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Chemical modulation of VLA integrin affinity in human breast cancer cells

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ARTICLE INFORMATION

ABSTRACT

The fact that disruption of integrin–extracellular matrix contacts leads to cell death, has converted cell adhesion into a potential target for the control of invasive cancer. In this work, we studied the functional consequences of the interference with the activity of the very late activation antigen (VLA) family of integrins in human breast cancer cell lines of distinct malignancy. The $\alpha2\beta1$-mediated adhesion reduced the entry of highly malignant, hormone-independent breast cancer cells into apoptosis. Adhesion of breast cancer cells through the VLA integrins $\alpha2\beta1$ and $\alpha5\beta1$ was significantly reduced by an apoptosis-inducing natural triterpenoid, dehydrothyrsiferol (DT), when studied on low amounts of extracellular matrix. This effect was dose-dependent, not related to cell toxicity and not shared with apoptosis-inducing standard chemotherapeutics, such as doxorubicin and taxol. The compound did not affect either the cell surface expression level of VLA integrins or cell distribution of vinculin and actin during cell spreading. In addition, neither phosphorylation of the focal adhesion kinase pp125FAK on Tyr397 nor the protein kinase B (Akt/PKB) on Ser473 was significantly altered by DT. The integrin activation level, assessed by binding of soluble collagen to the $\alpha2\beta1$ integrin, was reduced upon cell treatment with DT. Importantly, the TS2/16, an anti-$\beta1$ activating monoclonal antibody was able to rescue DT-treated cells from apoptosis. Since the activation state of integrins is increasingly recognized as an essential factor in metastasis formation, findings presented herein reveal that the chemical regulation of integrin affinity may be a potential therapeutic strategy in cancer therapy.

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Introduction

The formation of tumor metastasis is the consequence of a coordinated sequence of events, still not well understood. Initially, tumor cells acquire properties that allow them to detach from primary tumors. The metastatic cascade continues with the intravasation of tumor cells, circulation in lymph and blood vascular systems, arrest in distant sites, extravasation, and finally cancer cell proliferation into metastatic foci [1]. All these steps require profound changes of the adhesive properties of cancer cells [1]. The main adhesion molecules in epithelial cells are members of two families: integrins and cadherins, both of which have been implicated in metastasis formation. Integrins are cell surface αβheterodimers, involved in cell–cell and cell–matrix interactions, with bidirectional signalling properties [2]. The faculty of integrins to dynamically change their molecular conformation in response to intracellular signals (inside-out) enables them to modify their ligand binding affinity level from low to high, and vice-versa [3]. Integrins connect cells to components of the ECM, generating essential signals (outside-in) for migration, proliferation and survival [4]. Adhesion via integrins is regulated, additionally to changes in ligand binding affinity, by integrin rearrangements in the cell membrane which increases the avidity of the interaction [5,6].

It has been suggested that invasive cancer cells modify their adhesive interactions with the ECM. These changes seem to be the result of variations in the integrin expression profile, integrin activation state and remodeling of the ECM through metalloproteinase secretion [7]. Several findings support participation of integrins in tumorigenesis and metastasis formation. Recently, it has been shown in a mouse model that β1 integrin function is essential for both the initiation of mammary tumorigenesis and the maintenance of the proliferative capacity of late stage tumor cells [8]. The grade of invasiveness of breast cancer cells seems to correlate with changes in integrin expression and localization [9], and metastatic cancer cells have been shown to use integrins for matrix adhesion in the new environment [10,11]. Although, the functional implications of the dynamic switch of integrins between high and low affinity in cell motility have been extensively studied, especially in non-tumor cells [12], very little information is available addressing the role of changes in the activation state of integrins in tumor metastasis formation [13].

A failure of integrin–ECM contact promotes anoikis, a subtype of apoptosis that controls the cell number in high turnover tissues. However, some epithelial malignancies, including mammary tumors, have been shown to develop anoikis resistant cells thus, increasing their survival time in the absence of matrix attachment, principally while circulating in the blood or lymph flow [14]. There are additional forms of integrin-mediated death (IMD) which can occur through any of several apoptotic cascades [15]. IMD may result from the selective blockade of a single integrin on adherent, tissue associated cells [15]. Therefore, inducing IMD by modulating cell specific integrin properties provides a promising therapeutic target for the control of invasive malignancies.

In a previous report, we found that the marine triterpenoid, dehydrothyrsiferol (DT) induces apoptosis in breast cancer cell lines [16]. DT-treated cells suffered detachment of whole clusters before apoptosis was observed (Pec, M. K., unpublished observation). This finding prompted us to explore this effect and use the compound as a tool to study the modulation of integrin-mediated adhesion in these cell types. In this study, we show that invasive breast cancer cell lines require adhesion to survive. DT significantly reduces α2β1 and α5β1 mediated-adhesion of both hormone-responsive and hormone receptor negative breast cancer cells. Most importantly, the compound selectively modulates the affinity state of the respective integrin without affecting neither integrin surface expression levels nor cell surface distribution or cell spreading. Additionally, phosphorylation of both focal adhesion kinase pp125FAK (FAK), an important integration point of integrin-mediated cell spreading and migration, and protein kinase B (Akt/PKB), an enzyme with a key role in cellular processes such as cell proliferation and apoptosis seem to be circumvent in the anti-adhesive effect of DT. The presence of TS2/16, an anti-β1 monoclonal antibody (mAb) that induces high affinity state in VLA heterodimers [17] was able to prevent apoptosis in DT-treated cells. These data suggest that IMD in cancer cells might be induced by chemical antagonists that specifically interfere with inside-out integrin signalling, which might serve as a novel therapeutic strategy in the management of metastatic tumors.

Materials and methods

Antibodies and reagents

R2-8C8, a generous gift from Dr. Mark H. Ginsberg (The Scripps Research Institute), is a function-blocking monoclonal antibody (mAb) against the human α2 integrin subunit [18] and was used as a dilution from ascites fluid. The purified anti-human α5β1 functional mAb, Ab16 was a kind gift from Dr. Kenneth M. Yamada [19]. Purified anti-human β1 activating mAb, TS2/16 [17] was a kind gift from Dr. Francisco Sánchez-Madrid. Antibodies to phospho-FAK (Tyr[397]), total FAK, phospho-Akt (Ser[473]) and total Akt protein were from BioVision. Phalloidin Alexa Fluor 568 conjugate was purchased from Molecular Probes and the anti-vinculin rabbit polyclonal antibody from Santa Cruz Biotechnology. DT was isolated as described [20] and stock-diluted in dimethylsulfoxide (DMSO). Doxorubicin was purchased from Pharmacia and dissolved in water. Taxol (Sigma) was reconstituted in DMSO.

Cells and cell cultures

T47D human breast cancer cells were maintained in RPMI 1640 medium and MDA MB 231 human breast cancer cells were cultured in DMEM with 4.5 g/l glucose. Both media were supplemented with 10% bovine serum, 2 mM glutamine, 50 UI/ml penicillin, and 50 μg/ml streptomycin. All cell culture reagents were supplied from PAA Laboratories.

Adhesion assays

The adhesion of cells to immobilized collagen I and fibronectin was assayed as previously described [21]. Briefly, 96 well tissue culture plates (Nunclon, Nunc) were coated with 100 μl of soluble collagen or fibronectin (both from Sigma) for 1 h at 37 °C. Both ECM proteins were diluted in Ca/Mg-free phosphate-buffered saline (PBS), pH 7.4 to a concentration, which permitted adhesion of 50% of the maximum cell adhesion. This was 3–7 μg/ml of protein, depending on the cell line and the batch of the ECM substrate tested. After a wash with PBS, wells were blocked with 1% BSA in 200 μl PBS for 1 h at 37 °C. Cells were detached with 0.5 g/l trypsin, 0.2 g/l EDTA in PBS (PAA), rinsed in PBS with Ca/Mg, and resuspended in DMEM or RPMI 1640 in the presence or absence of mAbs, test compounds or solvents. A pre-incubation at room temperature with the respective mAb was carried out 10 min prior to cell seeding. T47D cells were plated at a concentration of 2.2×10^5/well and MDA MB 231 at 1.8×10^5/well in quadruplicates and incubated at 37 °C for 30 min. Non-adherent cells were then washed off with medium applying four rounds of gentle pipetting. Residual cells were checked by visual inspection and quantified with the colorimetric XTT tetrazolium assay (Roche Molecular Biochemicals) following the manufacturer's instructions. The tetrazolium salt XTT is metabolized by mitochondrial dehydrogenases of viable cells to a formazan dye. Numerical values of color densities were obtained in an enzyme-linked immunosorbent assay plate reader (BioRad) at a wavelength of 490 nm/reference 690 nm. Background values, determined by the adhesion of cells to wells coated with 1% BSA, were subtracted from each point. A direct modulating effect of the test compounds on mitochondrial XTT metabolization was excluded by incubating cells for 1 h in the presence and absence of the different compounds.

