Regulation of Breast Cancer Response to Chemotherapy by Fibulin-1

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Abstract

Doxorubicin treatment was found to augment the expression of the extracellular matrix (ECM) protein fibulin-1 in cultured human breast cancer cell lines and in MDA-MB-361 tumors grown in athymic mice. Doxorubicin was also found to augment tumor expression of the fibulin-1–binding proteins fibronectin and laminin-1. Growth of breast cancer cell lines on Matrigel, an ECM extract containing fibulin-1 and laminin-1, resulted in lower levels of doxorubicin-induced apoptosis as compared with controls. Moreover, tumors formed by injection of athymic mice with MDA-MB-361 cells mixed with Matrigel were significantly more doxorubicin resistant and displayed lower levels of apoptosis compared with those that formed in the absence of Matrigel. Monoclonal antibodies against fibulin-1 reversed Matrigel-dependent doxorubicin resistance. Furthermore, small interfering RNA–mediated suppression of fibulin-1 expression in breast cancer cells resulted in a 10-fold increase in doxorubicin sensitivity as compared with control cells. Together, these findings point to a role for fibulin-1 in breast cancer chemoresistance. [Cancer Res 2007;67(9):4271–7]

Introduction

Adjuvant chemotherapy and/or hormone therapy has improved the probability of survival of breast cancer patients by 10% to 15%. Nonetheless, the incomplete understanding of the biological mechanisms underlying chemoresistance has hampered further improvement in the treatment and outcome of breast carcinoma. To date, the biological classification of tumors as "responsive" or "nonresponsive" to treatment remains controversial, and even extensively studied conventional pathobiological markers of breast cancer such as hormone receptors, grade, p53, and HER-2 are not useful in predicting tumor responsiveness to chemotherapy (1). This likely reflects the multifactorial origin of chemoresistance in most solid tumors.

Mechanisms involved in drug resistance include the presence of a range of drug transporters (such as P-glycoprotein or multidrug resistance–associated protein); changes in the expression of topoisomerases; alterations in metabolic pathways that influence drug metabolism, DNA repair, or apoptosis; delivery and distribution of anticancer drugs to tumor cells; imbalance of the ratio of bax to bcl-2; high levels of constitutive nuclear factor-κB activity, cyclin D1 overexpression, and the microenvironment (2–6).

Recent evidence increasingly points to the important role of stromal extracellular matrix (ECM) components in the success of chemotherapy (7). Indeed, the disruption of integrin-mediated cell-ECM interactions can influence cancer cell sensitivity to apoptosis and affect drug resistance (1). Several studies have shown that small-cell lung cancer cells (8), myeloma cells (9), glioma cells (10), chronic myelogenous leukemia cells (11), lymphoma (12), uveal melanoma (13), and ovarian (14), bladder (15), and colon (16) carcinomas were protected from the apoptosis induced by various anticancer drugs when the cells were plated on ECM proteins such as fibronectin, laminin-1, and collagens, which directly bind integrin receptors. However, it has remained unclear whether other ECM proteins including those that do not directly engage integrins also contribute to chemoresistance. Among the latter proteins are the fibulins, a family of six proteins thought to function as bridges in the organization of ECM supramolecular structures (17). Fibulin-1 (FBLN-1), the prototype member of this family, has four splice variants (17) and binds to many ECM proteins, including fibronectin, laminin-1, fibrinogen, nidogen, and the proteoglycans aggrecan and versican (18). Several studies have reported that FBLN-1 is expressed in various human neoplasias and it is implicated in processes such as invasion, motility, and *in vivo* tumor growth (19–22). We recently showed that FBLN-1 is a breast cancer–restricted antigen aberrantly expressed in 35% of 528 breast cancer specimens analyzed (23); it induces both specific B-cell– and T-cell–mediated responses in breast cancer patients (23, 24); and it can be exploited as a tool for early detection of breast cancers (25). Here, we report that FBLN-1 acts to promote breast cancer cell survival during doxorubicin treatment.

Materials and Methods

Cell lines. Human breast carcinoma cell lines SKBR-3, MCF-7, MDA-MB-157, MDA-MB-231, MDA-MB-361, MDA-MB-468, MDA-MB-453, and MDA-MB-175 were obtained from the American Type Culture Collection (ATCC). The MDA-MB-435 cell line, which shows both breast-specific and melanocyte-specific markers (26), was also obtained from ATCC. Cell lines were routinely maintained in RPMI 1640 (Sigma Chemical Co.) supplemented with 10% (v/v) FCS (Sigma Chemical) and L-glutamine in a humidified chamber (95% air, 5% CO_2) at 37°C. Human breast tumor MCF-7-DXR (doxorubicin-resistant variant) and MCF-7B (parental) cell lines were kindly provided by Elena Monti [Department of Structural and Functional Biology, Section of Pharmacology, University of Insubria, Busto Arsizio (VA), Italy; ref. 27].

Antibodies. The mouse monoclonal antibodies (mAb) to human FBLN-1, P5B1, P3B4, P1B2, MEM-2, and 3A11 have previously been described (28, 29). Rabbit polyclonal antibodies to human laminin-1 and human fibronectin and mouse mAbs against α-actin and vinculin were purchased from Sigma Chemical.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Indirect immunofluorescence and fluorescence-activated cell sorting analysis. The cytoplasm-associated form of FBLN-1 was detected with the mAb MEM-2 in doxorubicin-treated and untreated permeabilized tumor cells after fixation and permeabilization with BD Cytofix/Cytoperm (BD Biosciences PharMingen) as described (23).

