Clusterin mediates TRAIL resistance in prostate tumor cells

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Abstract

One of the major obstacles in curing prostate cancer is the development of drug resistance to docetaxel, which is the gold standard for the treatment of this disease. It is not only imperative to discover the molecular basis of resistance but also to find therapeutic agents that can disrupt the resistant pathways. Based on initial findings that docetaxel-resistant PC3-DR and DU145-DR prostate tumor cell lines express tumor necrosis factor–related apoptosis inducing ligand (TRAIL) receptors, we examined whether TRAIL could be used as an alternative method to kill PC3-DR and DU145-DR cells. However, these tumor cells were found to be TRAIL resistant. Because PC3-DR and DU-145-DR cells were previously shown by us to be clusterin positive, we examined if clusterin could play a role in TRAIL resistance. We found that resveratrol could sensitize docetaxel-resistant tumor cells to TRAIL, and it worked by blocking clusterin expression. In particular, small interfering RNA clusterin expression in the cell lines was sufficient to produce apoptosis by TRAIL. Further analysis indicated that resveratrol functions as an effective tyrosine kinase inhibitor, similar to its analogue, piceatannol, and could inhibit Src and Jak kinases, thus resulting in loss of Stat1 activation. We have shown earlier that Stat1 is essential for gene transcription of clusterin. These results, taken together, show that resveratrol could be a useful new therapeutic agent to combat docetaxel resistance. [Mol Cancer Ther 2007;6(11):2938–47]

Introduction

Apoptosis is an intrinsic homeostasis mechanism that ensures proper functional development in multicellular organisms. This machinery is a main field of interest among cancer researchers whose goals are to decipher aberrant apoptotic mechanisms that can lead to tumorigenesis and to produce leads that could target these apoptotic pathways for cancer therapy. Members of the tumor necrosis factor family, including tumor necrosis factor FasL and tumor necrosis factor–related apoptosis inducing ligand (TRAIL), which are three major immune effector molecules, have potent apoptotic activities and have triggered keen interest in their potential for tumor eradication in vivo (1). However, tumor necrosis factor and FasL administration are hampered by severe toxicity (2, 3), and current focus is now on TRAIL, especially because of the reported selectivity in its ability to spare normal cells but induce apoptosis in a variety of tumor types (4).

TRAIL is a type II membrane protein that can be cleaved for release, and both membrane-bound and soluble forms are equally capable of inducing apoptosis (5). TRAIL binds to either of two membrane receptors, death receptor-4 (DR4) or DR5, on target cells to transmit a signal that results in cell death. Three other receptors, DcR1, DcR2, and osteoprotegerin, act as decoy receptors. TRAIL, as a homotrimer, binds DR4 or DR5, resulting in trimerization of these receptors that can then assemble a death-inducing signaling complex at the cytoplasmic tail. At the death-inducing signaling complex, the binding of Fas-associated death domain protein recruits caspase-8, which initiates the activation of downstream caspases to result in DNA fragmentation. Animal studies have shown that TRAIL alone or in combination with chemotherapeutic agents can suppress tumor xenografts without obvious toxicity (6, 7), raising expectations in its efficacy in man. Clinical trials have been initiated using either TRAIL itself or agonistic monoclonal antibodies against DR4 or DR5 in cancer patients with non–small cell lung and colorectal carcino- ma, as well as with non–Hodgkin’s lymphoma (8).

However, resistance to TRAIL has begun to surface, and antiapoptotic molecules, such as c-FLIP, Bcl-2, Bcl-xL, and IAP, have been implicated. Survivin, which is related to IAP, has also emerged as a barrier to TRAIL-mediated apoptosis (9). We report here that clusterin, which is unrelated to any of these survival proteins, is a key mediator of TRAIL resistance in prostate tumor cells.

In prostate cancer, clusterin expression has been associated with progressive disease (10). The up-regulation of clusterin corresponds to an increasing Gleason score (11). Additionally, it has been shown that clusterin-transfected LNCaP cells are highly resistant to paclitaxel-induced apoptosis (12). The initial controversial data that clusterin was found to display both proapoptotic and antiapoptotic activities has been resolved in that there are two functional isoforms of clusterin that are produced by use of alternative AUG start codons in the same gene (13, 14). The heterodimer of two 40-kDa α and β chains derived from different RNA transcripts served as the dominant form, whereas the single-chain form was found in tumors harboring tumors with low clusterin expression.

