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Cancer by Overexpression of XIAP

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Introduction

The failure to eradicate advanced prostate cancer that is resistant to conventional therapies, such as chemo and hormonal therapies, has led to the exploration of novel therapeutic applications such as immunotherapy. One form of immunotherapy is to generate anti-tumor cytotoxic lymphocytes that can recognize and eradicate resistant tumor cells. Cytotoxic lymphocytes mediate their killing by various mechanisms including the perforin granzyme pathway and by members of the TNF- γ superfamily. Among the TNF- γ superfamily, TRAIL has been shown to be selectively cytotoxic to cancer cells and poorly cytotoxic to normal cells and is, therefore, considered as a good candidate for prostate cancer therapy. However, the development of drug/hormonal resistant prostate cancer results in the development of tumor cells resistant to immune killer cells and, indeed, prostate cancer cell lines have been shown to be relatively resistant to TRAIL-induced apoptosis. Resistance is under the regulation of apoptotic regulatory gene products in the cancer cells. We have demonstrated that prostate cancer cells resistant to TRAIL are due in large part to the overexpression of the anti-apoptotic gene product, XIAP. Downregulation of XIAP expression or inhibition of its activity reverses the resistant tumor cells and becomes sensitive to TRAIL-induced apoptosis. The objective of this research proposal is to delineate the role of XIAP in the resistance of CaP cells to TRAIL-induced apoptosis and investigates the underlying mechanisms of the regulation of XIAP expression in prostate cancer cells. The following specific aims were proposed for investigation: 1) The role of XIAP in protecting prostate cancer cells from TRAIL-mediated apoptosis 2) The regulation of XIAP by NF- κ B and NF- κ B regulation by XIAP and 3) The role of endogenous TNF- γ and IL-6 in the regulation of XIAP and resistance of tumor cells to TRAIL-induced apoptosis. This progress report describes the findings of the year 2003-2004.

Body

This research proposal investigated the following tasks: Task 1- The role of XIAP in protecting CaP cells from TRAIL-mediated apoptosis. Task 2- The role of constitutively activated NF- κ B (survival factor) in the regulation of both resistance to TRAIL and to XIAP expression. Task 3- The role of constitutive and exogenous TNF- α and IL-6 in the regulation of NF- κ B, XIAP expression and sensitivity to TRAIL. We have made significant progress in addressing the remaining questions in these tasks and we have also made several novel findings that emanated from the studies. Overall, manuscripts have been completed for submission for publication and several abstracts have been presented in 2004 at the AACR Annual Meeting and additional ones are to be presented in the 2005 at the AACR and ASCO meetings.

Findings on Task 1- The role of XIAP in protecting CaP cells from TRAIL-mediated apoptosis

In Task 1c we have proposed to examine the expression of XIAP and Smac/DIABLO in freshly derived normal, benign, and prostate tumor cells at different stages and grades and correlation with prognosis. We have completed the staining for XIAP expression in CaP tissue microarrays. The studies suggest that XIAP is overexpressed and its expression is elevated as a function of disease progression. An abstract has been submitted for consideration at the AACR meeting in 2005 (Hongo *et al.*, 2005, Appendix 1). The present findings corroborate studies reported by Krajewska *et al.*, (2003) who reported the increased levels of IAP2 in malignant prostate cancer cells and that were associated with shorter relapse free survival. We have examined the expression of Smac/DIABLO in tumor cell lines by immunohistochemistry (IHC). We have established the optimal conditions and specificity of the antibody against Smac/DIABLO and demonstrate successfully that the antibody is applicable for IHC in addition to its usage for Western blot in our publications. Studies of Smac/DIABLO expression in CaP tissue microarrays are in progress.

We have made an original observation showing that in addition to the regulation of XIAP expression by NF- κ B and the regulation by XIAP of TRAIL-resistance, another gene product regulated by NF- κ B, namely Yin Yang 1 (YY1), has been shown to negatively regulate DR5 expression and regulates TRAIL resistance (See more detail below in Task 2). Accordingly, we have also analyzed the expression of YY1 in CaP tissue microarrays and established its overexpression and prognostic significance (Seligson *et al.*, to be submitted, Appendix 2).

Findings on Task 2- The role of constitutively activated NF- κ B (survival factor) in the regulation of both resistance to TRAIL and to XIAP expression.

We and others have also demonstrated that prostate cancer cell lines exhibit constitutively active nuclear factor kappa B (NF- κ B) (Suh *et al.*, 2002; Huerta-Yeppez *et al.*, 2004, Appendix 3). NF- κ B regulates the transcription of many anti-apoptotic gene products, including XIAP and Bcl- κ L. We examined the role and mechanism of NF- κ B-induced resistance to TRAIL apoptosis. We used the nitric oxide donor DETANONOate and the NF- κ B inhibitor Bay 11-7085 to inhibit NF- κ B activity, and treated PC-3 cells resulted in downstream inhibition of both XIAP and Bcl- κ L expression. The inhibition of NF- κ B resulted in sensitization to TRAIL apoptosis. Further, the role of Bcl- κ L in the regulation of TRAIL resistance was corroborated by the use of the chemical inhibitor 2-methoxyantimycin A which sensitized PC-3 cells to TRAIL-induced apoptosis. We further examined the apoptotic-signaling pathways following treatment of PC-3 cells with the combination of NF- κ B inhibitors and TRAIL, and demonstrate that the combination, but not single agents alone, activate the mitochondrial pathway and the activation of caspases 9 and 3 and the induction of apoptosis. The above findings have been recently reported (Huerta-Yeppez *et al.*, 2004; Appendix 3).

We examined the potential mechanism by which NF- κ B activation, aside from the expression of XIAP and Bcl- κ L, regulates PC-3 cells' resistance to TRAIL. We have found that inhibitors of NF- κ B, which induced sensitization of PC-3 to TRAIL induced apoptosis, correlated with the upregulation of the TRAIL receptor DR5. The upregulation of DR5 was determined by flow, RT-PCR and western. We then examined the upregulation of DR5 expression using a luciferase reporter system that we have obtained from our collaborator Dr. Sakai in Kyoto, Japan (Yoshida *et al.*, 2001). We analyzed transfectants and demonstrate that NF- κ B inhibitors such as DHMEQ (Ariga *et al.*, 2002) significantly augmented luciferase activity and confirmed the above findings. The mechanism by which NF- κ B negatively regulates DR5 expression was examined. We hypothesized that a gene regulated by NF- κ B with DR5 repressor activity may be responsible for the

upregulation. Based on studies that we have previously reported on the negative regulation of Fas by NF- κ B and more directly by the transcriptional NF- κ B regulated transcription repressor YY1 (Garban and Bonavida, 2001), we examined the role of YY1 in the regulation of DR5. The inhibition of YY1 (e.g. by NO, NF- κ B inhibition and YY1 siRNA) resulted in upregulation of DR5 expression and sensitization to TRAIL apoptosis. The direct role of YY1 in the negative regulation of DR5 was demonstrated by using a DR5 reporter system in which constructs with deletion of the promoter region containing the putative YY1 binding region or a construct with a mutation of YY1 binding sites, following transfection of PC-3 cells, resulted in significant augmentation of basal level of luciferase activity. In addition, transfection with YY1 siRNA resulted in upregulation of DR5 and sensitization to TRAIL apoptosis (Huerta-Yepez *et al.*, 2005, Appendix 4; Neshat *et al.*, 2005, Appendix 5). These findings demonstrated that YY1 negatively regulates DR5 expression and regulates resistance.

We have demonstrated that the NO donor, DETANONOate, inhibited YY1 DNA-binding activity and function and resulted in upregulation of DR5 expression and sensitivity to TRAIL-induced apoptosis. The mechanism by which NO inhibits YY1 was examined. We have found that NO chemically modifies YY1 by S-nitrosylation and results in inhibition of its DNA-binding activity (Hongo *et al.*, 2004, Appendix 6; Hongo *et al.*, to be submitted, Appendix 7).

Findings on Task 3- The role of constitutive and exogenous TNF- α and IL-6 in the regulation of NF- κ B, XIAP expression and sensitivity to TRAIL

We have previously reported that PC-3 cells synthesize and secrete TNF- α , and TNF- α is a resistant factor (Borsellino *et al.*, 1995). Since TNF- α is a major inducer of NF- κ B activity, we hypothesized that tumor-derived TNF- α may activate NF- κ B in PC-3 cells via an autocrine/ paracrine loop. The activation of NF- κ B by TNF- α will result in the activation of the anti-apoptotic gene products regulated by NF- κ B, such as YY1 Bcl-xL and XIAP. The expression of these gene products will then maintain the resistance of the tumor cells to TRAIL-induced apoptosis. This hypothesis was tested experimentally and validated. We demonstrate that treatment of PC-3 cells with exogenous TNF- α activates NF- κ B in PC-3 cells and augments resistance to TRAIL. The role of endogenous TNF- α in the regulation of NF- κ B activity was demonstrated in studies in which the PC-3 tumor cells were treated with recombinant soluble TNFR1 (sTNFR1) in order to neutralize the secreted TNF- α and inhibits its signaling and activation of NF- κ B. The findings demonstrate that such treatment resulted in significant inhibition of NF- κ B activity and downstream gene products and the cells were sensitized to TRAIL-induced apoptosis. These findings suggested that TNF- α secreted by PC-3 cells play a major role in the constitutive activation of NF- κ B and resistance to TRAIL via expression of XIAP and YY1 (Huerta-Yepez *et al.*, to be submitted, Appendix 8).

We have also found that NF- κ B regulation of XIAP and Bcl-xL expression regulates chemotherapy-induced apoptosis. We have found that prostate cancer tumor cell lines (PC-3, CL-1, LNCaP) are resistant to CDDP-mediated apoptosis. We examined whether the resistance is due in part to the constitutive activation of NF- κ B and downstream regulation of XIAP and Bcl-xL expression similar to the resistance observed against TRAIL. We also hypothesized that tumor-derived cytokines (e.g. TNF- α) that regulates the constitutive activity of NF- κ B and downstream anti-apoptotic gene products like XIAP and Bcl-xL will result in the regulation of tumor cell resistance to CDDP. Hence, interfering with this pathway should sensitize the cells to CDDP apoptosis. This hypothesis was tested and verified experimentally. We have found that inhibition of endogenous TNF- α by recombinant sTNFR1 sensitizes PC-3 cells to CDDP-induced apoptosis. Further, inhibition of NF- κ B by Bay 11-7085 mimicked the neutralization of TNF- α and sensitized the cells to CDDP apoptosis. The direct role of XIAP in the inhibition of CDDP-induced apoptosis was examined by the use of actinomycin D which we have earlier reported selectively inhibits XIAP expression (Ng and Bonavida, 2002). Treatment of PC-3 with Act D resulted in sensitization of PC-3 cells to CDDP-induced apoptosis. These findings demonstrate that NF- κ B and gene products XIAP and Bcl-xL regulate the resistance of PC-3 cells to CDDP-induced apoptosis (Huerta-Yepez *et al.*, 2004, Appendix 9).

Key Research Accomplishments

1. XIAP overexpression in prostate cancer cells is in part regulated by constitutive NF- κ B activity.
2. We have demonstrated that prostate cancer cell lines express constitutively, activated NF- κ B. NF- κ B regulates the transcription of XIAP and Bcl-x_L and inhibition of NF- κ B downregulates their expression and sensitizes the cells to TRAIL-induced apoptosis.
3. XIAP overexpression in human CaP tissue microarrays increases as disease progresses and was found to be a prognostic factor.
4. We have demonstrated that NF- κ B negatively regulates the expression of the TRAIL receptor, DR5. Inhibition of NF- κ B by chemical inhibitors or by nitric oxide donor (which S-nitrosylates p50) resulted in the upregulation of DR5 expression and sensitization to TRAIL-induced apoptosis. These findings were corroborated in PC-3 cells transfected with the DR5 promoter and treatment with NF- κ B inhibitors or by nitric oxide resulted in significant increase in the luciferase activity.
5. We have found that negative regulation of DR5 by NF- κ B is mediated by the transcription repressor YY1. The DR5 promoter has a YY1 consensus binding site which inhibits DR5 transcription. Inhibition of YY1 upregulates DR5 expression and sensitizes cells to TRAIL-apoptosis.
6. YY1 overexpression in human CaP tissue microarrays and was augmented as disease progresses and was found to be a prognostic factor.
7. We have demonstrated that PC-3 cells secrete TNF- α which acts by autocrine/paracrine fashion as an activator of NF- κ B and XIAP. Inhibition of TNF- α by anti-TNF- α antibody or by soluble TNFR-1 downregulates NF- κ B activity and the expression of YY1 XIAP and Bcl-x_L and sensitizes the cells to TRAIL-induced apoptosis.
8. We have found that NF- κ B also regulates Bcl-x_L expression in prostate cancer cells and both XIAP and Bcl-x_L overexpression render the cells resistant to chemotherapeutic drugs-induced apoptosis. This finding suggests that there is cross-resistance between immune-mediated and drug-induced apoptosis.