Immunoprecipitation and Western blotting. Tumor specimens from doxorubicin-treated and untreated athymic mice were minced, lysed for 1 h on ice in lysis buffer [50 mmol/L Tris-HCl (pH 7.2), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 0.1% SDS and 0.5% deoxycholic acid] containing protease inhibitors, 2 mmol/L phenylmethylsulfonylfluoride, and 10 μg/mL aprotinin, and specifically processed as described (23). Specific proteins were detected by enhanced chemiluminescence according to the manufacturer's recommendations (GE Healthcare Amersham). Autoradiographic signals were measured using a Bio-Rad scanning densitometer (Bio-Rad, ChemiDoc/XRS). Data were acquired and analyzed using Quantity One v 4.6.1 software. The expression of proteins and transcripts was assessed relative to housekeeping gene expression in the given sample.

In-gel tryptic digestion, matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry, and peptide mass fingerprinting. In-gel digestion was done following the protocol of Shevchenko et al. (30, 31) with modification. Reflector matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) spectra were acquired on a Voyager STR (Applied Biosystems) and used to search nonredundant protein sequence databases with Aldente and ProFound software.[5]

Reverse transcription-PCR analysis. RNA was extracted with RNAzol B isolation solvent (Tel-Test, Inc.). Reverse transcription was carried out at 42°C for 60 min using 2 μg of total RNA, 75 pmol of oligo(dT) primer, 0.5 mmol/L of each deoxynucleotide triphosphate, and 50 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR was done in a total volume of 50 μL using the following sequence-specific primer pairs: FBLN-1, (+) primer 5′-CCGGAGTGGACGCGGATGTCCTCC-3′ and (−) primer 5′-CCTCCAGCTGGCTGTGGCAGCACT-3′ (expected product 149 bp) and (+) primer 5′-ATATGCTACGGAATCCAAAG-3′ and (−) primer 5′-TTGATGCATGTATGCCCAAT-3′ (expected product 821 bp; upstream and downstream oligonucleotide primers both taken from a region of fibulin cDNA common to the four isoforms). After an initial denaturation step at 95°C for 3 min, PCR was carried out at 95°C for 1 min, 56°C for 2 min and 72°C for 2 min for 30 cycles, followed by final extension at 72°C for 10 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts were amplified in a 20-cycle PCR using (+) primer 5′-TCCACCACCCTGTTGCTGTA-3′ and (−) primer 5′-ACCACAGTCCATGCCATCAC-3′.

Cell treatments. Evaluations of FBLN-1 transcript and protein expression in response to chemotherapy were done on breast tumor cell lines first grown at 70% confluence and then treated with doxorubicin (Pharmacia Upjohn) or vehicle for 72 h. The following growth inhibition concentrations (IC_{30}) of doxorubicin were used: 122 nmol/L for MCF-7, 114 nmol/L for SKBR-3, and 132 nmol/L for MDA-MB-157, as previously calculated (32).

ELISA analysis of ECM-associated FBLN-1. ELISA was used to quantify levels of FBLN-1 associated with ECM. Cell lines were treated as above, the culture medium was removed, and cells were repeatedly washed in PBS. After lysing cells by two incubations in 20 mmol/L NH_4OH for 10 min, the plates were extensively washed with PBS and then with distilled water and allowed to air dry, as described (33). The ECM proteins were recovered in collection buffer [10 mmol/L Tris-HCl (pH 7.0), 0.1% SDS, 100 mmol/L β-mercaptoethanol]. After 15 min at 37°C, the fluid was collected with a cell scraper. The recovered ECM-associated proteins were used as targets for ELISA as described (34).

Silencing of FBLN-1 by small interfering RNA transfection. MCF-7 cells (3×10^5 per well) were seeded in six-well plates and grown to 60% to 80% confluence and the cell layers washed with serum-free medium. The cell layers were transfected either with a pool of two small interfering RNA (siRNA) oligonucleotides specific for human FBLN-1 (final concentration, 50 nmol/L; Dharmacon) or a pool of control RNA duplexes (Dharmacon) using transIT-TKO transfection reagent (10 μL/well; Mirus). After overnight incubation at 37°C, culture medium was replaced with fresh complete medium containing 10% FCS. Cells were harvested after 48 h, processed for immunofluorescence staining, and treated with escalating doses of doxorubicin for cytotoxicity assays or solubilized for Western blot analysis of FBLN-1 silencing.

Proliferation assay. To study doxorubicin sensitivity of FBLN-1–silenced tumor cells, 3×10^4 MCF-7 cells per well were seeded in 96-well plates in RPMI supplemented with 10% FCS, transfected with FBLN-1–specific siRNA sequences or with control reagents for 48 h, and treated with escalating doses of doxorubicin (six replicates for each drug dose for each cell treatment). Doxorubicin was removed after 72 h and cell proliferation was assessed as described (35).

***In vitro* apoptosis assays.** Human breast cancer cell lines MCF-7, MDA-MB-361, SKBR-3, MDA-MB-435, MDA-MB-175, MDA-MB-231, MDA-MB-453, and MDA-MB-468 in 2 mL of RPMI 1640 supplemented with 1% FCS were seeded at 2×10^5 per well in six-well plates. Replicate cultures were set up in wells precoated or not with a thin layer of Matrigel (BD Biosciences PharMingen; 2 mL of a 1:5 dilution of Matrigel stock solution). In some experiments, cells were seeded in the presence or absence of the FBLN-1 mAbs P3B4, P1B2, and P5B1, used individually or pooled. After 24 h, doxorubicin (200 nmol/L) was added to replicate wells containing cells with or without Matrigel. Apoptosis was evaluated after 72 h of doxorubicin treatment by staining tumor cells with Annexin V-FITC and propidium iodide as described (36) followed by flow cytometry analysis with FACScalibur flow cytometer (BD Biosciences PharMingen).