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from cleavage of a full-length 60-kDa protein is translated from the first AUG start codon, resides in the cytoplasm, and is associated with survival. This form is heavily glycosylated and can also be secreted (sCLU). On the other hand, an uncleaved 49-kDa protein, derived from a downstream AUG start codon of the same gene, is recruited to the nucleus and posttranslationally glycosylated to a 55-kDa proapoptotic protein upon treatment of cells with cytotoxic agents (nCLU). It is not understood how these proteins mediate their proapoptotic and antiapoptotic functions. Survival of certain malignancies, such as prostate cancer, could depend on the balance of nCLU and cytoplasmic sCLU (15).

In recent years, there has been a great amount of study devoted to understanding the anticancer abilities of naturally occurring antioxidant compounds that are present in our everyday diet (16). Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a phytoalexin that is commonly found in the skin of grapes and other plants (17). Resveratrol is a powerful antioxidant and chemopreventive agent against the three major stages of carcinogenesis: tumor initiation, promotion, and progression (18). Recently, resveratrol has been found to be a potent sensitizer for TRAIL-induced apoptosis in many cancer cell lines by down-regulating the IAP survivin (9). However, the mechanism for resveratrol’s sensitizing ability is not clearly understood.

In our search to identify agents that could kill prostate tumor cells, we investigated the role of TRAIL based on our preliminary findings that drug-resistant prostate tumor cells, we investigated the role of TRAIL based on our preliminary findings that drug-resistant prostate tumor cell lines express high levels of the TRAIL receptors DR4 and/or DR5. We made the unexpected discovery that DR4/DR5 expression was evaluated using sense primer (5'-CAA- AAGGGTCATCATCTCTGC-3') and antisense 5'-AACGTCCGAGTCA- AACGTCCGAGTCA-3'). As a control, expression of human glyceraldehyde-3-phosphate dehydrogenase mRNA expression was evaluated using sense primer (5'-CAA-AAGGGTCATCATCTCTGC-3') and antisense primer (5'-GAGGGGCATCCACAGTCTTC-3'). RT-PCR products were analyzed by agarose gel electrophoresis.

Materials and Methods

Cell Culture and Selection of Docetaxel-Resistant Clones

The human androgen-independent prostate carcinoma cell lines DU145 and PC3 were obtained from American Type Culture Collection and were maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS) with 100 units/mL penicillin and 100 μg/mL streptomycin. Docetaxel-resistant cell lines of DU145 and PC3 were developed by culturing the cells in docetaxel in a dose escalation manner. Initial culture was in 1 nmol/L docetaxel. After the cells sensitive to 1 nmol/L docetaxel were no longer present and the surviving DU145 and PC3 cells repopulated the flask and continued to divide through four passages, the concentration of docetaxel in the medium was increased to 5.5 nmol/L and, subsequently, to 11 nmol/L. The same selection methodology was followed with each increase in docetaxel concentration. Once DU145 and PC3 cells were freely dividing in 11 nmol/L docetaxel mediums, they were considered as resistant and labeled DU145-DR and PC3-DR. All the cell cultures were maintained at 70% confluency, and medium was changed every 48 h. Both of our DR cell lines were constantly maintained in 11 nmol/L docetaxel medium.

Apoptosis Assays

Docetaxel-resistant PC3 and DU145 cells were seeded in a six-well plate at a concentration of 5.0 × 10^3 cells per well and were allowed to adhere overnight. The cells were either untreated, treated with 100 μm resveratrol, 1 or 3 ng of TRAIL, or a combination of the two reagents. The plates were incubated for either 12 or 24 h at 37°C and analyzed for apoptosis using the Annexin V apoptosis kit (BD Pharmagen). Each well was trypsinized and resuspended in 1 × binding buffer at a concentration of 1.0 × 10^6 cells per milliliter and stained with Annexin-FITC and propidium iodide.