Cell spreading

Trypsinized and washed MDA MB 231 cells were seeded in DMEM with solvent alone or with DT at a concentration of 20 μg/ml on tissue culture plates precoated with low concentration of collagen (4 μg/ml). After 1 h of incubation at 37 °C, cells were fixed with 5% formaldehyde (Sigma) and visualized using phase-contrast microscopy (Zeiss). Fully spread (flattened cells with prolongations), partially spread (flattened cells without prolongations), and round (non-spread) cells were evaluated by two independent observers through direct counting.

Immunofluorescence staining

Permanox chamberslides (Lab-Tek, Nalge Nunc International) were coated with 4 μg/ml collagen or 7 μg/ml fibronectin in PBS for 1 h at 37 °C, followed by blocking for an additional hour with 1% BSA in PBS at 37 °C. Cells were detached with trypsin/EDTA as described before, rinsed in PBS with Ca/Mg, resuspended in serum free culture medium in the presence and absence of 20 μg/ml of DT or its solvent control and seeded in the PBS rinsed chamberslides. After 60 min at 37 °C, cells attached to the chamberslide were fixed with 3.7% formaldehyde (methanol free; Sigma) for 10 min at room temperature, rinsed with PBS, permeabilized for 5 min at room temperature with 0.2% Triton X-100, rinsed again with PBS and incubated with 10% rabbit serum for 30 min at room temperature to block unspecific binding. For immunofluorescence staining of VLA2 integrins, chamberslides were incubated with the mAb R2-8C8 in 10% rabbit serum for 45 min at 37 °C, rinsed five times with PBS, incubated for another 45 min at 37 °C with a fluorescein isothiocyanate-conjugated rabbit anti-mouse secondary antibody in 10% rabbit serum and rinsed another five times. For vinculin staining, cells were preincubated in 10% goat serum for 30 min at room temperature. Then, chamberslides were maintained for 45 min at 37 °C in the presence of the anti-vinculin polyclonal antibody in 10% goat serum. After washing, cells were incubated another 45 min at 37 °C with goat anti-rabbit Alexa 488 conjugated secondary antibody. Slides were then incubated in PBS containing Phalloidin-Alexa Fluor 568 conjugate for 30 min at room temperature. After washing, chambers were removed from the slides and samples were mounted in FITC-Guard mounting medium (Testog). The slides were examined with an Olympus FluoView 1000 confocal microscope. Digital images were mounted using Adobe Photoshop CS software.

Flow cytometry analysis

Flow cytometry analysis was performed at a Coulter EPICS XL flow cytometer. A total of 1×10^4 cells was analyzed for each sample in logarithmic scale unless indicated otherwise. For integrin expression analysis, 5×10^5 cells were incubated with mAbs R2-8C8 or Ab16 as primary antibodies on ice for 30 min, washed and subsequently incubated for another 30 min on ice with a fluorescein isothiocyanate-conjugated rabbit anti-mouse (Dako) secondary antibody and an Alexa 488-conjugated goat anti-rat (Molecular Probes) secondary antibody, respectively. Cells incubated with an isotype matching control mAb were used as negative control. Pelleted and resuspended cells were then analyzed by flow cytometry. To discriminate dead cell, cells were incubated with the indicated compound or medium alone for 60 min at 37 °C in disposables polypropylene tubes (Falcon Labware). After washing, cells were resuspended in PBS and then incubated with propidium iodide (Sigma) at a final concentration of 2 μg/ml for 5 min on ice. The cell suspension was analyzed on the flow cytometer for spontaneous uptake of propidium iodide.

To characterize apoptotic cells, 1×10^6 detached cells were incubated in DMEM with 0.1% of bovine serum containing 10 μM of the pan caspase inhibitor FITC-VAD-FMK (Promega) for 30 min at 37 °C. The inhibitor freely moves in and out of the cells except for when it binds irreversibly to activated caspases 1, 3, 4 or 7 thus, indicating the executor phase of apoptosis. Then, cells were washed and fixed with 0.5% paraformaldehyde in filtered PBS for 30 min at room temperature. After another wash, cell fluorescence intensities were determined on the flow cytometer.

Additionally, apoptosis was assessed by terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL method) using the APO-BRDU™ kit (BD Pharmingen). Following the manufacturer's instructions, $1–2 \times 10^6$ detached cells were fixed in 1% (w/v) paraformaldehyde in PBS (pH 7.4) for 60 min on ice.

After two wash steps in PBS the same amount of cells per ml was fixed in 70% (v/v) of ice-cold ethanol at least over night at −20 °C. The staining procedure was carried out as given in the manufacturer's protocol except for a lengthening of the incubation period with the labeling solution to 4 h in the CO_2 incubator at 37 °C. Cells stained for 30 min at room temperature with the FITC-labeled anti-BrdU antibody were washed with PBS, followed by analysis of the fluorescence intensities on the flow cytometer.

To assess whether TS2/16 mAb could rescue MDA MB 231 cells from adhesion-dependent apoptosis, cells were cultured for 3 days in DMEM containing 0.1% bovine serum on low amount of collagen in the presence or absence of DMSO or DT 15 μg/ml, with or without purified TS2/16 (10 μg/ml). The capability of TS2/16 to prevent apoptosis induced by lack of adhesion was confirmed in cells plated on BSA. Apoptosis was determined by TUNEL method, as described above.

Analysis of the phosphorylation state of FAK and Akt

MDA MB 231 and T47D breast cancer cells were plated in DMEM or RPMI 1640 in the absence or presence of DT or DMSO alone on tissue culture plates coated with 4 μg/ml of collagen in PBS as described for the adhesion assays. After incubation at 37 °C for 30 min, cells were rinsed in cold PBS. Attached and supernatant cells were pooled and lysed in cold Weinberg
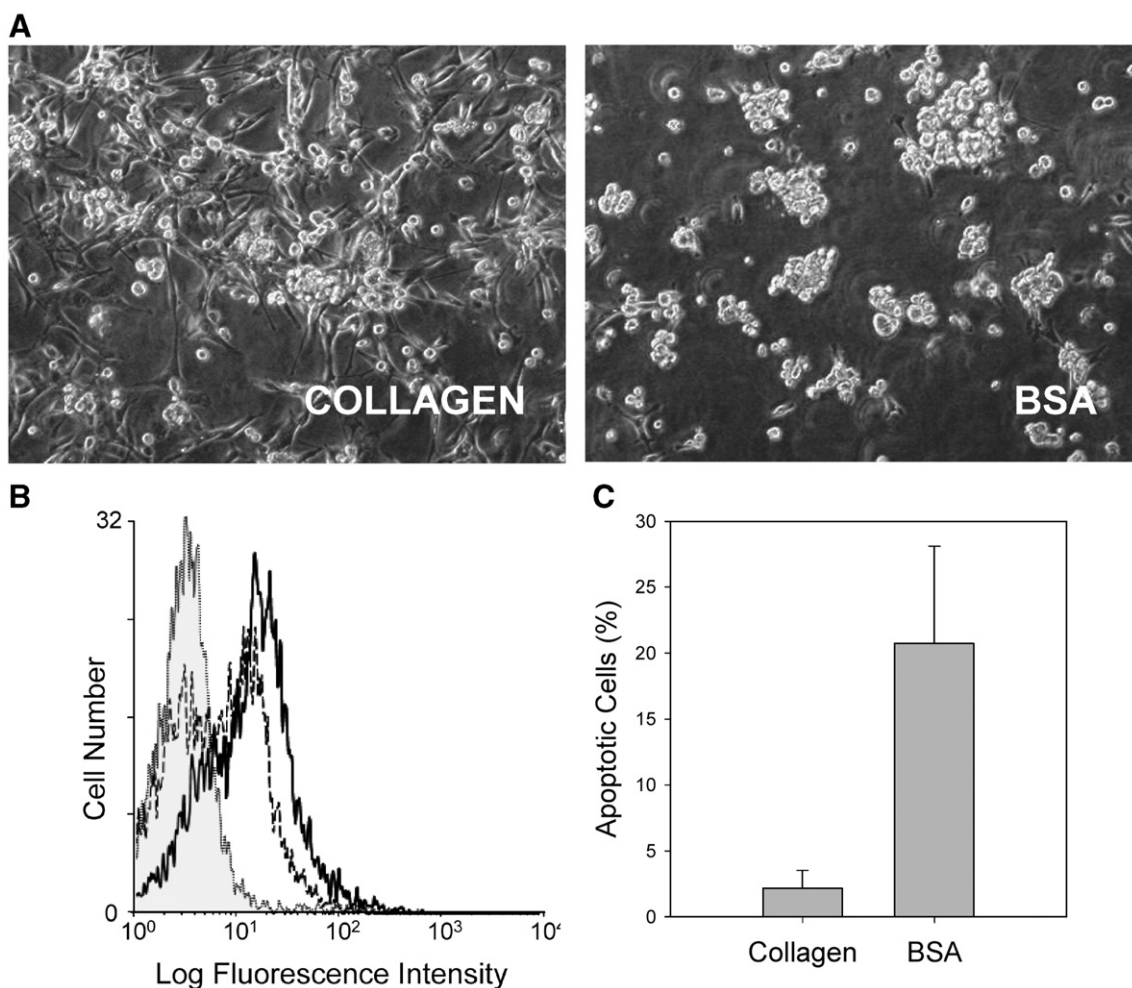

