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Expression of the multidrug transporter P-glycoprotein is inversely related to that of apoptosis-associated endogenous TRAIL

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Abstract

Multidrug resistance (MDR) has been associated with expression of ABC transporter genes including P-glycoprotein (Pgp, MDR1, ABCB1). However, deregulation of apoptotic pathways also renders cells resistant to chemotherapy. To discover apoptosis-related genes affected by Pgp expression, we used the HeLa MDR-off system. We found that using doxycycline to control Pgp expression has a significant advantage over tetracycline, in that doxycycline caused less endogenous gene expression modification/perturbation, and was more potent than tetracycline in suppressing Pgp expression. Cells overexpressing Pgp have lower TNFSF10 (TRAIL) expression than their parental cells. Controlled downregulation of Pgp increased endogenous TRAIL protein expression. Also, ectopic overexpression of TRAIL in Pgp-positive cells was associated with a reduction in Pgp levels. However, cells expressing a functionally defective mutant Pgp showed an increase in TRAIL expression, suggesting that Pgp function is required for TRAIL suppression. Cells in which Pgp is knocked down by upregulation of TRAIL expression are less susceptible to TRAIL ligand (sTRAIL)-induced apoptosis. Our findings reveal an inverse correlation between functional Pgp and endogenous TRAIL expression. Pgp function plays an important role in the TRAIL-mediated apoptosis pathway by regulating endogenous TRAIL expression and the TRAIL-mediated apoptosis pathway in MDR cancer cells.

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Conflicts of Interest

No potential conflicts of interest were disclosed.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version.

Keywords

ABCB1; MDR1; TRAIL; apoptosis; TET-Off; multidrug resistance; TNFSF10

Introduction

Among the most common factors that adversely affect efficacy of cancer treatments, acquired drug resistance is one of the most important. The ATP-dependent drug efflux pump, P-glycoprotein (Pgp, ABCB1, MDR1) and other ATP-binding cassette (ABC) transporters are found to be overexpressed on the surface of cancer cells, functioning as drug efflux pumps. This contributes to the development of multidrug resistance (MDR)[1, 2]. Although ABC transporters have important physiologic functions, clinical insensitivity to anticancer agents, attributed to an elevated expression of Pgp, may be associated with higher remission and lower survival rates in patients with leukemia and solid tumors [3].

Besides drug efflux, other cellular mechanisms are related to MDR, such as evasion of drug-induced cell death, due to disruption of apoptotic pathways [4]. Pgp has been reported to affect apoptotic pathways. The resistance induced by Pgp in some cell types has been associated with a lack of caspase-dependent apoptosis *in vitro* and increased resistance to spontaneous apoptosis in clinical samples [5-7].

Apoptosis can be activated in response to various stimuli. Conventional chemotherapy usually triggers apoptosis through intrinsic pathway activation [8, 9]. However, the extrinsic pathway, which is mediated by a death signal transduction from specific ligands binding to death receptors on the cell surface, has been a promising therapeutic approach [10]. Among apoptotic ligands, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) has been used in clinical trials for cancer therapy. Recombinant human TRAIL and TRAIL agonistic antibodies against death receptors – TRAIL-R1/DR4 and TRAIL-R2/DR5 (for review, see [11]) – have been tested as treatments for advanced solid tumors, lymphomas and other cancers as a single agent or in combination with conventional chemotherapy [12-14].

In terms of physiology, studies in mice and humans have shown that endogenous TRAIL expression can be highly stimulated by IFNs and interleukins on the surface of immune cells [15, 16]. Besides TRAIL's role in the innate immune response, the expression of TRAIL in neutrophils and monocytes has been associated with the cytotoxic activity against TRAIL-sensitive tumor cells [17, 18]. Furthermore, studies have shown that TRAIL^{-/-} mice have a greater predisposition to develop tumors and metastases, suggesting that TRAIL is a tumor suppressor [19, 20].

It has been reported that the co-expression of Pgp and anti-apoptotic proteins may contribute to the development of the MDR cancer phenotype, although those proteins might be modulated by chemotherapeutic agents [21, 22]. We hypothesized that Pgp, in addition to its function as a drug transporter, might actually play an additional role by modulating apoptotic protein expression. To test this hypothesis, tumor cell lines overexpressing Pgp through the TETOff system were submitted to TaqMan Apoptosis Array analysis. After

querying the transcriptome of 95 apoptosis related genes, we verified a correlation between Pgp and TRAIL using gene silencing, gene expression, loss-of-function mutation studies and chemical inhibition function assays, apoptosis measurements, and Western blots, as well as confocal and ELISA analysis.

Materials and Methods

Cell lines

Human cervical cancer HeLa MDR-Off [23], HeLa TET-off (Clontech Laboratories, Mountain View, CA) and human embryonic kidney 293T cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM) in high glucose (4.5 g/l), supplemented with 2 mM L-glutamine, 100 I.U./mL penicillin, 100 µg/mL streptomycin and 10% tetracycline approved FBS (Clontech). The parental KB-3-1 cells (a subclone of HeLa) and their drug-resistant sublines KB-8-5, KB-8-5-11 and KB-C1 were grown with colchicine 10 ng/mL, 100 ng/mL and 1 µg/mL, respectively. All variant sublines were also cultured in DMEM supplemented with 10% FBS, penicillin and streptomycin (Gibco, Grand Island, NY). Cells were cultured at 37 °C with 5% CO₂ and relative humidity maintained at 95%. Cell cultures beyond passage 10 were discarded.