To determine activation of caspase-3, cells were seeded in six-well plates as described above. The cells were either untreated, treated with 100 μmol/L resveratrol, 1 or 3 ng/mL of TRAIL, 50 μmol/L of the broad range caspase inhibitor zVAD.fmk (EMD Biosciences), zVAD-AMC (Bachem), or a combination of the reagents, as indicated in the experiments. The plates were incubated for either 12 or 24 h at 37°C. The cells were trypsinized and resuspended in Cytofix/Cytoperm solution and then incubated on ice for 20 min. The cells were washed twice with perm/wash buffer and stained with 20 μL of FITC-conjugated monoclonal rabbit anti-active caspase-3 antibody (BD Pharmagen). The cells were incubated with the antibody for 30 min at room temperature in the dark. Each sample was washed with 1 mL of perm/wash buffer and then resuspended with 0.5 mL perm/wash buffer. All samples were analyzed by flow cytometry using a FACScan within 30 min of staining.

Isolation of RNA and Reverse Transcription—PCR

Reverse transcription—PCR (RT-PCR) was used to determine mRNA expression for clusterin in DU145-DR and PC3-DR cells. Briefly, total RNA was prepared using TRIzol reagent (Invitrogen). A total amount of 1 μg of RNA was converted to cDNA by Omniscript reverse transcriptase in a solution containing random hexanucleotide, deoxynucleotide triphosphate, Rnase inhibitor, and RT buffer (Qiagen). Aliquots of 1 μL of RNA resulting from each RT reaction were then subjected to PCR. The temperature profiles of PCR were as follows: an initial denaturation step of 94°C for 5 min, followed by 25 cycles of 94°C for 15 s, 50°C for 15 s, 72°C for 30 s, and a final elongation step of 72°C for 7 min. PCR was done in reactions containing Taq DNA polymerase, deoxynucleotide triphosphate PCR buffer, and the clusterin primer (sense 5'-CCTGATG-CCTTCTCCTCGTA-3'; antisense 5'-AAGCTCCGACTCA-GAAGTGTTG-3'). As a control, expression of human glyceraldehyde-3-phosphate dehydrogenase mRNA expression was evaluated using sense primer (5'-CAA-AAGGGTCATCATCTCTGC-3') and antisense primer (5'-GAGGGGCAATCCACAGTCTTC-3'). RT-PCR products were analyzed by agarose gel electrophoresis.
Western Blotting Analysis

Resveratrol-treated or nontreated PC3-DR and DU145-DR cells were solubilized by incubation at 4°C for 30 min in 1% NP40, 10 mM/L Tris, 140 mM/L NaCl, 0.1 mM/L phenylmethylsulfonyl fluoride, 10 mM/L iodoacetamide, 50 mM/L NaF, 1 mM/L EDTA, 1 mM/L sodium orthovanadate, 0.25% Na deoxycholo, 100 µL/L ALA, and 100 µL of phosphatase inhibitor cocktails I and II (Sigma). Whole-cell lysates were centrifuged at 12,000×g for 10 min to remove nuclei and cell debris. The protein concentration of the soluble extracts was determined by using the BioRad protein assay (Bio-Rad). Separation of 50 µg of total protein was done on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane before immunoblotting with primary antibodies, as indicated. The monoclonal antibody-specific against clusterin was purchased from Upstate Cell Signaling Solutions. PC3-DR cells were treated with 100 µM/L of resveratrol for 0 to 24 h to evaluate its effect on clusterin expression. Antibodies against total and active phosphorylated forms of Stat1, Jak1, Jak2, and Src were obtained from Cell Signaling. The specific proteins were detected by the enhanced chemiluminescence detection system (Amersham). To determine the effect of resveratrol on Jak1, Jak2, Src, and Stat activation, PC3-DR cells were starved in serum-free medium overnight to knockdown the endogenous level of phosphorylated kinases. PC3-DR cells were then treated with 25 to 100 µM/L of resveratrol for 4 h before being stimulated with FBS for 15 min. Cells were harvested after FBS stimulation and were subject to Western blotting, as specified above. The equal loading of protein samples was verified with antibodies against total Stat1, Jak1, Jak2, and Src (Cell Signaling).