Fig. 1 – Survival of MDA MB 231 breast cancer cells is ECM adhesion dependent. (A) Phase contrast microscopy of cells maintained on immobilized BSA or collagen. Cells were cultured in DMEM containing 0.1% bovine serum on wells coated with collagen or BSA. After 72 h, the majority of cells seeded on collagen were flattened with regular margins, whereas most of the cells on BSA maintained a round phenotype and stayed in suspension. (B) Flow cytometry histograms of cells incorporating a fluorescein isothiocyanate labeled pan caspase inhibitor (FITC-VAD-FMK) which binds irreversibly to activated caspases 1, 3, 4, and 7 and thereby indicates the executor phase of apoptosis. Cells maintained on cell culture plastic in medium supplemented with 10% bovine serum were used as a negative control of apoptosis (shaded histogram). The higher fluorescence intensity of cells plated on BSA (solid line) indicates more apoptotic cells compared to cells cultured on immobilized collagen (dotted line). A representative experiment out of three is shown. (C) Bar graph showing the percentage of apoptotic cells assessed by TUNEL and flow cytometry analysis. MDA MB 231 cells plated on BSA or on collagen for 72 h in DMEM containing 0.1% bovine serum. The data confirm that collagen-mediated adhesion rescues MDA MB 231 breast cancer cells from apoptotic DNA cleavage. Data represent the means ± S.D. of the percentage of apoptotic cells from three independent experiments.

Buffer (50 mmol/l HEPES pH 7.0, 0.5% Nonidet P-40, 250 mmol/l NaCl, 5 mmol/l EDTA, 5 mmol/l NaF, 0.2 mmol/l Na_3VO_4, 50 mmol/l β-glycerophosphate, 1 mmol/l PMSF, 2 mmol/l benzamidine, 10 μg/ml aprotinine, 10 μg/ml leupeptine), additionally applying a freeze thaw cycle. After removing cellular debris by centrifugation (15,000×g, 10 min, 4 °C), aliquots of the supernatant, containing 10 μg total protein (as determined by BCA assay from BioRad according to the manufacturer's description), were subjected to SDS polyacrylamide gel electrophoresis and subsequently transferred onto nitrocellulose membranes (Schleicher and Schüll). Blocking of unspecific binding sites with non-fat dry milk (5% in Tris Buffered Saline containing 0.05% Tween-20) was followed by an incubation with the polyclonal rabbit anti-human primary antibodies (P-FAK 1:500, FAK 1:250, P-Akt 1:100, Akt 1:250) which were detected with HRP-conjugated anti-rabbit IgG
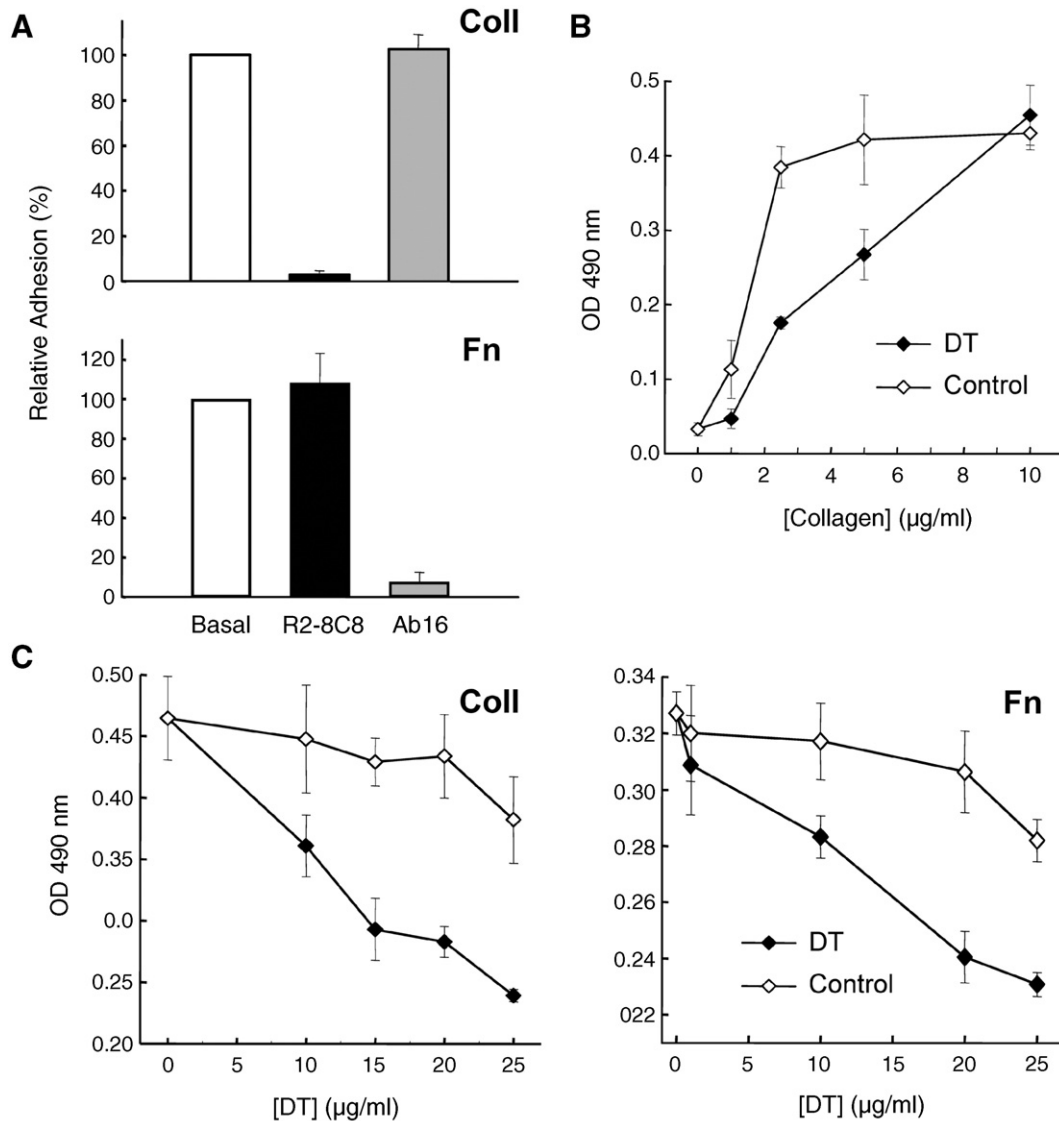

Fig. 2 – Effect of DT on MDA MB 231 breast cancer cell adhesion to collagen and fibronectin. (A) MDA MB 231 cells adhered to collagen (Coll) and fibronectin (Fn) exclusively through α2β1 and α5β1 integrin, respectively. In adhesion assays, the blocking anti-α2β1 mAb R2-8C8 completely abolished MDA MB 231 cell adhesion to immobilized collagen compared to untreated or Ab16 treated cells, whereas the blocking anti-α5β1 mAb Ab16, but not R2-8C8 blocked the binding of these cells to fibronectin. Data from three independent experiments are presented as means±S.D. of relative adhesion with respect to cells maintained in medium (Basal), which was considered 100%. (B) DT interferes with α2β1-mediated adhesion of MDA MB 231 cells at low-range concentrations of immobilized collagen. DT at 20 μg/ml reduced largely cell adhesion when cells were plated on wells precoated with PBS containing 2 to 5 μg/ml of collagen. Data are expressed as means±S.D. of quadruplicate measures of optical density at 490 nm. An example out of three independent adhesion assays is shown. (C) DT interferes with cell adhesion via α2β1 and α5β1 in a dose-dependent manner. Adhesion assays with MDA MB 231 cells were carried out on wells coated with limited availability of substrate (4 μg/ml collagen and 5 μg/ml fibronectin). The maximum effective dose was observed at 20 μg/ml of DT on both substrates. Data are expressed as means±S.D. of quadruplicate measures of optical density at 490 nm. Representative experiments out of four independent ones are shown.

