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Ecteinascidin-743 drug resistance in sarcoma cells: transcriptional and cellular alterations

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Abstract

A human chondrosarcoma cell line, CS-1, was treated successively with increasing concentrations of the marine chemotherapeutic Ecteinascidin-743 (ET-743), yielding a variant cell line displaying a significant degree of resistance to the cytotoxic action of this drug. Various experiments were performed to discern molecular aberrations between the parent and resistant cell line, and also identify potential molecular markers indicative of drug resistance. Although no significant differences in the levels of membrane transporters such as P-glycoprotein or multidrug resistance protein 1 (MRP1) were detected, the cell migratory ability of the ET-743-resistant cell variant was reduced, as was its attachment capability to gelatin-coated cell culture dishes. Staining of the actin-containing cytoskeleton with fluorescent-labeled phalloidin revealed marked differences in the cytoskeleton architecture between the parent and ET-743-resistant CS-1 cell lines. Comparison of serum-free conditioned medium from both cell lines showed conspicuous differences in the levels of several proteins, including a quartet of high molecular weight proteins ($\geq$140 kDa). The protein sequences of two of these high molecular weight proteins, present at significantly higher concentrations in conditioned medium obtained from the parent cell line, corresponded to subunits of types I and IV collagen. Analysis of type I collagen α1 chain mRNA revealed a significantly lower level in the ET-743-resistant CS-1 cell line. Thus, prolonged exposure to ET-743 may cause changes in cell function through cytoskeleton rearrangement and/or modulation of collagen levels.

Keywords: Ecteinascidin-743; Chondrosarcoma; Drug resistance; Chemotherapy; Collagen; Extracellular matrix

1. Introduction

The investigation of naturally occurring marine compounds as potential anticancer agents has yielded promising candidates, including the tetrahydroisoquinoline alkaloid ET-743, which has shown efficacy in clinical trials for treating sarcomas [1–3]. By binding to the minor groove of DNA and alkylating the N2 position of guanine, ET-743 produces a bending of the DNA toward the major groove as well as single-strand DNA breaks [4,5]. Thus, ET-743 appears to act differently mechanistically with respect to other DNA minor groove alkylators. Recent reports have demonstrated that ET-743 can interfere with induced but not basal transcription of various genes, possibly by forming covalent adducts that act as an impediment to transcription factors, or by interfering with assembly of transcription complexes [6–8].

Multidrug resistance (MDR), a phenomenon displayed by many tumor types, is characterized by various molecular adaptations in cancer cells (typically after continuous exposure to a chemotherapeutic) that allow them to withstand increasingly higher doses of multiple mechanistically distinguishable cytotoxic drugs. Although extensive

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Abbreviations: BSA, bovine serum albumin; CHSA, chondrosarcoma; CRS, competitive reference standard; ET-743, Ecteinascidin-743; FITC, fluorescein isothiocyanate; MMP, matrix metalloproteinase; MRP1, multidrug resistance protein 1; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate.

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studies addressing mechanistic aspects of MDR and aberrant apoptosis in cancer cells have yielded valuable insights, MDR is still considered a significant obstacle for patients receiving chemotherapy. This is further exacerbated by the inability to detect early manifestations of chemoresistance. Recent data have uncovered an intriguing mechanism of action mediating chemoresistance of particular cancer cell lines to ET-743 *in vitro* [4,9–11]. Tumor cells lacking the full complement of nucleotide excision repair enzymes were found to be significantly less sensitive to ET-743. Interestingly, a specific cell line that was developed for resistance to ET-743 contained a nonsense mutation in the xeroderma pigmentosum nucleotide excision repair enzyme (also designated XPG or ERCC5) [11]. The drug resistance exhibited by these cells could be substantially reversed by transfection of the appropriate repair enzyme. The absence of either the ERCC1 or ERCC3 repair enzymes also yielded an ET-743-resistance phenotype. These results reveal another mechanism other than efflux via membrane pumps such as P-glycoprotein and MRP1 that underlie chemoresistance to ET-743 [12].

CHSA refers to a heterogeneous group of tumors of mesenchymal origin that develop in bone and display chondrocytic characteristics [13–15]. These tumors frequently respond poorly to conventional treatments such as chemotherapy and radiation, and prognosis is related to tumor grading and differentiation status [16,17]. The sensitivity of sarcoma cells to ET-743 has led us and others to consider this compound as an important component of future treatment options for sarcomas (including CHSA) [18,19]. However, as with other chemotherapeutics, the propensity of tumor cells to develop resistance to ET-743 poses a significant challenge for employing this drug over an extended period of time as a cancer treatment. We have initiated studies on the response of a high-grade CHSA cell line to ET-743, and have explored possible mechanisms related to the development of drug resistance in this cell line to ET-743. Our data reveal multiple effects of prolonged exposure to ET-743, including alterations in gene transcription, cytoskeleton reorganization, and modulation of cell invasion, with a concomitant change in the repertoire of secreted proteins. These data may help in devising new molecular detection methods for identifying the onset of chemoresistance in cancer patients receiving chemotherapy.

2. Materials and methods

2.1. Chemotherapeutics

ET-743 (supplied by PharmaMar USA) was dissolved in DMSO at a stock concentration of 6.7 mM and diluted with serum-free RPMI-1640 medium before addition to cells. Doxorubicin (Sigma) was dissolved in PBS and diluted with serum-free RPMI-1640 medium before use.

2.2. Cell line and culture conditions

Explant cultures of a human CHSA cell line, propagated since 1999 and designated CS-1, were established from a surgically resected human high grade CHSA that was not previously exposed to chemotherapy or radiation therapy, and grown in monolayer [20]. Briefly, tissue was aseptically obtained immediately following resection, placed in RPMI-1640 tissue culture medium supplemented with 10% fetal bovine serum, and the tissue cultured at 37° in a humidified atmosphere containing 5% CO_2. Cell growth surrounding the tissue was observed within 7 days. Unless otherwise indicated, cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (designated as complete medium). In separate experiments, a stable form of ascorbic acid, ascorbic acid 2-phosphate (Wako Chemicals USA), was tested for effects on collagen expression at a concentration of 0.2 mM. We have performed a detailed analysis of the CS-1 cell line, and these data will be reported elsewhere.[4] Active gene expression of the type II collagen gene, considered a marker for cartilage cells, has been confirmed by RT-PCR analysis, and cytogenetic analyses performed twice over a span of 1 year on 20 cells has shown a near triploid nature, with identical gene rearrangements.

2.3. Development of an ET-743 resistant cell line

CHSA cells resistant to ET-743 were derived from the CHSA parent cell line by periodic exposure to a sub-lethal concentration of ET-743 for 10 min in serum-free medium followed by incubation in complete medium lacking drug for 20 days. Resistance cells were obtained after repeating this treatment protocol for 10 times with increasing concentrations of ET-743. Prior to performing cytotoxicity assays, the cells were kept in drug-free medium for at least 14 days.

2.4. Cytotoxic assay

Sensitivity of CHSA cells to ET-743 and doxorubicin was evaluated using a modified clonogenic assay or an adherent cell cytotoxicity assay. For the clonogenic assay, exponentially growing parent and resistant CHSA cells were placed in suspension and treated for 10 min in serum free medium with different concentrations of chemicals. After treatment, cells were washed twice with PBS, and 3000 cells/well of control or treated cells were plated in 6-well plates with 3 mL/well of fresh complete medium. The colonies were allowed to develop for 10 days. Cell number was determined by the crystal violet method [21]. Briefly, the colonies were fixed with 1% glutaraldehyde and stained with 0.5% crystal violet, washed four times with PBS, and then the dye was eluted with 0.2% Triton X-100. Four wells were tested for each concentration, and

[4] Weissbach L, Fletcher JA, Hornicek FJ, in preparation.

the data expressed ± SD. The adherent assay involved plating 20,000 cells/well in a 96-well plate, culturing for 24 hr, and then performing the drug treatment in complete medium for 30 min. The cells were cultured for an additional 5 days, and cell viability measured with the 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt reagent (i.e. MTS reagent; Promega). Color formation was monitored in a microplate reader.

