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Oxidative stress sensitizes bladder cancer cells to TRAIL-mediated apoptosis by downregulating anti-apoptotic proteins

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Abstract

Purpose—TRAIL, an endogenous protein involved in immunosurveillance and a novel drug in clinical trials, is of particular interest as a cancer therapeutic because it can induce apoptosis in cancer cells but not in normal cells. Since some cancers develop resistance to TRAIL, safe and effective methods of TRAIL sensitization are of great clinical interest. This study explores how chemotherapy and oxidative stress affect TRAIL sensitivity and expression of proteins in the apoptotic pathway.

Materials and Methods—Sensitivity to TRAIL was assessed in viability assays. Apoptosis was measured by caspase-3/7 activity and/or nuclear condensation using Hoechst staining. Western blotting was used to determine cleavage, phosphorylation, or alterations in protein expression.

Results—TRAIL reduced the viability of 5637 but not J82 and T24 bladder carcinoma cells. Chemotherapy with doxorubicin or cisplatin decreased the expression of the anti-apoptotic protein cFLIP_S and increased caspase-8 cleavage, reversing TRAIL resistance in T24 cells. Specific targeting of cFLIP_S by siRNA was insufficient for sensitization to TRAIL in T24 cells. However, chemotherapy-mediated TRAIL sensitization was mimicked by low concentrations of hydrogen peroxide, which resulted in phosphorylation of translation elongation factor 2 (EF2) and reduced expression of several short half-life, anti-apoptotic proteins including FLIP_S, XIAP, and survivin.

Conclusions—Induction of oxidative stress by low concentrations of hydrogen peroxide may reverse TRAIL resistance and warrants the further exploration of hydrogen peroxide as an adjuvant intravesical treatment to lower the apoptotic threshold of bladder cancer cells.

Keywords

TNF-related apoptosis inducing ligand (TRAIL); Apoptosis; Urinary bladder neoplasms; Oxidative stress; Hydrogen peroxide

Introduction

Bladder cancer is the fourth largest cause of cancer in men and the American Cancer Society estimates that a total of 68,810 new cases of bladder cancer will be diagnosed in the U.S. in 2008. A large proportion of bladder cancer cases consists of noninvasive, superficial tumors, which can be effectively treated by transurethral resection of the malignant lesions followed

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by intravesical administration of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG)¹. Recently, BCG therapy was found to induce the release of TNF-related apoptosis inducing ligand (TRAIL) from neutrophils infiltrating the bladder². Importantly, TRAIL secretion into voided urine correlated with responsiveness to BCG therapy, suggesting that TRAIL plays a role as a mediator of anti-tumor action of BCG².

TRAIL is a death inducing ligand that binds to its receptors on the cell surface and can selectively induce apoptosis in tumor cells without affecting normal cells. TRAIL-mediated apoptosis is initiated by the extrinsic pathway via caspase-8 cleavage and the signal can be amplified via the intrinsic (mitochondrial) pathway³. Clinical trials indicate that recombinant TRAIL can safely be administered to humans, although its efficacy appears to be enhanced when used in combination with other therapies^{3, 4}.

In this study we analyzed TRAIL susceptibility of three bladder cancer cell lines and evaluated the ability of two commonly used chemotherapeutic agents, doxorubicin and cisplatin, to reverse TRAIL resistance. Since TRAIL resistance in bladder cancer cell lines correlates with expression of the anti-apoptotic protein cFLIP⁵ and studies in prostate cancer cells confirm the involvement of the short isoform (cFLIP_S) in TRAIL resistance^{6, 7}, we initially focused on the effect of chemotherapy on cFLIP_S. Using T24 cells as a model, we sought to analyze the mechanism by which chemotherapeutic agents enhanced susceptibility to TRAIL. Although chemotherapy decreased cFLIP_S expression, downregulation of the protein was not sufficient for sensitization to TRAIL in the absence of doxorubicin or cisplatin. These results led us to explore the broader hypothesis that oxidative stress may be the underlying cause of TRAIL sensitization.

Materials and Methods

Cell Lines and Transfections

The bladder cancer cell lines 5637, J82 and T24 were purchased from the American Type Culture Collection (Rockville, MD), cultured in Primaria plasticware (Falcon, Bedford, MA) and propagated in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with heat-inactivated 10% fetal bovine serum (Hyclone, Logan, UT). Cells were maintained at 37°C in a 5% CO₂ atmosphere.

