Selective Modulation of Collagenase 1 Gene Expression by the Chemotherapeutic Agent Doxorubicin

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ABSTRACT

Matrix metalloproteinases (MMPs) play a crucial role in tumor cell invasion and metastasis due to their ability to digest basement membrane and extracellular matrix components, thereby facilitating cell movement through connective tissues. At noncytotoxic concentrations, i.e., concentrations lower than those normally used in cancer chemotherapy, the anthracycline doxorubicin specifically inhibited collagenase 1 (MMP-1) gene expression in the highly invasive and metastatic human melanoma cell line A2058. This inhibition was specific for collagenase 1 because it did not affect the expression of two other MMPs, gelatinase A (MMP-2) and gelatinase B (MMP-9). The reduction in collagenase 1 expression correlated with a decrease in the invasive ability of tumor cells through a collagen type I matrix and was independent of the cytotoxic and antiproliferative effects usually associated with this anticancer drug. The selective modulation of collagenase 1 expression by nontoxic doses of doxorubicin suggests a novel application for this chemotherapeutic agent, perhaps in combination therapy, because it decreases the invasive/metastatic potential of melanoma cells that are otherwise unaffected by this drug.

INTRODUCTION

MMPs\(^3\) are a family of at least 15 secreted and membrane-bound zinc endopeptidases. Numerous studies have shown an association between the expression of MMPs and the invasive behavior and metastatic potential of tumors (1–3). Most of the attention in the cancer research field has been focused on the gelatinases, gelatinase A (MMP-2) and gelatinase B (MMP-9), due to their ability to cleave collagen type IV, which is the major component of the basement membrane (3, 4). However, the interstitial collagenases, collagenase 1 (MMP-1), neutrophil collagenase (MMP-8), collagenase 3 (MMP-13), and MT1-MMP (MMP-14), also play an essential role in tumor invasion and metastasis because of their unique ability to cleave the major components of the interstitial stroma, collagens type I and III (4–6). Therefore, MMPs are important pharmacological targets, and a number of synthetic inhibitors have been developed (7). These compounds are potent, reversible, broad-spectrum inhibitors of MMPs and bind to the active site of the enzymes. Although clinical trials using synthetic MMP inhibitors are in progress, some of these agents are insoluble and may have poor bioavailability when administered p.o. (7).

Doxorubicin (Adriamycin), an anthracycline antibiotic with antitumor activity, is extensively used in the chemotherapy of solid cancers and cancers of the hematopoietic system (8, 9). The principle mode of action of the anthracyclines seems to be the ability of these agents to cross-link DNA and RNA, thereby affecting DNA and RNA synthesis (10–13). However, recent studies have demonstrated that genotoxic (i.e., DNA damaging) agents, including many important cancer chemotherapy drugs, can have significant and selective effects on the expression of certain inducible genes (11–15). It has also been demonstrated that nontoxic doses of the DNA cross-linking cancer chemotherapy drugs MMC, cisplatin, and carboplatin were effective at significantly altering the expression of the MDR1 gene coding for the multidrug resistance protein P-glycoprotein (13). Therefore, we were interested in whether genotoxic chemotherapy agents might be similarly able to preferentially alter the expression of the inducible collagenase 1 gene, thereby potentially altering tumor invasiveness.

We report that doxorubicin selectively inhibits collagenase 1 expression in the highly invasive and metastatic human melanoma cell line A2058 without affecting the expression of two other MMPs, gelatinase A and gelatinase B. Collagenase 1 inhibition correlated with a decrease in the ability of the A2058 cells to invade a collagen type I matrix. This decrease in invasiveness occurred at concentrations of doxorubicin that were nontoxic and did not affect the expression of P-glycoprotein. These results suggest a novel application for doxorubicin due to its ability to decrease the invasive/metastatic potential of melanoma cells that are otherwise unaffected by this drug.

