical benefit is an empirical process, often using cell culture cytotoxicity assays and animal tumor models that generally do not reflect complex human cancer syndromes. In this scenario, two observations might be important to take into account to prevent metastasis: (i) metastatic cells lodged in different organs might have a different sensitivity to therapy; therefore,
tumor cell sensitivity is not a good referent in global metastatic response. (ii) Genetic variability among cells from different metastatic foci argue that the driving force for metastasis development is selection of cells with the best conditions for survival in each microenvironment, including a protective effect against chemotherapy.

These results may have important clinical implications, in addition to understanding basic determinants of drug responsiveness in human cancer cells. Metastatic cells might become resistant to treatment by modifying expression levels or function of proteins involved in apoptosis signaling pathways, with the generation of genotypic changes and selection that allow an increase in malignant capacities and enables cells to take advantage of microenvironmental factors (25,44,45). To predict therapy response to combined chemotherapy, we should bear in mind that it probably derives its efficacy from coordinated regulation of specific gene products associated with apoptosis (46–48), modulators of the host microenvironment (49), and the prognosis signature defined in the current microarray-based studies (1,50–53).

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