(Amersham Biosciences) using Super Signal Substrate (Pierce). To verify equal loading, blots were stripped and reprobed with the antibody against the respective total protein. Western blots were exposed to Kodak XAR5-Omat films, and semi-quantitative evaluation of visualized bands was carried out by densitometry (gel documentation system, MWG Biotech) using Gene Profiler 3.56 for Windows (Scanlytics) over multiple exposures to verify that readings were in the linear range.

Binding of soluble fluorescent collagen

The standard buffer used in the ligand-binding assay was Tyrode's Salts (Sigma) adjusted to pH 7.4. Cells in suspension were incubated with buffer alone, in the presence of the mAb R2-8C8, 20 μg/ml of DT or in 1:1000 DMSO. Then, cells suspended in these four conditions were incubated with 20 μg/ml soluble Oregon Green labeled collagen IV (Molecular Probes) at 37 °C. After 30 min, cells were washed at RT and cell fluorescence intensities were determined by flow cytometry in linear scale. To obtain numerical estimates of the integrin affinity state, an activation index (AI) was calculated based on the one previously defined by Hughes et al. [22]: $AI = 100 \times (F_{VEC} - F_{DT})/(F_{COLL} - F_{Ab})$, where F_{VEC} is the mean fluorescence intensity (MFI) of cells maintained in presence of DMSO; F_{DT} is the MFI of cells upon DT treatment; F_{COLL} is the MFI of cells maintained in buffer alone, and F_{Ab} is the MFI of cells exposed to blocking mAb in buffer. Under our experimental conditions, $F_{COLL} - F_{Ab}$ represents the maximum possible variation in the binding of soluble collagen to cells through $\alpha2\beta1$ integrin whereas, $F_{VEC} - F_{DT}$ represents the reduction in the collagen binding to cells attributed to DT. This AI represents a percentage of binding capability, in which 0% indicates no changes of the basal activation state of $\alpha2\beta1$ integrin and 100% represents the maximum possible reduction in the affinity of the integrin under our experimental conditions.

Statistical analysis

Data are expressed as arithmetic means ± standard deviation (S.D.) or standard error of the mean (S.E.M.). Wilcoxon signed rank test or Chi-square test were used as indicated in the text to determine significant differences between means. $P < 0.05$ was considered statistically significant.

Results

Lack of ECM adhesion induces apoptosis in human breast cancer cell lines

It has been well-demonstrated in non-malignant adherent cells that loss of integrin-mediated adhesion leads to cell death by apoptosis [15,23]. However, several lines of evidence suggest that metastatic cancer cells are refractory to survival signals provided by cell–ECM interactions [24,25], a point that might contribute to an increment in malignancy [26]. To study the role that ECM adhesion plays in the resistance of breast cancer cells to undergo apoptosis, we maintained hormone-independent, highly malignant MDA MB 231 cells under limiting serum conditions on both BSA and collagen pre-coated (20 μg/ml) tissue culture plastic. Fig. 1A shows cells after 3 days in culture under these conditions. It is evident that MDA MB 231 cells plated on BSA exhibited less cell spreading
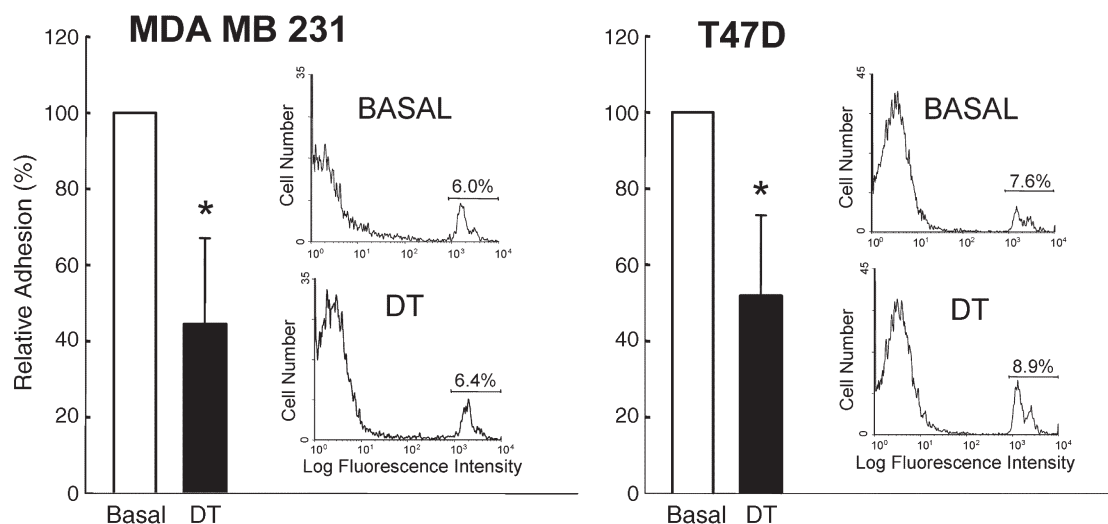

Fig. 3 – DT inhibits adhesion of both hormone-dependent and hormone-independent breast cancer cell lines. Adhesion assays of MDA MB 231 and T47D in the presence and absence (basal) of 20 μg/ml of DT on collagen under the experimental conditions described in Fig. 2C. Bar graphs represent date from four independent experiments represented as means ± S.D. of relative adhesion with respect to cells maintained in vehicle (Basal), which was considered 100%. *p < 0.01 for both cell lines using Wilcoxon signed rank test. Histograms shown in the insets represent the spontaneous uptake of propidium iodide by cells incubated in the absence (basal) and presence of DT at 20 μg/ml for 60 min, an incubation period that duplicates cell exposure during adhesion assays. Numbers correspond to the percentage of positive cells (dead cells). A representative experiment out of three independent ones is shown.

and a higher number of round cells with irregularly shrunk profiles than those maintained on collagen. The percentage of apoptotic cells, assessed by flow cytometry analysis using both a fluorescent pan caspase inhibitor (FITC-VAD-FMK) and the TUNEL method, was found to be reduced in cells cultured on collagen-coated plastic (Figs. 1B, C), which was in accordance with the phenotypical changes. These results show that collagen provides an anti-apoptotic signal for MDA MB 231 cells *in vitro* which indicates the involvement of the $\alpha2\beta1$ integrin as survival receptor in this tumor cell line.

$\alpha2\beta1$ and $\alpha5\beta1$ integrin-mediated adhesion of MDA MB 231 cells is decreased by an apoptosis-inducing compound

Our group has previously isolated and characterized the marine, polyether triterpenoid, DT, a natural compound that preferentially induces apoptosis in hormone-independent human breast cancer cell lines [16]. This chemical caused the detachment of cell clusters without losing cell–cell contact (Pec, M.K., personal observation) suggesting a potential effect on cell–ECM interactions. To further study the capability of this compound to interfere with the adhesion of tumor cells to ECM, we established the conditions for adhesion assays to collagen and fibronectin for two breast cancer cell lines of distinct malignancy. The basal adhesion of MDA MB 231 cells to immobilized collagen type I and fibronectin (20 µg/ml of protein in the coating solution) was completely abolished by R2-8C8, a blocking anti-$\alpha2\beta1$ mAb and Ab16, a blocking anti-$\alpha5\beta1$ mAb, respectively (Fig. 2A). These results confirm that this tumor cell line adhered to immobilized collagen and fibronectin exclusively via $\alpha2\beta1$ and $\alpha5\beta1$, respectively (Fig. 2A). The same behavior was observed in T47D, a hormone-dependent breast cancer cell line (data not shown).