MATERIALS AND METHODS

Cell Culture and Western Analysis. The human A2058 melanoma cell line was kindly provided by Dr. W. G. Stetler-
Stevenson (NIH, Bethesda, MD; Ref. 16). Cell cultures were propagated in DMEM (Life Technologies, Inc.) supplemented with 10% FCS (Life Technologies, Inc.), penicillin (100 units/ml), and streptomycin (100 mg/ml). At confluence, the cells were washed with HBSS (Life Technologies, Inc.) and subsequently treated with or without DNA cross-linking agents (13, 15) in the presence of DMEM and 0.20% LH for the indicated time periods. Culture medium was removed and replaced with DMEM/LH. After 18 h, the culture medium (200 μl) was precipitated with 100 μl of 10% TCA for 30 min on ice. Proteins were pelleted and resuspended in SDS-PAGE sample buffer, electrophoresed on 7.5% SDS-PAGE minigels, and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a Trans-Blot Cell (Bio-Rad). Collagenase protein was detected as described previously (17). The membranes were blocked with 1% BSA for 1 h with 10% FCS and then probed overnight with an anti-human collagenase antibody at a dilution of 1:10,000. The membranes were then washed, and specific antibody binding was detected with Vetcastain ABC kit (Vector Laboratories, Burlingame, CA).

ELISA. Quantification of collagenase 1 protein was performed using a human collagenase 1 ELISA system (Calbiochem). Standards and samples were incubated in microtiter wells precoated with anti-collagenase 1 antibody. After washing, the second antibody recognized by antirabbit horseradish peroxidase was added. The amount of peroxidase bound was determined by the addition of the substrate 3,3’-5,5’-tetramethylenediamine/hydrogen peroxide. The reaction was stopped by the addition of 1 M sulfuric acid. The assay was quantified using a Molecular Devices Vmax spectrophotometer measuring the absorbance at 450 nm.

Gelatin Zymography. Culture medium was electrophoresed on a 7.5% SDS-PAGE gel impregnated with 0.1% gelatin (w/v) as described previously (18). Cells were washed three times for 30 min in 50 mM Tris-HCl (pH 7.5) plus 2.5% Triton X-100 followed by an overnight incubation at 37°C in 50 mM Tris-HCl (pH 6.5), 150 mM NaCl, and 0.05% NaN3. Gels were stained with Coomassie Brilliant Blue R-250 and then destained.

Northern Analysis. Total cellular RNA was isolated using Trizol LS reagent (Life Technologies, Inc.). Cells were scraped from the plates, pelleted, and lysed in Trizol LS reagent. After chloroform extraction, the RNA was recovered by precipitation with isopropanol. Total RNA was quantitated by absorbance, and 10 μg of RNA were subjected to Northern analysis as described previously (17). Northern blots were hybridized with a [α-32P]dCTP-labeled probe for 20 h at 56°C (0.2× SSC and 0.5% SDS). Blots were washed twice at room temperature with 2× SSC followed by two 30-min washes at 56°C (0.2× SSC and 0.5% SDS) and autoradiographed. The human collagenase probe has been described previously (17).

Invasion Assay. Invasion assays were performed using a modification of the classical Boyden-Chamber (19). Briefly, nitrocellulose filters (Schleicher & Schuell AE100) were coated with collagen type I (1 mg/ml) diluted in sterile DMEM without serum in the presence of 1% antibiotics and successively applied to the membrane (3 × 150 μl and 1 × 550 μl) allowed to gel at 37°C for 30 min, and air dried for 1 h at room temperature.

Cells were grown to confluence and detached by trypsin treatment, and the number of living cells was counted by trypsin blue exclusion. The invasion chambers were placed in 60-mm tissue culture dishes containing 5 ml of conditioned medium obtained by culturing monolayers of human foreskin fibroblasts in serum-free DMEM/LH for 18–24 h (17). A 1-ml cell suspension (1 × 105 cells) was pipetted to the upper compartment in the presence or absence of doxorubicin at the concentrations and times indicated in the text.