2.5. Actin staining

The parent or ET-743-resistant CS-1 cells were seeded at 5000 cells/well in an 8-well Lab-Tek chamber slide system, grown for 24 hr, washed with PBS, and fixed with 1% glutaraldehyde. After removing the fixative by washing with PBS, the cells were permeabilized with 0.2% Triton X-100 for 15 min, washed with PBS, and incubated 2 hr at room temperature with 10 μM FITC-phalloidin in PBS containing 3% BSA and 0.5% Triton X-100. Actin staining was visualized by fluorescence microscopy using a SPOT digital camera (Diagnostic Instruments) attached to a Nikon Microphot-FX fluorescent microscope.

2.6. Flow cytometric analysis of P-glycoprotein and MRP1

For P-glycoprotein analysis, resistant CS-1 cells were detached from the tissue culture flasks by trypsinization, washed with PBS and then counted. The resistant CS-1 cells (1×10^6) were incubated with either anti-P-glycoprotein antibody (clone JSB-1, Chemicon) or a control isotypic IgG. The cells were then washed and incubated with a FITC-conjugated goat anti-mouse IgG (Sigma). Cells were analyzed on a Coulter Epic flow cytometer. The T-cell lymphoblastic leukemia cell line CEM was used to generate CEM-vinblastine (VLB)-resistant cells according to a previous report [22], and was used as a control.

MRP1 was evaluated in CEM cells, CEM-epirubicin resistant cells (prepared as previously described [23]), parent CS-1 cells, or resistant CS-1 cells after fixation in 0.5% formaldehyde and permeabilization in 0.1% Triton X-100. Cells (1×10^6) were washed in PBS/1% BSA and incubated with anti-MRP1 antibody (clone QCRL-1, kindly supplied by Dr. Susan P. Cole) [24,25] diluted 1:20 in PBS/1% BSA containing 0.1% Triton X-100, or a control isotypic IgG. Cells were then processed as described above for P-glycoprotein.

2.7. Cell attachment assay

Cell attachment to gelatin-coated tissue culture plates was assessed as described previously, with minor modifications [26]. The 96-well tissue culture plates were first coated with a 10 μg/mL gelatin solution. The parent and ET-743-resistant CS-1 cells were harvested and added to the wells. After incubation at 37° for 2 hr, the plate was washed three times with PBS, and the cells were fixed with 20% methanol for 15 min at room temperature. Attached cells were quantified by crystal violet staining. Data represent the average ± SD.

2.8. Tumor cell invasion assay

Cell invasion assay was performed as described, with minor modifications [27]. Cell culture inserts (Falcon, 8.0 μm pore size) were first coated with gelatin and placed into wells of a 24-well plate. Parent and resistant cells were harvested and suspended in RPMI-1640 containing 0.1% BSA at a concentration of 1×10^5 cells/mL. A cell suspension was added to each gelatin-coated insert and 300 μL of RPMI-1640 medium containing 0.1% BSA was added to the well underneath the insert. After a 4 hr incubation at 37° in a humidified atmosphere of 5% CO_2/95% air, cells on the upper side of the filters were removed by scraping with cotton swabs, while the cells migrating to the lower side were fixed in absolute ethanol, stained with Trypan blue and counted. At least 9 microscopic fields were counted per condition and averaged.

2.9. Conditioned medium collection and analysis

Both parent and ET-743-resistant CHSA cells in exponential growth phase were washed twice with PBS and incubated in serum-free medium for 2 hr. After removing the medium, the cells were again washed twice with PBS, fresh serum-free medium added, and the cells incubated for 48 hr at 37°. The 48 hr conditioned medium was collected and centrifuged and the supernatant frozen for future use. For SDS–PAGE and immunoblotting analysis, the conditioned medium was concentrated with a Biomax-100k centrifuge concentrating device (Millipore). Protein concentrations were determined with the Coomassie Blue Plus reagent (Pierce). After concentrating, equal amounts of protein were reduced and denatured and then applied to a 5% SDS–PAGE, and visualization of proteins was accomplished with Coomassie blue R-250.

2.10. Protein quantification

Since collagen represents a significant portion of the total secreted protein in the parent cell line, we needed to modify our Bradford assay employing the Coomassie Plus Protein Reagent (Pierce), because this method is very poor at detecting collagen, based on our data and that of others [28]. By adding 0.0035% SDS to the reagent, the detection of collagen is much improved relative to BSA.

2.11. Immunoblotting analysis

Concentrated conditioned medium samples (10 μg protein/sample) were resolved by 5% SDS–PAGE as

described above and transferred to a PVDF membrane. Immunoblotting was performed using an affinity purified rabbit polyclonal antibody to human type I collagen (Rockland Immunochemicals), which recognizes native and denatured type I collagen [29]. The blot was then incubated with a goat anti-rabbit horseradish peroxidase-labeled secondary antibody (Chemicon), and detection was accomplished using the SuperSignal West Pico Chemiluminescent Detection Kit (Pierce).

2.12. Northern blot analysis

Total RNA was isolated from nearly confluent cultured cells with RNAqueous-4PCR kit (Ambion), and 8 μg total RNA separated on a 1% agarose gel and transferred to BrightStar-Plus positively charged nylon membrane (Ambion). The immobilized RNA was hybridized to biotin-labeled cDNA probes for human collagen α1(I) mRNA (626 nt, recognizes full-length mRNA species of 4.8 and 5.8 bp; see below for RT-PCR description) and human glyceraldehyde-3-phosphate dehydrogenase mRNA (200 nt, recognizes full-length mRNA of 1.3 bp). Biotin labeling was performed using the BrightStar Psoralen-Biotin labeling kit (Ambion), according to the manufacturer's instructions. Binding of the probes to the membrane was detected with a streptavidin-alkaline phosphatase conjugate and chemiluminescence (BrightStar BioDetect Kit, Ambion).

2.13. Competitive RT-PCR

A comparison of the steady state level of type I collagen α1 chain mRNA between the parent CS-1 cells and the ET-743-resistant cells was accomplished by a competitive RT-PCR procedure. Total RNA was purified as described above for Northern blot analysis and used as a template for RT-PCR, employing the Titanium One-Step RT-PCR kit (Clonetech). The type α1(I) collagen antisense primer used in the RT reaction was: 5′-GAATCCATCGGTCATGCTCT, corresponding to nt 4102–4121 of the α1(I) collagen mRNA (GenBank identifier NM_000088.2) [30]. The PCR was then performed by addition of the following primers: (sense) 5′-GAGAGAGGCTTCCCTGGTCT (corresponding to nt 3024–3043) and (antisense) 5′-ACAGGACCAGCATCACCAGT (corresponding to nt 3630–3649). The RT-PCR product was 626 bp in length, and spanned 7 introns in the corresponding genomic DNA. Thermal cycling conditions were as follows: RT: 50° for 1 hr; PCR: 94° for 5 min followed by 24 or 43 cycles (for amplification of cDNA corresponding to the parent and ET-743-resistant CS-1 cell lines, respectively) of 94° for 30 s, 55° for 30 s and 68° for 1 min, followed by a final extension at 68° for 7 min. To create the vector containing the CRS α1(I) collagen sequence, the collagen α1(I) RT-PCR product generated as described above was ligated into TOPO-TA pCR4 by the TA cloning method (Invitrogen). An internal 225 bp BstEII fragment was excised from this insert and the vector re-ligated to produce a 400 bp CRS insert in the competition vector, allowing amplification of the CRS and target collagen cDNA with the same PCR primer set. Initial diagnostic experiments determined the cycle numbers in which amplification of the α1(I) collagen cDNA occurred exponentially as well as the approximate concentration of CRS vector necessary for effective competition with the RT-PCR product. Competitive PCR was conducted using the same temperature cycling parameters described except that the CRS vector was added to the RT reactions immediately prior to the PCR reactions. RT-PCR products were separated on a 2% agarose gel and the relative intensity of bands determined using densitometry with a Kodak Digital Imager 440 CF. To compensate for the increase in ethidium bromide incorporation in the larger target PCR product relative to the CRS, the densitometric values for the CRS products were multiplied by 1.56. A control semi-quantitative RT-PCR reaction to determine the relative levels of β-actin mRNA in both samples was conducted as described by the manufacturer (Clontech), using an 18-mer oligo(dT) RT primer, with the sense PCR primer 5′-GTGGGCCGCTCTAGGCACCAA and the antisense primer 5′-CTCTTTGATGTCACGCACGATTTC, producing a 540 bp product. Thermal cycling conditions were as follows: RT: 50° for 1 hr; PCR: 94° for 5 min followed by 24 cycles of 94° for 30 s, 65° for 30 s, and 68° for 1 min. Data were analyzed at 24 cycles because this was determined to be within the exponential segment of the amplification reaction.