Preparation of total RNA and reverse transcription

Total RNA was prepared using the RNeasy Kit (Qiagen, Gaithersburg, MD, USA) and RNA concentration was measured by a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Synthesis of cDNA from 1 µg total RNA reaction volume was carried out using the High Capacity cDNA kit with RNase inhibitor (Applied Biosystems, Grand Island, NY) according to the manufacturer's instructions.

TaqMan Low Density Arrays (TLDA) and Real Time quantitative PCR (RT-qPCR)

For expression levels of the 380 MDR-associated genes detected by a custom-made TLDA [24] and a TaqMan Array Human Apoptosis 96-well plate, both from Applied Biosystems, procedures were performed according to the manufacturer's instructions. RT-qPCR and signal detection were performed by the 7900HT Fast Real-Time PCR System (Applied Biosystems). Raw data from SDS (Sequence Detection System) files were analyzed by RQ (Relative Quantification) Manager software.

Human Pgp/*ABCB1* and TRAIL/*TNFSF10* mRNA relative quantification were measured by RT-qPCR using Hs00184491_m1 and Hs00234356_m1 probes, respectively. GAPDH (Hs99999905_m1) mRNA was used as an endogenous gene control. All data were recorded in the 7900HT Fast Real-Time PCR System and analyzed by 2^{-Ct} method using SDS RQ Manager.

Western blot analysis

Cell lysate and Western blot were performed as described earlier [25]. For Pgp analysis (monoclonal anti-Pgp clone C219 at 1:2000 dilution), total protein was separated on a 3-8% gradient NuPAGE Novex Tris-acetate gel (Invitrogen, Grand Island, NY) and for other proteins (anti-TRAIL clone C92B9; anti-caspase-3, -8 and -9 poly and monoclonal antibody

at 1:1000 dilutions; anti-DcR2 clone D13H4 all from Cell Signaling; and anti-DcR1 clone LS-C154605 from LSBio), crude protein lysate was loaded on 12% Tris-bis gels (Invitrogen).

Cytotoxicity assay

The cytotoxicity of drugs on HeLa TET-Off and HeLa MDR-Off cells was measured by a colorimetric viability assay using MTT. Briefly, 5000 cells were seeded in each well of a 96-well multiwall plate. After 24 hours, cells were treated with drugs and further cultured for 72 hours. Cells were then incubated with MTT reagent for 4 hours and solubilized in 0.1M HCl/ethanol (1:4 v/v). Absorbance at 570 nm in each well was measured by multiplate reader (Spectra Max Molecular Devices, Sunnyvale, CA). Cytotoxicity (IC₅₀) was defined as the drug concentration that reduced cell viability to 50% of the untreated control.

Detection of cell surface proteins

To detect cell surface Pgp expression, 2×10^5 /200 μ l cells (per condition) were incubated with 1 μ g MRK-16 monoclonal antibody (Abnova, Taipei, Taiwan) diluted in Iscove's Modified Dulbecco medium (IMDM) supplemented with 5% FBS at 37° C for 30 min. After washing with IMDM, cells were incubated with FITC-labeled anti-mouse secondary antibody (Jackson Immuno Research Laboratories, West Grove, PA), 0.5 μ g in 2×10^5 /200 μ l cells at 37° C for 30 min. For TRAIL (CD253-PE, clone N2B2, eBiosciences, San Diego, CA) DR4 (CD261-PE, clone DJR1, eBioscience) and DR5 (CD262-PE, DJR2-4, eBioscience) protein expression analysis, the procedures were performed as recommended by the manufacturer. All samples were analyzed by FACS Calibur (Becton Dickinson) flow cytometer. Isotype controls (IgG2a from R&D, and IgG1 K from eBioscience) were used in these FACs experiments.

Pgp transport assay

For Pgp efflux analysis by Fluorescence Activated Cell Sorting (FACS), 2×10^5 cells were incubated in 1 mL IMDM with Pgp substrate 0.5 μ M Calcein-AM (Sigma, St. Louis, MO) or 0.5 μ g/mL rhodamine 123 (Sigma) in the presence or absence of the Pgp inhibitor, 1 μ M tariquidar (TXR9576) or 20 μ M cyclosporine (Sigma) for 10 min at 37° C in the dark. Fluorescence intensity was measured by a FACS Calibur (BD, San Jose, CA) flow cytometer and analyzed by FlowJo version 7.6.4 software.