CLSM. Invasion assays were terminated after 24 h. The membranes were washed in PBS and dehydrated with 2-propanol (10 min) followed by a 30-min RNase treatment (1 mg/ml) at room temperature. After washing with PBS, the filters were stained for 30 min with PI (0.01 μg/ml) at room temperature. Filters were washed with H2O and dehydrated with 2-propanol. The specimens were then treated with 100% xylene (4 × 4 min), which leads to complete translucence of the filters. Samples were mounted on slides and sealed with Canada balsam (Sigma). A Bio-Rad MRC-1024 CLSM with a Zeiss Axiostop microscope and a Zeiss Plan Neofluor 40 × 1.3NA objective were used to assay PI fluorescence in the membranes (Carl Zeiss, Inc., Thornwood, NY). Excitation light was 488 + 568 nm, with fluorescence measured through a 605 ± 16 nm filter using a 0.7 confocal iris. Images were 240 × 240 μm and were captured at 2-μm steps starting at the top of the collagen matrix. For analyses, the stack of images was processed with Molecular Dynamics Image Space software (Molecular Dynamics, Sunnyvale, CA) in a modification of the method described by Schoenmark (19). A threshold was set to eliminate background fluorescence, and the total PI fluorescence (related to the total double-stranded nucleic acid content) of each section was determined as the sum of the intensity of cell fluorescent pixels in that section. Results were plotted as the percentage of total fluorescence in each section. This is equivalent to the numbers of cells in each section.

Cytotoxicity and Cell Proliferation Assays. Cytotoxicity was measured using the CytoTox 96® kit from Promega. This assay measures the release of LDH in culture supernatants. The release of LDH in culture supernatants was measured with a coupled enzymatic assay that results in the conversion of tetrazolium salt into a red formazan product. The conversion of tetrazolium salt into red formazan is measured at 490 nm. The amount of color formazan is proportional to the number of cells lysed.

Cell proliferation was measured using the Promega CellTiter 96® AQueous cell proliferation assay. Cells were grown to 50% confluence in a 96-well microtiter plate in the presence of 10% serum. The number of viable cells in proliferation was measured by the conversion of MTS (Owen’s reagent) to the aqueous soluble formazan. Cell proliferation product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in the culture.

Reverse Transcription-PCR. One μg of total RNA from A2058 cells was reverse-transcribed using the RNA PCR Core Kit (Perkin-Elmer Corp.). The final concentration of the reagents used per reaction was 5 mM MgCl2, 1 × PCR buffer, 1 mM deoxynucleotide triphosphates, 1 μl of RNase inhibitor (1 unit/μl), 2.5 μM random hexamers, and 0.5 μl of Moloney murine leukemia virus reverse transcriptase (2.5 units/μl) in a
RESULTS

Selective Inhibition of Collagenase 1 Expression by Doxorubicin. To study the effect of doxorubicin on MMP expression, we used a highly invasive and metastatic human melanoma cell line, A2058, which constitutively produces high levels of collagenase 1 (16). A2058 cells were treated with a variety of cytotoxic drugs such as carboplatin, MMC, and doxorubicin. Among these agents, only doxorubicin specifically repressed collagenase 1 expression at both the protein and mRNA level (Fig. 1A), with no effect on gelatinase A and gelatinase B (Fig. 1B). Quantification of the protein by ELISA indicated that a 3 μM doxorubicin treatment resulted in a 50% inhibition of collagenase 1 protein secreted from A2058 cells (Fig. 1C).

Cells were also treated with cisplatin, but no effect was observed for this anticancer drug on either collagenase 1, gelatinase A, or gelatinase B expression (data not shown). In contrast, treatment of human glioblastoma cells with cisplatin decreased gelatinase A expression (18), suggesting that the repression of MMPs by chemotherapeutic agents may be drug and cell type specific.

Time and Dose-dependent Repression of Collagenase 1 Expression by Doxorubicin. To determine the minimum dose and incubation time needed for collagenase 1 repression without affecting cell viability, cells were treated with concentrations of doxorubicin ranging from 0.5–3.0 μM, and the pre-incubation time was reduced to 1 h. Doxorubicin inhibited collagenase 1 protein and mRNA levels in a dose-dependent manner (Fig. 2, A and B). An effect on protein levels was seen with as little as 2 μM, whereas mRNA levels were affected by concentrations as low as 0.5 μM. The discrepancy between the effects on mRNA and protein expression may be due to the time required for protein synthesis and the fact that levels of protein were cumulative in the culture medium over a 24-h period, whereas mRNA was harvested at the end of 24 h. We did not see any difference in the level of repression of collagenase 1 protein or mRNA between the 1- or 2-h treatments (Fig. 2; data not shown). Similar to the data shown in Fig. 1, none of the concentrations of doxorubicin used had any effect on the expression of gelatinase A or B (Fig. 2C), arguing against non-specific cytotoxicity and further supporting the concept that doxorubicin specifically targets collagenase 1 repression.