Plasmids encoding c-FLIP-GFP fusion proteins were obtained from Dr. Harald Wajant (University of Stuttgart, Germany)⁸. DNA was prepared using the Endo-Free kit (Qiagen Inc, Valencia, CA) linearized with DraIII (New England Biolabs, Beverly, MA) and transfected using Lipofectamine Plus (Invitrogen). At 48 hours post-transfection, cells were plated into selective media containing 600 µg/ml G418. Individual colonies were screened for GFP expression by flow cytometry and western blot for expression of the fusion protein.

Transfections with siRNA were performed as described⁹. The RNAi against cFLIP_S is targeted against exon 7 (5' AAC ACC CTA TGC CCA TTG TCC), which is included only in the short isoform of the protein.

Apoptosis and Viability Assays

Cells were seeded into 96-well plates at 5×10³ (J82) or 1×10⁴ (5637, T24) cells/well, incubated overnight and subsequently treated with chemotherapeutic agents and/or TRAIL (Killer-TRAIL™, Alexis, San Diego, CA). Doxorubicin and cisplatin were from Ben Venue Laboratories, Bedford OH. The CellTiter® Aqueous One Solution Cell Proliferation and Apo-ONE™ homogeneous caspase 3/7 assays were obtained from Promega, Madison, WI). The LDH assay was obtained from Roche (Basel, Switzerland). Assays were performed according

to manufacturer's directions. Percent cytotoxicity was calculated as follows: % cytotoxicity = $1 - [(\text{OD of experimental} / \text{OD of control}) \times 100]$.

Antibodies and Western Blot Analysis

Cells were allowed to adhere overnight before treating with chemotherapeutic agents or H_2O_2 in the absence or presence of TRAIL. Protein lysates were prepared and analyzed as previously described⁷. The monoclonal c-FLIP antibody NF-6 was generously provided by Dr. Marcus Peter, University of Chicago, IL. NF-6 hybridoma supernatant was used at 1:5 in TBS-Tween at room temperature overnight. Antibodies against caspase-8 (1:2000) and caspase-3 (1:1000) were purchased from Alexis Corp, San Diego, CA. XIAP, survivin, EF2 and phospho-EF2 antibodies were all obtained from Cell Signaling Technology (Danvers, MA) and were used at a dilution of 1:1000. Anti-actin (1:1000) was from Sigma, St. Louis, MO.

Hoechst staining

Cells were plated 2×10^5 in 12-well plates and the next day treated with growth media alone, H_2O_2 , TRAIL, or H_2O_2 and TRAIL for 22 hours. Hoechst dye was then added (final concentration 10 $\mu\text{g}/\text{ml}$) for 10 minutes at 37°C and cells were visualized using a fluorescent microscope with an ultraviolet filter (Zeiss Axiovert 200, magnification 200 $\times$). Images of three representative fields from each well were captured, the condensed nuclei were counted and the percentage of condensing nuclei out of the total count was calculated for each field.

Results

Chemotherapy reduces the expression of cFLIP_S facilitating processing of caspase-8

Susceptibility of cancer cells to TRAIL, a novel anticancer therapeutic currently in clinical trials, varies and does not generally correlate with TRAIL receptor expression. We confirmed this observation in three bladder cancer cell lines. Despite similar surface expression of agonistic TRAIL receptors, only 5637 but not T24 and J82 cells were sensitive to TRAIL (supplementary figure 1). Numerous agents can enhance susceptibility to TRAIL^{3, 4}. Treatment of J82 (Fig. 1 A, C) and T24 cells (Fig. 1 B, D) with either doxorubicin or cisplatin rendered T24 cells sensitive to TRAIL-induced apoptosis but had little effect on viability or caspase-3/7 activity in J82 cells (Fig 1E). Others have previously shown that interaction between doxorubicin and TRAIL is synergistic and we have confirmed these observations with both doxorubicin/TRAIL and cisplatin/TRAIL (not shown)¹⁰.