Reportable Outcomes

Published Paper(s)

S. Huerta-Yepez *et al.*, *Oncogene* **23**, 4993 (2004).

Manuscripts in Preparation

F. Hongo, S. Huerta-Yepez, M. Vega, H. Garban, A. Jazirehi, Y. Mizutani, T. Miki, B. Bonavida. Nitrosylation of the transcription repressor Yin-Yang 1 (YY1) mediates upregulation of Fas expression in cancer cells: nitric oxide (NO)-induced sensitization to Fas-mediated apoptosis. (To be submitted)

S. Huerta-Yepez, M. Vega, S.E. Escoto-Chavez, B. Murdock, T. Sakai, B. Bonavida. The transcription repressor YY1 negatively regulates DR5 expression and controls cancer cells resistance to TRAIL-induced apoptosis: Reversal of resistance by inhibitors of YY1. (To be submitted)

S. Huerta-Yepez, M. Vega, H. Garban, B. Bonavida. TNF- α controls the expression and activity of the transcriptional repressor Yin-Yang 1 (YY1) via NF- κ B. (Under revision).

D. Seligson, S. Huerta-Yepez, S. Horvath, S. Hanna, T. Shi, H. Garban, D. Chia, L. Goodglick, B. Bonavida. Nuclear expression of transcription factor YY1 in prostate cancer. (To be submitted)

Abstracts Presented in 2004

F. Hongo *et al.*, *Proceedings of the AACR* **45**, Abstract #4356 (2004).

S. Huerta-Yepez *et al.*, *Proceedings of the AACR* **45**, Abstract #4826 (2004).

Abstracts to be Presented in 2005

F. Hongo, S. Huerta-Yepez, H. Yu, L. Goodglick, D. Chia, S. Horvath, Y. Mizutani, T. Miki, D. Chatterjee, D. Seligson, B. Bonavida. Overexpression of inhibitor of apoptosis protein XIAP in human prostate cancer. Control/Tracking No: 05-AB-9166-AACR (2005).

M.S. Neshat, S. Huerta-Yepez, S. Baritaki, S.E. Escoto-Chavez, B. Murdock, K. Umezawa, T. Sakai, K.C. Yeung, D. Chatterjee, B. Bonavida. Inhibition of constitutive NF- κ B or YY1 activity sensitizes prostate cancer cells to TRAIL-induced apoptosis via upregulation of DR5 expression. Control/Tracking No: 05-AB-20368-ASCOPCS (2005).

Conclusions

The studies have resulted in significant findings of clinical relevance. The *in vitro* laboratory findings indicated that CaP cell lines that are drug resistant are also resistant to cytotoxic immunotherapy. The cross-resistance is in large part due to the development of anti-apoptotic mechanisms that prevent cell death from apoptotic-induced stimuli (e.g. chemotherapeutic drug, toxins, cytotoxic immunotherapy, etc.). Since immunotherapy is being actively considered in the treatment of drug hormone-resistant CaP, mechanisms underlying resistance to immunotherapy must be examined. This study investigated the role of the anti-apoptotic gene product XIAP, a member of the anti-apoptotic gene product IAPs family, whose overexpression inhibits the mitochondrial pathway of apoptosis by inhibiting the activation of caspase 9 and consequently the activation downstream of effector caspases leading to apoptosis. The role of XIAP in resistance was corroborated in studies in which its activity was inhibited by inhibitors of the constitutively activated NF- κ B, which regulates XIAP transcription. The constitutive NF- κ B activation in certain CaP cell lines was found to be mediated in large part by tumor-derived cytokines, like TNF- α which activate NF- κ B by an autocrine-paracrine loop. New findings also demonstrated that resistance to TRAIL is also regulated at the level of the TRAIL death receptor DR5 via the transcription repressor YY1. The DR5 promoter was shown to contain one consensus binding site for YY1 and its binding inhibits DR5 transcription and regulates TRAIL resistance. The inhibition of YY1 by chemicals (e.g. NO) or by inhibitors of NF- κ B resulted in the upregulation of DR5 expression and sensitization to TRAIL apoptosis. The findings indicate that constitutive NF- κ B activity negatively regulates TRAIL sensitivity by at least 2 non-mutually exclusive mechanisms, namely via the upregulation of XIAP expression and by upregulation of YY1. Thus interference with the TNF- α / NF- κ B/ XIAP/ YY1 pathways by various inhibitors reverses the resistance of CaP to TRAIL-induced apoptosis. The identification of such regulatory targets offer opportunities for the development of new therapeutic agents for intervention which should result in the sensitization of CaP cells to TRAIL-induced apoptosis. While the studies above were primarily done with established CaP cell lines, there was validation with clinical specimens using human CaP tissue microarrays and which were examined by immunohistochemistry. It was found that CaP overexpressed both XIAP and YY1 and their expression increases as a function of disease progression and was shown to be prognostic markers.

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 5. Neshat MS, Huerta-Yepez S, Baritaki S, Escoto-Chavez SE, Murdock B, Umezawa K, Sakai T, Yeung KC, Chatterjee D, Bonavida B. Inhibition of constitutive NF- κ B or YY1 activity sensitizes prostate cancer cells to TRAIL-induced apoptosis via upregulation of DR5 expression. Control/Tracking No: 05-AB-20368-ASCOPCS (2005).
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APPENDIX 1

Control/Tracking No: 05-AB-9166-AACR

Overexpression of inhibitor of apoptosis protein XIAP in human prostate cancer.

Short Title: XIAP expression in prostate cancer

Fumiya Hongo, Sara Huerta-Yepez, Hong Yu, Lee Goodglick, David Chia, Steve Horvath, Yoichi Mizutani, Tsuneharu Miki, Devasis Chatterjee, David Seligson and Benjamin Bonavida

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The X-linked inhibitor of apoptosis protein XIAP is a member of a family of proteins that suppress cell death by apoptosis. We have reported that several prostate cancer (CaP) cell lines are resistant to TRAIL-induced apoptosis and several chemotherapeutic drugs, like actinomycin D (Act D), sensitized the CaP tumor cells to TRAIL-induced apoptosis. Sensitization by Act D was shown to selectively inhibit the expression of XIAP, which was overexpressed in the CaP cell lines (Zisman *et al.*, *J Immunother.* 24: 459, 2002; Ng *et al.*, *Prostate.* 53: 286, 2003). Immunotherapy by TRAIL or agonist antibodies to DR4 or DR5 are currently being considered clinically in the treatment of various drug-resistant tumor cells, including CaP. However, CaP may not respond to such immunotherapy due to tumor cell resistance to TRAIL-induced apoptosis. Therefore, it may be useful to identify cancer patients who may benefit from such immunotherapeutic strategies. We hypothesized that the level of expression of XIAP in cancer cells may be a predictable marker for clinical response to TRAIL-mediated immunotherapy. In this study, we examined the expression of XIAP by immunohistochemistry in human prostate cancer using prostate cancer tissue microarrays (TMA). Formalin-fixed paraffin embedded tissue was collected from primary radical prostatectomy specimens performed between the years 1984 and 1995. At least three replicate tumor samples and one benign tissue sample was taken from each surgical case. A total of 246 prostatectomy specimens were assayed, resulting in a total of 1,102 non-metastatic informative tissue spots for analysis. These include 680 prostate cancer samples, 48 PIN, 121 BPH, and 253 morphologically normal samples. Staining for XIAP was done with anti-XIAP antibody (R&D System, Minneapolis, MN). The major Gleason grade was determined for each tissue spot. Statistical analysis of the data indicated that there was a significant increase of XIAP expression in CaP with an early elevation seen in PIN. Noteworthy, XIAP expression in BPH was lower than normal. The difference in XIAP expression between BPH and NL, NL and PIN, and PIN and CaP was statistically significant. These data are consistent with findings in a recent report (Krajewska *et al.*, *Clin. Can. Res.* 9: 4914, 2003). Based on these results, we are currently assessing the hypothesis that overexpression of XIAP may be one predictor of patients responsiveness to TRAIL-mediated immunotherapy. *This study was supported in part by DOD/US ARMY DAMD 17-02-1-0023 (BB), the Jonsson Comprehensive Cancer Center Shared Resource Core Grant (JCCC) at UCLA NIH NCI 2 P30 CA16042-29 (DS), and the Early Detection Research Network (LG, DC) NCI CA-86366.*

APPENDIX 2

Expression of Transcription Factor Ying-Yang 1 in Prostate Cancer

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Running Title: YY1 in prostate cancer

Keywords: YY1, Yin Yang 1; Tissue Microarray, prostate cancer, diagnostic marker, tumor marker, prognostic indicator.

Abstract

The transcription repressor Yin Yang 1 (YY1) is expressed in several human cancer cell lines and its expression correlates with resistance to immune-mediated apoptosis. This study used tissue microarrays to investigate the expression and localization of YY1 in 246 hormone naïve prostate cancer patients who underwent radical prostatectomy. Tissue microarrays containing representative prostate tissue samples from these patients were prepared consisting of 1364 total tissue samples. Staining intensity and frequency measures for both YY1 nuclear and cytoplasmic expression were higher in neoplastic tissues and in PIN samples compared to matched benign cells ($p < 0.0001$ for all comparisons). Expression of YY1 is predominantly elevated in early malignancy (PIN), as well as in tumors of intermediate to high morphologic grade (Gleason's grade 3-5). Using multivariate Cox proportional hazards analysis, we observed that low nuclear YY1 staining is an independent predictor of a shorter time to recurrence ($p = 0.012$). Based on these results, we hypothesize that YY1 may play a role in prostate cancer development; however, decreased YY1 may give metastatic cells a survival advantage. These results may also implicate YY1 as a useful diagnostic and prognostic marker.

Introduction

Prostate cancer is the most common cancer of males in the U.S, with an age-adjusted incidence of 170 per 100,000, and 29,900 associated deaths estimated for 2004.^{1,2} Sixteen percent of patients with localized invasive prostate cancer who undergo radical prostatectomy surgery, will develop recurrence of malignancy within 5 years.³ When detected at locally advanced or metastatic stages, no consistently curative treatment regimen exists. Treatment for metastatic prostate cancer includes hormonal ablation, chemotherapy and combination therapies. Unfortunately, there is frequent relapse of an aggressive androgen-independent disease that is insensitive to further hormonal manipulation or to treatment with conventional chemotherapy.⁴ Therefore, there is a need for alternative therapies.

One such alternate approach is immunotherapy. This strategy depends on enhancing the recognition of tumor cells by components of the immune system including cytotoxic T lymphocytes (CTL) and natural killer (NK) cells.⁵⁻¹⁰ This strategy also predicts that tumors which have become resistant to chemotherapeutic drugs, may still be targets for NK or CTL-mediated killing. However, this approach has only been modestly successful in part due to acquired cross-resistance by tumor cells to immune-mediated surveillance thus ultimately leading to tumor progression and metastasis of the resistant cells.¹¹ The mechanism(s) responsible

for the anti-apoptotic phenotype, if identified, may be both a useful prognostic indicator as well as an important therapeutic target.

Our recent findings reveal a novel mechanism of tumor cell resistance to immune-mediated cytotoxicity. We show that resistance to Fas-mediated apoptosis of ovarian and prostate cancer cells is in large part due to the transcription repressor Ying Yang 1 (YY1) that inhibits Fas expression. The inhibition of YY1 up-regulates Fas expression and the cells become sensitive to Fas-mediated apoptosis.¹²

YY1 is a multifunctional DNA binding protein, which can activate, repress, or initiate transcription depending on the context in which it binds.^{13,14} In addition, YY1 can modulate protein levels or activity through protein-protein interaction.¹⁵ Through DNA binding and/or protein interaction YY1 has been identified as a potential repressor factor for gene which include human interferon- γ ,^{16,17} IL-3,¹⁸ GM-CSF,^{16,19} and p53.¹⁵ Significantly, we have identified a relevant putative repressor cluster at the silencer region of the human Fas promoter that matched the consensus sequence that binds the transcription factor YY1.¹²

To start to address the role of YY1 in regulating the sensitivity of prostate cancer cells to apoptosis, we have initiated a study to characterize the expression level and location of this factor in normal and malignant prostate cancer cells. Preliminary findings has demonstrated a relatively high level of YY1 in the human prostate cell line PC3, and in a limited studies, increased expression of YY1 in malignant compared to nonmalignant human prostate tissue. Here we have constructed and utilized a high density prostate tissue array to more fully characterize the level and subcellular localization (i.e., cytoplasmic versus nuclear) of YY1 during different stages of malignant progression. Notably, our results strongly indicate that YY1 expression potentially has both diagnostic and prognostic value for prostate cancer.