2.14. Zymography assay

Gelatin zymography was performed as described, employing a 10% SDS–PAGE gel impregnated with 0.1% gelatin [31,32]. Briefly, non-reduced/non-denatured protein samples were loaded onto a 10% SDS–PAGE gel impregnated with 0.1% gelatin. Following electrophoresis, the gel was washed in 2.5% Triton X-100 for 20 min, and then incubated in a buffer consisting of 20 mM Tris–HCl, pH 7.8, 1 mM calcium chloride, and 150 mM NaCl overnight at 37°. After staining with Coomassie blue, areas of gelatinolytic activity were observed as clear areas set against the background of the stained gelatin.

2.15. Protein sequencing analysis

Samples of concentrated serum-free conditioned medium (20 μg total protein) were separated on a 5% SDS–PAGE gel and stained with 0.1% Coomassie blue. The protein bands of interest were excised and protein identification by MALDI-TOF mass spectrometry and microsequencing of tryptic peptides was performed at the University of Massachusetts Laboratory for Protein Microsequencing and Proteomic Mass Spectrometry Center.

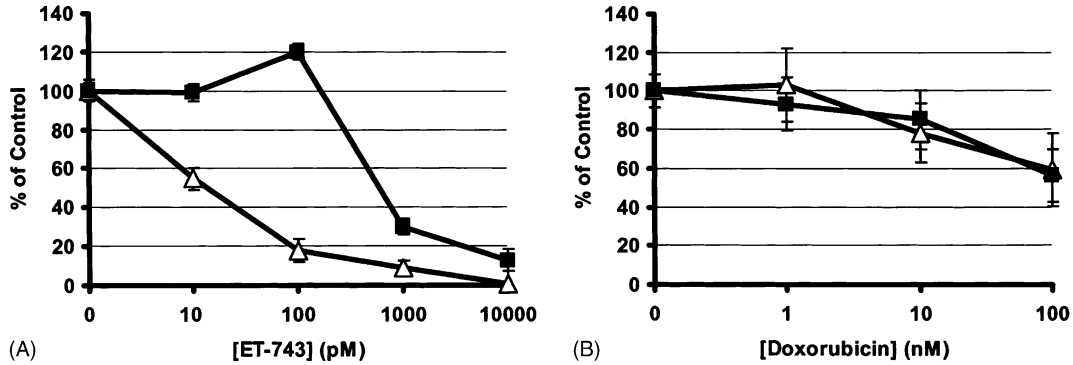

Fig. 1. Sensitivity of parent and resistant CS-1 CHSA cells to ET-743 and doxorubicin. Both parent (open triangles) and ET-743-resistant CS-1 cells (solid boxes) in suspension were exposed to either ET-743 (A) or doxorubicin (B) at the indicated concentrations for 10 min in the absence of serum, and then analyzed by a 10-day clonogenic assay. Cell number was determined by crystal violet staining. Each value represents the average of four determinations ± SD.

2.16. Statistical analysis

All values are given as means ± SD. Statistically significant differences between experimental groups were determined using either two factor ANOVA analysis or a two-tailed Students *t*-test; statistical significance was set at a confidence level of >95% ($P < 0.05$).

3. Results

3.1. Selection and characterization of resistance cells

As a first step toward investigating the phenomenon of drug resistance in CHSA cells, we developed a variant of the CS-1 human CHSA cell line that displays resistance to the cytotoxic effects of ET-743. After exposing the parent CS-1 cell line to increasing concentrations of the drug over several months, a subpopulation of cells prospered that could withstand the effects of the drug at higher concentrations than the parent cell line. As shown in Fig. 1, the effects of ET-743 and doxorubicin were monitored on both parent and ET-743-resistant CS-1 cells. The response of the parent CS-1 cells and the variant ET-743-resistant cell line to ET-743 in a cytotoxicity assay is shown in Fig. 1A. The data demonstrate that the variant cell line is approximately 36-fold more resistant to the cytototxic effect of ET-743, as judged by a clonogenic assay. The degree of resistance dissipates unless the ET-743-resistant cells are continually re-exposed to the drug, although the cells never completely revert to a sensitive phenotype, based on cell viability assays. Figure 1B shows that the ET-743-resistant CHSA cell line does not display cross-resistance to doxorubicin, suggesting that at the biochemical level, the CHSA cells respond differently to ET-743.

3.2. Morphology and actin staining

A morphological comparison of the parent and ET-743-resistant CS-1 cell lines by microscopic examination revealed distinct differences between the two cell populations. The parent cells appear rounder, while the resistant cells displayed a more spindle shape (Fig. 2). The differences in cell morphology between the two cell lines suggested differences in the cytoskeletal architecture. To address this hypothesis, we incubated the two cell lines

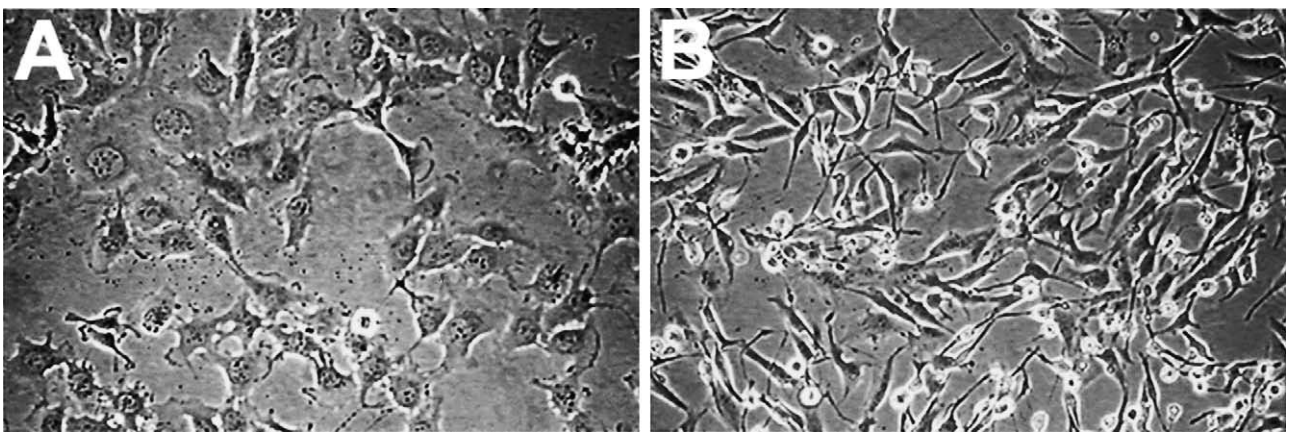

Fig. 2. Cell morphology comparison of parent and resistant CS-1 CHSA cells. Photomicrographs of each cell line were taken with a Zeiss photomicroscope equipped with a camera (100×). (A) Parent CS-1 cells; (B) resistant CS-1 cells.