Since cFLIP levels have been shown to correlate with TRAIL sensitivity among bladder cancer cell lines⁵, and we have previously shown a correlation between doxorubicin-mediated sensitization to TRAIL and loss of cFLIP_S expression in prostate cancer cells⁶, we investigated the effect of chemotherapy on cFLIP expression and caspase-8 cleavage. cFLIP exists in long and short isoforms, which form heterodimers with initiator caspase-8 and interfere with processing to its active form. Cisplatin and doxorubicin primarily decreased the expression of cFLIP_S, which correlated with processing of caspase-8 into its active form (Fig 2). These data indicate that chemotherapy-induced loss of cFLIP_S was necessary for caspase-8 activation and also suggest that the lesion in the TRAIL apoptotic pathway in J82 cells is downstream of caspase-8. J82 cells express low levels of Apaf1, a protein involved in apoptosome formation downstream of caspase-8 activation¹¹. Low Apaf1 expression is expected to result in poor apoptosome formation and failure to amplify the TRAIL signal, which offers an explanation why chemotherapeutic agents in combination with TRAIL generated a greater than additive effect on cytotoxicity and caspase-3/7 activity only in T24 cells. T24 cells were used as a model to further investigate mechanisms that alter TRAIL responsiveness.

Overexpression of cFLIPs protects against TRAIL-induced apoptosis but targeting cFLIPs by siRNA is insufficient for sensitization

To address the role of cFLIP in chemotherapy-mediated sensitization to TRAIL, we modulated cFLIP expression in T24 cells. Overexpression of cFLIP_S (or cFLIP_L) resulted in inhibition of TRAIL-mediated caspase-3/7 activation in chemotherapy-sensitized T24 cells (Fig. 3A). A similar effect was observed when we measured cytotoxicity (supplementary figure 2). In contrast, specific downregulation of cFLIP_S by siRNA failed to sensitize T24 cells to TRAIL (Fig 3B). Similar results were obtained when a plasmid, expressing an shRNA against the same target site in cFLIP_S, was stably transfected into T24 cells (data not shown). These data indicate that although overexpression of cFLIP protects from TRAIL-mediated apoptosis, a decrease in cFLIP_S is not sufficient for sensitization of T24 cells.

Oxidative stress sensitizes T24 cells to TRAIL and reduces the expression of short half-life anti-apoptotic proteins

While chemotherapeutic agents and RNAi both decreased the expression of cFLIP_S, only chemotherapeutic agents resulted in sensitization to TRAIL in T24 cells. We therefore adopted a broader hypothesis to explain the sensitizing effect of chemotherapeutic agents. One mechanism of action that doxorubicin and cisplatin share is the generation of free radicals. In order to test the hypothesis that free radicals are sufficient to sensitize T24 cells to TRAIL, we co-treated cells with a low dose of hydrogen peroxide and TRAIL. Only cells receiving both hydrogen peroxide and TRAIL underwent apoptosis as evidenced by increased nuclear condensation (Fig. 4A) and caspase-3/7 activity (Fig 4B).

We previously demonstrated that low concentrations of hydrogen peroxide enhance TRAIL susceptibility in PC3 prostate carcinoma cells by inducing phosphorylation (and thereby inactivation) of elongation factor 2 (EF2), which is the enzyme responsible for elongation of nascent peptides⁷. Here we investigated whether this mechanism can also be activated in T24 bladder cancer cells. As shown in Fig 5A, escalation of hydrogen peroxide concentrations results in a dose-dependent increase in phosphorylation of EF2. This increase was accompanied by a decline in several short half-life anti-apoptotic proteins such as cFLIP_S, XIAP, and survivin without altering the expression of pro-apoptotic proteins such as caspase-8, -9, and -3 (Fig. 5B). Taken together, these results indicate that oxidative stress induced by hydrogen peroxide results in the phosphorylation and inactivation of EF2 and a subsequent decrease of short half-life, anti-apoptotic proteins. Since proteins with more stable half-lives such as caspases are not immediately affected by translational arrest the TRAIL apoptotic pathway is left unopposed.

We also evaluated the effect of hydrogen peroxide on sensitivity of 5637 cells. 5637 cells are sensitive to TRAIL and as shown in Figure 6A, TRAIL alone induced nuclear condensation in about 40% of cells. However, in the presence of H₂O₂, nuclear condensation approximately doubles while H₂O₂ alone had very little effect. We confirmed these results in a caspase-3/7 activity assay (Fig. 6B). These data indicate that hydrogen peroxide not only sensitizes T24 cells to TRAIL but also increases the sensitivity of cells that are spontaneously susceptible to TRAIL.