Materials and Methods

Western Blot Analysis

PC-3 cells were obtained from the ATCC (Manassas, VA) and cultured as a monolayer in RPMI 1640 (Life Technologies, Bethesda, MD) supplemented with 5% heat-inactivated fetal bovine serum (Life Technologies). Cells were lysed at 40 °C in RIPA buffer (50mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150mM NaCl), and supplemented with one tablet of protease inhibitor cocktail, Complete Mini Roche (Indianapolis, IN). Protein concentration was determined using a DC protein assay kit (Bio-Rad, Hercules, CA). An aliquot of total protein lysate was diluted in an equal volume of 2X SDS sample buffer (6.2 mM Tris (pH 6.8), 2.3% SDS, 5% mercaptoethanol, 10% glycerol, and 0.02% bromophenol blue) and boiled for 10 minutes. The cell lysates (40 μ g) were then electrophoresed on 12% SDS-PAGE gels (Bio-Rad) and were subjected to Western blot analysis as previously reported.²⁰ The mouse anti-YY1 antibody was purchased from Geneka Biotechnology (Montreal, Quebec), and the mouse monoclonal anti- β -actin was purchased from Chemicon (Temecula, CA). Levels of β -actin were used to normalize the YY1 expression. Relative concentrations were assessed by densitometric analysis of digitized autographic images using public domain NIH Image J Program (<http://rsb.info.nih/ij/>).

Prostate Tissue Microarray (TMA)

The prostate tissue microarray (TMA) was constructed using formalin-fixed, paraffin-embedded prostate tissue samples provided through the Department of Pathology and Laboratory Medicine at the UCLA Medical Center under IRB approval. Primary radical prostatectomy cases from 1984-1995 were randomly selected from the pathology database. The original H&E stained slides were reviewed by a pathologists (D.S.) utilizing the Gleason histological grading²¹ and the 1997 AJCC/UICC TNM classification systems.²² Case material from 246 prostatectomies was arrayed into 3 blocks encompassing a total of 1,364 individual tissue cores. All cases were of the histological type adenocarcinoma, conventional, not otherwise specified.²³

TMAs were constructed as previously described.²⁴ At least 3 replicate tumor samples were taken from donor tissue blocks in a highly representative fashion. Tumor samples were accompanied by matching benign (morphologically normal or hypertrophic) and in situ neoplastic lesions (PIN), when available. Table 1 shows the clinicopathologic data for the 190 patients included in the outcomes analysis. The median age at the time of surgery was 65 (range 46 to 76). 112 (59%) patients were low grade (Gleason score 2-6); 78 (41%) were high grade (Gleason score 7-10). Approximately half of the tumors, (51%) were confined to the prostate (organ

confined here = T2a or T2b with negative lymph nodes, no capsular extension and with negative surgical margins). 128 (67%) patients were margin negative, 62 (33%) margin positive, 32 (17%) had seminal vesicle invasion (pT3b). Regarding capsular invasion, 44 (23%) had no invasion, 107 (56%) had invasion, and 39 (21%) had capsular extension. Concurrent regional lymphadenectomy accompanied 187 (98%) cases, only 9 of which (5%) were positive for metastases. The maximum pre-operative serum PSA was known for 169 patients (89%), with a median value of 8.9 ng/ml, (range 0.6-76.0).

A retrospective analysis for outcome assessment was based on detailed anonymized clinicopathologic information linked to the TMA tissue specimens. Recurrence, defined as a postoperative serum PSA of 0.2 ng/ml or greater, was seen in 65 (34%) patients. Total follow-up, defined as the time to recurrence or to last contact in non-recurring patients, had a median of 53.5 months (range 0.1-163). The median follow-up time within the recurring and non-recurring groups was 21 (1.0-115) and 66 months (range 0.1-163), respectively.

Each case was represented by an average of 3.2 informative tumor spots. Tissue spots from all 246 cases were included in the histological distribution analysis of YY1; 79% of these spots were informative (i.e., contained benign and/or malignant epithelial cells). Patients that were treated preoperatively with neoadjuvant hormones were excluded from the clinical analysis (n=20). An additional 23 cases were not evaluated predominantly due to a lack of target tumor tissue. For thirteen cases, we had no associated outcomes data. Therefore, of 246 total cases, 190 (77%) were available for outcomes studies.

Immunohistochemistry

A standard 2-step indirect avidin-biotin complex (ABC) method was used (Vector Laboratories, Burlingame, CA). Tissue array sections (4 μ m-thick) were cut immediately prior to staining using the TMA sectioning aid (Instrumedics, NJ). Following deparaffinization in xylenes, the sections were rehydrated in graded alcohols and endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol at room temperature. The sections were placed in 95°C solution of 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval, and then blocked with 5% normal goat serum for 30 minutes. Endogenous biotin was blocked with sequential application of avidin D then biotin (A/B blocking system, Vector Laboratories, Burlingame, CA). Primary rabbit anti-human YY1 polyclonal IgG1 antibody (Geneka Biotechnology, Inc., Montreal, Quebec, Canada) was applied at a 1:1000 dilution (0.2 μ g/ml) for 60 minutes at room temperature. After washing, biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) was applied for 30 minutes at room temperature. The ABC complex was applied for 25 minutes followed by the chromogen diaminobenzidine (DAB). PBS (10 mM, pH 7.4) was used for all wash steps and dilutions. Incubations were performed in a humidity chamber. The sections were counterstained with Harris' Hematoxylin, followed by dehydration and mounting.

Antibody specificity was tested by concentration-dependent inhibition of staining using the immunizing YY1 peptide (Geneka Biotechnology, Inc.). Anti-YY1 antibody was preincubated for 3 hours at room temperature with a 0X, 5X, or 10X molar excess of peptide. The antibody in the presence or absence of the peptide was then added to a mini-prostate array (16 spots) and stained as described above.

Scoring of Immunohistochemistry

Semi-quantitative assessment of antibody staining on the TMAs was performed by a study pathologist (A.R.) blinded to the clinicopathologic variables. Random spots were double scored for quality control purposes by one of the study pathologists (D.S.). The target tissue for scoring was the prostatic glandular epithelium, scoring of benign tissues did not include basal cells. Tissue spot histology and grading was confirmed on Hematoxylin and Eosin (H&E) stained TMA slides, as well as on the counterstained study slides. The staining intensities of the nuclear and cytoplasmic cellular compartments were scored separately, each on a 0-3 scale (0 = negative; 1 = weak; 2 = moderate; 3 = strong staining) as previously described.²⁵ In addition, the frequency of positive target cells (range 0-100%) at each intensity level was also scored for each TMA spot.

For outcome analyses, we considered 190 tumor cases for which we had recurrence data. The following analysis of data gave the most significant results: i) First, the percentage of tumor cells staining (i.e., frequency) was quantified for each tissue spot. ii) Second, we focused on those tissue spots within each case that represented the lowest region of YY1 expression. We examined all 190 cases in this fashion (i.e., as a continuous variable) as well as dichotomized population (i.e., dichotomized variable). For the dichotomized

data, we divided cases based on spots in which = 50% of the cells expressed nuclear YY1 (referred to as "YY1-low" cases; n=42) or in which > 50% of cells expressed nuclear YY1 (referred to as "YY1-high" cases; n=148).

Statistical Analysis

Associations between YY1 expression groups and clinicopathologic variables were tested using the Pearson chi-square and Mann-Whitney tests. We used the Pearson correlations and corresponding p-values to study the relationship between nuclear and cytoplasmic staining intensities and frequencies on a per-spot level. Recurrence was defined as a rising total PSA >0.2 ng/ml status post prostatectomy, and time to recurrence was calculated from the date of the primary surgery. Patients without recurrence at last follow-up were censored. Kaplan-Meier plots were used to visualize recurrence-free time distributions and the log rank test was used to test for differences between them. To assess which covariates associate with recurrence-free time, we fit both univariate and multivariate Cox proportional hazards models. For each covariate, we list the 2 sided p-value, the hazard ratio and its 95% confidence interval. A p-value smaller than 0.05 was accepted as significant. The proportional hazards assumption was checked through the use of Schoenfeld residuals. All analyses were conducted with the freely available software package R (<http://www.r-project.org>).

Results

YY1 Expression in PC3

YY1 is a transcription factor that demonstrates context-specific repression or activation activity.¹³ Recently, we have demonstrated that nitric oxide indirectly up-regulates the expression of Fas by blocking the silencing effect of YY1.¹² The apparent role of YY1 in modulating Fas expression, combined with postulated role of TNF receptor family members in tumor progression and resistance,^{11,26} prompted us to examine the expression distribution of YY1 in normal and malignant prostate tissue. To initiate this study, we first examined the expression of YY1 in an androgen-independent human prostate cancer cell line, PC3, by Western blot then immunocytochemical analyses. For Western blot analysis, cell extracts were prepared, electrophoresed, transferred, and probed as described in Materials and Methods. Abundant YY1 expression was detected in these cells as demonstrated by a prominent 68 kDa band (Figure 1). Immunocytochemical results were consistent with Western Blot data as = 95% of the cells expressed YY1, predominantly within the nucleus (Figure 2). These findings also established a positive control for subsequent immunohistochemical analyses in whole prostate tissues and tissue microarrays.

YY1 Expression in Prostate Tissue Sections

The relatively high expression in PC3 cells prompted us to embark on a study examining YY1 expression in human prostate tissue. We first examined YY1 expression by immunohistochemistry in three morphologically benign (normal and BPH) human prostate whole tissue sections. Staining was observed in the glandular epithelium, basal cells, and occasionally in stromal fibromuscular cells; Figure 3A shows a representative example. Approximately 90% of the prostatic epithelium stained positive with typically weak to moderate intensities. Staining was predominantly in the nucleus consistent with the expression pattern seen in PC3 cells (Figure 3A). Negative control sample incubated with non-immune sera had no staining (Figures 3B). In addition, preincubation of the anti-YY1 antibody with varying doses of immunogen peptide, displayed a dose-dependent inhibition of staining culminating in complete inhibition (Figures 3C).

We next examined the spectrum of YY1 expression patterns on whole tissue sections from a panel of ten human prostate carcinomas (Figure 4). Compared to the typically pronounced nuclear staining seen in non-malignant epithelium (Figure 4A), two low-grade tumors demonstrated weak or minimal YY1 staining (example in Figure 4I) while another low-grade tumor exhibited strong nuclear staining and diffuse cytoplasmic staining (Figure 4E). Two high-grade tumors were also examined; one demonstrated weak to moderate nuclear staining (Figure 4C), while the other showed relatively strong nuclear and cytoplasmic staining (Figure 4G). This complex set of staining patterns prompted us to examine a larger sample population using tissue microarray (TMA) technology.

YY1 Expression is Increased in Malignant Prostate Samples

We next evaluated the protein expression of YY1 in clinical prostate samples using a TMA platform. We examined YY1 expression across histological categories on 1061 informative primary site tissue spots (data for 12 lymph node metastases were not included).

Nuclear YY1 staining. Figures 5A and 5B show distribution graphs of nuclear YY1 staining intensity (i.e., percentage of array spots with negative-weak or moderate-strong nuclear YY1 staining) and staining frequency (i.e., percentage of array spots that showed 0-49% or 50-100% of the cells positive for nuclear YY1), respectively. We observed a significant increase in YY1 staining in tumor and PIN samples compared to nonmalignant samples (morphologically normal and BPH tissues, $p < 0.0001$; Table 2). As a group, 82% of tumor-containing and 76% of PIN-containing spots showed moderate to strong nuclear staining, whereas only 57% of normal and 34% of BPH tissue spots displayed equivalent nuclear staining (Figure 5A). Interestingly, the proportion of tumor spots displaying moderate to strong staining increased abruptly with grade = Gleason grade 3 (graph not shown). Compared to Gleason grades 3, 4 and 5, for which 84%, 87% and 79% of tissues stained at that level, only 65% of low grade tumor spots (Gleason grades 1 and 2) stained the same (grade 1-2 versus grade = 3, $p < 0.0001$).

The frequency of cells with nuclear YY1 staining followed the same trend as was seen with staining intensity; a higher proportion of tumor and PIN tissue spots stained with higher frequency (50-100% category) compared to normal and BPH (Figure 5B; $p < 0.0001$). In summary, in the neoplastic lesions, there is a concomitant increase in both the amount of nuclear YY1 expression per cell and in the proportion of cells with nuclear staining.