To test the potential effect of DT on integrin-mediated breast cancer cell adhesion, we plated DT-treated MDA MB 231 cells to a concentration range of immobilized collagen and fibronectin. We observed a maximal inhibitory effect of 20 µg/ml DT, with respect to controls, in the wells that were precoated with a PBS solution containing between 2 and 5 µg/ml of collagen (Fig. 2B). Increasing the ECM substrate concentration could overcome the inhibitory effect of DT (Fig. 2B). A similar adhesion profile was obtained when MDA MB 231 were plated on fibronectin (data not shown). The effect of DT on MDA MB 231 adhesion to wells pre-coated with 4 µg/ml of collagen or 5 µg/ml of fibronectin was dose-dependent, with a maximum effect of 15–20 µg/ml of DT with respect to control cells plated in presence of DMSO (Fig. 2C).

Since there is little information about cell line specific integrin properties, we decided to test the behavior of a less invasive, hormone-dependent breast cancer cell line (T47D) in adhesion assays to collagen and fibronectin. Fig. 3 shows that DT, at 20 µg/ml significantly inhibited the basal adhesion of MDA MB 231 and T47D cells to a limited concentration of immobilized collagen (4 µg/ml). Given that DT is a potent inducer of apoptosis, we examined the possibility that the reduced adhesion merely reflected decreased cell viability. We exposed both cell lines to the maximal effective dose of DT for 1 h, which duplicated the incubation period in adhesion assays. Cell viability was determined by flow cytometry through the spontaneous

uptake of propidium iodide. Insets of Fig. 3 show flow cytometry histograms in which the percentage of dead cells was similar in the MDA MB 231 and T47D cell lines, independently of the presence or absence of DT. These data indicate that DT interferes in a dose-dependent manner
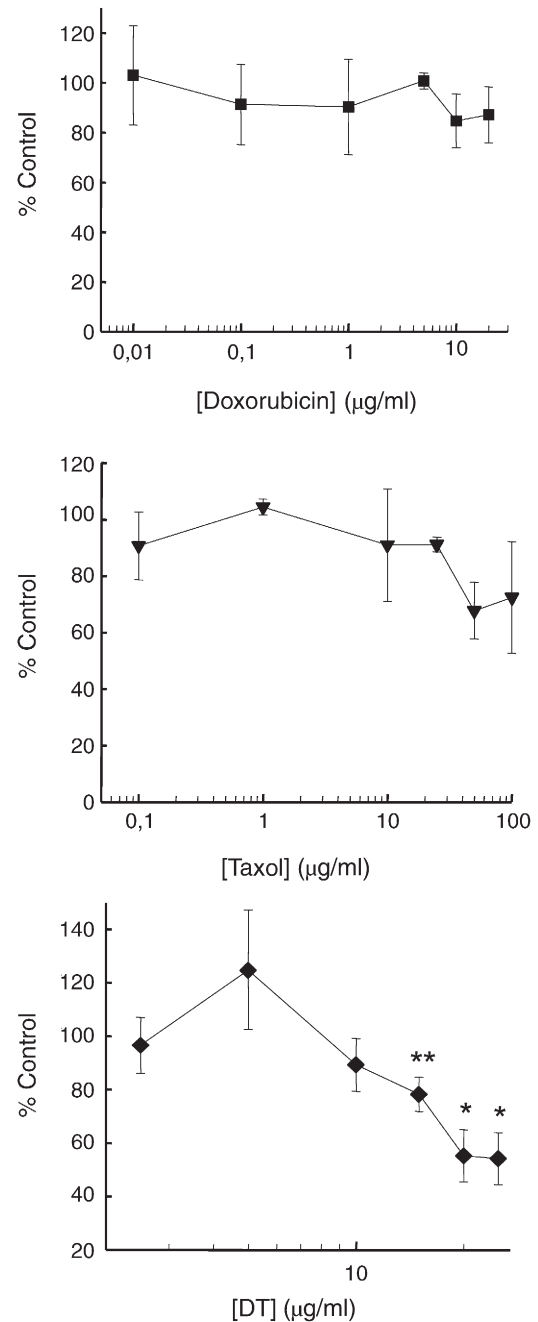

Fig. 4 – **Effect of chemotherapeutics on adhesion of MDA MB 231 cells. Doxorubicin and taxol, at doses as high as 20 and 100 µg/ml did not significantly inhibit adhesion of this cell line to low amount of immobilized collagen (4 µg/ml). However, the presence of DT diminished the adhesion of MDA MB 231 in a dose-dependent profile under the same conditions. Results are expressed as means±S.E. with respect to controls of four independent adhesion assays. *p<0.01, **p<0.001, evaluated with Wilcoxon signed rank test.**
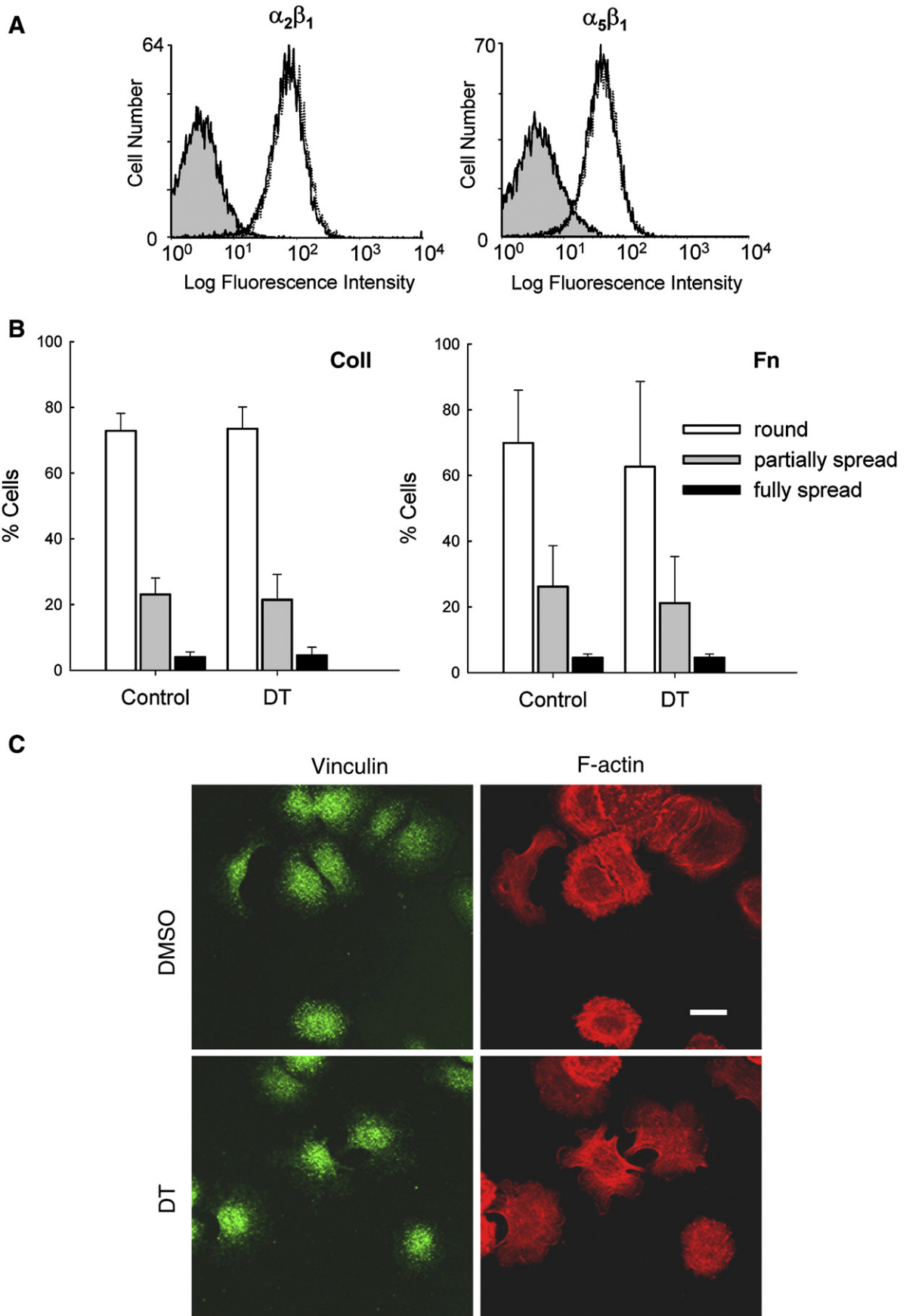
with VLA integrin-mediated adhesion in hormone-dependent as well as hormone-independent breast cancer cell lines. This effect was cell toxicity-independent.