Treatment of PC3-DR Cells with Clusterin Small Interfering RNA

To inhibit clusterin expression, a clusterin small interfering RNA (siRNA) expression plasmid was purchased from Dharmacon. PC3 cells were seeded at 5 × 10⁵ cells in each well of a six-well plate with 2 mL of growth medium 24 h before transfection and were 80% to 90% confluent at the time of transfection. The following mixture was made to perform the transfection: 4 µg containing clusterin siRNA (siRNA-clusterin) or the negative control (siRNA-control) and 10 µL LipofectAMINE 2000 reagent (Invitrogen-Life Technologies, Inc.) were diluted in 600 µL of Opti-MEM I (Life Technologies). The transfection complexes were preincubated with LipofectAMINE reagent in Opti-MEM I for 30 min. Cells were transfected with LipofectAMINE + siRNA-clusterin, LipofectAMINE + siRNA-control, or left untransfected. The transfection was terminated after 5 h by aspirating the transfection medium and adding fresh culture medium. After another 48-h incubation, an aliquot of the transfected cells was harvested with Trizol for evaluation of mRNA expression. Another aliquot was lysed after 72 h of incubation for Western blot analysis to evaluate clusterin knockdown. A third aliquot, after 48-h incubation, was seeded at 5 × 10⁵ cells per well and treated with 3 ng/mL of TRAIL for an additional 12 h to measure apoptosis.

Treatment of PC3-DR Cells with PP2 and AG490

PP2 and AG490 (Src inhibitor). After the treatment, cells were stimulated with FBS for 15 min to induce activation of phosphorylated proteins. Cells were harvested, lysed, and Western blotted for phosphorylated Src, Jak1, Jak2, and Stat1. Equal loading controls were done by blotting with antibodies to unphosphorylated forms of the same proteins. To determine the effect of the two inhibitors on clusterin expression, PC3-DR cells were seeded in a six-well plate and treated with 50 µM/L of the two inhibitors for 48 h. After treatment, cells were harvested, lysed, and Western blotted for clusterin expression. To check for equal loading, membranes were stripped and blotted with β-actin.

Statistical Analysis

For all apoptosis assays, the graphs represent the mean of three separate experiments and the error bars represent the SD.

Results

DR5 Expression in Docetaxel-Resistant PC3 and DU145 Cells

In our quest to find an alternative means to kill docetaxel-resistant prostate tumor cells PC3-DR and DU145-DR, we investigated the potential for TRAIL in inducing cell death. Based on earlier reports of the expression of DR4 and DR5 in paclitaxel-treated prostate tumor cells (19), we set out to determine if expression of these TRAIL receptors was present in our docetaxel-resistant cell lines. Flow cytometric analysis of PC3-DR and DU145-DR indicated that DR5 was expressed in >90% of both PC3-DR and DU145-DR cells (Fig. 1A and B). DR4 was expressed in PC3-DR cells (Fig. 1C), but was minimally expressed in DU145-DR cells (Fig. 1D). Thus, both docetaxel-resistant cell lines displayed at least one death receptor, DR5. To further characterize the expression of DR4/DR5 in prostate tumor cell lines, we examined the expression of these receptors between our parental and resistant cell lines. As shown in Fig. 1, the expression of both DR5 and DR4 was similar between the parental and the resistant cell line.

TRAIL-Induced Apoptosis in PC3-DR and DU145-DR Cells

Due to the significant expression of DR5, TRAIL seemed an excellent choice as an alternative drug choice to chemotherapy. TRAIL offers some therapeutic advantages over chemotherapy because it has been reported to have a minimal toxic effect on normal cells, whereas conventional chemotherapy has significant toxicity on both normal and malignant cells (20). To analyze the apoptotic response to TRAIL treatment, both parental and docetaxel-resistant PC3 and DU-145 prostate tumor cells were treated with 1 or
3 ng/mL of TRAIL for 12 h. TRAIL induced apoptosis in a time- and dose-dependent manner (Fig. 2A and B). However, the extent to which apoptosis was induced was less than expected, and this was especially evident in our docetaxel-resistant cell lines. Upon 12-h treatment with TRAIL, the maximum amount of apoptosis that was induced by 3 ng/mL of TRAIL was only 13% in PC3-DR cells, whereas 40% in parental PC3 cells (Fig. 2A). This was surprising because >90% of both PC3 and PC3-DR cell lines were positive for DR5. Due to this apparent TRAIL resistance, we investigated the effects of the compound resveratrol, which has been reported to sensitize tumor cells to TRAIL-induced apoptosis by down-regulating IAP proteins (9). Treatment with 100 μmol/L of resveratrol alone induced a minimal amount of apoptosis in PC3-DR and DU145-DR cells (Fig. 2A and B). However, resveratrol sensitized both parental and docetaxel-resistant PC3 and DU145 prostate tumor cells to TRAIL-induced apoptosis.