***In vivo* tumorigenesis assay.** Six- to eight-week-old BALB/c athymic mice were purchased from Charles River. Care and use of the animals were in accordance with institutional guidelines. Mice were injected s.c. with 1×10^6 MDA-MB-361 cells alone; 100 μL of Matrigel alone; cells and Matrigel; cells and doxorubicin (7 mg/mL), administered i.p. at 72 h following tumor cell injection; or cells and Matrigel and doxorubicin, with six mice in each group. Tumors were calibrated twice weekly for 58 days and tumor volume was calculated as $0.5 \times d_1^2 \times d_2$, where d_1 and d_2 are the larger and smaller diameters, respectively.

Terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assay and immunohistochemistry. Apoptotic cell death was evaluated in paraffin-embedded sections of xenografts surgically removed from athymic mice 10 days after s.c. injection of MDA-MB-361 cells, suspended in 0.1 mL of Matrigel or in PBS. Doxorubicin treatment was done 72 h postinjection of cells. Control mice received no injection. A terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)–based assay with the *In situ* Cell Death Detection Kit, POD (Roche), was carried out according to the manufacturer's instructions. Sections were analyzed as described (37) by acquiring digital images of 10 areas of each tissue section (at ×400) on a Zeiss Axiovert 100 microscope (Carl Zeiss). Other immunohistochemical stainings were done on cryosections using a peroxidase-streptavidin method as previously described (23).

Statistical methods. The results of ELISA and of *in vitro* apoptosis assay of cells cultured on Matrigel layer and involving the use of FBLN-1 mAbs were evaluated by ANOVA followed by Student-Newman-Keuls test. Statistical significance was annotated as *, $P < 0.05$; ***, $P < 0.001$. The results of *in vitro* apoptosis assays of breast tumor cell lines and of MDA-MB-361 tumor cells admixed or not with Matrigel and injected in mice treated with doxorubicin were evaluated by two-tailed paired and unpaired *t* test, respectively. Comparison of FBLN-1 gene expression levels in pre- and post-doxorubicin treated breast cancers was evaluated by Mann-Whitney test. Differences were considered significant at $P \leq 0.05$.

Results

Modulation of ECM components on doxorubicin treatment. Analyses using reverse transcription-PCR (RT-PCR; Fig. 1*A*), fluorescence-activated cell sorting (FACS; Fig. 1*B*), and ELISA

[5] For more information about MALDI-TOF data analysis, visit (http://www.expasy.org/tools/aldente/) and (http://www.expasy.org/tools/genomicsolutions/).