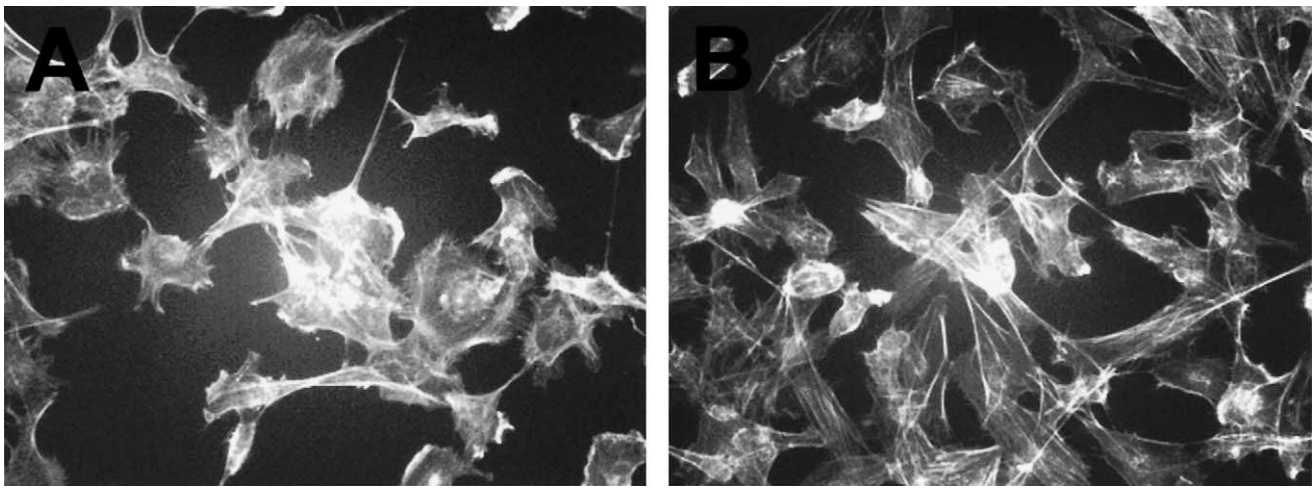

Fig. 3. Staining of actin filaments. The parent (A) or ET-743-resistant CS-1 cells (B) were plated and cultured in wells within a chamber slide system, fixed with 1% glutaraldehyde, and permeabilized with 0.2% Triton X-100. The cells were then exposed to 10 μM FITC-phalloidin, and the actin staining visualized by fluorescence microscopy (100×).

with a phalloidin-FITC conjugate, and monitored the binding to actin by fluorescence microscopy. As shown in Fig. 3, the parent cell line displayed a much more diffuse staining throughout the cytoplasm, without significant numbers of actin stress fibers. Moreover, numerous actin-containing cell surface protrusions such as lamellipodia were evident. However, the ET-743-resistant cells lacked the irregular membrane surface characteristic of the parent cells, and were more elongated, exhibiting a concentrated rim of stained material located near the plasma membrane along with numerous actin stress fibers. These data suggest that prolonged exposure to ET-743 resulted in a substantial reorganization of the actin cytoskeleton and alteration of the cell shape.

3.3. Drug transporters

One cellular mechanism that may account for the ET-743 chemoresistance is MDR mediated by various ATP-dependent cell surface transporters. Two transporters commonly associated with MDR are P-glycoprotein and MRP1. We first determined if P-glycoprotein was present on the ET-743-resistant cells. As shown in Fig. 4, there was virtually no difference in immunoreactivity of a control

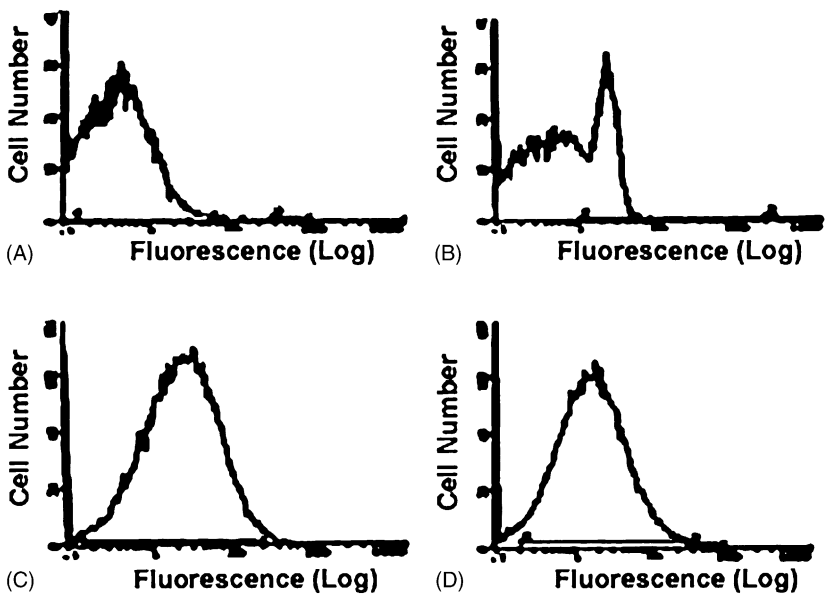

Fig. 4. Flow cytometric analysis of P-glycoprotein expression in ET-743-resistant CS-1 CHSA cells. Control cells (CEM-VLB) and ET-743-resistant CS-1 cells were incubated with either a control IgG or a monoclonal P-glycoprotein antibody, followed by a FITC-labeled secondary antibody, and fluorescence determined by flow cytometry. Panel A, CEM-VLB cells exposed to control IgG; Panel B, CEM-VLB cells exposed to P-glycoprotein antibody; Panel C, ET-743-resistant CS-1 cells exposed to control IgG; Panel D, ET-743-resistant CS-1 cells exposed to P-glycoprotein antibody.

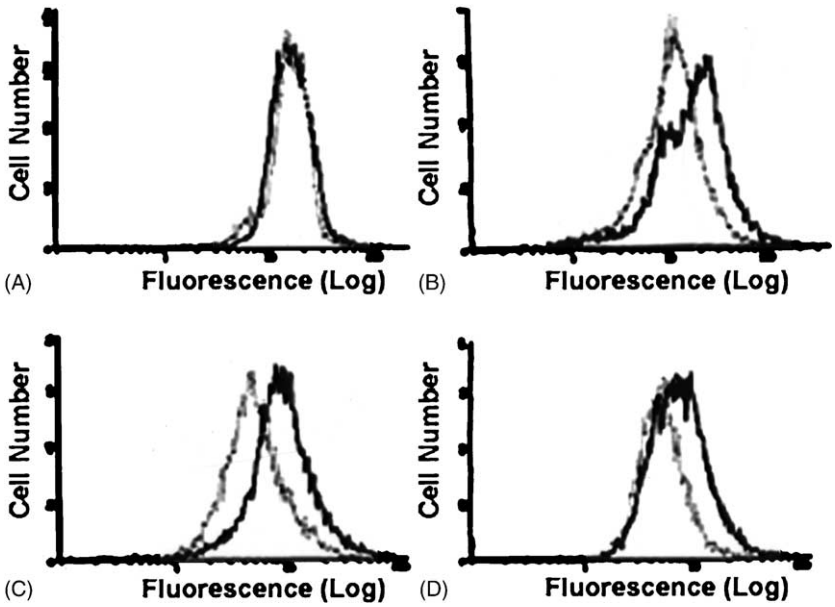

Fig. 5. Flow cytometric analysis of MRP in parent and ET-743-resistant CS-1 cells. Different cell lines were incubated with either a monoclonal MRP antibody (darker peak) or an isotypic IgG control antibody (lighter peak) and fluorescence intensity determined as described in Fig. 4, except that the two peaks of fluorescence intensity (i.e. control antibody and MRP antibody) were superimposed. Panel A, CEM cells; Panel B, CEM-MRP cells; Panel C, parent CS-1 cells; Panel D, ET-743-resistant CS-1 cells.

and P-glycoprotein antibody on the ET-743-resistant cells (Fig. 4C vs. D), in contrast to CEM leukemia cells resistant to vinblastine (Fig. 4A depicts control antibody reactivity and Fig. 4B the corresponding reactivity with the P-glycoprotein antibody). We conclude from these data that P-glycoprotein is not significantly expressed in either cell line. Alternatively, it was possible that MRP1 was active in mediating efflux of ET-743. Employing a monoclonal antibody to MRP1, we analyzed both the parent (Fig. 5C) and ET-743-resistant CS-1 cells (Fig. 5D) for differences in MRP1 expression. As a positive control, we again used the CEM cell line and generated MRP1-resistant CEM cells (Fig. 5B) from the parent CEM cells (Fig. 5A) by prolonged exposure to epirubicin. The degree of fluorescence corresponding to MRP1 (darker peak) is virtually identical between the parent and ET-743-resistant cells, suggesting that MRP1, in addition to P-glycoprotein, is most likely not involved in the mechanism of ET-743 drug resistance displayed by the ET-743-resistant CS-1 cells.