Figure 1. Expression of DR4 and DR5 in parental and docetaxel-resistant PC3 and DU145 cells. Cells were stained with antibodies against DR5 or DR4, and flow cytometric analysis was done on these cells to determine cell receptor expression. A and B, DR5 expression in parental and docetaxel-resistant PC3 and DU145 cells compared with isotype control. C and D, DR4 expression in parental and docetaxel-resistant PC3 and DU145 compared with isotype control. Points, representative from three separate experiments.
This synergy was much more pronounced in the parental cell lines than in the resistant cell lines (Fig. 2A and B). From our previous work (21), we found that clusterin expression was up-regulated in our DR cell lines (Fig. 2C). We therefore hypothesized that clusterin was responsible for TRAIL resistance and that resistance was much greater in DR cells due to the higher expression of clusterin. However, parental cell lines were also sensitive to resveratrol treatment because there is a background expression of clusterin (Fig. 2C). At 24-h treatment, the combination of 1000 μmol/L resveratrol and 3 ng/mL TRAIL produced an enhanced synergistic effect inducing apoptosis up to 75% of PC3-DR cells and 65% of DU145-DR cells (Fig. 2D). Even at 12-h treatment, as little as 1 ng/mL of TRAIL was sufficient to produce a substantial apoptotic effect when combined with resveratrol (Fig. 2A and B). To further validate that the extrinsic apoptosis pathway is occurring, we evaluated the percentage of cells that were positive for active caspase-3 expression upon treatment with resveratrol and TRAIL. Flow cytometric analysis using anti-active caspase-3 antibody indicated that cotreatment with resveratrol and TRAIL dramatically increased the percentage of cells that had activated caspase-3 at 12 h and an even higher percentage at 24 h (Fig. 3A and B). In contrast, treatment with either agent alone produced only a small effect. To confirm that TRAIL sensitization by resveratrol occurred in a caspase-dependent manner, zVAD.fmk, which is a known caspase inhibitor, was used. It effectively blocked caspase-3 activation during TRAIL/resveratrol treatment (Fig. 3A and B). Treatment with zVAD.AMC, which is a caspase-1 inhibitor, was used as a negative control and showed no effect on TRAIL/resveratrol activation of caspase-3.

Clusterin Expression Decreases after Treatment with Resveratrol

To understand the molecular mechanism behind TRAIL resistance in our prostate tumor cells, we investigated the function of clusterin in correlation with the sensitization effect of resveratrol. We have recently shown that docetaxel resistance in prostate tumor cells was dependent upon the overexpression of clusterin (21). Therefore, we hypothesized that TRAIL resistance is also dependent upon clusterin. Because clusterin expression is greater in our DR cell lines and they were less sensitive to TRAIL treatment, we investigated the mechanism of resveratrol treatment using DR cell lines. Both resistant cell lines were

Figure 2. TRAIL resistance and induction of apoptosis through the combination treatment of TRAIL (T) and resveratrol (R) and differential response between parental and docetaxel-resistant tumor cell lines. A and B, cells were either left untreated (ctrl), or treated with 100 μmol/L resveratrol, 1 ng of TRAIL, 3 ng of TRAIL, or a combination of TRAIL and resveratrol for 12 h. C, whole-cell lysates were made of all cell lines, and Western blot analysis with specific antibodies was done to detect the protein levels of clusterin and β-actin. D, using the same drug combinations as in A, PC3-DR and DU145-DR cells were treated for 24 h. Viability was assessed by Annexin V–propidium iodide staining for apoptotic cells on a FACScan. Columns, data from three separate experiments; bars, SD.
treated with resveratrol for 0, 6, 18, or 24 h and then analyzed for clusterin mRNA expression by RT-PCR amplification using primers specific for clusterin. Clusterin mRNA expression was significantly reduced in a time-dependent manner upon treatment with resveratrol in both PC3-DR and DU145-DR cells (Fig. 4A and B). To determine if the decreased mRNA expression corresponded to a decrease in the amount of protein, a Western blot was done. The protein levels of clusterin were analyzed after treatment with resveratrol from 0 to 24 h. In both PC3-DR and DU145-DR cells, clusterin expression was dramatically down-regulated in a time-dependent manner by resveratrol (Fig. 4C and D).