Modulation of integrin-mediated cell adhesion to ECM is not a common feature of apoptosis-inducing chemotherapeutics

Doxorubicin and taxol are compounds with a well-known apoptosis-inducing capability. Both are commonly used for the treatment of human breast cancer [27,28]. As mentioned before, integrins can mediate various apoptotic pathways. This raises the possibility that interference with integrin-mediated adhesion might be part of the general characteristics of cell death-inducing, anti-neoplastic compounds. To test this hypothesis, we compared, under our experimental conditions, the effects of concentration ranges of doxorubicin, taxol, and DT on MDA MB 231 cell adhesion to low amount ECM. As shown in Fig. 4, both chemotherapeutics failed to modify significantly the basal adhesion of MDA MB 231 cells to collagen (4 μg/ml). In contrast, DT caused a significant decrease in the basal adhesion of this cell line at a concentration of 15 μg/ml or higher. Similar results were obtained on fibronectin (data not shown). These findings suggest that, although integrins have been related to several apoptotic pathways, the apoptotic effects of anti-cancerous compounds do not necessarily affect integrin-mediated adhesion.

DT does not modify integrin cell surface expression and distribution, cell spreading and vinculin and F-actin distribution

A potential explanation for the capability of DT to reduce breast cancer cell adhesion could be given in changes of the expression levels of integrins. Thus, we studied the effect of DT on the surface expression of $\alpha2\beta1$ and $\alpha5\beta1$ by flow cytometry. The expression levels of both integrins after 1 h of exposure to DT (20 μg/ml) were equivalent to basal surface expression in MDA MB 231 (Fig. 5A) and T47D cells (data not shown). This data ruled out changes in VLA cell surface expression as an explanation for the decreased cell adhesion caused by DT in these cell lines.

Yauch et al. have demonstrated that fibronectin binding can occur by avidity regulation via $\beta1$ integrin multimerization in the absence of an increase in receptor affinity [29]. Consequently, and as we had not found changes in integrin cell surface expression, we decided to examine possible effects of the compound on integrin outside-in signalling reflected by cell spreading [18,30]. To this end, we quantified the number of fully spread, partially spread, and round (non-spread) MDA MB 231 cells by direct counting after an exposure time of 1 h to a limiting concentration of immobilized collagen or fibronectin. We did not observe any significant differences between treated and control MDA MB 231 cell spreading on both ECM proteins (Fig. 5B). Additionally, we analyzed $\alpha2\beta1$ and $\alpha5\beta1$ cell membrane distribution of collagen and fibronectin adhered MDA MB 231 cells by immunofluorescence staining. We observed diffuse staining but no differences in the cell surface distribution of both VLA integrins between DT-treated and solvent control cells (data not shown). No focal adhesion formation was observed [31]. In addition, we also examined intracellular vinculin and actin stress fiber distribution by confocal microscopy. Consistently with our previous observation, we did not find any obvious differences between solvent control and DT-treated cells in the vinculin and actin fiber organization in MDA MB 231 cells plated on low amount of collagen and fibronectin (Fig. 5C and data not shown). These observations suggest that DT does not affect either basal surface expression levels or avidity regulation of VLA integrins in the tested cancer cell lines.

DT treatment neither modifies adhesion-induced phosphorylation of FAK nor Akt/PKB

Activation of FAK plays a dominant role as an integration point of integrin and growth factor signalling. Autophosphorylation of FAK on tyrosine 397 affects integrin affinity and avidity [32]. Therefore, we analyzed FAK phosphorylation by Western blotting of MDA MB 321 and T47D cell extracts. No significant differences with respect to total protein content could be detected in both cell lines plated on a limiting concentration of collagen and fibronectin, in the absence or presence of DT or DMSO after 30 min (Fig. 6A and data not

Fig. 5 – DT did not modulate either VLA integrin cell surface expression, cell spreading capability or vinculin and actin intracellular distribution. (A) $\alpha2\beta1$ and $\alpha5\beta1$ cell surface expression in MDA MB 231 cells. MDA MB 231 cells were treated for 60 min with 20 μg/ml of DT, doubling the exposure time applied in adhesion assays. Representative flow cytometry histograms of integrin $\alpha2\beta1$ and $\alpha5\beta1$ surface expression are shown. Dotted lines represent integrin expression in the presence of DT; solid lines indicate basal integrin expression; and shaded histograms stand for negative controls. Histograms are representative for three independent ones each. (B) Spreading of MDA MB 231 cells on collagen and fibronectin. MDA MB 231 cells were plated on collagen or on fibronectin coated wells (4 and 7 μg/ml, respectively) at 37 °C for 1 h in the absence (solvent Control) or presence of 20 μg/ml of DT. After fixation, fully spread, partially spread and round cells were counted using phase-contrast microscopy. No significant differences were found (Chi-square test) in MDA MB 231 cell spreading on any of the two substrates by the presence of DT. Data represent the means±S.D. of the percentages of round, fully and partially spread cells from three independent experiments. (C) Immunofluorescence of vinculin and F-actin fibers distribution in MDA MB 231 breast cancer cells during cell spreading. Cells were plated in serum free culture medium on collagen (4 μg/ml) coated chamberslides for 1 h at 37 °C in the absence (solvent control, DMSO) or presence of 20 μg/ml of DT followed by immunofluorescence staining for vinculin (green labeling) and actin (phalloidin, red labeling), as described in Materials and methods. Micrographs of confocal microscopy of the horizontal cell section plane closest to the substrate did not show any relevant differences in intracellular vinculin distribution and stress fiber formation between solvent control and DT-treated cells. Scale bar is 10 μm.

shown). Thus, we further analyzed phosphorylation of Akt/PKB on serine 473, a downstream target of both integrin and survival signalling pathways under the same conditions. Surprisingly, western blot quantification did not reveal any significant differences between Akt phosphorylation in solvent control and DT-treated T47D (Fig. 6B) cells and MDA MB 231 (not shown). Thus, the DT-induced decrease in breast cancer cell adhesion does not significantly affect neither FAK nor Akt phosphorylation-mediated signalling.