Discussion

In bladder cancer cells resistance to TRAIL has been associated with the expression of the anti-apoptotic protein cFLIP⁵. Here we show that in T24 bladder cancer cells doxorubicin and cisplatin each reduce cFLIP_S expression and enhance TRAIL sensitivity, yet specific targeting of cFLIP_S was insufficient to achieve the same results. Therefore, we hypothesized that ROS generated by the chemotherapeutic drugs may have a broader effect.

Using low concentrations of hydrogen peroxide as a source of ROS, we were able to recapitulate the effect of the chemotherapeutic agents on TRAIL sensitivity. In a previous study, we had confirmed that the mechanism by which doxorubicin mediated sensitization to TRAIL involves generation of ROS via the Fenton reaction in PC3 prostate cancer cells. Our data indicated that pretreatment with the iron chelator desferoxamine, which inhibits interaction of doxorubicin with intracellular iron and thereby generation of ROS, prevented the decrease of cFLIP β and XIAP in a dose dependent manner. Desferoxamine also inhibited EF2 phosphorylation and TRAIL-mediated apoptosis in doxorubicin treated cells. Taken together these data indicated that ROS generation is an important mechanism by which doxorubicin enhances TRAIL sensitivity⁷. However, ROS have also been associated with treatment related toxicities, such as cardiotoxicity and nephrotoxicity resulting from doxorubicin and cisplatin, respectively. Concerns about using TRAIL in combination with systemic chemotherapy have been raised^{12–14}. Thus bladder cancer in which therapies could be administered locally by intravesical instillation may be a good candidate for combination therapies.

ROS have dose-dependent effects on both normal and cancer cells. At low doses, ROS stimulate cell-repair and apoptosis inhibiting proteins, thus promoting cell survival. Transient exposure to oxidative stress allows cells to survive and to subsequently downregulate stress response proteins. In contrast, chronic exposure to low dose ROS-generating agents can lead to permanent activation of cell survival mechanisms, mutations, and malignant transformation^{15–17}. For instance, exogenous hydrogen peroxide was sufficient to induce a malignant transformation in an immortalized rat urothelial line¹⁷. It has also been shown that elevated endogenous ROS resulting from a mitochondrial gene mutation was sufficient for increasing the tumorigenicity of bladder cancer cells¹⁶. Increased endogenous hydrogen peroxide as a result of elevated Sod2 expression has also been associated with a metastatic phenotype in bladder cancer¹⁵. Experimental and epidemiological evidence links chronic inflammation, and its accompanying ROS production, to tumor induction, especially in the bladder urothelium^{15, 17}.

While ROS are associated with tumor promotion and progression, they are also associated with cell death. According to a threshold model of cancer therapy proposed by Kong and co-workers, non-surgical methods of cancer therapy elevate ROS to a level that malignant cells may not be able to survive¹⁸. Tumor cells, by virtue of their more rapid growth and subsequent increased energy requirements, generate more ROS than their untransformed counterparts¹⁹. Therefore, cancer cells would be expected to be more sensitive to additional ROS than untransformed cells due to the fact that they subsist nearer to the lethal threshold. This idea has been substantiated in a study comparing malignant T24 cells with the immortalized, non-transformed urothelial cell line UROtsa²⁰. In addition, combination of doxorubicin with hydrogen peroxide had a greater than additive effect on reduced viability of MBT-2 bladder cancer cells²¹. The hypothesis that hydrogen peroxide enhanced cytotoxicity by increasing lipid peroxidation was investigated but not substantiated²¹. In the current study we demonstrate that chemotherapeutic agents known to exert their anti-neoplastic effects at least in part through ROS generation can also synergize with TRAIL, if the pro-apoptotic machinery is intact. We provide evidence that the effect of chemotherapeutic agents can be recapitulated by hydrogen peroxide as a source of free radicals and that the mechanism by which ROS affect the apoptotic threshold of cells involves phosphorylation (inactivation) of EF2. EF2 is emerging as a novel mediator of cell death. In yeast a search for suppressors of Vpr-induced apoptosis led to EF2²². Furthermore, taxol a widely used anti-tubulin drug was found to mediate translational inhibition via phosphorylation and inhibition of EF2²³. Transient inhibition of EF2 is expected to result in reduced expression of short half-life proteins, such as cFLIP, XIAP, and survivin thereby increasing susceptibility to death ligands like TRAIL whereas prolonged inhibition would by itself interfere with cell survival.