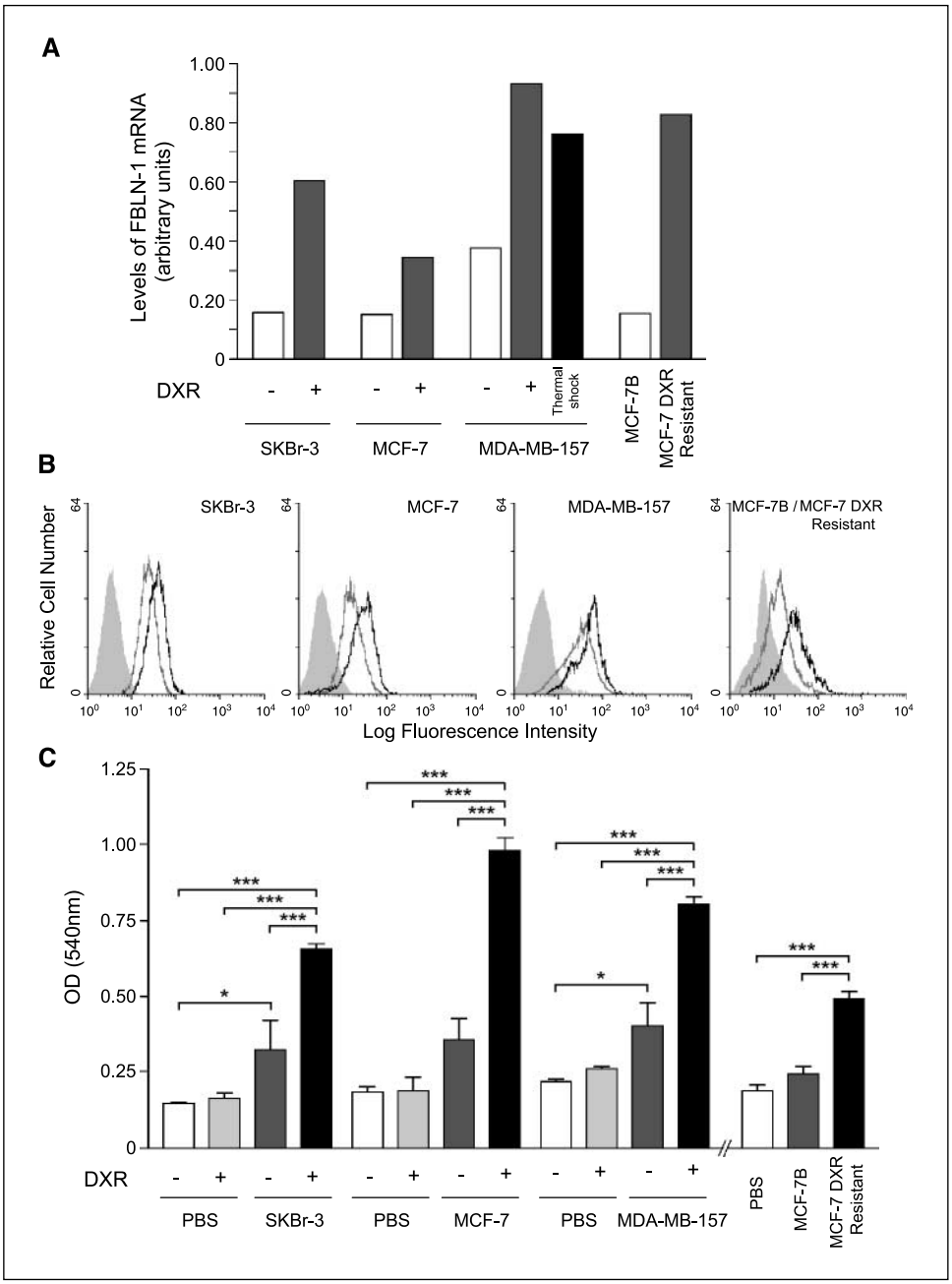

Figure 1. Doxorubicin modulation of FBLN-1 RNA and protein expression in cultured breast cancer cells. *A,* densitometric analysis of FBLN-1 mRNA levels evaluated by RT-PCR before (−) and after (+) doxorubicin treatment (IC_{30} doses) for 72 h of human breast cancer cell lines SKBR-3, MCF-7, MDA-MB-157, MDA-MB-157 (thermally shocked at 42°C for 4 h), MCF-7B, and MCF-7 doxorubicin (*DXR*)-resistant cell variant. *B,* cytofluorimetric analysis of cytoplasm-associated FBLN-1 expression before (*gray empty histograms*) and after (*black empty histograms*) doxorubicin treatment of SKBR-3, MCF-7, MDA-MB-157, MCF-7B (*gray empty histogram*), and MCF-7 doxorubicin-resistant (*black empty histogram*) cells using the FBLN-1 MEM-2 mAb. *Gray filled histograms,* internal negative control data. *C,* ELISA analysis of ECM-associated FBLN-1 released *in vitro* by SKBR-3, MCF-7, MDA-MB-157 treated (+) or not (−) with doxorubicin, as indicated above, and MCF-7B and MCF-7 doxorubicin-resistant cell variant using MEM-2 mAb. *White* and *light gray columns,* internal negative control (PBS); *dark gray* and *black columns,* FBLN-1 expression of each tumor cell line tested before (−) and after (+) doxorubicin treatment, respectively. *, $P < 0.05$; ***, $P < 0.001$ (ANOVA followed by Student-Newman-Keuls multiple comparison test).

(Fig. 1*C*) revealed increased FBLN-1 expression at both the transcript and protein levels in SKBR-3, MCF-7, MDA-MB-157, and MCF-7-DXR (doxorubicin-resistant) tumor cells as compared with untreated control cells. Densitometric analysis of the expression data indicated that doxorubicin induced 2-, 3-, and 4-fold increases in the expression of FBLN-1 RNA and protein (Fig. 1*A*). Because a 2-fold increase in FBLN-1 mRNA levels was also observed after a nontoxic physical stress in the form of thermal shock at 42°C for 4 h (Fig. 1*A*), the doxorubicin-induced increases in FBLN-1 are likely related to drug-induced stress and not to selection of drug-resistant cell variants. The MCF-7 doxorubicin-resistant variant cell line was found to constitutively overexpress FBLN-1 compared with the parental MCF-7B line (Fig. 1*A*–*C*).

To verify the findings from *in vitro* experiments, FBLN-1 levels were evaluated in xenografts of MDA-MB-361 cells grown in athymic mice treated with doxorubicin or buffer 72 h after tumor cell injection. As shown in Fig. 2, FBLN-1 mRNA and protein were increased by 2-fold in tumors grown in mice treated with doxorubicin compared with controls (Fig. 2*A* and *B*). In addition, immunohistochemistry analysis of the above-mentioned samples, done with MEM-2 mAb, revealed higher levels of FBLN-1 in surrounding tumor stroma after doxorubicin treatment compared with control (Fig. 2*C*).

To ascertain whether the FBLN-1 observed in tumor specimens was of tumor or host origin, FBLN-1 was immunoaffinity purified from xenotransplants from untreated and doxorubicin-treated mice and subjected to peptide mass fingerprinting based on MALDI-TOF mass spectrometry (Supplementary Fig. S1*A, lanes 1* and *2*, respectively). Human FBLN-1 was identified with a high score in the anti–FBLN-1 immunoprecipitates (see peptides listed with bold