Cytotoxic Effects of Doxorubicin on A2058 Cells. At a concentration of 3 μM, doxorubicin treatment resulted in a 50% inhibition of collagenase 1 protein secreted from A2058 cells (Fig. 1C). Although a slight decrease in cell viability was noted at this doxorubicin concentration (see below), the fact that the expression of gelatinase A and gelatinase B (Fig. 2B) was not decreased when compared to that of untreated cells suggests that the decrease in collagenase 1 protein and mRNA was not due to generalized cytotoxicity of the drug. To confirm the absence of cytotoxic effects, we measured the release of LDH into the culture medium. Pretreatment of the A2058 cells with doxorubicin for 1 h with concentrations ranging from 1.5–2.5 μM did not result in cell death, although an increase in cytotoxicity was observed when the cells were pretreated with doxorubicin for 4 h (Fig. 3). Specifically, treatment for 1 h at a concentration of 2.5 μM reduced collagenase 1 mRNA levels by about 70% (Fig.
but had no effect on the release of LDH. In contrast, a 4-h treatment with this concentration resulted in a 30% increase in LDH release. Thus, these observations demonstrate that the down-regulation of a specific member of the MMP family by low doses of the chemotherapeutic agent doxorubicin without apparent toxicity depends on the doxorubicin concentration used and the time of treatment.

**Doxorubicin Inhibits the Invasion of A2058 Cells through a Collagen Type I Matrix.** Next we determined the ability of doxorubicin to suppress the invasive phenotype of A2058 melanoma cells. Cells were treated with concentrations of doxorubicin that inhibited collagenase 1 mRNA but were not cytotoxic. We found that about 38% of untreated cells invaded the matrix of type I collagen to a depth of 8 μm. In contrast, after treating cells with 0.5 μM doxorubicin, which reduced collagenase 1 mRNA levels by about 30% (Fig. 2B), only about 12% of the cells invaded to this depth (Fig. 4). Furthermore, in the presence of 1.5 μM doxorubicin, only about 2% of the cells invaded the collagen type I matrix of 8 μm. These experiments indicate that doxorubicin, even at low concentrations, is able to inhibit the invasive phenotype of A2058 cells, and it does so in a dose-dependent manner.

**Effect of Doxorubicin on Cell Proliferation.** To exclude that the observed decrease in invasiveness upon treatment with doxorubicin is due to the effect of this compound on cell proliferation, A2058 cells were incubated in the presence of varying concentrations of doxorubicin for the indicated time periods. Concentrations of doxorubicin from 1.5–2.5 μM had no effect on cell proliferation when cells were pretreated for 1 or 2 h. Therefore, the reduction in collagenase 1 expression and the decrease in invasiveness are not due to a decrease in cell proliferation (Fig. 5). The decreased invasive potential of A2058 cells in the collagen matrix, as seen in Fig. 4, is most likely a result of the decrease in collagenase 1 expression and the subsequent suppression of the invasive phenotype of this highly metastatic melanoma cell line.

**Doxorubicin Inhibits Collagenase 1 without the Induction of P-Glycoprotein Expression.** In contrast to the DNA cross-linking agents that reduce the expression of P-glycoprotein, doxorubicin itself is a substrate for P-glycoprotein. It is therefore of clinical importance to determine the effect of doxorubicin on the expression of P-glycoprotein in A2058 cells. Interestingly, we found that low concentrations of doxorubicin (0.5 and 1.0 μM) that inhibit both collagenase 1 mRNA and tumor cell invasion do not affect the expression of P-glycoprotein (Fig. 6). These data suggest that low doses of doxorubicin...
can prevent the observed drug resistance associated with this drug when used at higher concentrations (10).