3.4. Cell attachment and invasion

The different morphology of the parent and ET-743-resistant CS-1 cells prompted us to investigate other cellular phenomena that might distinguish these two cell populations. We first studied the ability of each cell type to adhere to gelatin-coated dishes. Cell attachment is thought to involve a variety of cell surface molecules such as the integrins, as well as cytoskeleton rearrangements. As shown in Fig. 6, the parent CS-1 cells display a significantly greater capacity to adhere to gelatin when compared to their ET-743-resistant counterpart. These data suggest that the two cell lines are functionally as well as morphologically different. To illustrate this point further, we performed a cell invasion assay, employing a modified Boyden chamber, using gelatin as the protein barrier on the filter insert. The data presented in Fig. 7 indicate that the parent cell line is also significantly more adept at migrating through the gelatin film and transversing the membrane.

3.5. Analysis of secreted proteins

Based on the cellular data presented above, we wanted to compare the cell lines at a more molecular level, and therefore attempted to identify differences in the repertoire

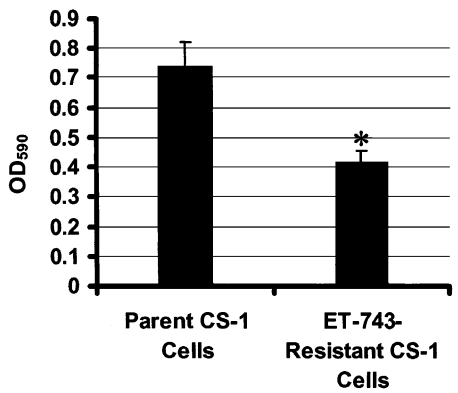

Fig. 6. Effect of ET-743 drug resistance on cell adhesion. CS-1 cells or ET-743-resistant CS-1 cells were washed in serum-free medium and placed in gelatin-coated cell culture dishes for 2 hr at 37°. Cells were washed, fixed with methanol, and adherent cells were quantified by crystal violet staining. Each condition represents the average of 7 wells ± SD. The asterisk denotes a P value of <0.001.

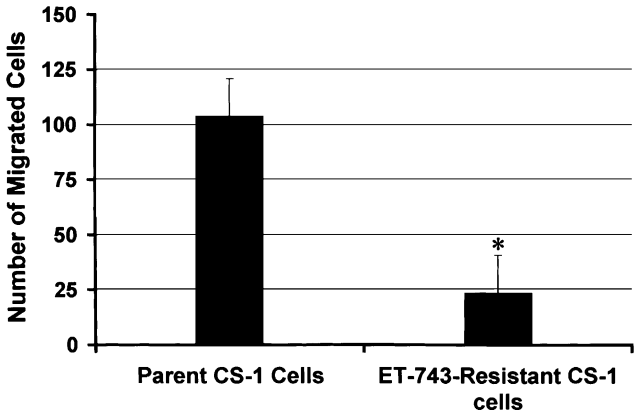

Fig. 7. Effect of ET-743 drug resistance on cell invasion. CS-1 and ET-743-resistant CS-1 cells were tested for their ability to transverse a gelatin-coated polycarbonate filter by employing a modified Boyden chamber. Serum-free medium was placed beneath the cell culture insert, while cells were placed in serum-free medium above the insert. After incubating for 4 hr at 37°, the cells on the top of the filter were removed with a cotton swab, and the cells on the bottom of the filter fixed in absolute ethanol and counted after staining with Trypan blue. A total of 11 microscopic fields were used for counting per filter, and the data represent the average ± SD. The asterisk denotes a P value of <0.001.

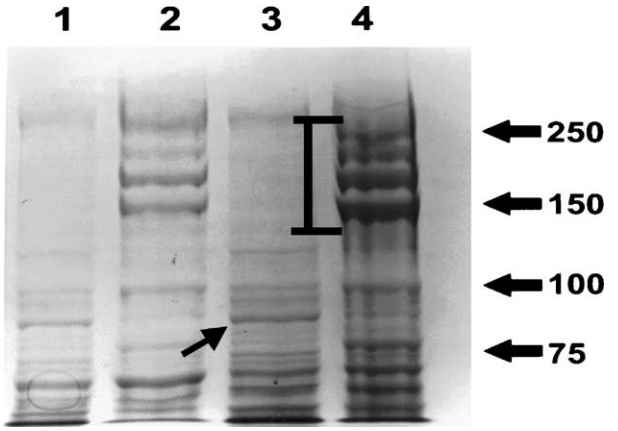

Fig. 8. Analysis of conditioned medium from CS-1 parent and ET-743-resistant CS-1 cells by SDS–PAGE and Coomassie blue staining. Serum-free conditioned medium was collected from CS-1 cells and ET-743-resistant CS-1 cells at different time points, and equal amounts of protein analyzed on a 5% SDS–PAGE. Lane 1: serum-free conditioned medium from the ET-743-resistant CS-1 cells collected after 24 hr incubation; lane 2, conditioned medium from the parent CS-1 cells collected after 24 hr incubation; lane 3, conditioned medium from ET-743-resistant CS-1 cells collected after 48 hr incubation; lane 4, conditioned medium from the parent CS-1 cells collected after 48 hr incubation. Molecular weights in kDa are indicated to the right. The bracket denotes the quartet of protein bands that are in higher concentration in the parent CS-1 cells, while the arrow indicates an 85 kDa protein band that is in higher amounts in the ET-743-resistant CS-1 cells.

of secreted proteins present in serum-free conditioned medium between the parent and ET-743-resistant CS-1 cell lines. Cells were extensively washed to remove serum, and serum-free conditioned medium from each cell line was collected after 24 hr (Fig. 8, lanes 1 and 2) and 48 hr (Fig. 8, lanes 3 and 4), concentrated by Centricon centrifugation (molecular weight cut-off of 100 kDa, although a significant number of proteins <100 kDa were retained), and subjected to SDS–PAGE analysis and Coomassie blue staining. As shown in Fig. 8, the presence of four prominent protein bands ranging from 140 to 240 kDa are apparent in the conditioned medium prepared from the parent CS-1 cell (lanes 2 and 4), but almost completely absent from the corresponding ET-743-resistant CS-1 cell conditioned medium (lanes 1 and 3). In contrast, a protein band migrating at approximately 85 kDa is seen in the ET-743-resistant CS-1 cell conditioned medium (lanes 1 and 3) but is significantly reduced in the parent CS-1 cell conditioned medium. The same results are found if conditioned medium from an equal number of cells are compared (data not shown). The 170 and 190 kDa protein bands were excised from the gel, and analyzed by mass spectrometry and protein sequencing of tryptic peptides. Sequences of six tryptic peptides (ranging in size from 13 amino acids to greater than 20 amino acids) from the 190 kDa protein band corresponded perfectly with the α2 chain of human type IV collagen. The same analysis of the 170 kDa protein species revealed a match with the α1 chain of human type I collagen, as judged by sequences of 10 tryptic peptides ranging in size from 9 amino acids to greater than 20 amino acids.

The secreted collagens appear to be primarily in the triple helical form, based on gel analysis. The four prominent high molecular weight bands (two of them definitively identified as collagen subunits) seen under reducing/denaturing conditions and SDS–PAGE analysis disappear almost completely when conditioned medium from the parent cell line is electrophoresed under non-reducing conditions, with a concomitant appearance of a high molecular weight species of >300 kDa (data not shown). This >300 kDa band is not generated from the ET-743-resistant cell conditioned medium under these same conditions, as expected.