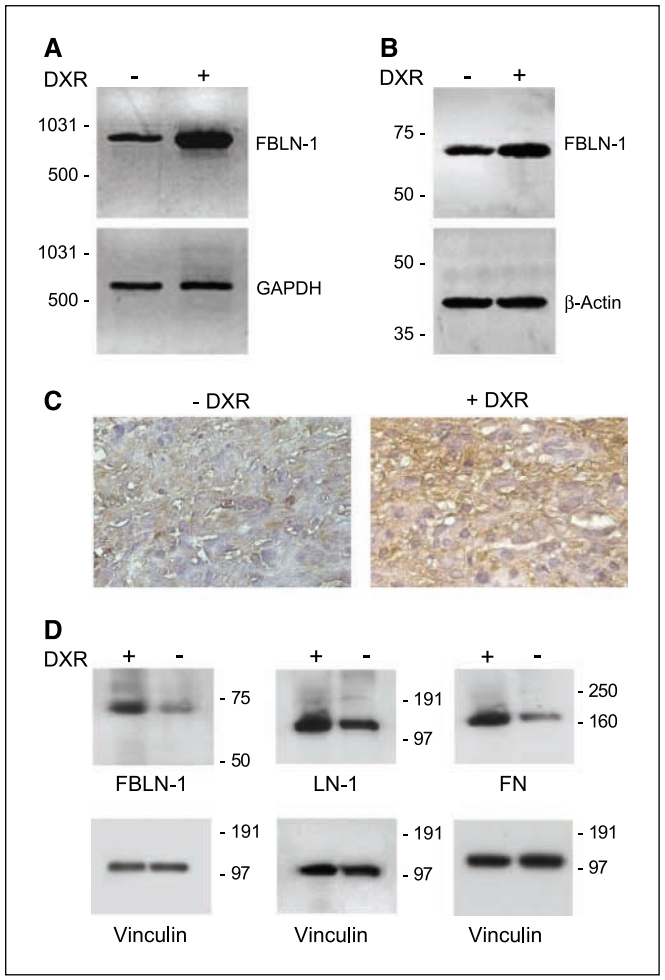

Figure 2. Modulation of *in vivo* expression of FBLN-1, fibronectin, and laminin-1 by doxorubicin treatment in breast tumor xenografts. *A,* RT-PCR analysis of FBLN-1 mRNA expression in MDA-MB-361 breast tumor xenografts grown in athymic mice treated (+) or not (−) with doxorubicin (administered i.p. after 72 h from tumor cell injection). cDNAs were tested for integrity by amplification of GAPDH transcripts as an internal control. Densitometry reveals a 2-fold increase in FBLN-1 transcript biosynthesis after drug treatment compared with the control. *B,* Western blot analysis of soluble extracts from MDA-MB-361 tumor biopsies grown in athymic mice treated (+) or not (−) with doxorubicin as above using the FBLN-1 MEM-2 mAb. β-Actin was used to normalize protein loading. Densitometry reveals a 2-fold increase in FBLN-1 protein biosynthesis following drug treatment compared with the control. *C,* immunohistochemical analysis of FBLN-1 expression in a cryosection from a biopsy from a MDA-MB-361 tumor grown in athymic mice treated (+) or not (−) with doxorubicin. The primary antibody used was FBLN-1 mAb MEM-2. *D,* Western blot analysis of soluble extracts from MDA-MB-361 xenografts grown in doxorubicin treated (+) and control (−) athymic mice using FBLN-1 MEM-2 mAb and antihuman laminin-1 (*LN-1*) and fibronectin-1 (*FN-1*) polyclonal sera. The level of the anti-vinculin polypeptide was used to normalize protein loading. Densitometry reveals a 2-, 2.1-, and 3.6-fold increase in expression of FBLN-1, laminin-1, and fibronectin, respectively, in xenotransplants of drug-treated mice as compared with controls.

characters, Supplementary Fig. S1*B*). Copurification of human and mouse FBLN-1 could not be excluded. In fact, due to high sequence similarity, a number of the tryptic peptides found in mass spectra were consistent with FBLN-1 of both species (Supplementary Fig. S1*B*).

Additional analysis of *ex vivo* changes in ECM composition on doxorubicin treatment revealed 3.6- and 2-fold increases in levels of fibronectin and laminin-1, two ECM proteins capable of binding to FBLN-1 (Fig. 2*D*). These findings suggest that tumor cells respond