Creation of shRNA-mediated gene knockdown cell lines

To stably knock down expression of Pgp, pLKO.1-puro plasmid-based shRNA targeting the sequence CCGAACACATTGGAAGGAAAT (clone ID: TRCN0000059683, Sigma) was employed (Pgp-shRNA). Similarly, to stably knock down expression of TRAIL, pLKO.1-puro plasmid-based shRNAs targeting the sequences: GCTGTAACTTACGTGTACTTT, CCATTCTACAGTTCAAGAAA and GTAACAAATGAGCACTTGATA (clone IDs: TRCN0000005925, TRCN0000005927 and TRCN0000005928, respectively, Sigma) were employed (TRAIL-shRNAs 25, 27 and 28). Additionally, a non-targeting shRNA vector (-NS-shRNA) that targets no known human sequence was utilized as a control. A primer containing the target sequence (CTGGTTACGAAGCGAATCCTT) along with a stem loop

followed by the reverse target sequence was annealed to a complimentary primer and inserted into the EcoRI and AgeI sites of the pLKO.1-puro vector (Addgene number 10878). Lentiviral particles were produced via Lipofectamine 2000 (Invitrogen)-mediated triple transfection of 293T cells with either the Pgp-shRNAs, TRAIL-shRNA or the -NS-shRNA along with the lentiviral envelope plasmid (pMD2.G, Addgene number 12259) and the lentiviral packaging plasmid (psPAX2, Addgene number 12260). Target cells (KB-3-1) were transduced individually with either NS-shRNA, Pgp-shRNA or TRAIL-shRNA containing lentiviral particles in the presence of 8 µg/mL polybrene and stable cells were selected 2 days later using 3 µg/mL puromycin (Sigma).

Transient transfection of TRAIL and BacMam-Pgp baculovirus transduction

For the pCMV6-XL5 cloning vector (OriGene, Rockville, MD, USA), transfection was performed using a Nucleofector II machine (LONZA, Cologne, Germany). 6×10^5 HeLa MDR-Off, KB-8-5-11 and KB-C1 cells were used for each point transfection through Amaxa® Cell Line Nucleofector® Kit R and the protocol was performed as recommended by the manufacturer. Empty vector with or without GFP was used as a control for transfection.

HeLa cell transductions with BacMam-Pgp virus wild-type and E556Q/ E1206Q mutants were performed as described previously [26, 27].

Immunofluorescence and confocal microscopy

HeLa MDR-Off cells in the absence and presence of doxycycline and KB-C1 shABCB1 and shNS were immunolabeled with primary antibody TRAIL at 1:100 and then with secondary antibody Alexa 488-conjugated goat anti-rabbit IgG at 1:500 (Molecular Probes, Grand Island, NY). The procedures were performed as previously described [22] and cell staining was visualized in a Zeiss LSM 510 NLO Meta confocal microscope.

Promoter Luciferase assay

HeLa TET-Off and HeLa MDR-Off cells were transfected with pTF-Luc reporter vectors from the apoptosis luciferase reporter vector set (Signosis, Santa Clara, CA). Transfection was performed by using FuGENE6 following the manufacturer's suggested protocol (Promega, Madison, WI). For luciferase activity detection, cells were lysed by Glo-lysis buffer (Promega), following incubation with steady-Glo® firefly luciferase (Promega). Transfection efficiency was standardized by measuring renilla luciferase activity using renilla Glo luciferase Assay System in cells co-transfected with pRL renilla luciferase control reporter vectors (Promega). Firefly and renilla luciferase activities were measured by the Victor3 multiplate reader according to the manufacturer's instructions (Perkin Elmer, Waltham, MA).

ELISA against TRAIL

HeLa MDR-Off cells in the presence or absence of doxycycline, and after transient transfection with pCMV6-XL5 or empty vector and KB-C1 shABCB1 and shNS cells were cultured for 24 h. After that, cell-free supernatant was assessed for TRAIL protein levels

using human TRAIL/TNFSF10 DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Statistical analysis

The Student's *t* test or one-way analysis of variance (ANOVA) was applied for all data analysis. *P* < 0.05 was considered statistically significant.

Results

Doxycycline alters fewer endogenous genes and has fewer off-target effects on MDR-linked gene expression than tetracycline during downregulation of Pgp in the HeLa TET-Off system

We previously developed the HeLa MDR-Off cell line to control Pgp expression [23]. This cell line was created from the HeLa TET-Off cell line by transfecting a plasmid containing the MDR1 gene into the HeLa cell line. HeLa MDR-Off cells express human Pgp by binding the TET-controlled transactivator (tRE) to the Tet-responsive element (TRE). In the presence of tetracycline, Pgp expression is blocked, since the tRE is bound to tetracycline, which prevents the complex from binding to TRE. In our previous study, 2 µg/mL tetracycline was used to turn off Pgp expression. In addition to tetracycline, the TET-responsive element in HeLa MDR-Off cells is also responsive to doxycycline. To optimize the method to control Pgp expression, HeLa MDR-Off cells were treated with 0.002, 0.02, 0.2 and 2 µg/mL tetracycline and 0.0001, 0.001, 0.01 and 0.1 µg/mL doxycycline. After 4 days, Pgp mRNA (Figure 1A) and protein levels (Figure 1B) were measured. Tetracycline was able to suppress Pgp mRNA and protein from 0.02 to 2 µg/mL. However, doxycycline could turn Pgp protein expression off at lower concentrations, starting from 0.1 ng/mL (Figure 1B and C). After examining the time and dosage effect on Pgp expression in HeLa MDR-Off cells, 0.005 µg/mL doxycycline for 3 days was chosen to turn Pgp expression off (Supplemental Figure S1A-B). Using a CaAM drug efflux assay, it was shown that drug efflux function of Pgp was lost when the MDR-Off cells were treated with doxycycline (Figure 1D). We also tested whether Pgp expression could be recovered by doxycycline wash-out. However, removal of doxycycline for 4 days resulted in only partial Pgp recovery, as shown by rt-qPCR, Western blot analysis and flow cytometry (Supplemental Figure S1C-E).