DT modulates collagen receptor binding capability in MDA MB 231 cells

Having ruled out the other possible reasons for the DT mediated decrease in breast cancer cell adhesion, we decided to test the hypothesis that this compound would specifically affect the affinity of VLA integrins. Integrins in a high affinity state can bind to soluble and immobilized ECM proteins, while low affinity receptors only bind to immobilized substrates [21].
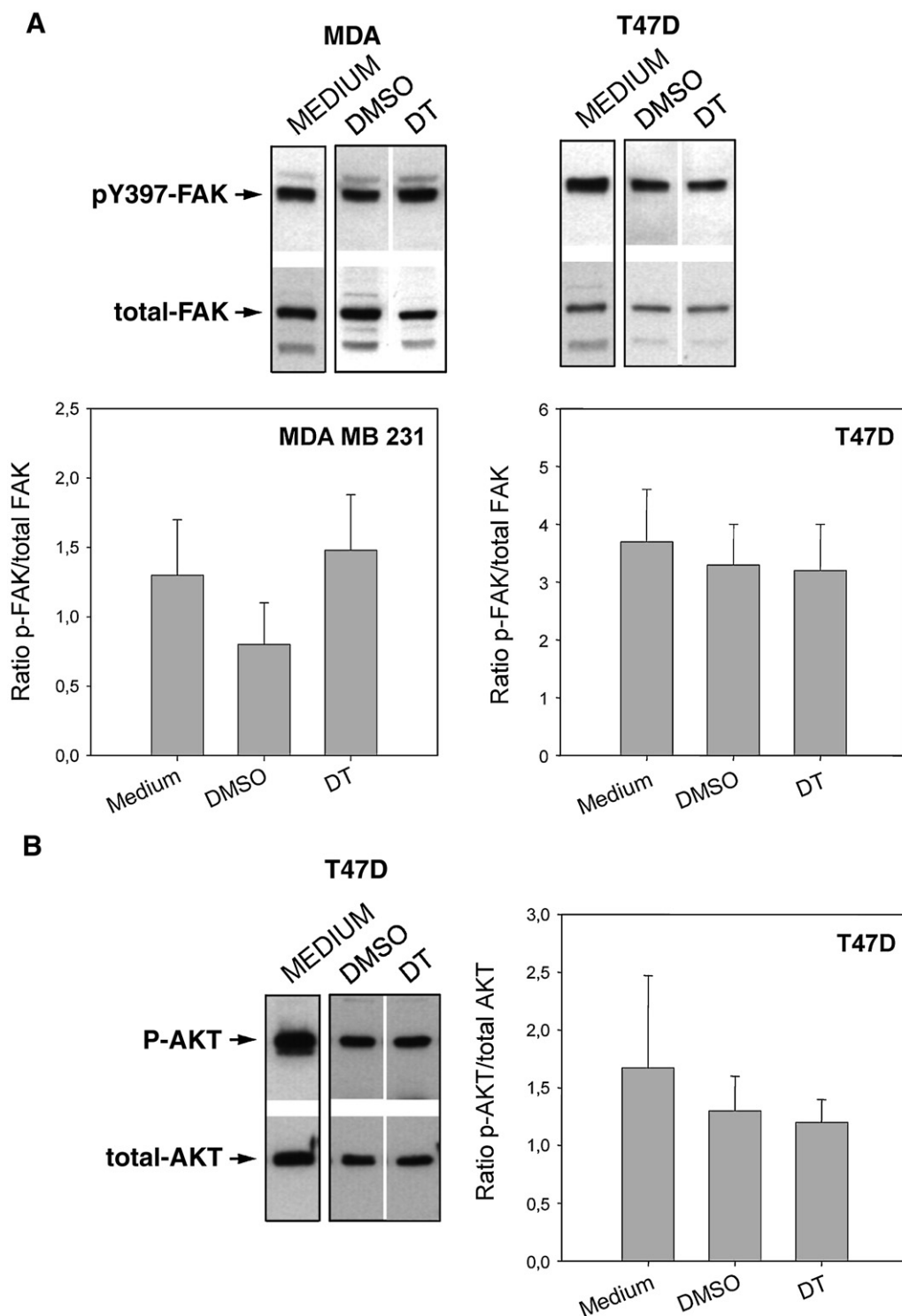
To determine whether DT interfered directly with the integrin activation state, we established a binding assay with fluorescent, soluble collagen. We incubated MDA MB 231 cells with the fluorescent substrate and analyzed by flow cytometry fluorescence intensities of cells incubated in medium alone, in the presence of the mAb R2-8C8, DT at 20 μg/ml or DMSO. To minimize interexperimental variations, we normalized data by calculating an activation index (AI) as described in Materials and methods. The mean AI was 43±12 S.E.M., which means that the presence of DT reduced α2β1 integrin activation by 43% in this cell line. We found significantly reduced MFI in DT-treated cells compared to vehicle controls or medium alone (Fig. 7). These results suggest that DT decreases breast cancer cell binding to collagen by selective modulation of the α2β1 integrin affinity state.

A β1 activating antibody can prevent DT-induced apoptosis

To further investigate the role of the interference with integrin-mediated cell adhesion in the DT-induced apoptosis, we tested the effect of TS2/16, an anti-β1 mAb which induces the high affinity state of VLA heterodimers. We cultured MDA MB 231 cells plated on BSA for 72 h, experimental conditions we had found to induce extensive apoptosis in this cell line with or without 10 μg/ml TS2/16 mAb. As shown in Fig. 8, TS2/16 mAb reduced the induction of apoptosis to a great extend in BSA plated cells. Most importantly, TS2/16 mAb was able to completely prevent DT-induced apoptosis in MDA MB 231 cell under our experimental conditions (Fig. 8). This result suggests that, at least part of the DT-induced apoptosis in breast cancer cell lines is because of IMD.

Discussion

In this work, we describe the specific modulation of VLA integrin activation in breast cancer cell lines by DT, a marine, apoptosis-inducing compound. This chemical reduced adhesion of MDA MB 231 and T47D tumor cell lines to low amounts of immobilized ECM and the binding of soluble collagen to α2β1, without a significant effect on cell surface expression or distribution of integrins. DT affected neither cell spreading,

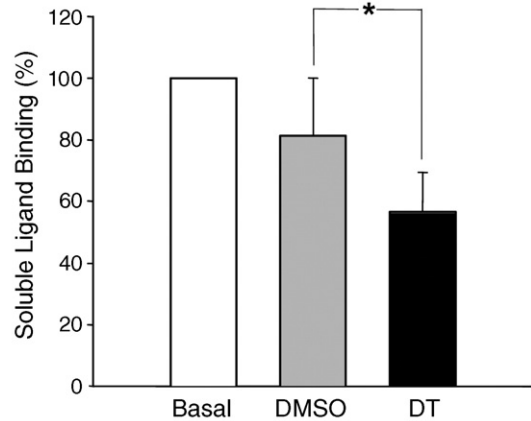Fig. 7 – DT modulates the α2β1 integrin affinity in MDA MB 231 cells. The binding of soluble fluorescent collagen was used to assess the activation state of the α2β1 integrin in MDA MB 231 cells, as described in Materials and methods. Data from seven independent experiments are presented as means±S.E.M. of relative MFI of cells maintained in DMSO or DT 20 μg/ml with respect to the MFI of cells maintained in medium alone (Basal) that was considered 100%. Differences between DT and solvent (DMSO) treatment were significant (p=0.0156, evaluated with Wilcoxon signed rank test).

intracellular distribution of vinculin and actin, nor phosphorylation of FAK and Akt/PKB in these cells. Interestingly, addition of the β1-integrin activating antibody (TS2/16) prevented the apoptosis induced by DT in these tumor cell lines.

Insufficient cell–matrix contacts promote IMD in anchorage-dependent cells [15]. Specific integrins have been shown to protect non-tumor cells from apoptosis by mediating bcl-2 up-regulation [33]. However, the role of adhesion in the regulation of apoptosis in tumor cells is not completely understood. Carcinoma cells can become anchorage-independent with increased survival time and invasiveness in the absence of matrix attachment [14]. To assure that the highly invasive MDA MB 231 breast cancer cell line conserved its susceptibility to IMD, we maintained these cells without ECM contacts for 3 days. Exposure to immobilized collagen rescued