Cytoplasmic YY1 staining. In addition to nuclear staining, we were somewhat surprised to see tissue samples with a relatively high cytoplasmic expression of YY1 (Figure 4G). This staining appeared specific as it was dose-dependant, present in some, but not all cells, and inhibited by the immunizing peptide (data not shown). Figures 5C and 5D show the staining distribution and frequency of cytoplasmic YY1 staining in the TMA. Similar to nuclear YY1 staining, there was a higher intensity of cytoplasmic YY1-staining cells in PIN and tumor tissue spots compared to benign histologies ($p < 0.0001$; Table 2). The majority of all tissue spot histology categories showed $>50\%$ of the cells staining positively for cytoplasmic YY1 (Figure 5D).

Interestingly, there was a strong correlation between cytoplasmic and nuclear YY1 staining (i.e., the trends observed with regard to histologies or outcomes were similar for either cytoplasmic or nuclear staining). Thus, we show only one set of data, nuclear YY1 staining, below.

YY1 Expression and Cancer Recurrence

We next examined whether nuclear YY1 expression was associated with tumor recurrence following prostatectomy (as detected by a postoperative recurrence of serum PSA). Recurrence data was available for 190 patient cases from patients in the tissue array. All spots from a given case were pooled and analyzed for the percentage of invasive malignant cells expressing nuclear YY1 as described in Materials and Methods. Results were examined both as a continuous and dichotomized population. Surprisingly, the most significant results were obtained when we considered cases that contained regions with fewer positive cells (defined in Materials and Methods). Figure 6 shows the frequency distribution of these YY1 weak/negative cells as well as the dichotomized cutoff. As continuous variable, YY1 was a significant predictor of recurrence in both univariate and multivariate Cox proportional hazards models ($P=0.011$; 0.99; 95% CI 0.98-0.99 for both models; Table 3).

We further analyzed the dichotomized cases. As shown in Figure 6, cases were divided based on spots in which = 50% of the cells expressed nuclear YY1 (referred to as "YY1-low" cases) or in which $> 50\%$ of cells expressed nuclear YY1 (referred to as "YY1-high" cases). These two distinct patient groups did not associate with traditional clinicopathological covariates (Table 1). However, the YY1-high cases had a longer time to cancer recurrence than the YY1-low cases (Table 3; $P = 0.016$; hazard ratio 0.53; 95% CI 0.31-0.89, univariate). Figure 7 shows a Kaplan Meier estimate of recurrence (cancer)-free time for all 190 patients (Log Rank $P=0.014$). The median recurrence-free time was 90 months in YY1-low cases, compared to >163 months in YY1-high cases. Moreover, only 30% of the YY1-high cases had a tumor recurrence (70% were censored), compared to 50% of the YY1-low cases (50% censored cases).

The dichotomized YY1 groups proved to be independent predictors of recurrence in multivariate Cox proportional hazards model including all 190 patients ($P=0.012$, hazard ratio 0.47, 95% CI 0.27-0.85). However, the associations did not remain significant when patients were substratified by tumor grade (Table 3).

In summary, while the majority of prostate tumors examined expressed ample YY1 (Figure 6), if any part of a tumor showed a significant decrease in the percentage of cells staining, then there was an increased risk of disease recurrence.

Discussion

In this study we have examined the expression pattern of YY1 in normal and malignant prostate tissue using a prostate tissue microarray. The genesis of this investigation stemmed from results using a cell culture system which demonstrated that YY1 expression contributed to the inhibition of Fas expression and thus decreased sensitivity to Fas-mediated apoptosis.¹² Based on these initial studies, we started to examine the YY1 expression level and pattern in human normal and malignant prostatic tissue. To do this, we took advantage of tissue microarray technology, constructing an array of case material from 246 prostatectomies. This tissue microarray contains all relevant histologies / pathologies linked to outcomes data when available, thus allowing us to examine YY1 expression pattern in a relatively large cohort of patients. When we examined YY1 protein expression levels using immunohistochemistry, we observed an increase in nuclear and cytoplasmic YY1 expression in tumor and PIN samples compared to histologically normal or BPH tissues. This was the case for both staining intensity and for the percentage of cells that stained positively. Notably, this represents that first association of YY1 expression with prostate cancer progression.

YY1 Transcription Regulation.

While YY1 has been described as a context-specific positive or negative regulator of transcription, the exact mechanism of action of YY1 is currently unknown.^{13,14} Proposed models for YY1 function include context-specific activation or repression, interaction with other transcription factors or modulating proteins (e.g., transcription factor IIB (TFIIB), Sp1, c-myc, Rb, the notch receptor, YY1AP, p300, and CBP), regulation of p53 ubiquitinylation, and/or chromatin modification (e.g., histone acetylation or deacetylation).^{13-15,27-36} Relevant to processes such as inflammation, immune responses, and tumor initiation/progression, and cell cycle progression, YY1 can modulate the expression of genes such as c-myc,³⁷ c-fos,^{38,39} p53,^{15,40} human IFN- γ gene,^{16,17} Fas,¹² IL-3,¹⁹ IL-4,⁴¹ GM-CSF,^{16,19} IFN- γ ,⁴² histone,⁴³ and CCR5.⁴⁴ Whether these and/or other gene(s) are primarily influenced or modulated by YY1 in prostatic epithelial cells in vivo remains to be determined.

Of particular note is the recently described role of YY1 in regulating levels of the tumor suppressor p53 by affecting its ubiquitination by Mdm2.¹⁵ In this study, increased expression of YY1 promoted the ubiquitination and resultant steady-state reduced expression of p53. Alterations in p53 with the subsequent loss of wild type function is one of the most common events in human cancers.⁴⁵⁻⁴⁸ Although the data is still somewhat ambiguous, as much as 94% of prostate cancer cases have some alteration in p53.⁴⁹⁻⁵¹ There are reports that indicate that alterations in p53 can be both an early and/or a late event in prostate cancer development.⁵⁰⁻⁵² It is an intriguing possibility that one of the consequences of changes in YY1 expression and/or localization is disruption of p53 or p53-dependent pathways thus contributing to the malignant process.

Although in our in vitro studies YY1 was inversely associated with Fas expression, there was no obvious correlation in vivo by immunohistochemical analysis (data not shown). There are many potential explanations for this. First, it is possible that this merely represents a technical limitation of immunohistochemistry to simultaneously detect localized changes in Fas and YY1. Second, in vivo, YY1 activity as opposed to YY1 expression levels, might be the determinant of Fas expression. YY1 activity would not be accurately measured by the assays utilized in this study. Finally, potential discordances between in vitro and in vivo observations could reflect differences in cellular milieu. The in vitro system modeled IFN- γ - induced up-regulation of Fas; this up-regulation occurred due to inactivation of YY1 by NO.¹² These conditions may not exist in these clinical samples where the relative abundance and influence of IFN- γ are unknown. Nevertheless, we continue to explore the interplay between YY1, Fas, NO, and IFN- γ in this as well as other systems.

YY1 Expression pattern in Malignant Prostate Tissue.

In non-malignant prostatic epithelium, YY1 was present predominantly in the nucleus of glandular epithelium and basal cells consistent with its activity in transcription regulation. Interestingly, there was often staining in the cytoplasm of these cells as well; cytoplasmic became more pronounced in PIN and more malignant cells. For example, greater than 95% of the malignant samples examined displayed significant cytoplasmic staining (score of 2-3) (Figure 5). Recently, Palko et al. reported that YY1 transits from the cytoplasm to nucleus at various stages of the cell cycle.⁵³ Specifically, YY1 was localized primarily in the nucleus during late G1-early S phase, but primarily distributed in the cytoplasm during G1 and late S phase.⁵³ A limited number of other groups have similarly observed localization of YY1 in both the cytoplasm and nucleus in various model systems.^{54,55} In this light, the observation in our study that the majority of tumor samples contained malignant cells with relatively high levels of both nuclear and cytoplasmic staining is intriguing. The mechanism that regulates the migration of YY1 from the cytoplasm to the nucleus is unknown but may be dependent on nuclear localization signals, specific protein interactions such as observed with I κ B, and/or intracellular shuttling proteins. In regard to the later mechanism, the nuclear / nucleolar shuttle protein nucleophosmin / B23 is known to bind to YY1.^{56,57} It is interesting to consider that the high correlation of expression of YY1 in both nuclear and cytoplasmic compartments in malignant cells may contribute to dysfunction of YY1 activity. Such a mechanism awaits further definition.

Regions of Low YY1 Expression Predict a Poorer Outcome

The results that we obtained when examining the potential relationship between YY1 expression / distribution and tumor recurrence were interesting and somewhat surprising. Rather than high or more abundant levels of YY1 being a predictor for more rapid tumor recurrence, tumors that were heterogeneous and displayed regions of minimal YY1 staining correlated with poorer outcome (Figure 7). Tumor recurrence is defined as an increase in PSA levels following a prostatectomy thus indicating the presence of metastatic tumor cells. Although formal proof is required, these data raise the possibility that decreased YY1 expression may enhance the survival of metastatic prostate cancer cells. Although there are numerous potential reasons for why decreased YY1 expression could provide a survival advantage for metastatic cells, it is conceivable that expression or repression of a new repertoire of genes would be required to survive in a new milieu. Such a mechanism requires further examination.

Our results are similar to those observed by Hofer et al., for the expression of metastasis-associated gene 1 (MTA1) in prostate cancer.⁵⁸ MTA1 was originally identified from differential screening of the rat mammary adenocarcinoma non-metastatic cell line, MTC.4, versus the metastatic cell line, MTLn3; MTA1 was overexpressed in the latter.^{59,60} Subsequently, MTA1 was found to be overexpressed in invasive lesions of various human cancers.^{58,61-65} Hofer et al., observed that MTA1 was expressed at highest levels in metastatic prostate cells versus either non-malignant or clinically localized malignant cells.⁵⁸ Moreover, they found that higher expression levels of MTA1 correlated with a longer PSA-free period following prostatectomy, whereas negative or weak MTA1 expression correlated with an increased time to tumor recurrence.⁵⁸ While the exact function of the MTA protein family has yet to be determined, there is recent evidence suggesting that MTA1 is part of the nucleosome remodeling histone deacetylation (NuRD) complex.⁶⁶ That decreased expression of both MTA1 and YY1 is correlated with a more rapid time to tumor recurrence, and that both proteins share histone deacetylation function may be more than mere coincidence. Studies are currently underway to test this hypothesis.

Conclusion. YY1 joins an expanding list of proteins whose expression or activity is altered during the course of prostate cancer progression.^{67,68} The general increase in YY1 expression in malignant compared to benign cells, as well as the associated increase survival in patients with tumors displaying decreased YY1 expression is intriguing. We predict that YY1 and/or proteins present in YY1-dependent pathways, will become part of a profile of proteins that may be useful diagnostic or prognostic tools as well as potential therapeutic targets.

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Footnotes

8Abbreviations: YY1, Yin Yang 1; PIN prostatic intraepithelial neoplasia; BPH benign prostatic hypertrophy; CTL, cytotoxic T lymphocytes; NK, natural killer cells; GM-CSF, granulocyte-macrophage colony stimulating factor; H & E, Hematoxylin and Eosin; ABC avidin-biotin complex; TMA, tissue microarray; PSA, prostate-specific antigen; TNF, tumor necrosis factor; TFIIB, transcription factor IIB; MTA1, metastasis-associated gene 1; NuRD, nucleosome remodeling histone deacetylation.

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TABLES

Table 1
Relationship of YY1 nuclear expression with clinicopathologic parameters

YY1 Nuclear Expression Frequency	All Patients	"Low" YY1 Minimum Positivity =50% (% of Total)	"High" YY1 Minimum Positivity > 50% (% of Total)	P-value ^a
Total Cases (n=190)		42 (22)	148 (78)	
Age At Surgery (n=190)				0.12 (NS) ^b
Median (Range)	65 (46-76)	65 (50-76)	65 (46-74)	
Gleason Score (n=190)				0.65 (NS)
2-6	112 (59)	23 (55)	89 (60)	
7-10	78 (41)	19 (45)	59 (40)	
Pathology pT Stage^c (n=190)				0.51 (NS)
PT2-pT3a	158 (83)	33 (79)	125 (84)	
PT3b	32 (17)	9 (21)	23 (16)	
Lymph Node Status (n=187)				0.42 (NS) ^b
Positive	9 (5)	1 (2)	8 (5)	
Negative	178 (95)	40 (98)	138 (95)	
Tumor Margins (n=190)				0.94 (NS)
Positive	62 (33)	13 (31)	49 (33)	
Negative	128 (67)	29 (69)	99 (67)	
Capsular Invasion (n=190)				0.23 (NS)
No Invasion	44 (23)	6 (14)	38 (26)	
Invasion	107 (56)	28 (67)	79 (53)	
Extension	39 (21)	8 (19)	31 (21)	
Organ Confined^d (n=190)				0.74 (NS)
Yes	97 (51)	20 (48)	77 (48)	
No	93 (49)	22 (52)	71 (52)	
High Risk (n=187)				0.79 (NS)
Yes	36 (19)	9 (22)	27 (18)	
No	151 (81)	32 (78)	119 (82)	
PreOpPSA ng/ml (n=169)				0.47 (NS) ^b
Median (Range)	8.9 (0.6-76.0)	9.1 (1.8-76.0)	8.9 (0.6-60.7)	
Mean	13.3	15.8	12.6	
Recurrence^e (n=190)				0.024f
Yes	65 (34)	21 (50)	44 (30)	
No	125 (66)	21 (50)	104 (70)	
Total Follow-up^g, months				0.21 (NS) ^b

Median (Range)	49.5 (0.1-163.0)	43.0 (1.0-120.0)	51.0 (0.1-163.0)	
Mean	53.5	46.7	55.4	
Total Follow-up in Recurred group (n=65)				
Median (Range)	21.0 (1.0-115.0)	16.0 (1.0-115.0)	27.0 (1.0-98.0)	
Mean	28.0	26.1	28.9	
Total Follow-up in Non-Recurring group (n=125)				
Median (Range)	66.0 (0.1-163.0)	65.0 (9.0-120.0)	71.5 (0.1-163.0)	
Mean	66.7	67.2	66.6	

^a P-value was determined by the Pearson chi-squared test with Yates continuity correction unless otherwise specified

^b P-value was determined by the Mann-Whitney test

^c pT3b indicates seminal vesicle invasion. There are no pT4 cases.