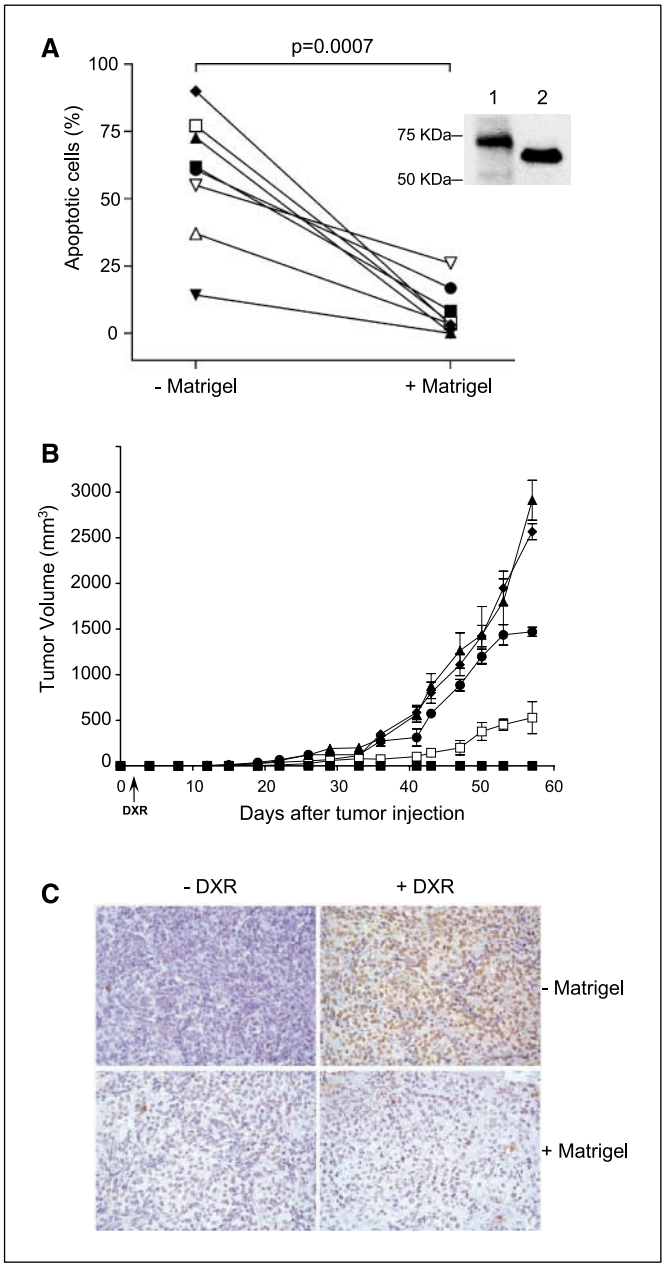

Figure 3. Matrigel protects breast cancer cells from doxorubicin toxicity. *A,* human breast cancer cell lines MDA-MB-361 (♦), MDA-MB-435 (□), MDA-MB-468 (▲), SKBR-3 (■), MCF-7 (●), MDA-MB-453 (▽), MDA-MB-231 (△), and MDA-MB-157 (▼) were seeded onto plastic or Matrigel and treated with doxorubicin (200 nmol/L) for 72 h. Apoptosis was evaluated by flow cytometry after staining cell samples with Annexin V and propidium iodide. Statistical significance was evaluated by two-tailed paired *t* test and globally annotated as $P = 0.0007$ for apoptosis protection mediated by Matrigel. *Inset,* Western blot analysis of human FBLN-1 from the SKBR-3 cell extract (*lane 1*; tested as internal control) and of murine FBLN-1 (*lane 2*) from Matrigel (used in all experiments) probed with the pool of P5B1, P3B4, and P1B2 mAbs. *B,* tumor growth rate of MDA-MB-361 cells injected s.c. alone or combined with Matrigel in athymic mice treated or not with doxorubicin (7 mg/mL) administered 72 h from tumor cell injection. Mice were divided into five experimental groups of six animals per group: (*a*) tumor cells only (▲); (*b*) Matrigel only (■); (*c*) tumor cells and doxorubicin (□); (*d*) tumor cells and Matrigel (♦); and (*e*) tumor cells, doxorubicin, and Matrigel (●). Statistical significance was evaluated by two-tailed unpaired *t* test. The difference between mouse group no. 3 (□) and no. 5 (●) was significant ($P = 0.05$). *C,* TUNEL staining of xenografts from athymic mice 10 d postinjection with MDA-MB-361 cells in the presence or absence of Matrigel and doxorubicin treatment. Doxorubicin was administered for 72 h. Surgically removed MDA-MB-361 xenografts were evaluated by TUNEL assay for apoptotic cell death 10 d from tumor cell injection. Magnification, ×400.

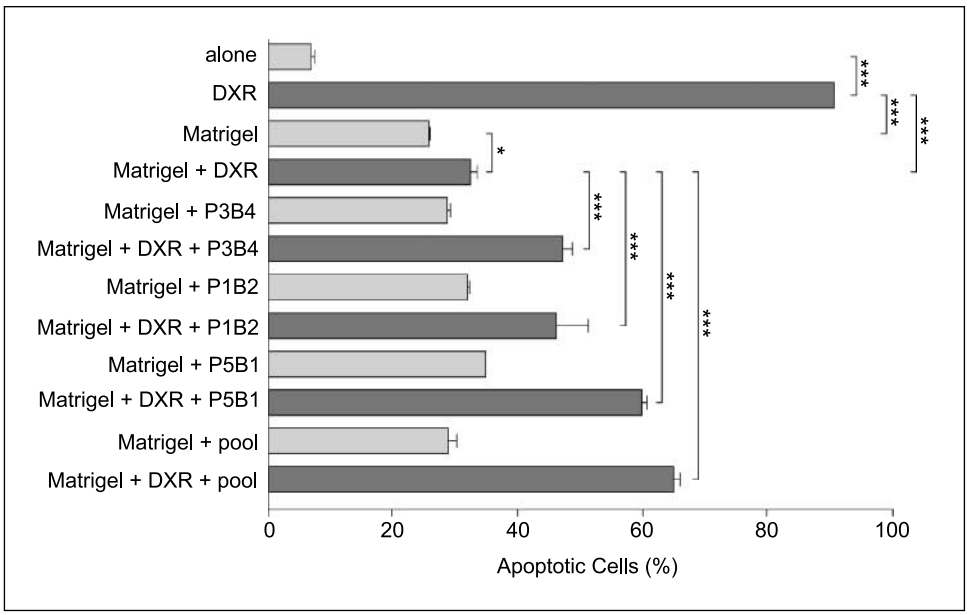

Figure 4. FBLN-1 mAbs block the protective effects of Matrigel against doxorubicin-induced apoptosis. MDA-MB-361 cells were cultured on plastic or Matrigel and then treated (*dark gray columns*) or not (*light gray columns*) with doxorubicin (200 nmol/L) for 72 h in the presence or absence of FBLN-1 mAbs P3B4, P1B2, and P5B1, alone or pooled. Apoptosis was evaluated by flow cytometry after staining cell samples with Annexin V and propidium iodide. *, $P < 0.05$; ***, $P < 0.001$, ANOVA followed by Student-Newman-Keuls test.

to doxorubicin treatment by remodeling the ECM in their microenvironment.

Role of ECM in sensitivity to doxorubicin. To assess the role of ECM proteins in sensitivity to doxorubicin, eight human breast cancer cell lines were seeded on plastic or on a thin layer of Matrigel, a solubilized basement membrane matrix preparation containing high levels of FBLN-1 (Fig. 3*A, inset, lane 2*) and laminin-1, as indicated in the BD Matrigel data sheet. The cells were then exposed to doxorubicin (Fig. 3*A*). Overall, cells cultured on Matrigel showed a significant reduction in doxorubicin-induced apoptosis ($P = 0.0007$), with the level of protection ranging from 53% with MDA-MB-453 cells to 95% with MDA-MB-435, MDA-MB-361, MDA-MB-468, and MDA-MB-175 cells.