To further elucidate the influence of tetracycline on cell physiology and gene expression, we first determined sensitivity of doxycycline and tetracycline in HeLa MDR-Off and HeLa-TETOff cell lines. There is no significant difference in sensitivity to increasing concentrations of doxycycline between HeLa MDR-Off and HeLa TET-Off cells. With tetracycline, however, the HeLa-MDR-Off cells display higher resistance than HeLa-TET-Off cells (Figure 1E). We also tested the effect of colchicine in HeLa MDR-Off cells, since colchicine (50 nM) was used to maintain Pgp expression [23]. The HeLa MDR-Off cells, which express Pgp, were significantly more resistant than the HeLa TET-Off cells (IC₅₀ 268 ± 27 nM vs 14.7 ± 0.8 nM, RR: 18.23 fold, *P* < 0.05) (Figure 1F). We found that Pgp expression in HeLa MDR-Off cells was unaffected when colchicine was not added to the

culture medium (data not shown). Therefore, the HeLa MDR-Off cells were cultured without colchicine in this study.

We examined the effect of tetracycline (2 µg/mL) or doxycycline (0.05 µg/mL) on expression of 380 MDR-related genes in HeLa TET-off parental cells. Using this customized TLDA array [24], expression of seventeen genes was significantly changed (more than two-fold difference) by tetracycline but expression of only nine genes was changed by doxycycline (Supplemental Table S1). Also, for the genes whose expression was modulated by doxycycline or tetracycline (e.g. PDGFRB), doxycycline caused less gene induction than tetracycline (482 fold by tetracycline versus 97 fold by doxycycline) (Table 1). Taken together, these data indicate that doxycycline can control Pgp expression in HeLa MDR-Off cells with less off-target gene expression than tetracycline. We therefore decided to treat HeLa MDR-Off cells with doxycycline for further analysis.

Analysis of apoptosis-related gene expression in Pgp-positive cells reveals an inverse correlation between Pgp and TNFSF10/TRAIL expression

To elucidate the effect of Pgp expression on apoptosis, we utilized a TLDA array with primers for 95 human apoptosis-related genes. We first compared the HeLa MDR-Off cells in the absence of doxycycline (Pgp^{ON}) to HeLa MDR-Off cells in the presence of doxycycline (Pgp^{OFF}), with HeLa TET-Off parental cells as a control. Expression of eleven genes was significantly changed when Pgp levels decreased upon doxycycline treatment. Among these genes, eight were also changed in control HeLa TET-Off cells (Figure 2A; Supplemental Table S2). When we compared drug-selected cells (KB-8-5, KB-8-5-11 and KB-C1) with KB-3-1 parental cells, 34 apoptosis-related genes correlated with Pgp expression in one or more MDR1-expressing cell lines. Among these genes, 12 have altered expression in all three pairs of cells. (Figure 2B; Supplemental Table S3).

Endogenous TRAIL expression levels are dependent on Pgp expression

Analysis of TLDA data displayed in Tables S2 and S3 demonstrate TNFSF10 (TRAIL) as a gene whose expression is consistently increased when Pgp expression decreases (Fig 2). To elucidate whether Pgp has an effect on TRAIL expression, both genes were validated by rt-qPCR using TaqMan probes. Comparing HeLa MDR-Off cells with (Pgp^{OFF}) or without doxycycline (Pgp^{ON}) showed that Pgp mRNA and protein levels were downregulated significantly (Figure 3A-3B). Using the same sample, an elevated level (14.1 ± 0.9 fold) of TRAIL/TNFSF10 mRNA was found (Figure 3A). In contrast, we observed that doxycycline induced only ~8-fold more expression of TNFSF10/TRAIL mRNA in the parental HeLa TET-Off cells (Figure 3A). Using confocal microscopy, TRAIL protein was found in MDR-Off cells upon incubation with doxycycline (Figure 3B). Also, a time course study confirmed that induction of TRAIL mRNA is time-dependent upon doxycycline treatment (Figure 3C). Next, the correlation between Pgp and TRAIL expression was analyzed in HeLa MDR-Off cells transiently transfected with a TRAIL expression vector. Western blot analysis confirmed that overexpression of TRAIL suppresses Pgp expression levels (Figure 3D).

The reciprocal association between Pgp and TRAIL expression was further analyzed using cell lines with Pgp expression knocked down. KB-8-5-11 and KB-C1 are drug-resistant sublines of the KB-3-1 parental cell line that express Pgp. We transfected *ABCB1*-specific shRNA into KB-8-5-11 and KB-C1 cell lines in order to knockdown Pgp expression. Pgp protein levels were significantly reduced in *ABCB1* shRNA transfected cells (Figure 3E). Both KB-8-5-11 sh*ABCB1* and KB-C1 sh*ABCB1* cell lines demonstrated increased TRAIL expression compared to their scrambled shRNA control cell lines (Figure 3E). Moreover, we performed TRAIL transient transfection in KB-8-5-11 and KB-C1 wild type cell lines. Ectopic overexpression of TRAIL induced a reduction in Pgp protein level (Figure 3F). The above evidence suggested a tight correlation between Pgp and TRAIL.