^d Organ Confined = no capsular extension and/or seminal vesicle and/or lymph node involvement, and margins are negative.

^e Recurrence = PSA elevation raising >0.2 ng/ml status post radical prostatectomy

^f As a continuous variable, YY1 minimum positivity is associated with recurrence by logistic regression; (P=0.0097; 0.99; 95% confidence interval 0.98-0.99)

^g Follow-up = time to recurrence or last follow-up

Table 2

Association of Benign^a and Neoplastic^b Tissue Groups by Nuclear or Cytoplasmic YY1 Expression Variables (per spot comparison; N=1061)

	Benign versus Neoplastic Expression ^c	
YY1 Expression Scoring Method	Chi Square	P value
Nuclear Intensity	107.5	<0.0001
Nuclear Positivity ^d	216.3	<0.0001
Cytoplasmic Intensity	199.6	<0.0001
Cytoplasmic Positivity	34.6	<0.0001

^a n=333 array spots

^b n=728 array spots

^c Kuskal-Wallis Test

^d variable measure used for clinical outcomes studies

FIGURE LEGENDS

Figure 1. Western blot, PC-3 cell line. PC-3 cells were grown in RPMI with 10% of FBS. Total cellular protein was extracted from the culture and then separated by SDS-PAGE and transferred onto the nitrocellulose membrane as described in Materials and Methods. The membrane was stained with anti YY1 antibody (1:1500 and 1:3000) or IgG control (1:1500). The β -actin antibody (1:10,000) was used as a loading control. The findings revealed that PC-3 express YY1 constitutively. The blot represents one of two separate experiments.

Figure 2. YY1 Protein Expression in a prostate cancer cell line (PC3). Distinct nuclear and light cytoplasmic staining of YY1 protein is seen by immunohistochemistry (A). Replacing primary anti-YY1 antibody with non-immune pooled rabbit IgG at an equivalent concentration serves as negative control (B), note a complete absence of staining. (400 X magnification)

Figure 3. Typical YY1 Protein Expression Localization, Normal Prostate Wholes Tissues. Demonstration of the typical staining pattern of YY1 protein by immunohistochemistry (A), showing predominantly nuclear staining of glandular (thick arrow) and basal cells (thin arrow), as well as stromal fibromuscular cells (triangle). Negative controls include non-immune IgG primary antibody substituted for YY1 (B), and primary YY1 antibody staining after competitive inhibition with immunogen peptide (C). (400 X magnification)

Figure 4. Spectrum of YY1 Protein Expression Patterns in Prostate Cancer. Immuno-histochemical staining for YY1 protein is seen on prostate tissue samples. (A) Normal tissue included for comparison shows crisp diffuse nuclear staining; (C) High grade tumor with finely granular nuclear staining; (E) low grade tumor with nuclear and diffuse cytoplasmic staining, note the normal gland in the lower left (arrow) showing nuclear staining only; (G) High grade tumor with neuroendocrine features showing coarsely granular nuclear and diffuse cytoplasmic staining; (I) low grade tumor with minimal to absent nuclear staining. (B, D, F, H, J are all non-immune pooled rabbit IgG negative controls). (400X magnification)

Figure 5. YY1 Protein Expression Distribution on the Prostate TMA Stratified by Histological Category.

Shown are the proportional distributions of YY1 protein staining by immunohistochemistry with attention to the maximal nuclear and cytoplasmic staining intensity, (A and C, respectively), and the total proportion of nuclear and cytoplasmic positivity at any intensity (B and D, respectively) of the target cells of the appropriate histologic category of each spot. 12 informative spots representing metastases are not included here.

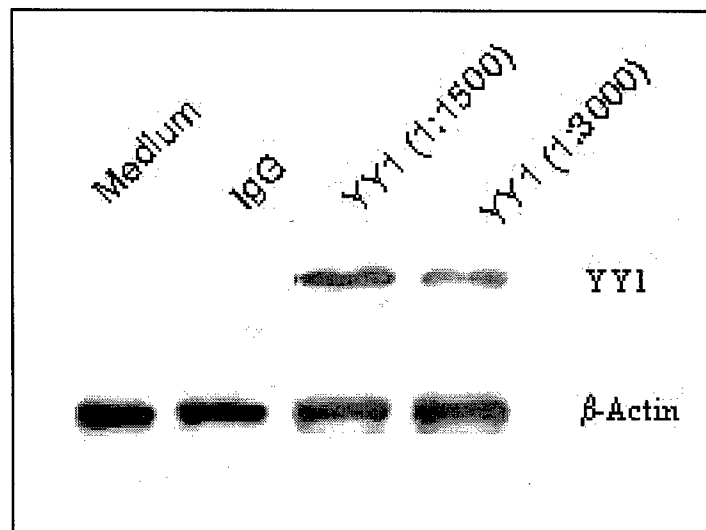
Figure 6. Distribution of Nuclear YY1 Protein Expression Used for Outcomes Analyses.

Outcomes data was available for 190 cases. The most significant correlation was derived by identifying the tissue array core from each case which showed the minimal level of nuclear YY1 staining (described in Materials and Methods). The x-axis shows the percentage of YY1 nuclear positivity in these selected cores. The y-axis shows the number of cases. The cutoff value shown (= 50% positive cells) was used to generate dichotomized patient groups, 42 cases (22%) had low YY1 expression ("YY1-low cases") and 148 cases (78%) had high YY1 expression ("YY1-high cases"). Note that most of the cases (i.e., 129 cases; 68% of total cases) had predominantly high nuclear YY1 staining; these samples are shown to the right of the cutoff line.

Figure 7. Kaplan Meier Curve for Time to Recurrence.

Kaplan Meier Curve for time to tumor recurrence stratified by nuclear YY1 protein expression status (n=190 patients). The upper and lower curves contain groups of individuals whose tumors demonstrated a minimal staining frequency of >50% or =50%, respectively. Censored patients are indicated as open circles (70% censored) or triangles (50% censored). A low nuclear YY1 expression phenotype is significantly associated with a higher risk to develop recurrent disease.