3.6. MMP analysis by zymography

One explanation for the discrepancy in collagen levels between the two conditioned medium samples is differences in collagen-degrading metalloproteinase activity. Therefore, we performed a zymographic analysis, comparing the gelatinase activities present in the two conditioned medium samples. Two collagen cleaving metalloenzymes that can recognize types I and IV collagen as substrates are MMP-2 and MMP-9, also referred to as gelatinases A and B [33]. Therefore, we performed a gelatin zymographic analysis, comparing the gelatinase activities present in the two conditioned medium samples (enzymatic activity is seen as clear areas set against the background of stained gelatin impregnated within the gel). As shown in Fig. 9, pro-MMP-2 (migrating at approximately 68 kDa, while mature MMP-2 is seen as the faint band at 62 kDa) is the principal gelatinase degrading enzyme in both samples, and there appears to be enhanced activity in the parent cell

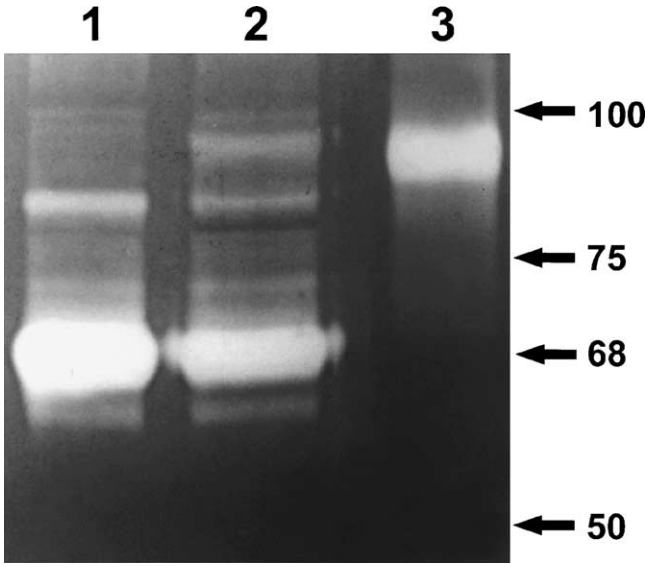

Fig. 9. Analysis of gelatinase activity by gelatin zymography. Serum-free conditioned medium from either parent or resistant CS-1 CHSA cells were concentrated and applied to 10% SDS–PAGE gelatin-impregnated gels without denaturation or boiling. Gels were incubated in calcium-containing buffer to allow proteolysis to occur, and gelatinase activity visualized after Coomassie blue staining as clear areas set against the background of stained gelatin. Lane 1, 10 μg parent CS-1 conditioned medium; lane 2, 10 μg ET-743-resistant CS-1 conditioned medium; lane 3, 10 ng purified pro-MMP-9. Numbers to the right indicate molecular weight standards.

sample (lane 1). Moreover, increased amounts of mature MMP-9 (seen at 82 kDa) are also present in the parent cell conditioned medium, suggesting that processing of pro-MMP-9 is inefficient in the resistant cell medium (compare the 92 kDa pro-MMP-9 band between lanes 1 and 2; purified pro-MMP-9 was electrophoresed in lane 3 as a control). These data suggest that rampant MMP activity is most likely not responsible for the absence of collagen in the resistant cell conditioned medium.

As a further way of testing for unrestrained proteolysis in the ET-743-resistant cell conditioned medium, we performed a swapping experiment. Conditioned serum-free medium from the resistant cells was used to culture the parent CS-1 cells, and further conditioned according to our standard procedure. Collagen accumulated to virtually the same levels seen as typically observed with the parent cells, again suggesting that uncontrolled proteolytic degradation of collagen in the ET-743-resistant cell conditioned medium was not occurring (data not shown).

3.7. Ascorbic acid

We also considered the possibility that low levels of ascorbic acid in our medium might be affecting our results. Assembly of collagen is dependent on hydroxylation of particular prolyl residues in collagen monomers, a modification that helps stabilize triple helix formation. This reaction is catalyzed by prolyl hydroxylase, which uses ascorbic acid as an essential cofactor [34,35]. Ascorbic acid has also been reported to affect transcriptional activity of the type I collagen gene [36]. The culture medium used for the CS-1 parent and ET-743-resistant cell lines does not contain ascorbic acid, and fetal bovine serum has limiting amounts, so ascorbic acid deprivation might be involved in the differential collagen gene expression that we observe [37]. Therefore, we tested the effects of a stable form of ascorbic acid, L-ascorbic acid 2-phosphate, on the accumulation of type I collagen in the serum-free culture medium [35,37]. We found that addition of ascorbic acid did not alter the levels of secreted collagens for the two cell lines observed when cultured without ascorbic acid supplementation, suggesting that the altered collagen metabolism in the ET-743-resistant CS-1 cells was not due to limiting amounts of ascorbic acid (data not shown). Most likely, an ascorbate-independent proline hydroxylation reaction occurs in these cell lines, as shown for other cell lines [38].

3.8. Collagen expression

To gain more information concerning the regulation of collagen metabolism by ET-743, we performed both

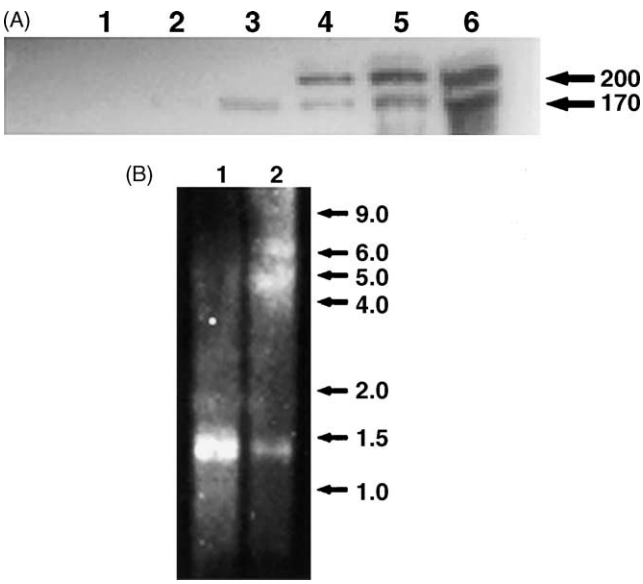

Fig. 10. Measurement of collagen α1(I) mRNA and protein levels by Western and Northern Blot analysis. For immunoblotting (A), the serum-free conditioned medium from the parent and ET-743-resistant cell lines was separated on a 5% denaturing SDS–PAGE gel, transferred to nylon, and incubated with a rabbit collagen α1(I)-specific polyclonal antibody followed by a horseradish peroxidase-labeled secondary antibody, and detection accomplished by chemiluminescence. Lanes 1–3: 1, 3, and 15 mg of conditioned medium from ET-743-resistant cells; lanes 4–6, 1, 3, and 15 mg of conditioned medium from the parent CS-1 cells. Numbers to the right indicate molecular weights in kDa. For Northern blot analysis (B), 8 μg of total RNA from either the ET-743-resistant cells (lane 1) or the parent CS-1 cells (lane 2) were separated on a 1% agarose gel, transferred to nylon, and simultaneously hybridized with biotin-labeled probes for both collagen α1(I) mRNA (4.8 and 5.8 kbp) and glyceraldehyde-3-phoshpate dehydrogenase mRNA (1.3 kbp). Binding of the probes to the membrane was detected with a streptavidin–alkaline phosphatase conjugate and chemiluminescence.

Western and Northern blot analysis for determination of collagen protein and mRNA levels, respectively. When we tested the levels of soluble collagen α1(I) protein in the two conditioned medium samples by Western blot analysis, employing an antibody specific for this collagen subunit [29], the level of the processed (i.e. lacking propeptides) collagen α1(I) chain (170 kDa, bottom band in Fig. 10A) was significantly lower in the ET-743-resistant cell conditioned medium (lanes 1–3) as compared to the parent cell conditioned medium (lanes 4–6). Identical immunoblotting results were obtained for the collagen α2(IV) chain (data not shown). Interestingly, the top band of approximately 200 kDa, which most likely represents partially processed collagen α1(I) containing one of its propeptides, is completely absent in the ET-743-resistant cell conditioned medium (this band has also been observed previously with this particular antibody [29]). Our Northern blot results were consistent with the immunoblotting data. As shown in Fig. 10B, the collagen α1(I) mRNA doublet of approximately 4.8 and 6 kbp is seen in the RNA sample isolated from the parent CS-1 cells but absent from the corresponding RNA sample isolated from the ET-743-resistant cells. The doublet is due to alternate polyadenylation signals in the collagen α1(I) gene [39]. Thus, the ET-743-resistant cells appear to have a deficiency in transcription in several of the collagen genes, ultimately resulting in severely depressed protein levels.