Functional Pgp plays a critical role in TRAIL expression

To explore the correlation between Pgp and TRAIL, we determined whether Pgp efflux function is associated with TRAIL expression. Blocking Pgp activity with the Pgp-specific inhibitors tariquidar and PSC833 induced increased TRAIL expression in HeLa MDR-Off cells. However, verapamil, which inhibits Ca²⁺ channels and many other membrane functions, including Pgp, did not alter TRAIL expression (Figure 4A). To further confirm the link between Pgp function and TRAIL expression, we compared TRAIL expression in HeLa cells transfected with wild-type *ABCB1* and with a functional mutant *ABCB1* (E556Q/E1206Q). This mutant *ABCB1* was reported to encode Pgp on the cell surface but it was unable to efflux its substrates, such as CaAM (Figure 4C)[27]. While cell surface and total Pgp expression on HeLa-EQ cells and HeLa-WT cells are similar, cells expressing a Pgp mutant (HeLa-EQ) did not show suppression of TRAIL protein (Figure 4B). These experiments demonstrate that Pgp activity is a critical requirement to suppress TRAIL expression.

Endogenous TRAIL contributes to TRAIL ligand resistance in Pgp-negative cells

TRAIL ligand (sTRAIL) activates the apoptosis extrinsic pathway, which ultimately results in active caspase proteins. We assessed these apoptosis genes induced by TRAIL ligand in Pgp-expressing cell lines with their parental pairs through caspase cleavage. After treatment with increasing concentrations of sTRAIL (a gift from Dr. Stanley Lipkowitz), we found that the HeLa MDR-Off (Pgp^{ON}) and KB-C1 cells had more active caspase 8, 9 and 3 compared to the control HeLa TET-off and KB-3-1 cell lines (Figs. 5A and 5B). When the KB-C1 shNS and KBC1 sh*ABCB1* cell lines were treated with sTRAIL, the KB-C1 sh*ABCB1* cells were less sensitive to TRAIL ligand than the KB-C1 shNS cells (Figure 5C). MTT cytotoxicity studies also revealed that the Pgp knockdown KB-C1 cells are less sensitive to sTRAIL when compared with their parental cells (Figure 5D). However, we found that there are no differences in IC₅₀ values between parental and Pgp knockdown KB-C1 cell using TNF α and IFN γ (data not shown). The above data confirmed that MDR cells with lower levels of Pgp expression, and consequently increased levels of endogenous TRAIL expression, are more resistant to sTRAIL-mediated cytotoxicity.

TRAIL receptors play an important role in activating apoptosis. The TRAIL receptors DR4/DR5 elicit the apoptotic pathway but the decoy receptors DcR1/DcR2 attenuate their function. We sought to profile the TRAIL receptors in Pgp-positive and Pgp-null cells. For

DR4 or DR5, no differential expression was found between HeLa MDR-Off (Pgp^{ON}) and HeLa MDR-Off (Pgp^{OFF}) or between KB-C1 shNS and KB-C1 shABCB1 cell lines (data not shown). For the decoy receptors, however, we observed an increase in DcR1 expression in HeLa MDR-Off + doxycycline (Pgp^{OFF}) cells and KB-C1 shABCB1 cells. Expression of DcR2 was decreased in comparison to HeLa MDR-Off/Pgp^{ON} and KB-C1 shNS, respectively (Supplemental Figure S2). These data suggest that the decoy receptors might play a role in sTRAIL sensitivity in drug-resistant cells.

Finally, we assessed the impact of Pgp downregulation on transactivation activity and also localization of endogenous TRAIL. Using a promoter-luciferase activity assay, we assessed whether NFkB, p53, NFAT, Myc or AP1 activity was changed in HeLa-TET-Off and MDR-Off cells in the presence of doxycycline. In Figure 6A, we show that the p53 and Myc activities in MDR-Off cells were significantly increased when doxycycline was added, compared to the HeLa TET-Off cells. In contrast, NFkB activity was suppressed in both HeLa TET-off and MDR-Off cell lines, suggesting that doxycycline suppresses NFkB activity. NFAT and AP1 activities were not significantly altered. We then determined whether endogenous TRAIL induced by Pgp suppression is membrane-bound. Confocal microscopy images revealed small amounts of TRAIL protein in Pgp-expressing cells (HeLa MDR-Off (Pgp^{ON}) and KB-C1 (shNS). However, in doxycycline-treated HeLa MDR-Off (Pgp^{OFF}) and KB-C1 shABCB1 Pgp knockdown cells, TRAIL protein was increased and was found in the cytoplasm (Figure 3B, 6B). Cell surface immunolabeling flow-cytometry experiments did not detect the presence of TRAIL in doxycycline-treated HeLa MDR-Off (Pgp^{OFF}) and KB-C1 shABCB1 Pgp knockdown cells (Figure 6C). Since TRAIL might be secreted from cells, we sought to determine whether TRAIL protein could be found in culture medium. Using TRAIL ELISA we found no difference in secreted TRAIL protein levels in Pgp-expressing and non-Pgp-expressing cells (Figure 6D). These results indicate that endogenous cytosolic TRAIL is not secreted or membrane-bound, suggesting a differential role for endogenous TRAIL regarding apoptosis.