**DISCUSSION**

The incidence of malignant melanoma has steadily increased from a risk of 1 in 250 in 1980 to a projection of 1 in 90 by 2000 (20). Despite early diagnosis, surgery, and chemotherapy, cure rates have not been improved, and death rates continue to rise (20, 21). Doxorubicin is not commonly used as a therapeutic agent for malignant melanoma. However, in animal studies, doxorubicin in combination with the chemopreventive agent NAC (22, 23) decreased tumor formation and metastasis in a murine model of melanoma, an effect attributed, at least in part, to the selective inhibition of gelatinase A and gelatinase B by NAC (24). In addition, doxorubicin-induced inhibition of melanoma cell invasion correlated with decreases in tumor cell motility and increases in focal contact formation in the mouse melanoma cell line K1735-M2 (25). Thus, there is precedence for examining the effect of this drug on collagenase 1 gene expression. In this study, we found that low concentrations of doxorubicin, i.e., doses lower than those used to inhibit cell growth, have a selective inhibitory effect on expression of the collagenase 1 gene by A2058 melanoma cells. These inhibitory concentrations prevented the ability of these melanoma cells to invade a matrix of type I collagen. Thus, the selective inhibition of collagenase 1 by low, noncytotoxic doses of doxorubicin suggests that this chemotherapeutic agent may be useful as a chemopreventive agent in melanoma dysplastic nevus syndrome and perhaps in other tumors as well, due to its ability to decrease invasion of the extracellular matrix. In addition, our observations suggest that low concentrations of traditional chemotherapeutic agents inhibit the invasive behavior of malignant melanoma, which is not currently considered for treatment by agents such as doxorubicin. Indeed, it is possible that in combination with a second cytotoxic agent, such as NAC, doxorubicin may provide a more efficacious treatment for this highly malignant disease by decreasing its invasive potential.

Previous studies have shown that DNA-damaging agents such as cisplatin and MMC display targeted effects on certain inducible genes (11–14). These effects occur principally at the level of gene transcription and are closely correlated with DNA damage. Chromatin structure also seems to play an important role in these effects (14). The precise mechanism by which these effects occur is not clear. However, these effects take place at doses that do not cause any overt cytotoxicity and do not affect total DNA or protein synthesis, the transcription of RNA, or the expression of constitutive genes (11–12). The doses at which these effects occur are also well below those required to activate general signaling pathways, because there is no effect on the nuclear levels or activities of transcription factors nuclear factor kB or activator protein 1 or on the expression of mRNAs for fos, jun, gadd45, or gadd153, all of which are hallmarks of the UV response to lethal doses of genotoxic agents (15). We observed that low concentrations of doxorubicin inhibit collagenase 1 expression without affecting the expression of P-glycoprotein. In similar studies on gene expression, treating cancer cells with DNA cross-linking agents such as MMC, cisplatin, and carboplatin significantly suppressed MDR1 mRNA expression (13). This suppression led to a subsequent decrease in cellular P-glycoprotein protein levels, a reversal of the multidrug resistance phenotype, and a sensitization of cancer cells to killing by a second agent (13). Pretreatment of human breast cancer xenografted nude mice with a low dose of MMC or carboplatin also suppressed tumor P-glycoprotein expression in vivo and led to a significant enhancement in subsequent tumor growth suppression by a second agent such as paclitaxel.4 In contrast to the DNA cross-linking agents that reduce the expression of P-glycoprotein, doxorubicin itself is a substrate for P-glycoprotein. However, low concentrations of doxorubicin (0.5 and 1.0 μM) that inhibit both collagenase 1 mRNA and tumor cell invasion do not affect the expression of P-glycoprotein (Fig. 6). Thus, the use of these low doses avoids the induction of multidrug resistance often associated with higher doses of drug treatment (10). This general approach of using a genotoxic agent

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4 J. W. Hamilton, personal communication.
to selectively modulate the expression of certain genes to manipulate targeted genes expressed by cancer cells may therefore hold promise in a multiagent therapy regimen that can increase the responsiveness of a tumor to treatment and/or suppress the more malignant aspects of its phenotype.

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