To obtain more quantitative data on the repression of type α1(I) collagen gene expression in the ET-743-resistant cells, we performed a competitive RT-PCR experiment. The cloned CRS collagen DNA sequence was identical to the type I collagen α1 chain cDNA fragment used in the Northern blot analysis, but lacked an internal fragment, thus representing a cDNA fragment that could bind the type I collagen primers with equal efficiency yet could be discriminated from the wild-type fragment by size. This allowed us to estimate the amount of plasmid needed to inhibit amplification of a wild type collagen cDNA segment such that an equal amount of CRS and wild-type PCR product are produced. As shown in Fig. 11, the collagen α1(I) mRNA concentration in the parent CS-1 cells was approximately 117-fold greater in concentration when compared to the concentration in the ET-743-resistant cells, suggesting that prolonged exposure to ET-743

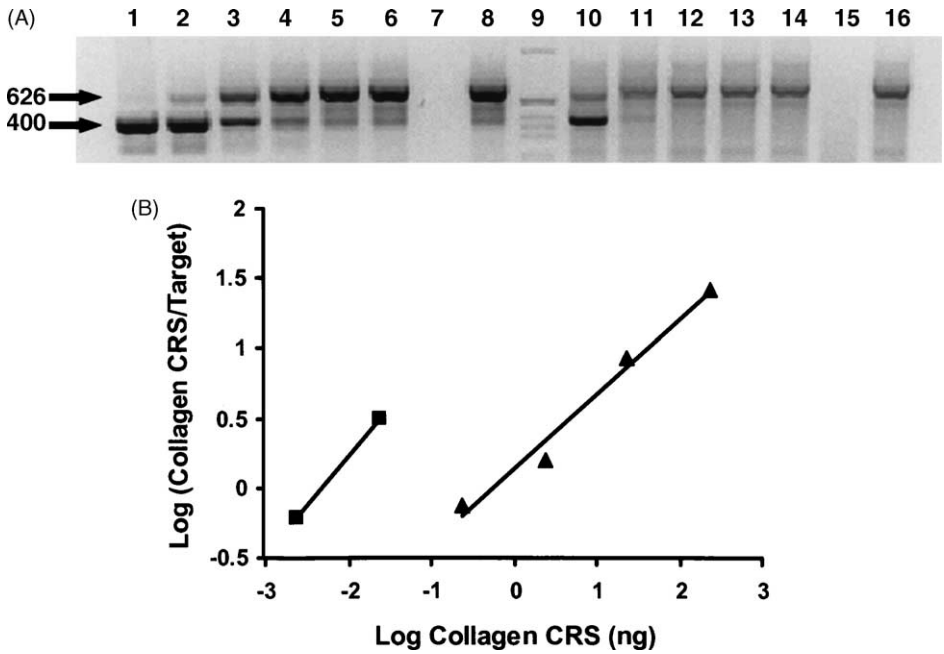

Fig. 11. Competitive RT-PCR for comparing collagen α1(I) mRNA levels in the parent and ET-743-resistant CS-1 cells. (A) Serial dilutions of a CRS consisting of a modified collagen α1(I) cDNA insert cloned into a plasmid were added to PCR reactions that employed collagen α1(I) cDNA derived from RT reactions, in order to estimate a relative amount of collagen α1(I) mRNA in total RNA isolated from the parent and ET-743-resistant CS-1 cells. PCR products were removed at either 24 cycles for the parent CS-1 RNA or 43 cycles for the ET-743-resistant CS-1 RNA and analyzed on a 2.0% agarose gel. The 400 bp band is the CRS product derived from the competitor plasmid, while the 626 bp band is the authentic collagen α1(I) PCR product. Lane 1: control PCR reaction with plasmid containing the CRS collagen α1(I) cDNA. Lanes 2–6: collagen α1(I) RT-PCR products from the parent CS-1 cells, with decreasing amounts of the CRS added into the PCR reactions: 240, 24, 2.4, 0.24, and 0.024 ng. Lane 7: control RT-PCR reaction with a preheating of the parent CS-1 RT reaction to test for dependence on RNA. Lane 8: RT-PCR reaction from the parent CS-1 cell total RNA, amplifying the collagen α1(I) mRNA in the absence of any CRS. Lane 9: molecular weight ladder. Lanes 10–14: collagen α1(I) RT-PCR products from the ET-743-resistant CS-1 cells, with decreasing amounts of the CRS added into the PCR reactions: 2.4×10^{-2}, 2.4×10^{-3}, 2.4×10^{-4}, 2.4×10^{-5}, 2.4×10^{-6} ng. Lane 15: control RT-PCR reaction with a preheating of the RT reaction to test for RNA dependence. Lane 16: RT-PCR reaction from the ET-743-resistant CS-1 cell total RNA, amplifying the collagen α1(I) mRNA in the absence of any CRS. (B) Amplified RT-PCR target and CRS were quantified by densitometry. The equivalence point where the CRS and target are present in equal mass amounts occurs when the log of the CRS/target ratio is equal to 0. Linear regression analyses of the data in the linear part of the two data sets were performed to draw the best fit line. Solid triangles, PCR product from template RNA isolated from the parent CS-1 cells; solid squares, template RNA isolated from ET-743-resistant CS-1 cells.

dramatically downregulated collagen α1(I) gene transcription. As a control, we performed semi-quantitative RT-PCR for β-actin mRNA levels in both cell lines, and found that the β-actin mRNA level was comparable for both cell lines (a 1.7-fold increase in the ET-743-resistant CS-1 cells) (data not shown).

4. Discussion

The well-studied marine chemotherapeutic ET-743 appears to act by a novel mechanism involving covalent DNA adducts and DNA bending toward the minor groove, and may be particularly effective against mesenchymal cell-derived tumors [1–4]. ET-743 is currently in Phase II clinical studies, and may have utility both alone and in combination with other chemotherapeutics or angiogenesis inhibitors [4,19,40]. The fact that tumors such as CHSAs, which are frequently refractory to standard therapies, are so exquisitely sensitive to ET-743 (Fig. 1) suggests that this compound may represent a significant advance in the treatment of chondrocytic and possibly other bone tumors. However, the ability of tumor cells to develop chemotherapeutic drug resistance is a formidable obstacle for successful eradication of neoplastic growth by chemotherapy. Drug resistance is not confined to traditional chemotherapeutics, for tumor cell resistance to the widely publicized kinase inhibitor Gleevec has also been reported [41]. Thus, we developed a variant CHSA cell line resistant to ET-743, and then initiated studies aimed at uncovering molecular features of this resistance. In addition to investigating molecular differences between the parent and ET-743-resistant CS-1 cells, we were also intent on uncovering biological markers that might be exploited for use in new clinical tests for diagnosing the onset of chemoresistance. Currently, clinicians rely heavily on measurements of tumor size and degree of necrosis for assessing response to chemotherapy. Thus, earlier diagnosis of chemoresistance would be extremely beneficial.

As shown in Fig. 1, the degree of resistance for the ET-743-resistant cell line is substantial, but cross-resistance to doxorubicin, a chemotherapeutic used to treat sarcomas, was not observed, suggesting that ET-743 may represent a new treatment paradigm for treating sarcomas such as chondrosarcoma [42]. Although previous data have correlated P-glycoprotein expression with doxorubicin chemoresistance in CHSA cell lines, the doxorubicin data presented in Fig. 1 are inconsistent with the notion that the drug efflux pumps such as P-glycoprotein and MRP1 play a significant role in the chemoresistance of the ET-743-resistant CS-1 cell line [43]. We directly addressed the potential involvement of both P-glycoprotein and MRP1 in mediating cell resistance to ET-743 by employing specific antibodies to these proteins and quantifying antigen molecules by flow cytometry (Figs. 3 and 4). The results demonstrate that these two ATP-dependent drug transporters do not seem to be responsible for the reduced sensitivity to ET-743 in the ET-743-resistant CS-1 cells, in contrast to previous data for an ET-743-resistant ovarian cancer cell line [44]. However, there have been many reports suggesting that alternative molecular mechanisms may be involved in the chemoresistance phenomenon, as illustrated by the recent data documenting the link between defects in nucleotide excision repair and resistance to ET-743 [4,9–11]. Importantly, studies with various mesenchymal soft tissue cell lines and carcinoma cell lines demonstrated no overexpression at the protein level of P-glycoprotein by prolonged ET-743 treatment [18,45]. Thus, there may be significant differences not only in sensitivity to ET-743 but also in drug resistance mechanisms between epithelial and mesenchymal-derived cell types.