Discussion

In this study, we utilize a tet-repressible expression system to regulate expression of Pgp in cultured HeLa cells in order to study the effect of Pgp on apoptotic pathways. Although the TETON/OFF gene expression system is widely used as a tool to examine gene function, we find that the off-tartet effects of tetracycline are greater than generally reported. One striking result of our detailed study of gene expression in Pgp-expressing cells using a TET-off expression system is that tetracycline (to a greater extent) and doxycycline (to a lesser extent) produce changes by themselves in many genes - the expression of which is associated with drug resistance and/or apoptosis. This important consideration should be factored into any *in vitro* or *in vivo* studies that utilize tetracycline-regulated gene cassettes to dissect the role of specific gene expression.

Although Pgp has been described as a major contributor to MDR, other phenomena such as evasion of apoptosis have also been observed in several cancers displaying the resistance phenotype [28]. The concomitant expression of Pgp and apoptotic pathway proteins correlates with a multifactorial resistance phenotype in cancer cells [7, 21, 22, 29]. In the

present study, we analyzed 95 apoptosis-linked genes, *via* TLDA, in both inducible Pgp cell lines and drug-resistant cell lines. The TNFSF10/TRAIL gene was identified as inversely correlated to Pgp expression, with Pgp-positive cells exhibiting low levels of TRAIL expression. In addition to Pgp expression, we also demonstrated that TRAIL expression in multidrug-resistant cells correlates with Pgp efflux activity. Furthermore, multidrug-resistant cells with ectopic TRAIL overexpression showed a reduction in Pgp expression levels. Factors that increase TRAIL/TNFSF10 expression levels were previously reported by Tanaka et al. [30]. This study found that *TNFSF10* mRNA expression levels were elevated in CD34-positive cells from CML patients treated with IFN[γ 302] than CD34-positive cells from CML patients at diagnosis or cells from normal individuals. Although Pgp expression was not assessed in their work, CD34-positive cells do express Pgp, which may contribute to low levels of *TNFSF10* expression in untreated cells [31]. We emphasize in our study that high TRAIL expression levels were independent of cytokine stimuli.

Pgp often reduces cellular toxicity by active efflux of its substrates. However, there is no direct evidence to demonstrate that sTRAIL is a substrate of Pgp. In this study, we demonstrate that Pgp expression could influence the cytotoxicity of sTRAIL in an alternate manner. We identified, for the first time, an inverse correlation between Pgp and TRAIL expression. We observed that cells with Pgp knocked down or cells with defective Pgp activity have upregulated TRAIL. Moreover, cytoplasmic TRAIL accumulation in Pgp-negative cells was found to be associated with a low apoptotic index induced by sTRAIL. In fact, these results suggest that Pgp-expressing cells show collateral sensitivity to sTRAIL, similar to other drugs such as NSC73306 and verapamil [32]. The mechanism(s) of this sensitivity have not been determined, but the data showing enhanced apoptotic responses in Pgp-expressing cells may contribute to our understanding of the underlying molecular mechanism leading to collateral drug sensitivity.

TRAIL is a potent anticancer agent that has been used in clinical trials as monotherapy or in combination with chemotherapy for several malignancies. In our present study, we observed that cells expressing Pgp are generally more sensitive to sTRAIL-induced apoptosis, compared with cells having Pgp knocked down. These data are in agreement with other published reports which demonstrate that Pgp-positive cells are more sensitive to TRAIL-induced apoptosis than Pgp-negative parental cells [33, 34]. In contrast, Galski et al. demonstrated that Pgp contributes to sTRAIL resistance [35]. Therefore, in the initial stages of this study we attempted to optimize the amount of inducers to prevent possible identification of unrelated Pgp off-targets. In fact, we found that a much lower concentration of doxycycline (0.005 $\mu\text{g/mL}$) could have the same effect on Pgp downregulation in MDR-Off cells as tetracycline (2 $\mu\text{g/mL}$). Also, TLDA analysis revealed that tetracycline could yield more off-target effects, suggesting that using doxycycline to knockdown Pgp is a more feasible approach than tetracycline. In fact, in Galski's study, tetracycline (2 $\mu\text{g/mL}$) was used to knockdown Pgp, and colchicine was used to maintain the HeLa MDR-Off cells. The off-targets induced by tetracycline might contribute to increased resistance to sTRAIL. Nevertheless, our study clearly demonstrates that two cell lines with acquired drug resistance (KB-8-5, KB-C1) showed higher caspase activation upon sTRAIL treatment than their parental (KB-3-1) cells. This implies that long-term treatment with drugs, such as

colchicine, could alter the sensitivity of sTRAIL. Also, genetic knockdown of *ABCB1* in these cell lines reversed this phenotype. These results suggest that drug-resistant cells expressing Pgp are more sensitive to sTRAIL.