To determine if the expression of clusterin is responsible for TRAIL resistance, we investigated the effect of clusterin depletion in docetaxel-resistant prostate cancer. PC3-DR prostate tumor cells were transfected with either a siRNA plasmid targeted for clusterin (siRNA-clusterin) or a random siRNA plasmid (siRNA-control), and at 48 h, RT-PCR amplification was done to ensure transfection efficiency. In addition to clusterin-specific primers, glyceraldehyde-3-phosphate dehydrogenase primers were also included as a control for equal loading. Treatment of PC3-DR cells with siRNA-clusterin effectively knocked down clusterin mRNA and protein expression compared with either untransfected cells or cells transfected with siRNA-control (Fig. 5A and B). Once clusterin is depleted, TRAIL alone should be able to induce apoptosis in PC3-DR tumor cells. Therefore, we added TRAIL to PC3-DR cells that were either untreated or treated with siRNA-clusterin or siRNA-control. TRAIL-induced apoptosis increased significantly in PC3-DR cells that were transfected with a siRNA plasmid targeted for clusterin compared with control-siRNA transfected cells or untreated medium control cells (Fig. 5C). The level of TRAIL-induced apoptosis in clusterin siRNA-treated PC3-DR cells was comparable with that seen with the combination treatment of TRAIL and resveratrol. Thus, clusterin depletion worked as effectively as resveratrol.

**Resveratrol Inhibits Activation of the Stat Pathway**

From our recent work, we found that Stat1 regulated clusterin expression (21). Src and Jak kinase have both been implicated in controlling the activation of the Stats (22, 23). Piceatannol, which is a natural analogue of resveratrol, has been shown to inhibit the activation of Src kinase (24). We therefore hypothesized that resveratrol might itself work as a tyrosine kinase inhibitor and mediate its inhibitory effect on PC3-DR cells via regulation of Stat1 through inhibition of an upstream tyrosine kinase. To test this hypothesis, we first examined if Src and Jak tyrosine kinases were activated in docetaxel-resistant cells and next evaluated the effect of resveratrol on these kinases. PC3-DR cells were serum-starved overnight to lower the endogenous level of phosphorylated proteins. The cells were then treated with 25 to 100 μmol/L of resveratrol for 4 h before stimulation with FBS for 15 min. Src phosphorylation was markedly reduced by resveratrol treatment in a dose-dependent manner (Fig. 6A). Similarly, Jak1 and Jak2 phosphorylation were effectively blocked by resveratrol treatment (Fig. 6A). Because phosphorylation of Jak1 and Src was inhibited by resveratrol and both of these kinases are known to control Stat activation, we next investigated the effects of

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**Figure 3.** Activation of caspase-3 after treatment with resveratrol and TRAIL. Cells were either left untreated or treated with 100 μmol/L resveratrol, 1 ng of TRAIL, 3 ng of TRAIL, 50 μmol/L of the broad range caspase inhibitor zVAD.fmk (fmk), 50 μmol/L of the caspase-1 inhibitor zVAD.AMC (amc), or a combination of these reagents. A and B, after treatment for 12 or 24 h, cells were permeabilized and stained with an antibody against active caspase-3, and activation was detected on a FACScan. Columns, data from three separate experiments; bars, SD.
resveratrol treatment on Stat1 activation. As with the
tyrosine kinases, resveratrol markedly suppressed the
phosphorylation of Stat1 in PC3-DR cells resulting in
the elimination of expression at the 100 μmol/L concentra-
tion (Fig. 6A). Thus, Src, Jak1, Jak2, and Stat 1 were all
inhibited by resveratrol. Reprobing with antibodies recog-
nizing total Src, Jak1, Stat1, and Stat3 indicated that the
protein levels were not affected by resveratrol.

Stat1 Activation Is Controlled by Jak and Src in PC3-
DR Cells

The results above indicated that resveratrol inhibited
both Src and Jak kinases, which have been associated with
Stat activation. To identify which tyrosine kinase is the
upstream regulator of Stat1 in PC3-DR cells resulting in
the elimination of expression at the 100 μmol/L concentra-
tion (Fig. 6A). Thus, Src, Jak1, Jak2, and Stat 1 were all
inhibited by resveratrol. Reprobing with antibodies recognizing total Src, Jak1, Stat1, and Stat3 indicated that the
protein levels were not affected by resveratrol.