The most striking gross difference between the parent and ET-743-resistant cell lines is cell shape, which formed the basis for a convenient method of discriminating the two cell populations (Fig. 2). The altered morphology of the ET-743-resistant CS-1 cells, consisting of more elongated spindle-like cells, suggested that repeated exposure to ET-743 generated cytoskeleton rearrangements. Staining of cells with FITC-labeled phalloidin supported this hypothesis, for actin filaments existed as a diffuse meshwork in the parent CS-1 cells, within the cytosol as well as lamellipodia, while the ET-743-resistant cells displayed a network of stress fibers as well as actin staining that was considerably more concentrated at the rim of the cell surface, without noticeable membrane protrusions (Fig. 3). Regulation of the actin cytoskeleton is complex, and involves various small GTPases, including Rho, Cdc42, and Rac [46]. It is not clear how ET-743 might alter the cytoskeleton architecture, but the implications may be important for tumor cell attachment to extracellular matrix, motility, and invasion through tissue barriers. ET-743 may be altering the synthesis or activity of actin regulatory molecules such as members of the thymosin protein family, which can modulate actin polymerization and also tumor cell invasion and metastasis [47,48]. In fact, one previous report has shown disruptive effects of ET-743 on the cellular microtubule network [49]. As a first step toward elucidating functional consequences of the cytoskeleton rearrangement, we compared the performance of the parent and ET-743-resistance CS-1 cells in cell invasion and adhesion assays. The marked difference in both invasion and cell attachment between the two cell lines corroborates the actin staining data. The ET-743-resistant cells are compromised in both their ability to adhere to and invade through gelatin (Figs. 5 and 6). These data are somewhat surprising, considering previous reports that have demonstrated enhanced cell attachment and migration for various drug resistant tumor cell lines, and the fact that enhanced cell adhesion (i.e. cell adhesion-mediated drug resistance or CAM-DR) has been linked to chemoresistance [50,51]. Cell adhesion and cell motility are connected through both protrusive and contractile cell matrix contacts, which

mediate the linkage of cells to the extracellular matrix. Protrusive cell matrix contacts such as lamellipodium are thought to be discrete cell extensions comprised of cell surface adhesion receptors linked to the actin cytoskeleton. Our data suggest that the mechanism of ET-743 drug resistance operating in CS-1 cells involves at some stage the reorganization and repartitioning of the actin cytoskeleton, by as yet unknown mechanisms. This rearrangement appears to have significant effects on cell attachment and invasion, and may in fact generate a CHSA cell compromised in its ability to migrate through tissue barriers. However, the relationship of these altered cell functions to metastatic capability *in vivo* remains to be determined.

Since our data did not support a role for transmembrane drug efflux pumps in the resistance to ET-743, we began to explore alternative mechanisms that might be invoked by CHSA cells to withstand the toxicity of ET-743 *in vitro*. We decided to study the repertoire of secreted proteins from the parent and ET-743 resistant cells, with the goal of discerning differences between the two samples. Our rationale was that this type of approach, in contrast to gene array hybridization experiments, might lead to the identification of secreted molecular markers indicative of the onset of cell resistance, and possibly illuminate mechanistic aspects of cell resistance. The most prominent alterations in the secreted protein profile of the resistant CS-1 cells as compared to the parent cell line were found in the high molecular weight range (greater than 100 kDa), so we tailored our experiments to effectively separate proteins in this size range. As shown in Fig. 8, the differences in accumulation of several of these proteins when comparing the two conditioned medium samples were significant, based on Coomassie blue staining. The proteins migrated as discrete bands, allowing identification by one-dimensional SDS–PAGE combined with sequencing of tryptic peptides derived from excised proteins.

Two of these proteins were analyzed and identified as the α1 subunit of type I collagen and the α2 subunit of type IV collagen. Type I collagen, the most abundant protein in the human body, is composed of two α1 chains and one α2 chain, and is synthesized in mesenchymal-derived cells, while the most common form of type IV collagen is also comprised of two α1 chains and one α2 chain, and is expressed by many cell types [52–55]. Fibrillar collagens such as types I and III play important structural roles in tissues subjected to mechanical forces, while network-like collagens such as type IV are key components of multifunctional basement membranes [52]. Collagens serve a variety of roles, such as comprising an extracellular matrix that provides for cell adhesion and migration, binding to and signaling through integrins and other cell surface receptors, and binding of various proteins such as the matrix molecules fibronectin and vitronectin and the gelatinase matrix metalloproteinase-9 [52,55–62]. Collagen expression is regulated at both the transcriptional and post-transcriptional level, and is modulated by cytokines such as TGF-β or reactive oxygen species [63–67]. Collagens and other extracellular proteins are linked to aspects of tumor growth, motility, angiogenesis, and metastasis [68–72]. Moreover, compelling evidence indicates that chemoresistance by cancer cells may be affected by collagen and other extracellular matrix proteins that are derived largely from surrounding stromal cells due to paracrine stimulation [71,73–76].

Northern blot and competitive RT-PCR data presented in Figs. 10B and 11 demonstrate that the differential regulation of collagen α1(I) gene expression in the parent and ET-743-resistant CS-1 cell lines occurs at the transcription level. Two alternatively spliced mRNA species for collagen α1(I) are present in the parent cells, but there is virtually no detectable level of steady state mRNA for this gene in the ET-743-resistant cells. This finding is not completely surprising, considering the fact that previous work from several laboratories has established that ET-743 can inhibit transcription of particular genes, particularly those dependent on the transcription factors NF-Y and Sp1 [6–8]. Interestingly, both of these transcription factors have been implicated in the transcription of the collagen α1(I) gene, and may be inhibited by ET-743, culminating in reduced collagen gene transcription [77,78]. Sp1 plays a role in TGF-α-mediated collagen β1(I) gene transcription as well as expression of the TGF-β gene itself, suggesting that repression of collagen gene expression by ET-743 may be achieved by either targeting Sp1 directly or indirectly via inhibition of the TGF-β signaling pathway, possibly by stabilizing endogenous inhibitors of TGF-β signaling [79–82]. It seems noteworthy that DNA intercalators other than ET-743, such as mitoxantrone and mithramycin, can compete for Sp1 binding to DNA as well as curtail collagen α1(I) gene transcription in human fibroblasts, raising the hypothesis that ET-743 may act in a similar fashion to these drugs [78,83]. Interestingly, genes encoding collagens as well as other extracellular matrix molecules require Sp1 for optimal transcriptional activity, suggesting that if ET-743 is found to inhibit collagen gene expression through an Sp1-dependent mechanism, the ability of the ET-743-resistant cells to locally deposit extracellular matrix may be severely compromised [84].

The synthesis of collagens by CHSA and by CHSA cell lines is well established. Although normal adult articular cartilage synthesizes primarily types II, IX, and XI collagens (or type X in hypertrophic chondrocytes), dedifferentiated chondrocytes, osteoblastic-like cells derived from chondrocytes, or CHSA cell lines propagated from high grade tumors display an altered pattern of collagen biosynthesis, and tend to secrete collagens types I, II, and IV, which is in agreement with our data [85–94]. The abrogation of types I and IV collagen expression in the ET-743 resistant cells suggests that collagen itself or some aspect of collagen metabolism may be linked to the ET-743 sensitivity in the CS-1 cells. For example, it has recently been reported that the phosphatidylinositol 3-kinase/Akt

pathway can mediate type I collagen gene expression in hepatic stellate cells [95]. Activation of this pathway has been identified in many studies as being involved in the development of chemotherapeutic drug resistance, and thus our collagen experimental data may be extremely useful in elucidating molecular mechanisms of drug resistance.

It may be noteworthy that type I collagen expression is primarily restricted to mesenchymal cells, and it is intriguing to speculate that the significant sensitivity of these cells to ET-743 may be related to collagen expression [96]. Moreover, collagen metabolism is used to assess bone turnover in diseases such as osteoporosis and bone tumors, by determining the blood level of various collagen fragments such as procollagen fragments (derived from extracellular liberation of collagen propeptides and thus indicative of new bone formation) or telopeptides (products of collagen degradation and thus bone resorption) [97]. It will be important to test whether currently used diagnostic tests employed to assess bone turnover by measuring collagen fragments can be adapted for the purpose of detecting chemoresistance of chondrosarcoma to ET-743 or other chemotherapeutics.

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