It is commonly believed that sensitivity to sTRAIL is influenced by genes in the extrinsic apoptotic pathway [36, 37]. Our data demonstrate that suppression of Pgp induces TRAIL and reduces sTRAIL toxicity. Cytotoxicity to TNF α and IFN γ were not affected, suggesting that only the TRAIL related extrinsic apoptotic pathway was affected. When Pgp was suppressed, TRAIL/DcR1 expression levels were increased. So the question remains: what is the link between Pgp and TRAIL? The promoter luciferase assay data showed that several transcription factors, including p53, were changed when Pgp was suppressed. Therefore, we suggest that induction of p53 might be an important factor to increase TRAIL and decoy receptor levels, once human TRAIL and DcR1 are regulated by p53 [38-40] (Figure 7). Expression of TRAIL and DcR1 only affects sTRAIL sensitivity, not sensitivity to TNF α and IFN γ , suggested that these proteins primarily affect the sTRAIL-mediated extrinsic apoptotic pathway.

The decoy receptors have been shown to be related to TRAIL resistance, as DcR1 could reduce sTRAIL-induced signal transduction. Our data suggest that higher expression of decoy receptor DcR1 in Pgp knockdown cells could play a role in resisting sTRAIL-mediated apoptotic activation, in contrast to the NT control shRNA-expressing cells. As for TRAIL, its exact function in cancer cells is not yet fully determined. TRAIL might be involved in tumor metastasis suppression, since TRAIL expression in neutrophils and monocytes is associated with cytotoxic activity against tumors [17, 18]. Also, TRAIL knockout mice are more susceptible to tumor metastasis than wild-type mice [19, 20]. Using TRAIL-resistant cell lines, Ehrhardt et al. also demonstrated that TRAIL inhibited spontaneous apoptosis in sTRAIL-resistant primary leukemic cells *in vitro* [41]. The above studies led to our hypothesis that TRAIL expression in Pgp-downregulated cells might inhibit signal transduction in the extrinsic pathway (Figure 7). Nevertheless, several hypotheses have been offered regarding sTRAIL resistance in Pgp-negative cells. Seo et al. suggested that high expression levels of DR4 and DR5 downregulate cFLIP and can contribute to sTRAIL sensitivity of Pgp-positive cells [34]. Park et al. also suggested that upregulation of DR5 can contribute to the sensitivity of Pgp-positive cells to sTRAIL [42]. In their work, inhibition of DR5 contributed to the low apoptosis index induced by sTRAIL in Pgp-positive cells. However, in our TET-off and KB-C1 drug resistance models, DR4 and DR5 expression levels remain unchanged following Pgp downregulation. In contrast, we observed that decoy receptor levels changed upon Pgp expression.

In fact, our results demonstrate that multidrug-resistant cancer cells with high endogenous TRAIL expression are less susceptible to sTRAIL-induced apoptosis than cells with lower TRAIL expression levels. This suggests that TRAIL is associated with cancer cell survival. Also, reduced TRAIL expression levels in multidrug-resistant cells and its cytoplasmic localization may contribute to a differential role for endogenous TRAIL in multidrug-resistant cells. In any case, a detailed exploration of such a pathway could decipher new anti-MDR strategies.

In summary, we demonstrate that an inverse correlation between Pgp and TRAIL expression promotes a differential response to the cytotoxic effect of sTRAIL-induced apoptosis. Pgp-expressing cells suppress endogenous TRAIL protein expression, which plays an important role in activation of apoptosis through the apoptotic extrinsic pathway. This may be one reason why drug-resistant cells are more sensitive to sTRAIL. Conversely, when Pgp expression is decreased, upregulation of TRAIL may explain the low apoptotic index induced by sTRAIL. Identification of genes that are tightly regulated by Pgp offers new alternatives to target drug resistance in cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Gene expression of tetra-/doxy-cycline-treated TetOFF-Pgp cells was compared.
- Doxycycline has less influence than tetracycline on Pgp downregulation.
- TNFSF10 is inversely correlated to Pgp in Pgp-expressing cells.
- Pgp activity is a critical requirement to suppress TNFSF10 expression.
- Silencing of ABCB1 led to poorer sensitivity to TRAIL.

Fig. 1.

Comparison of the effects of tetracycline and doxycycline on P-glycoprotein expression in HeLa MDR-Off cells. Minimal doses of doxycycline induced repression of (A) *ABCB1* mRNA by rt-PCR, (B) Pgp in total protein lysates by Western blot, and (C) Pgp expression on the cell surface by immunolabeling. (D) FACS histograms showing accumulation of rhodamine 123 in the presence (black) and absence (red) of cyclosporin A in MDR-Off cells. (E) Difference in doxycycline and tetracycline sensitivity between HeLa TET-Off and HeLa MDR-Off cells (F) Colchicine toxicity observed in both HeLa TET-Off and HeLa

MDR-Off cells. The relative *ABCB1* mRNA level was normalized to *GAPDH* mRNA. For comparison of Pgp protein, Na⁺/K⁺ATPase expression was used as a loading control.

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Fig. 2.