Discussion

Our purpose in this project was to investigate whether
TRAIL could provide an alternative treatment modality for
advanced prostate cancer. Despite high levels of DR5
expression, TRAIL only minimally induced apoptosis in
DU145-DR and PC3-DR cells, and TRAIL resistance was
found to be dependent on clusterin expression. The
combination of TRAIL and resveratrol had an increased
effect on parental cells than DR cells despite similar
expression of DR5, indicating that clusterin is responsible
for TRAIL resistance.

It is now known that the heavily glycosylated, secretory
form of clusterin provides resistance to a number of
different apoptotic stimuli (15). Consistent with this idea,
clusterin has been shown to be responsible for androgen
independence (10) and radiation resistance (25). From our
previous studies (21), we found that clusterin induction by
Stat1 activation was responsible for resistance to docetaxel.
Although our docetaxel-resistant cells lines were initially
only slightly affected by TRAIL treatment, cotreatment
with resveratrol significantly sensitized them to apoptosis.
We found that this sensitization effect by resveratrol
occurred through the down-regulation of clusterin. For
confirmation, we used a siRNA plasmid targeted for
clusterin to determine if clusterin expression was responsible for TRAIL resistance. Depletion of clusterin significantly sensitized PC3-DR cells to TRAIL-induced apoptosis compared with cells transfected with siRNA-control. Induction of apoptosis upon TRAIL treatment was similar between PC3-DR cells transfected with siRNA-clusterin and nontransfected cells that were cotreated with resveratrol and TRAIL, further indicating that the primary role of resveratrol is in the down-regulation of clusterin. Together, this information confirms that clusterin expression plays an important role in TRAIL resistance. Thus, clusterin seems to be a key survival agent of prostate cancer in blocking both the extrinsic and intrinsic apoptotic pathways.

To elucidate the mechanism of clusterin depletion by resveratrol, we analyzed the signaling events that occur during serum growth factor stimulation of PC3-DR cells. We hypothesized that resveratrol down-regulated clusterin by the inactivation of an upstream kinase, which resulted in the inhibition of Stat1, which is a key transcription factor for clusterin (21). This was based on the similarity of resveratrol to piceatannol, a tyrosine kinase inhibitor. In accordance with our hypothesis, we found that Jak1 and Src activation was blocked upon resveratrol treatment and that this inhibition correlated with the loss of phosphorylation of Stat1. These data and work from our previous study further elucidate the mechanism that clusterin overexpression in prostate cancer is the result of a Jak/Src-Stat1-clusterin pathway that mediates drug resistance to both docetaxel and TRAIL.

A recent study corroborates our findings, indicating that secretory clusterin expression in breast cancer is regulated by activation of insulin-like growth factor-1 receptor and its subsequent activation of Src (25). Src then activates other downstream kinases, such as mitogen-activated protein kinase, which result in clusterin induction and radiation resistance. Src is a well-known oncogene product and has been reported to play an important role in Stat3-mediated tumorigenesis (23). We now provide evidence that resveratrol can inhibit the Src/Jak-Stat1 pathway that has been shown earlier by us (21) to control clusterin gene expression. We also provide new evidence that resveratrol is a potent tyrosine kinase inhibitor. This latter finding is key to breaking docetaxel resistance in prostate cancer, and targeting of Src/Jak tyrosine kinases could be a potentially effective therapeutic means to overcome docetaxel and TRAIL resistance through the inhibition of clusterin expression.

Direct targeting of clusterin has been attempted in prostate cancer patients, and the prevailing methodology is in the use of antisense oligonucleotides against clusterin, such as OGX-011. These have been shown to increase the efficacy of chemotherapy and radiation, and phase II trials are undergoing to test the efficacy of this treatment in several tumor models (26). Resveratrol is a powerful therapeutic agent because it possesses multiple anticarcinogenic qualities, as well as having more than one mechanism in which to attack transformed cells. Although the mechanisms for resveratrol’s multiple functions are not clearly defined, there has been data shown that it can inhibit cyclooxygenase enzymes, cytochrome P450 complex, and promutagen bioactivation (27). Resveratrol has been shown to affect many cellular targets, such as nuclear factor-κB, mitogen-activated protein kinase, Akt, activator protein-1, and protein kinase C (16, 28). Our present study now provides the clue to how these seemingly diverse signal molecules can be all affected by resveratrol through tyrosine kinase inhibition.