Fig. 6 – Analysis of the phosphorylation state of focal adhesion kinase pp125FAK and Akt/PKB. (A) FAK phosphorylation. MDA MB 231 and T47D breast cancer cells were plated in medium alone, in DMSO or in the presence of 20 μg/ml of DT on tissue culture plastic coated with 4 μg/ml of collagen. After incubation for 30 min at 37 °C, proteins were extracted and subjected to Western blotting for phospho-FAK (Y397). To verify equal loading, blots were stripped and reprobed with the polyclonal antibody against total FAK protein as described in Materials and methods. Solvent control (DMSO) and DT samples were analyzed on the same blots while untreated cell extracts came from a different membrane (Medium). DT did not alter FAK Y397 phosphorylation in a significant way. Semi-quantitative densitometry evaluation of phospho-protein from three separate extractions was normalized to the respective total protein sample. Data are presented as means±S.D. of p-FAK/total FAK ratio for each cell line. (B) Akt/PKB phosphorylation. T47D breast cancer cells were plated for 30 min in pure medium or in the presence of 20 μg/ml of DT or solvent control (DMSO) on tissue culture plastic, coated with 4 μg/ml of collagen and subjected to the same procedure as described above. Blots were probed with a polyclonal antibody specific for phospho-Akt (Ser 473). Loading controls were again analyzed on the stripped blots and reprobed with the polyclonal antibody against total Akt protein. Solvent control (DMSO) and DT samples were analyzed on the same blots while untreated cell extracts were processed on a different membrane. Again, Akt phosphorylation did not change significantly upon DT treatment in this cell line. Semi-quantitative densitometry evaluation of phospho-protein from three separate extractions was normalized to the respective total protein sample. Data are presented as means±S.D. of p-Akt/total Akt ratio.
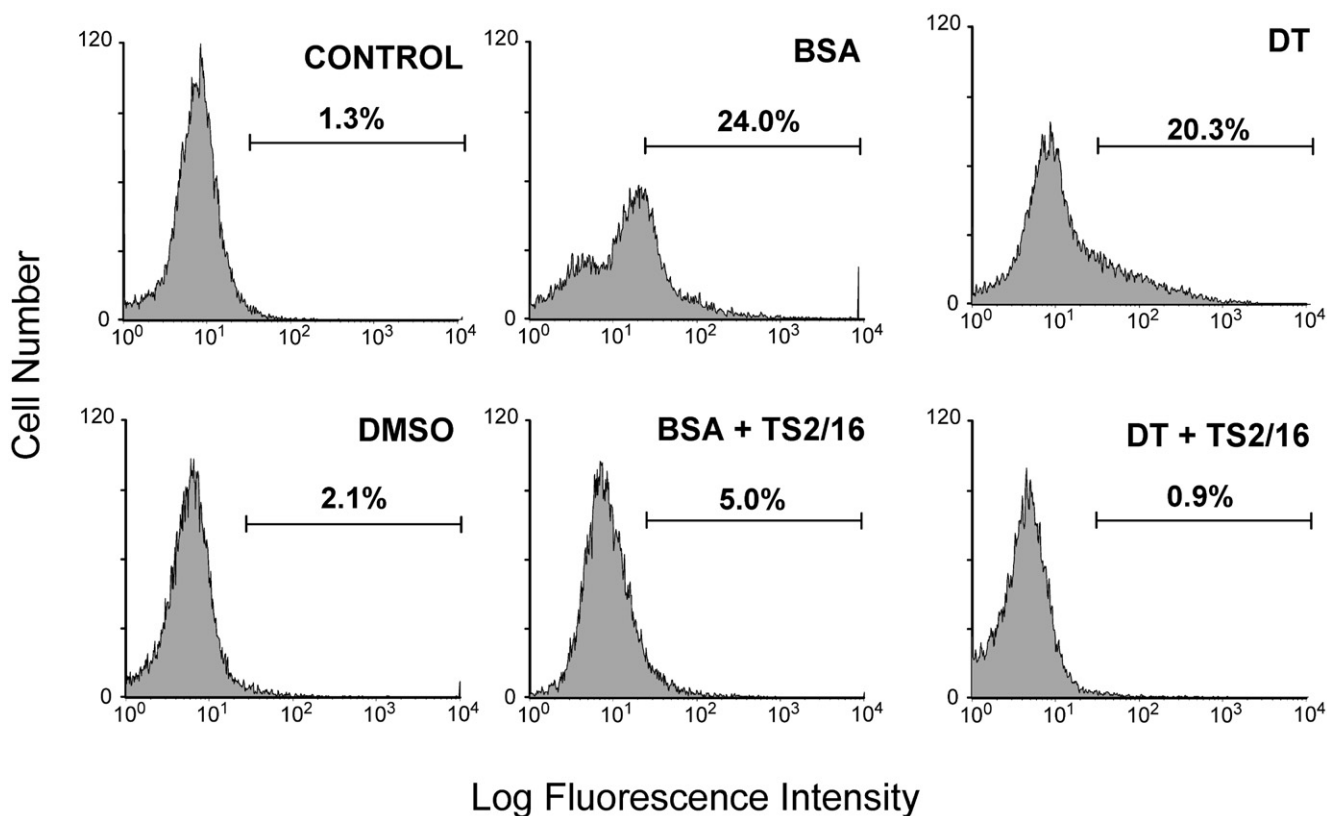
Fig. 8 – TS2/16, a VLA integrin activating monoclonal antibody rescues MDA MB 231 cells from DT-mediated apoptosis. Flow cytometry histograms showing the percentage of apoptotic cells assessed by TUNEL method. Cells were maintained in DMEM with 0.1% bovine serum either on BSA or collagen at 4 μg/ml for 72 h. Collagen exposed cells were cultured in medium alone (control) and in the presence or absence of DT (15 μg/ml) or DMSO with or without the β1 activating monoclonal antibody, TS2/16 (10 μg/ml). Apoptosis of adhesion deprived cells on BSA was reduced to a great extent by the activating antibody. Remarkably, DT-induced apoptosis of cells cultured on immobilized collagen in a limiting concentration was completely prevented by the TS2/16 antibody. Numbers correspond to the percentage of positive cells (apoptotic cells). Flow cytometry histograms are representative for three independent experiments.

most of the cells from entering into apoptosis. This demonstrates that a very aggressive, metastasis forming breast cancer cell line, such as MDA MD 231 can be susceptible to IMD.

The change from primary cancer to metastatic tumor cells is initially accompanied by modifications in cell surface expression profiles of integrins and variations in the release of ECM degrading proteases [7]. Distinct regions of the β1 cytoplasmic domain have been linked with adhesion, invasion, and metastasis formation [34]. However, conflicting works on integrin cell surface expression and their association with invasive carcinomas demonstrate the gap of information in this field [35–37]. Therefore, we decided to establish the experimental conditions in breast carcinoma cell lines to analyze specific interactions of α2β1 and α5β1 with collagen and fibronectin, components of the ECM that have been shown to play a role in the metastatic process [1]. Our data demonstrate that DT interferes, in a dose-dependent manner, with VLA-mediated breast cancer cell adhesion to low amount of immobilized substrate. This effect appears to be compound specific, since doxorubicin as well as taxol, which also induce apoptosis in breast cancer cells, did not affect β1-mediated cell adhesion. DT did not modify expression levels and cell surface

distributions of α2β1 and α5β1. MDA MB 231 cells did not show focal adhesion formation when plated on collagen or fibronectin, a fact previously observed by other groups [31] and the cell distribution of vinculin and actin fibers was unaffected by the presence of DT. Thus, DT, unlike other apoptosis-inducing compounds, interferes with adhesion of breast cancer cells through a mechanism more likely related to a modulation of the activation of integrins than to changes in the cell surface expression level or avidity of these adhesion molecules.

The most rapid means of regulating cellular adhesion is through dynamic modulation of receptor affinity, a key feature of the integrin family. Recently, it has been demonstrated that the activated form of an integrin (αvβ3) supports breast cancer cell arrest during blood flow. The authors showed that the constitutively activated integrin in the MDA MB 435 breast cancer cell line strongly promoted metastasis in a mouse model [13]. This work points at integrin affinity as an important factor in the metastatic process *in vivo*. Integrins in high affinity can be evaluated using soluble ligands [38]. In conditions of limited, immobilized substrate, cells are supposed to adhere mainly through their integrins in a high affinity state [18]. The fact that DT maximally inhibited

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β1 mediated adhesion of breast cancer cells when immobilized ECM was available in limiting concentrations, strongly suggested that this compound was preferentially targeting integrins in their high affinity state. The assumption that DT affects only VLA integrins in high affinity state was confirmed by the specific binding of a fluorescent, soluble collagen IV to the α2β1 integrin in MDA MB 231 cells maintained in suspension. Since, β1 integrins are mostly in low affinity when cells are in suspension, it was not surprising to find relatively high interexperimental variations with soluble ligand binding used to assess the integrin activation state. However, a reduction in α2β1 affinity state upon DT treatment was consistently observed in all experiments. Importantly, the inhibition of DT induced apoptosis by VLA activating TS2/16 confirmed that the conformational state of β1 integrins is affected by DT and, at least in part responsible of its apoptotic effects in breast cancer cell lines. Consequently, our data indicate that DT interferes with the α2β1 inside-out signalling in breast tumor cells.

Elevated FAK expression and activity have been correlated with the progression to a highly malignant and metastatic phenotype [39,40]. DT did not induce significant variations in basal FAK phosphorylation in collagen plated breast cancer cells. Several pathways of FAK-independent integrin-mediated signalling have been demonstrated [41,42]. Accordingly, inhibition of β1 integrin function in the mammary gland *in vivo* leads to increased cell death with FAK phosphorylation staying normal under these conditions [43]. Thus, the anti-apoptotic and survival promoting effects of integrin-mediated adhesion can be transduced by signalling cascades, which circumvent FAK activation (reviewed in [32]). This data suggests that either DT did not induce outside-in signalling or that it acts by a FAK-independent pathway in these cell lines. The fact that DT did not affect spreading of MDA MB 231 cells supports the hypothesis that DT does not interfere with the outside-in signalling of the antagonized integrins. Adhesion by means of β1 integrins induces the phospatidylinositol 3-kinase (PI3K) dependent-phosphorylation of Akt, a signalling pathway that participate in the cell migration, proliferation and apoptosis. This pathway can be activated in a FAK-dependent [44] and -independent [45] manner and it has been shown that their components are frequently altered in human cancers. Under our experimental conditions, Akt phosphorylation was not affected by DT treatment. Consequently, our data suggest that, at least in part, DT-induced apoptosis in breast cancer cells may be mediated by modulating VLA integrin signalling in a FAK-, Akt-independent way.

Our data suggest that DT acts as an integrin antagonist that interferes specifically with the inside-out signalling. This makes the compound different from most of the integrin antagonists currently developed, as they also act as agonists, inducing conformational receptor changes which produce undesired clinical effects [46].

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