Representative Venn diagram illustrating the commonly and exclusively apoptosis-related genes expressed in Pgp-expressing cells (Pgp/ABCB1). HeLa TET-Off and HeLa MDROff cells in the presence or absence of doxycycline (A) and Pgp-expressing drug-resistant cell lines (KB-8-5, KB-8-5-11 and KB-C1) (B) were analyzed by TLDA analysis.

Fig. 3. Analysis of P-glycoprotein (*ABCB1*) and TRAIL/*TNFSF10* expression in MDR-positive cells. Protein and mRNA analysis of Pgp and TRAIL expression in HeLa MDR-Off and HeLa TET-Off cells in the presence or absence of doxycycline (Dox) (A). Confocal microscope image of TRAIL localization in HeLa MDR-Off in the presence and absence of doxycycline. DAPI - Nuclear staining (blue) and anti-TRAIL staining (green). Time-dependent analysis of Pgp and TRAIL expression in HeLa MDR-Off cells in the presence of Dox (C). Pgp expression analysis in HeLa MDR-Off cells under transient overexpression of

TRAIL (D). TRAIL and Pgp expression analysis in KB-8-5-11 and KB-C1 Pgp knockdown cell lines (E), and comparison of Pgp expression in KB-8-5-11 and KB-C1 Pgp knockdown cell lines under transient overexpression of TRAIL (F). The mRNA expression level was normalized to GAPDH. Na⁺/K⁺ATPase was used as a loading control for Western blots.

Fig. 4.

Effect of P-glycoprotein efflux function on TRAIL/TNFSF10 expression. (A) *TRAIL/TNFSF10* mRNA expression analysis in HeLa MDR-Off cells incubated with Pgp inhibitors - verapamil (VRP), tariquidar (TXR) and PSC833 for 24 hours. (B) Analysis of TRAIL expression in HeLa cells expressing functionally defective Pgp (EQ) or functional Pgp (WT). Equal expression of cell surface Pgp WT and EQ cells by MRK16 immunolabeling. (C) Pgp EQ-expressing cells are unable to efflux Calcein. Pgp function was analyzed in nontransfected HeLa cells (Ctrl), HeLa expressing functional Pgp (wild-type – WT) and HeLa cells expressing a functionally defective Pgp (EQ) mutant. Cells were incubated with calcein-AM (0.5 μM) ± tariquidar (1 μM) for 10 minutes followed by FACS analysis. Fluorescence intensities emitted by calcein in the cells were recorded by flow cytometry. Fluorescence intensity was recorded by the FACS FL1 channel. The mRNA expression level was normalized to GAPDH expression. Na⁺/K⁺ATPase was used as loading control for Western blots.

Fig. 5.

Effect of TRAIL ligand (sTRAIL) on inducing apoptosis in Pgp-expressing and -non-expressing cells. (A) Analysis of apoptosis induced by sTRAIL in HeLa TET-Off and HeLa MDR-Off cells. (B) Analysis of apoptosis induced by sTRAIL in KB-3-1 (parental cells) and KB-C1. (C) Analysis of apoptosis induced by sTRAIL in KB-C1 Pgp knockdown cells. GAPDH was used as a loading control for Western blots. (D) MTT assay showing difference in sensitivity to sTRAIL among KB-C1 shNS (—◆—), KB-C1 shABCB1 (—●—) and KB-3-1 (dotted line) cells. Data points showed statistical significance between KB-C1 shNS and KB-C1 shABCB1 cells were marked with asterisk (*). GAPDH was used as a loading control for Western blots.

Fig. 6.

Effect on transactivation of NFκB, p53, NFAT, MYC and AP1 in HeLa TET-off (grey) and HeLa MDR-Off (dark grey) cells by luciferase activity assay. Each bar represents the ratio of luciferase activity of cells treated with doxycycline over the luciferase activity of the same cells without doxycycline. (B) Analysis of TRAIL localization. Confocal microscope image of TRAIL localization in KB-C1 Pgp knockdown cells and control cells transfected with scrambled shRNA. DAPI - Nuclear staining (blue) and anti-TRAIL staining (green). (C) Cell surface TRAIL expression analysis of HeLa TET-Off and HeLa MDR-Off cells with and without doxycycline THP-1 cells was used as a positive control (D) Quantitative analysis of soluble TRAIL through ELISA in HeLa TET-Off cells, HeLa MDR-Off and KB-C1 Pgp knockdown cells.

Fig. 7. Schematic diagram of endogenous TRAIL function in multidrug-resistant cancer cells. In drug-resistant cells expressing P-glycoprotein (e.g., HeLa MDR-OFF (Pgp-ON) and KB-C1 cells), its intracellular environment allows a better response upon sTRAIL binding to death receptors and apoptotic signal transduction, compared to the environment when Pgp activity is lost. When Pgp is suppressed, as in HeLa MDR-Off cells treated with doxycycline (Pgp-OFF) or KB-C1 cells with Pgp knockdown, changes in the intracellular environment favor transactivation of p53, which induces TRAIL and DcR1 proteins. While the decoy receptor reduces sTRAIL-mediated apoptotic signal transduction, endogenous TRAIL in the cytoplasm seems to inhibit the extrinsic pathway signal transduction. These genes collectively reduce sTRAIL-mediated apoptosis once MDR cells lose Pgp expression or function.