Tumor volume measurements were made on the tumors from athymic mice injected with MDA-MB-361 cells in the presence or absence of Matrigel and doxorubicin treatment. The results show that there were no differences in the volume of tumors from mice injected with MDA-MB-361 cells alone or those from mice injected with MDA-MB-361 cells mixed with Matrigel but not treated with doxorubicin. By contrast, tumors from mice injected with MDA-MB-361 cells and Matrigel and treated with doxorubicin were significantly larger ($P = 0.05$) than tumors from mice injected with MDA-MB-361 cells not combined with Matrigel but treated with doxorubicin (Fig. 3*B*). Tissue sections from xenografts in mice injected with MDA-MB-361 cells without Matrigel and treated with doxorubicin also displayed TUNEL staining. By contrast, little or no TUNEL staining was observed in tumor sections from mice injected with MDA-MB-361 cells and Matrigel but not treated with doxorubicin (Fig. 3*C*).

Inhibition of FBLN-1 restores sensitivity to doxorubicin. To further support *in vivo* data, we next evaluated the effect of FBLN-1 antibodies on the observed protective effects of Matrigel on doxorubicin-induced apoptosis. MDA-MB-361 cells were cultured on a thin Matrigel layer and treated with doxorubicin in the presence or absence of FBLN-1 mAbs P3B4, P1B2, and P5B1 (used individually or pooled together). As shown in Fig. 4, each FBLN-1 mAb produced a significant reduction ($P < 0.001$) in the protective effect of Matrigel against doxorubicin-induced apoptosis, with the highest level of cytotoxicity achieved with mAb P5B1 and the pooled mAbs.

In another approach to test the consequence of suppressing FBLN-1 expression on doxorubicin sensitivity, MCF-7 cells were transfected with FBLN-1–specific siRNAs or control siRNA duplexes and treated with doxorubicin for 72 h. Immunoblot analysis revealed a 2-fold decrease in FBLN-1 expression in cells transfected with a pool of FBLN-1 siRNAs (Fig. 5, *lane 3*) as compared with cells transfected with a pool of control RNA duplexes (Fig. 5, *lane 4*) or cells subjected to treatment with transfection reagent alone (Fig. 5, *lane 2*). By comparison to cells treated with medium alone, there was a 2.7-fold decrease in FBLN-1 expression in cells transfected with FBLN-1–specific siRNAs (Fig. 5, *lane 1*). FACS analysis using the same biological samples confirmed the decrease in FBLN-1–silenced cells, as reflected in decreased anti–FBLN-1 immunofluorescence intensity

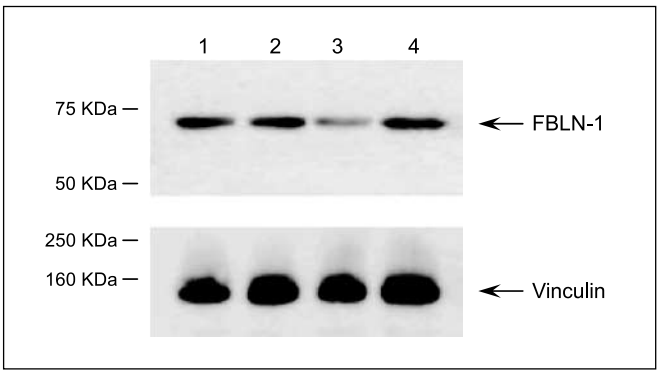

Figure 5. SiRNA-mediated suppression of FBLN-1 expression in breast cancer cells. Western blot analysis of extracts from untreated MCF-7 cells (*lane 1*), sham transfected MCF-7 cells (*lane 2*), MCF-7 cells transfected with a pool of two siRNA oligonucleotides specific for human FBLN-1 (*lane 3*), or MCF-7 cells transfected with a pool of unrelated RNA duplexes (*lane 4*). The blot was probed with MEM-2 mAb. Relative levels of FBLN-1 were measured by densitometry with the vinculin immunoreactive polypeptide bands used to normalize protein loading. Densitometry reveals a 2-fold decrease in FBLN-1 expression in *lane 3* compared with *lanes 2* and *4*, and a 2.7-fold decrease compared with *lane 1*.

Table 1. Inhibition of FBLN-1 biosynthesis leads to increased sensitivity to doxorubicin

Cell treatments	FACS analysis		Sensitivity to doxorubicin (IC_{50}), $\times 10^{-6}$ mol/L
	% FBLN-1–positive events	Fluorescence index	
Medium	68	10	1 ± 0.06
Sham transfection	69	8	1 ± 0.05
Transfection with FBLN-1 siRNA pool	41	2.5	0.09 ± 0.23
Transfection with control siRNA pool	74	9	0.8 ± 0.13

and decreased percentage of FBLN-1–positive events (Table 1). Dose dependence analysis of FBLN-1–silenced MCF-7 cells and controls incubated in the presence of varying concentrations of doxorubicin (0.002×10^{-6}–9×10^{-6} mol/L) revealed an IC_{50} dose of 10×10^{-6} mol/L for siRNA-silenced cells as compared with 1×10^{-6} mol/L for sham transfected cells or cells incubated in medium, and compared with 0.8×10^{-6} mol/L for MCF-7 cells transfected with scrambled oligonucleotides (Table 1). These data indicate that FBLN-1–silenced breast tumor cells display a 10-fold increase in doxorubicin sensitivity compared with controls.