![Figure 5](MolCancerTher.2007.6.11.Figure5.jpg)

Figure 5. Effective suppression of clusterin expression in PC3-DR cells after transfection with siRNA-clusterin results in TRAIL sensitization. PC3-DR cells were either left untreated (med), transfected with siRNA-control, or transfected with specific siRNA targeting clusterin expression (siRNA-clusterin) for 5 h in serum-free media. A, clusterin mRNA levels were evaluated by RT-PCR amplification using primers for clusterin and analyzed by agarose gel electrophoresis. Glyceraldehyde-3-phosphate dehydrogenase mRNA expression was used as a control for equal gel loading. B, using the same experimental conditions as above, Western blot analysis with specific antibodies was done to detect the protein levels of clusterin and b-actin. After the 5-h transfection, cells were cultured in 10% FBS media for 2 h before treatment with 3 ng of TRAIL. After a 12-h incubation, cells were harvested and stained with Annexin V–propidium iodide, and apoptotic cells were detected by a FACScan. A nontransfected sample was treated with with 3 ng of TRAIL + 100 μm resveratrol for comparison. Representative from three separate experiments.
In our investigations of docetaxel-resistant tumor cells, we have shown that resveratrol down-regulates clusterin expression. In our hands, clusterin may play a key role in survival by interfering with caspase activation. Previous studies have indicated that the apoptotic effects of TRAIL can be significantly increased upon cotreatment with chemotherapy agents (20, 29). Furthermore, a recent study showed that clusterin can offer resistance to TRAIL.

Figure 6. Resveratrol inhibits the activation of Jak and Src tyrosine kinases and their subsequent regulation of Stat1. PC3-DR cells were starved overnight to lower the endogenous level of kinase activation. A, PC3-DR cells were then cultured with 100 μmol/L of resveratrol for 4 h before stimulation with 10% FBS for 15 min, at which time lysates were made. Western blot analysis was done with specific antibodies to phosphorylated Src (P-Src), phosphorylated Jak1 (P-Jak1), and phosphorylated Stat1 (P-Stat1) to determine the affect of resveratrol treatment on kinase activation. Membranes were stripped and reprobed with total Src, Jak1, and Stat1 to check for equal loading. B, PC3-DR cells were pretreated with the specific Src inhibitor PP2 for 2 h before stimulation with 10% FBS for 15 min. As controls, cells were either left untreated (media) or DMSO was added to rule out DMSO toxicity from the inhibitors (DMSO). Cells were then lysed, and Western blot analysis was done using specific antibodies for P-Src and P-Stat1. Membranes were stripped and reprobed with total Src and Stat1. The same procedure was done using the specific Jak inhibitor AG490. Lysates were blotted for P-Jak1, P-Jak2, and Stat1. Membranes were stripped and reprobed for total Jak1, Jak2, and Stat1. C, PC3-DR cells were seeded in a six-well plate and treated with either 50 μmol/L of PP2 or AG490 for 48 h, and lysates were made. Western blot analysis was done to detect clusterin expression, and membranes were then stripped and blotted with b-actin to check for equal loading. These data are representative from three separate experiments. D, mechanism of tyrosine kinase inhibition upon resveratrol treatment. By blocking Src/Jak activation, Stat1 is not phosphorylated, which decreases the level of clusterin expression, leading to a decrease in cell survival, even in the presence of TRAIL.
chemotherapeutic agents that target the intrinsic apoptotic pathway by interfering with the function of Bax (30). Interestingly, a study has shown that TRAIL-induced sensitization after irradiation of prostate cancer cells was entirely dependent upon a Bax-mediated pathway (31). Understanding the mechanism of resistance is important to increase the biological affects of TRAIL. Currently, both phases I and II trials are being completed using both rhTRAIL and antibody agonists of DR4 and DR5 (32). Because these studies only showed response rates up to 30%, it will be important to investigate combination treatments with TRAIL, resveratrol, and docetaxel in prostate cancer. Of importance, there have been several derivatives of resveratrol that have been found to have more potent antitumor effects while having less toxicity to nonmalignant cells (33). Therefore, it will be worthwhile to investigate the sensitization effects of resveratrol in vivo because it might prove to greatly enhance the efficacy of the available treatment options of prostate cancer.

From this study, we have discovered a novel finding that resveratrol can mediate antitumor properties through inhibition of specific tyrosine kinase pathways. TRAIL resistance in docetaxel-resistant prostate cancer is mediated through clusterin expression and that resveratrol can successfully sensitize these cells through inhibition of the Src/Jak-Stat1 pathway.

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References

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