Figure 1



Figure

2

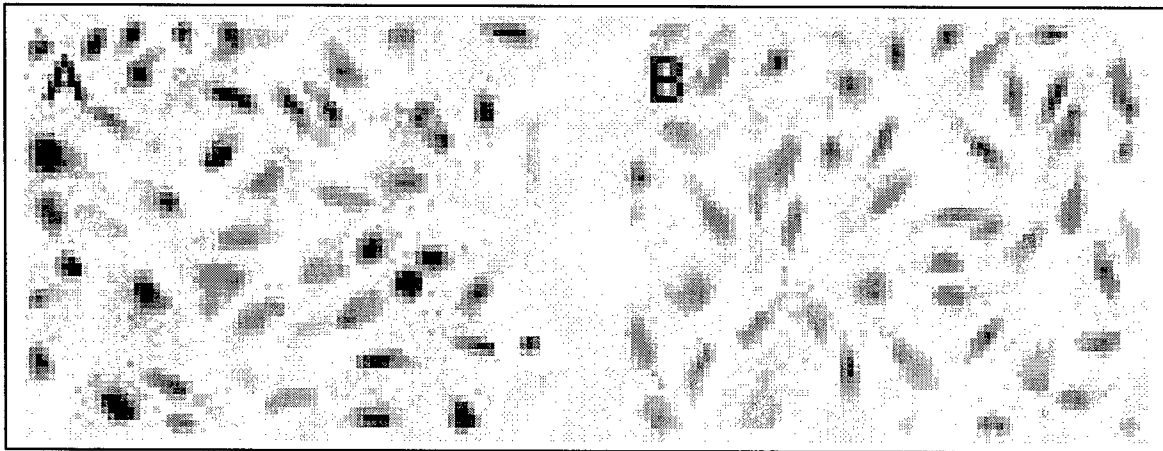


Figure 3

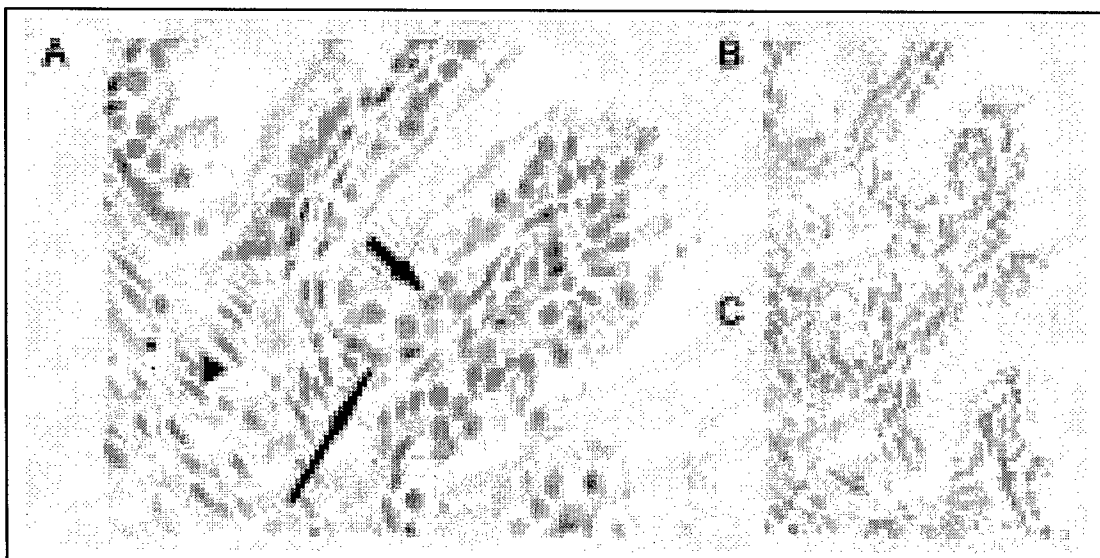


Figure 4

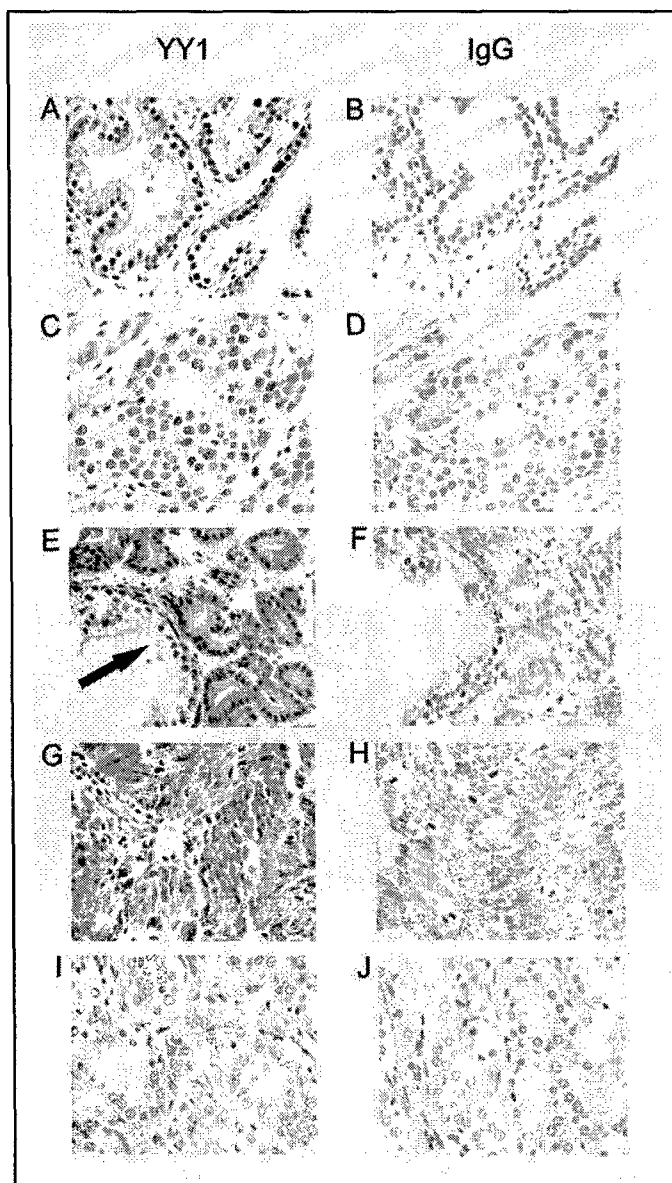


Figure 5

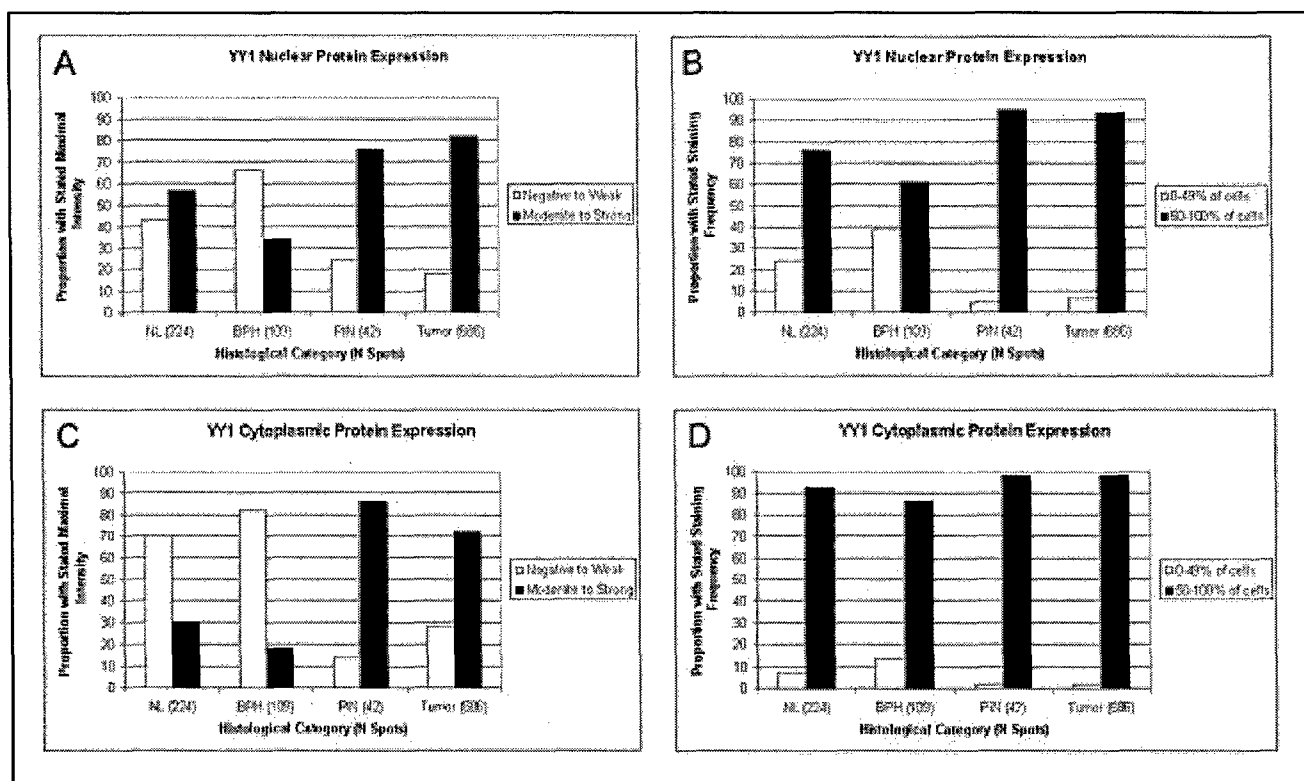


Figure 6

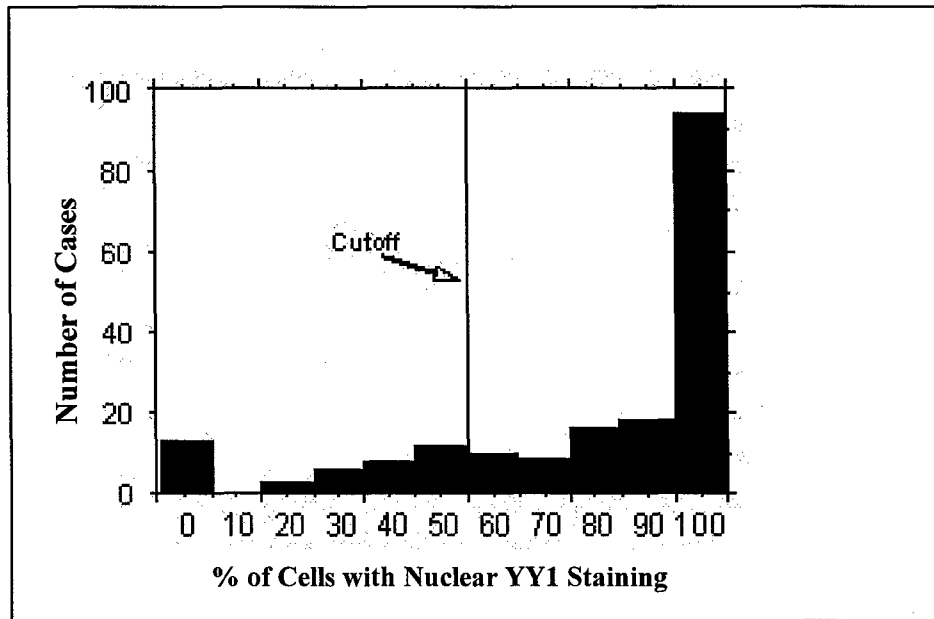
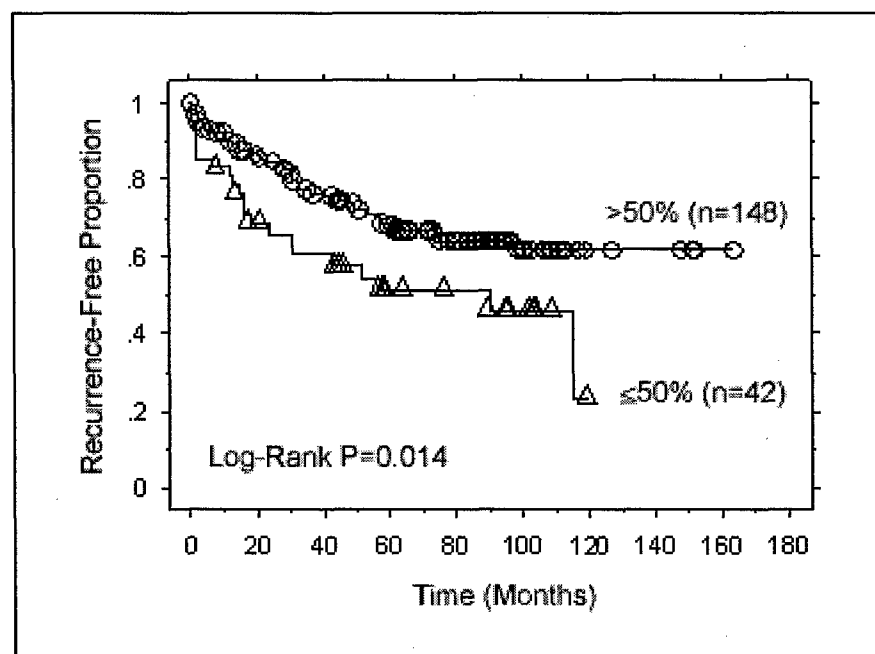


Figure 7



APPENDIX 3 (ALREADY PUBLISHED IN ONCOGENE 23:4993, 2004.)

Nitric oxide sensitizes prostate carcinoma cell lines to TRAIL-mediated apoptosis via inactivation of NF- κ B and inhibition of Bcl-xL expression

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Running title: NO-mediated inactivation of NF- κ B and inhibition of Bcl-xL expression

Key Works: NF- κ B, prostate cancer, nitric oxide, TRAIL, apoptosis

ABBREVIATIONS

Bcl-xL: Bcl-2 related gene

CaP: prostate cancer

DETANONOate: (Z)-1-[2-(2-aminoethyl)-N-(2-ammonio-ethyl)amino]diazene-1-ium-1, 2-diolate

DHT: 5- α dihydrotestosterone

DR: death receptor

DTT: 1,4-dithiothreitol

EDTA: ethylenediaminetetraacetic acid

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

IAP: inhibitor of apoptosis protein

IKK: I κ B kinase complex

JNK: c-Jun N-terminal kinase

2MAM-A3: 2-Methoxyantimycin A₃

NF- κ B: nuclear factor κ B

NO: nitric oxide

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PI: Propidium iodide

RIPA: radioimmunoprecipitation assay (buffer)

SDS: sodium dodecyl sulfate

Smac/DIABLO: Second mitochondria derived activator of caspase/direct inhibitor of apoptosis-binding protein with low PI

TNF- α : tumor necrosis factor alpha

TPA: 12-O-tetradecanoylphorbolacetate

TRAIL: tumor necrosis factor-related apoptosis-inducing ligand

XIAP: X-linked inhibitor of apoptosis

ABSTRACT

TRAIL has been shown to be selective in the induction of apoptosis in cancer cells with minimal toxicity to normal tissues and prompted its potential therapeutic application in cancer. However, not all cancers are sensitive to TRAIL-mediated apoptosis and, therefore, TRAIL-resistant cancer cells must be sensitized first to become sensitive to TRAIL. Treatment of prostate cancer (CaP) cell lines (DU145, PC-3, CL-1, and LNCaP) with nitric oxide donors (e.g. DETANONOate) sensitized CaP cells to TRAIL-induced apoptosis and synergy was achieved. The mechanism by which DETANONOate mediated the sensitization was examined. DETANONOate inhibited the constitutive NF- κ B activity as assessed by EMSA. Also, p50 was S-nitrosylated by DETANONOate resulting in inhibition of NF- κ B. Inhibition of NF- κ B activity by the chemical inhibitor Bay 11-7085, like DETANONOate, sensitized CaP to TRAIL apoptosis. In addition, DETANONOate downregulated the expression of Bcl-x_L which is under the transcriptional regulation of NF- κ B. The regulation of NF- κ B and Bcl-x_L by DETANONOate was corroborated by the use of Bcl-x_L and Bcl-x κ B reporter systems. DETANONOate inhibited luciferase activity in the wild type and had no effect on the mutant cells. Inhibition of NF- κ B resulted in downregulation of Bcl-x_L expression and sensitized CaP to TRAIL-induced apoptosis. The role of Bcl-x_L in the regulation of TRAIL apoptosis was corroborated by inhibiting Bcl-x_L function by the chemical inhibitor 2-MAM-A₃ and which resulted in sensitization of the cells to TRAIL apoptosis. Signaling by DETANONOate and TRAIL for apoptosis was examined. DETANONOate altered the mitochondria by inducing membrane depolarization and releasing modest amounts of cytochrome c and Smac/DIABLO in the absence of downstream activation of caspases 9 and 3. However, the combination of DETANONOate and TRAIL resulted in activation of the mitochondrial pathway and activation of caspases 9, 3, and induction of apoptosis. These findings demonstrate that DETANONOate-mediated sensitization of CaP to TRAIL-induced apoptosis is via inhibition of constitutive NF- κ B activity and Bcl-x_L expression.

INTRODUCTION

Tumor cells develop resistance to apoptotic stimuli induced by various therapeutics such as drugs, irradiation, and immunotherapy since most of their primary cytotoxic effects are through apoptosis (Ng and Bonavida, 2002a; Hersey and Zhang, 2003). Therefore, after the initial response to these therapies, tumor cells develop resistance and/or are selected for resistance to apoptosis. Therefore, new therapeutic strategies are needed to reverse resistance to apoptosis.