To our knowledge, this is the first study to establish a connection between oxidative stress and increased TRAIL sensitivity in bladder cancer cells. TRAIL is of particular relevance to bladder cancer, since the anti-neoplastic effects of intravesical BCG therapy, the current first line therapy for superficial lesions, may in part be directly mediated by release of TRAIL from neutrophils². Our results suggest that intravesical treatment with hydrogen peroxide may increase the efficacy of BCG therapy by inhibiting EF2, which leads to a decrease in the expression of anti-apoptotic proteins, thereby enhancing the sensitivity of tumor cells to the BCG-induced TRAIL release. Since BCG therapy leads to severe side effects in 5% of patients²⁴, exploration of intravesical treatment with hydrogen peroxide followed by recombinant TRAIL is also warranted. Future experiments will be needed to ascertain the safety as well as the most effective doses and timing for treatment regimens combining either BCG or TRAIL with hydrogen peroxide intravesically in vivo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Effect of chemotherapy and TRAIL on cytotoxicity and caspase-3/7 activity in bladder cancer cell lines

J82 (A, C) and T24 cells (B, D) were incubated with doxorubicin (A, B) or cisplatin (C, D) in the absence or presence of soluble TRAIL and cytotoxicity measured at 24 hours. (E) Cells were incubated with 1 μ g/ml doxorubicin or 6.6 μ g/ml cisplatin in the absence and presence of 10 ng/ml soluble TRAIL. Data shown are representative experiments performed in triplicate. Similar results were obtained at least three times.

Figure 2. The effect of chemotherapeutic agents on expression of c-FLIP and caspase-8 cleavage
Cells were treated with chemotherapeutic agents for 24 hours in the absence or presence of TRAIL. Protein lysates (50 ug/lane) were analyzed for c-FLIP (A) or caspase-8 (B, C) by western blotting. In panels B and C, the p54/55 band represents the zymogen (pre-pro) form of caspase-8. Detection of the p20 fragment is indicative of caspase-8 activation. Blots were reprobbed with actin as a loading control.

Figure 3. The effect of cFLIP modulation on cell death

(A) T24 cells were stably transfected with plasmids expressing GFP, FLIP_L-GFP or FLIP_S-GFP as described in the Materials and Methods. Transfectants, pooled from 10 individual clones to rule out clonal variability were incubated with 10 ng/ml sTRAIL in absence or presence of 1 µg/ml doxorubicin or 6.6 µg/ml cisplatin. Caspase activation was measured 20 hours after initiation of treatment. Similar results were obtained in an independent assay or when clones were tested individually. (B) cFLIP_S was downregulated by transient transfection of an siRNA targeted against exon 7 and downregulation verified by western blotting (insert). Untransfected T24 cells (none) or cells transfected with scrambled (scr) or cFLIP_S siRNA for 16 hours were incubated in media in the absence or presence of 10 ng/ml TRAIL. After 24

hours, cytotoxicity was assessed by LDH assay. The positive control indicates maximum LDH release that could have been achieved.

Figure 4. Hydrogen peroxide reverses TRAIL resistance in T24 cells

Cells were treated with 10 ng/ml TRAIL, 25 μ M hydrogen peroxide or a combination of both and nuclear condensation (A) or caspase-3/7 activity (B) assessed. Data shown are the mean $\pm$ SD from at three independent experiments.

Figure 5. Western blot analysis of protein expression following hydrogen peroxide treatment
T24 cells were treated with increasing concentrations of hydrogen peroxide for 4 hours. The status of phosphorylated and total EF2 expression (A) as well as caspases and anti-apoptotic proteins (B) was determined. A representative experiment is shown. Similar results have been obtained in other experiments.

Figure 6. Hydrogen peroxide enhances TRAIL-mediated apoptosis in 5637 cells
Cells were treated with 10 ng/ml TRAIL, 25 μ M hydrogen peroxide or a combination of both and nuclear condensation (A) or caspase-3/7 activity (B) assessed. Data shown are the mean $\pm$ SD from at two independent experiments.