Discussion

Findings presented here show a role for the ECM protein FBLN-1 in promoting resistance of breast cancer cells to the antitumor drug doxorubicin. This conclusion was based on several findings including those showing that FBLN-1 mAbs block the ability of Matrigel to protect tumor cells from doxorubicin-induced apoptosis. Furthermore, inhibition of FBLN-1 expression by FBLN-1 siRNA treatment markedly increased doxorubicin sensitivity of breast cancer cells.

Findings from *in vitro* and *in vivo* experiments examining FBLN-1 transcript and protein expression in doxorubicin-treated breast cancer cells suggest that FBLN-1 is part of an early response mechanism against drug-induced cell death. In fact, breast cancer cell lines exhibited a marked increase in FBLN-1 mRNA levels, as well as intracellular and extracellular FBLN-1 protein production, on doxorubicin treatment at 72 h. Moreover, analysis of xenografts showed a 2-fold increase in FBLN-1 expression levels in tumors grown in mice treated with doxorubicin compared with controls. In addition to FBLN-1, doxorubicin treatment also increased expression of the FBLN-1–interacting ECM proteins, fibronectin and laminin-1, suggesting a common mechanism activated by doxorubicin that increases tumor cell transcription of functionally interrelated ECM components. It is possible that doxorubicin induces transcription factors that act on the promoters of these ECM genes. Indeed, the promoters of fibronectin, laminin-1, and FBLN-1 genes harbor consensus binding sites for the transcription factor specificity protein 1 (Sp1; ref. 38), and doxorubicin directly induces Sp1 activation (39). Mechanisms involving activation of p53 following doxorubicin-induced DNA damage (40) seem to be less likely because we observed FBLN-1 up-regulation in three breast cancer cell lines differing in p53 status (wild-type in MCF-7, gain-of-function mutant in SKBR-3, and null in MDA-MB-157 cells).

Previous studies have suggested that long-term exposure of cancer patients to antitumor drugs is associated with up-regulation of ECM genes. Through analysis of a database (compiled by Perou and coworkers)[6] of breast cancer specimens isolated after doxorubicin chemotherapy and pretherapy samples from the same patients (41), we found increased expression of ECM genes in the post drug treatment group and mainly of FBLN-1 (see Supplementary Fig. S2). Similarly, Jazaeri et al. (42) also found statistically significant overrepresentation of ECM genes, including FBLN-1, in ovarian tumors after treatment with platinum-based chemotherapy compared with that of primary tumors from different patients (42). Taken together, these findings indicate that stromal-epithelial interactions or ECM may be involved in acquired chemoresistance of breast and ovarian cancers and that the link between FBLN-1 and drug response is not necessarily exclusive to doxorubicin. As noted by Damiano et al. (9), mechanisms of resistance selected by chronic drug treatment may differ from mechanisms of cell survival after acute exposure to chemotherapeutic drugs, so that a selection process might underlie the higher ECM gene expression in the former studies. Thus, in a heterogeneous tumor population, neoplastic cells with high and constitutive expression of ECM genes that exert a "protective" effect against drug-induced death may survive chemotherapy. However, our findings from short-term treatment (72 h) of neoplastic cells with doxorubicin suggest a direct modulation of FBLN-1 gene expression due to doxorubicin-induced stress and not due to selection of a doxorubicin-resistant cell clone, especially because thermal shock induced an altered expression of the FBLN-1 molecule without affecting viability of breast cancer cells. These two different models may yet be reconciled in light of early results of gene expression analysis in doxorubicin-resistant neoplastic cells, indicating increased expression and DNA-binding activity of the Sp1 transcription factor (43). Such a "doxorubicin-Sp1" pathway might explain the observed modulation of ECM genes after brief drug exposure while providing a mechanism that stably affects gene expression after long-term drug exposure of neoplastic cells.

The mechanism by which FBLN-1 exerts chemoresistance in breast cancer cells remains unclear. Based on the current knowledge of the biological activities of FBLN-1, the chemoresistance effects might be mediated through integrin adhesion receptor signaling. FBLN-1 has been shown to suppress the motility and adhesive-promoting effects of fibronectin (20), which are mediated by integrin $\alpha_5\beta_1$. In fact, fibronectin has been shown to deliver survival signals transduced by focal adhesion kinase, leading to suppression of p53-dependent apoptosis in normal cells (44). Furthermore, triggering of β_1 integrins by ECM interaction suppresses chemotherapy-induced apoptosis in small-cell lung

[6] For more information about gene expression, visit (http://genome-www.stanford.edu/molecularportraits/).

cancer cells (8). Moreover, integrin-dependent activation by ECM components of the Akt/mammalian target of rapamycin pathway and cell cycle arrest through $p27^{kip1}$ up-regulation are important mechanisms in promoting resistance of neoplastic cells to chemotherapy (45–47). It is possible that FBLN-1 plays an indirect role in promoting doxorubicin resistance by facilitating the interaction of integrins with other ECM components. FBLN-1 may act as a molecular scaffold assisting in the correct assembly of complex ECMs, thereby favoring the appropriate spatial orientation of ECM components needed for efficient engagement and triggering of integrins. The results described in this study, on FBLN-1 mAb–mediated inhibition of Matrigel protection against doxorubicin-induced apoptosis, are consistent with such model of FBLN-1 function.

In conclusion, our study provides evidence that FBLN-1 promotes chemoresistance in breast cancer cells, adding to the number of ECM components that provide such prosurvival function to neoplastic cells. Further studies are needed to determine the potential usefulness of assessing FBLN-1 expression in breast tumors as a predictive marker of drug response and as a therapeutic target to promote drug sensitivity.

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