TRAIL is a cytotoxic molecule that has been shown to exert, selectively, anti-tumor cytotoxic effects both *in vitro* and *in vivo* with minimal toxicity to normal tissues (Ashkenazi and Dixit, 1999; Ashkenazi et al., 1999). TRAIL has been considered a new therapeutic and preclinical studies demonstrate its anti-tumor activity alone or in combination with drugs (Ashkenazi et al., 1999; Wajant et al., 2002; Chawla-Sarkar et al., 2003; De Jongs et al., 2001). However, many tumor cells have been shown to be resistant to TRAIL (Zisman et al., 2001; Ng et al., 2002; Bouralexis et al., 2003; Tillman et al., 2003). We and others have reported that various sensitizing agents like chemotherapeutic drugs (Zisman et al., 2001; Munshi et al., 2002), cytokines (Park et al., 2002), and inhibitors (Nyormoi et al., 2003) are able to render TRAIL-resistant tumor cells sensitive to TRAIL apoptosis.

Prostate cancer cells have been shown to exhibit constitutive NF- κ B activity (Suh et al., 2002). It has been recently reported that NF- κ B can regulate the sensitivity of target cells to TRAIL apoptosis in hepatoma cells (Shigero et al., 2003). In addition, it has been reported that prostate cancer cells over-express Bcl- κ L which negatively regulates tumor cells sensitivity to drug-mediated apoptosis (Raffo et al., 1995). Studies on Bcl- κ L gene transcription demonstrate that Bcl- κ L is regulated in part by NF- κ B (Mori et al., 2000). Thus, constitutive expression of NF- κ B in CaP may regulate the constitutive expression of Bcl- κ L. We have reported that NO donors can sensitize tumor cells to FasL and TNF- α -mediated apoptosis (Garban and Bonavida, 2001a, b). Further, we (Huerta-Yepez et al., 2003) and others (Lee et al., 2001; Secchiero et al., 2001) reported that DETANONOate can also sensitize tumor cells to TRAIL-mediated apoptosis.

The mechanism underlying the NO-mediated sensitization to TRAIL is not known. We hypothesized that NO-mediated sensitization of CaP cells to apoptosis may be due to NO-induced inhibition of constitutive NF- κ B activity and which, in turn, will result in the down-regulation of Bcl- κ L transcription and expression. Hence, downregulation of the anti-apoptotic gene product Bcl- κ L will result in the sensitization of CaP cells to TRAIL-mediated apoptosis. This study was designed to test this hypothesis and the followings were investigated: 1) Does NO sensitize androgen-dependent and independent CaP cell lines to TRAIL-mediated apoptosis? 2) Does NO inhibit constitutive NF- κ B activity resulting in inhibition of Bcl- κ L expression? 3) Do inhibitors of NF- κ B and Bcl- κ L mimic NO and sensitize CaP to TRAIL-mediated apoptosis? And 4) By what mechanism does NO modify the apoptotic signaling pathway and sensitize CaP to TRAIL-mediated apoptosis?

RESULTS

Sensitization of CaP cell lines to TRAIL-mediated apoptosis by DETANONOate. Our previous findings have demonstrated that CaP cell lines (LNCaP, DU-145, PC-3, and CL-1) are relatively resistant to TRAIL-mediated apoptosis (Zisman et al., 2000; Ng et al., 2002) and also shown in Figure 1A. However, pretreatment of CaP cell lines with the nitric oxide donor DETANONOate resulted in significant potentiation of apoptosis by TRAIL for the four cell lines tested. The extent of potentiation was a function of the concentration of TRAIL used (Figure 1A). The sensitization by DETANONOate was synergistic as determined by isobologram analysis (Figure 1B). We selected PC-3 as a model system for further investigation. Treatment of PC-3 cells with various concentrations of DETANONOate sensitized the cells to TRAIL-induced apoptosis and the extent of apoptosis was a function of the concentration of DETANONOate used (Figure 1C). In addition to apoptosis, NO, TRAIL, and combination inhibited cell proliferation significantly (Figure 1D). These findings demonstrate DETANONOate sensitizes androgen-dependent and independent CaP tumor cell lines to TRAIL-mediated apoptosis and synergy is achieved. Previous findings demonstrated that the androgen DHT sensitizes LNCaP to TPA-induced apoptosis (Altuwaijri et al., 2003). We examined whether DHT also sensitizes LNCaP to TRAIL apoptosis. We observed that treatment of LNCaP with DHT sensitizes the cells to TRAIL (Table 1).

DETANONOate inhibits NF- κ B activity and inhibition of NF- κ B sensitizes PC-3 to TRAIL-apoptosis. We examined the effect of DETANONOate on NF- κ B activity in PC-3 cells. The cells were treated with DETANONOate (500 μ M and 1000 μ M) and tested for NF- κ B activity by EMSA. In addition, we used the NF- κ B inhibitor, Bay 11-7085, at different concentrations as control for inhibition of NF- κ B activity. Figure 2A demonstrates that DETANONOate inhibits NF- κ B activity significantly and the inhibition at 1000 μ M was much higher than the inhibition at 500 μ M. As expected, the Bay 11-7085 inhibitor also significantly inhibited NF- κ B activity and the inhibition was a function of the concentration of Bay 11-7085 used (Figure 2A).

It has been reported that the DNA-binding activity of NF- κ B p50 can be modified by DETANONOate and p50 becomes S-nitrosylated and inhibits NF- κ B activity (Matthews et al., 1996; Dela Torre et al., 1997; Marshall and Stamler, 2001). Thus, we examined whether NO treatment of PC-3 cells induces S-nitrosylation of p50. PC-3 cells were grown in the absence or presence of DETANONOate (500 μ M or 1000 μ M) for 18 h and total cell lysates were prepared and immunoprecipitation assay was performed as described in Methods. Using anti-S-nitrosylated antibody, the S-nitrosylated proteins were immunoprecipitated and were run on SDS-PAGE and immunoblotted with anti-NF- κ B p50 antibody. S-nitrosylation of p50 was significantly enhanced following DETANONOate treatment (Figure 2B).

The relationship between DETANONOate-mediated inhibition of NF- κ B and sensitization to TRAIL was examined. PC-3 cells were treated with various concentrations of Bay 11-7085 (1-5 μ M) and TRAIL (5 and 10 ng/ml). Treatment with Bay 11-7085 significantly potentiated the sensitivity of PC-3 to TRAIL-mediated apoptosis and the degree of apoptosis was a function of the concentration used (Figure 2C).

These findings demonstrate that DETANONOate inhibits NF- κ B activity and results in the sensitization of PC-3 to TRAIL-induced apoptosis. Further, the results suggest that DETANONOate-mediated sensitization is via inactivation of NF- κ B.

DETANONOate-mediated downregulation of Bcl-x_L expression and sensitization to TRAIL

DETANONOate selectively inhibited Bcl-x_L expression in PC-3 with little effect on other pro- and anti-apoptotic gene products examined (Figure 3A). TRAIL has no effect on any of the gene products examined. It has been reported that Bcl-x_L transcription is regulated in part by NF- κ B (Mori et al., 2001; Sevilla et al., 2001). Thus, it was possible that DETANONOate-mediated inhibition of NF- κ B (Figure 2A) was responsible for the observed DETANONOate-mediated inhibition of Bcl-x_L expression (Figure 3A). This was confirmed by demonstrating that treatment of PC-3 with the NF- κ B inhibitor Bay 11-7085, like DETANONOate, also inhibited Bcl-x_L expression (Figure 3B). Therefore, it was possible that sensitization of PC-3 by DETANONOate to TRAIL-induced apoptosis was due in part to downregulation of Bcl-x_L expression via inhibition of NF- κ B. Accordingly, inhibition of Bcl-x_L expression should sensitize PC-3, like NO, to TRAIL-induced apoptosis. Treatment of PC-3 with the Bcl-x_L inhibitor 2MAM-A3 (Tzung et al., 2001) resulted in significant sensitization of the cells to TRAIL-induced apoptosis. The potentiation was a function of the concentration of 2MAM-A3 used (Figure 3C). These findings suggest that Bcl-x_L is the dominant resistant factor in PC-3 cells for TRAIL-induced apoptosis and Bcl-x_L inhibition by DETANONOate via NF- κ B inactivation may be responsible for sensitization to TRAIL.

It has been reported that NF- κ B activity plays an important role in the transcriptional regulation of Bcl-x_L (Mori et al., 2001; Sevilla et al., 2001). To determine whether NF- κ B activity is required for Bcl-x_L transcription and to determine how DETANONOate induces selective inhibition of Bcl-x_L via NF- κ B, transient transfection assays were performed. PC-3 cells were transfected with the Bcl-x WT promoter and Bcl-x κ B promoter reporter plasmids. 24 h after transfection, the cells were treated with either Bay 11-7085 (2 or 3 μ M), DETANONOate (500 or 1000 μ M) or optimal concentrations of TNF- α (50 or 100 U/mL) for 18 h. Both DETANONOate treatment and Bay 11-7085 treatment induced significant inhibition of Bcl-x_L transcription and the extent of inhibition was concentration dependent. In contrast, activation of NF- κ B by TNF- α treatment induced significant increase in Bcl-x_L transcription above (Figure 4). The basal luciferase activity was significantly reduced in the mutant (5x) compared to wild type suggesting that Bcl-x_L transcription in PC-3 is primarily regulated by NF- κ B. In contrast to the findings in the wild type, the different treatments did not affect the cells transfected with the Bcl-x κ B -promoter (Figure 4). These results indicate that Bcl-x_L transcription in PC-3 is in large part regulated by NF- κ B and inhibition of NF- κ B by DETANONOate is responsible for DETANONOate-mediated downregulation of Bcl-x_L expression.

Mechanism of DETANONOate-mediated sensitization to TRAIL apoptosis. We investigated the mechanism by which DETANONOate signals the cells leading to sensitization to TRAIL-mediated apoptosis.

The effect of DETANONOate on the mitochondria was examined. DETANONOate significantly induced membrane depolarization of the mitochondria in PC-3 cells. In addition, TRAIL also significantly induced membrane depolarization and the combination resulted in membrane depolarization that was equivalent to either DETANONOate or TRAIL used alone (Figure 5A). The effect of DETANONOate and TRAIL on the release of cytochrome c and Smac/DIABLO from the mitochondria was also examined. Both DETANONOate and TRAIL induced the release of both cytochrome c and Smac/DIABLO from the mitochondria into the cytosol and the combination of DETANONOate and TRAIL resulted in more significant release of cytochrome c and Smac/DIABLO (Figure 5B). In addition, there was little activation of pro-caspase-8 and pro-caspase-9 by either DETANONOate or TRAIL used alone, although the combination resulted in significant activation of pro-caspase 8 and pro-caspase 9 (Figure 5C). These findings demonstrate that DETANONOate selectively inhibits Bcl-x_L expression (Figure 3A) and the activation of the mitochondria by both TRAIL and DETANONOate used in combination resulted in complementation and type II mitochondria-mediated sensitization of the cells to TRAIL-mediated apoptosis.

DISCUSSION

This study presents evidence that the NO donor, DETANONOate, sensitizes androgen-dependent and independent CaP cell lines to TRAIL-mediated apoptosis via inhibition of NF- κ B activity and down-regulation of Bcl-x_L expression. The inactivation of NF- κ B by DETANONOate was via S-nitrosylation of NF- κ B p50. The role of NF- κ B in the transcriptional activity of Bcl-x_L expression was demonstrated by the use of NF- κ B inhibitors and by the use of a luciferase reporter construct driving the Bcl-x_L promoter. Treatment with DETANONOate or Bay11-7085 inhibited significantly luciferase activity whereas TNF- α augmented the basal activity. In contrast, removal of the putative NF- κ B-binding sequence from the promoter resulted in low constitutive level of Luc activity and this basal level was not affected by DETANONOate or by the NF- κ B inhibitor. Inhibition of either NF- κ B or Bcl-x_L by chemical inhibitors sensitized significantly to TRAIL-mediated apoptosis. The synergy achieved in apoptosis by combination treatment was the result of complementation in the activation of the Type II mitochondrial pathway for apoptosis. Thus, both TRAIL and DETANONOate partially activate the mitochondria, with membrane potential depolarization and some release of cytochrome c and Smac/DIABLO, though each alone could not activate caspase 9. The combination of DETANONOate and TRAIL, however, resulted in caspase 9 and 3 activation and apoptosis. Altogether, these findings provide a novel mechanism of Bcl-x_L regulation by NO via NF- κ B inhibition and suggest that NO donors may be of potential therapeutic value as sensitizing agents when used in combination with TRAIL in the treatment of TRAIL-resistant tumor cells.

Our findings demonstrate that DETANONOate sensitized both androgen-dependent (LNCaP) and androgen-independent (DU145, PC-3, and CL-1) CaP cells to TRAIL-induced apoptosis and synergy was

achieved. Previous findings from our laboratory have demonstrated that subtoxic concentrations of chemotherapeutic drugs like Actinomycin D sensitized the above CaP tumor cells to TRAIL apoptosis (Zisman et al., 2001). Actinomycin D was shown to downregulate XIAP selectively and, thus, facilitated the TRAIL-induced apoptotic pathway (Ng et al., 2002). The role of XIAP in resistance was corroborated in experiments showing that transfection with Smac/DIABLO, which inhibits IAPs, sensitizes cells to TRAIL apoptosis in the absence of Actinomycin D (Ng and Bonavida, 2002b). The present findings with DETANONOate, however, are different such that NO selectively inhibits NF- κ B and Bcl-x_L expression in the absence of modification of XIAP expression and sensitizes the cells to TRAIL apoptosis. These findings demonstrate that the regulation of apoptosis by TRAIL in the CaP cell lines studied may be influenced by various anti-apoptotic members of the signaling pathway and the inhibition of one such member, such as XIAP or Bcl-x_L, was sufficient to reverse the resistance to TRAIL.

In prostate cancer, NF- κ B contributes to the progression to androgen-independence and increases invasive and metastatic properties (Palayoor S, et al. 1999; Rayet and Galinas, 1999). Basal levels of NF- κ B are detected in normal prostatic epithelial cells and the androgen-dependent prostate cancer cell line LNCaP (Huang et al., 2001; Payaloor et al., 1999). It has been reported that cross-talk occurs between NF- κ B signaling and steroid receptor signaling pathways (McKay et al., 2000; Palvimo et al., 1996). We show that treatment of LNCaP with DHT sensitized the cells to TRAIL via inhibition of NF- κ B. In contrast, androgen-independent prostate cancer cells PC-3 and DU-145 have elevated NF- κ B activity and was confirmed here (data not shown). In addition, PC-3 and DU-145 cells have constitutively active IKK, which activates NF- κ B (Gasparian et al., 2002). Thus, constitutive activation of NF- κ B play a central role in the resistance to prostate cancer cell line to therapeutic agents.

The present findings demonstrate that DETANONOate inhibits NF- κ B activity. It has been shown that high levels of NO inhibit NF- κ B activity by several mechanisms. For instance, DETANONOate inhibits the phosphorylation and subsequent degradation of I κ B- α which prevents nuclear localization of NF- κ B (Katsuyama et al., 1998). Also, NO may quench reactive oxygen species that are responsible for the activation of NF- κ B (Garban and Bonavida, 2001b). In addition, recent studies demonstrate that NO induces S-nitrosylation of NF- κ B p50 and reducing its DNA-binding activity (Marshall and Stamler, 2001; Connely et al., 2001). NF- κ B displays redox-sensitive DNA-binding activity (Tell et al., 1998; Chinenov et al., 1998). This redox sensitivity is conferred by a single cysteine residue within the DNA-binding site (Matthews et al., 1993; Marshall and Stamler, 2001). In this study we demonstrate that NF- κ B binding activity was significant decreased after treatment with DETANONOate (Figure 2A). We also demonstrate that DETANONOate induced strongly S-nitrosylation of NF- κ B p50 (Figure 2B) in agreement with the findings of Marshall et al (2001) and Connely et al (2001).

Recent studies demonstrated that Bcl-2 and Bcl-x_L block apoptosis induced by physiological agents such as TRAIL in PC-3, DU-145 and LNCaP prostate cancer cells (Rokhlin et al., 2001). In addition, overexpression of Bcl-x_L in LNCaP and PC-3 cells desensitized the cells to the effects of cytotoxic chemotherapeutic agents (Li et al., 2001). However, down-regulated endogenous levels of Bcl-x_L, but not Bcl-2, induced marked increase in chemosensitivity (Lebedeva et al., 2000). These results suggest the important role of Bcl-x_L in the resistance to apoptosis induced by cytotoxic agents like TRAIL in CaP. Noteworthy, our results demonstrate that DETANONOate treatment induces selective downregulation of Bcl-x_L expression and sensitizes the CaP cells to TRAIL-induced apoptosis. Further, inhibition of Bcl-x_L function by 2MAM-A3 sensitizes the cells to TRAIL apoptosis. These findings corroborate the role of Bcl-x_L in the regulation of resistance of CaP to chemotherapy and TRAIL.

The mechanism by which NO induces inhibition of Bcl-x_L expression was examined. Previous findings demonstrated that the Bcl-x_L promoter contains an element that binds NF- κ B transcription factors and supports transcriptional activation by members of this family (Lee et al., 1999). It was possible that DETANONOate inhibits NF- κ B and this, in turn, inhibits Bcl-x_L transcription. We demonstrate here that DETANONOate inhibits Bcl-x_L expression via inactivation of NF- κ B activity. This was shown by using a luciferase reporter construct driving the Bcl-x_L promoter. Treatment with DETANONOate or Bay 11-7085 [which selectively and irreversibly inhibits the induced phosphorylation of I κ B without affecting the constitutive I κ B- γ phosphorylation (Pierce et al., 1997) significantly inhibited the high constitutive luciferase activity. However, there was little luciferase activity following the removal of the putative NF- κ B-binding sequence from the promoter and neither DETANONOate nor Bay 11-7085 had any effect. These results directly demonstrate that Bcl-x_L expression in PC-3 is primarily regulated by NF- κ B and inhibition of NF- κ B, in turn, inhibits Bcl-x_L transcription.

Nitric oxide (NO), synthesized from L-arginine by NO synthase, is a small, diffusible, highly reactive molecule with dual regulatory roles under physiological and pathological conditions (Schmidt and Walter, 1994). NO can promote apoptosis (pro-apoptosis) in some cells, whereas it inhibits apoptosis (anti-apoptosis) in other cells. This dichotomy depends on the rate of NO production and the interaction with biological molecules such as iron, thiol, proteins, and reactive oxygen species (Schmidt, 1992; Stamler, 1994). High concentrations of NO and also long-lasting production of NO such as by DETANONOate used here act as proapoptotic modulators (Poderoso et al., 1996; Messmer et al., 1996; Jun et al., 1999a; So et al., 1998; Di Nardo et al., 2000). The present findings are consistent with the pro-apoptotic effects of the high levels of NO used to sensitize CaP cells.

NO binds to cytochrome c oxidase (complex IV) in the mitochondrial electron transfer chain (Poderoso et al., 1996). Under this condition, superoxide generated from mitochondria interacts with NO to form peroxynitrite, which induces mitochondrial dysfunction and cytochrome c release. NO also generates ceramide, which induces cytochrome c release from mitochondria (Ghafourifar et al., 1999). Our results clearly

show that DETANONOate induces activation of the mitochondria pathway, including mitochondrial membrane depolarization (Figure 3A) and some release of both cytochrome c and Smac/DIABLO (Figure 3B). The participation of the mitochondria is not complete because we demonstrate that downstream caspases are not activated. Caspase activation, however, resulted from the combination of DETNNOate and TRAIL. Recent studies have shown that caspase-8 activation is necessary but not sufficient for TRAIL-mediated apoptosis in prostate carcinoma cells (Rokhlin et al., 2002) suggesting the important participation of the mitochondria-dependent pathway in TRAIL-mediated apoptosis. Further, our findings with DETANONOate are consistent with those of Lee et al (2001) who reported that sodium nitroprusside enhances TRAIL-induced apoptosis via a mitochondria-dependent pathway.

This study demonstrates that the combination of NO donor and TRAIL can sensitize TRAIL-resistant CaP to TRAIL-induced apoptosis. This combination treatment is a result of two complementary signals induced by each agent alone (Ng and Bonavida, 2002; schematically diagrammed in Figure 6). Signal 1 results from NO-induced perturbation of the mitochondria, inhibition of NF- κ B activity and downregulation of Bcl-x_L expression. Signal 1 alone is not sufficient to promote the cells towards apoptosis. Signal 2 is induced by TRAIL which activates the mitochondria slightly, but not sufficient to activate the apoptosome and induce apoptosis. However, combination of the two signals results in complementation and activation of the mitochondrial pathway and activation downstream of caspases 9 and 3 resulting in apoptosis. Thus, the findings of this report reveal that NO can selectively inhibit the expression of the anti-apoptotic resistant factor, Bcl-x_L, via inhibition of NF- κ B activity. The findings also reveal new targets for intervention affecting NF- κ B activity or Bcl-x_L expression and whose modification may revert resistance of CaP to TRAIL apoptosis. Thus, NO donors or Bcl-x_L inhibitors may be useful in the treatment of TRAIL resistant tumors in combination with TRAIL or TRAIL agonists such as antibody against DR4/DR5.

MATERIALS AND METHODS

Reagents

The anti-Bcl-x_L and anti- β -actin monoclonal antibodies were purchased from Santa Cruz (California, USA) and from Calbiochem (San Francisco, CA), respectively. mAb anti-Bcl-2 was obtained from DAKO Corporation (Carpinteria, CA). The polyclonal antibodies anti-XIAP, anti-IAP-1, anti-IAP-2, anti-caspase-8, anti-caspase-9, and survivin were obtained from Cell Signaling (San Diego, CA, USA), anti-cytochrome c from Pharmingen (San Diego, CA, USA), and anti-Smac/DIABLO from Alexis (San Diego, CA, USA). The human recombinant TRAIL and TNF- α were obtained from PeproTech, Inc (Rocky Hills, NJ). FITC-conjugated anti-active caspase-3 and FITC-conjugated IgG was purchased from PharMingen (San Diego, CA). The NF- κ B inhibitor Bay 11-7085 (specific inhibitor of I κ B β phosphorylation (Pierce et al., 1997) was obtained from Calbiochem (San Francisco, CA), and the Bcl-x_L inhibitor 2-Methoxyantimycin A₃ (binds to the hydrophobic

groove of Bcl-2 and Bcl_{xL}) (Tzung et al., 2001) was obtained from Biomol (Plymouth, PA). The DETANONOate was obtained from Alexis (San Diego, CA).

Cells and Culture Conditions

The human androgen-independent PC-3 and DU145 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The androgen-dependent LNCaP and the androgen-independent (Tso et al., 2000) CL-1 (LNCaP-derived) cell lines were kindly provided by Dr. Arie Belldegrun at UCLA. Cells were maintained as a monolayer in 80mm² plates in RPMI 1640 (Life Technologies, Bethesda, MD), supplemented with 5% heat-inactivated fetal bovine serum (FBS) (to ensure the absence of complement), 1% (v/v) penicillin (100 U/ml), 1% (v/v) streptomycin (100 U/ml), 1% (v/v) L-glutamine, 1% (v/v) pyruvate, and 1% nonessential amino acids. FBS (Life Technologies) was charcoal-stripped to maintain CL-1 cells in an androgen-free medium. The LNCaP cell medium was supplemented with 0.1 nmol/L R1881 methyltrienolone (New Life Science Products, Boston, MA). The cell cultures were maintained as monolayers on plastic dishes and were incubated at 37°C and 5% carbon dioxide in RPMI 1640 Life Technologies (Bethesda, MD), supplemented with 5% heat-inactivated FBS (to ensure the absence of complement), 1% (v/v) penicillin (100 U/ml), 1% (v/v) streptomycin (100 U/ml), 1% (v/v) L-glutamine, 1% (v/v) pyruvate, and 1% nonessential amino acids (Invitrogen Life Technologies, Carlsbad, CA). For every experimental condition, the cells were cultured in 1% FBS, 18 h prior to treatments.

Cell treatments

Log phase prostate carcinoma cell lines cells were seeded into six-well plates at approximately 6 X10⁴ cells/ml and grown in 1 ml of medium as described above in 5% FBS for 24 h to approximately 70% confluence. The DU145, CL-1 and PC-3 cells were synchronized by treatment with 1 % FBS for 18 h prior to each experiment. The treatment of LNCaP cells was in medium with 1% of serum and the treatments of DU145, CL1 and PC-3 were in serum-free conditions. For experiments to measure TRAIL-mediated apoptosis by DETANONOate, the cells were treated with TRAIL, DETANONOate or combination for 18 h. For the experiments of sensitization to TRAIL-mediated apoptosis by the NF- κ B inhibitor Bay 11-7085, the cells were treated with different concentrations of Bay 11-7085 for 1 h and then treated with various concentrations of TRAIL for 18 h. For sensitization to TRAIL-mediated apoptosis by the Bcl_{xL} inhibitor 2MAM-A3, the cells were treated with different concentrations of 2MAM-A3 for 4 h, and then treated with TRAIL for 18 h.

Determination of apoptosis

After each treatment, the adherent cells and the floating cells were recovered by centrifugation at 1800 rpm for 8 min. Afterwards, the cells were washed once with ice cold 1XPBS and were re-suspended in 100 of the cytofix/cytoperm solution (PharMingen, San Diego, CA) for 20 min. Thereafter, the samples were washed twice with ice cold 1 $\times$ perm/wash buffer solution (PharMingen) and were stained with FITC-labeled anti-active-

caspase-3 mAb for 30 min (light protected). The samples were subsequently washed once with 1Xperm/wash buffer solution and 250 μ l of 1% PBS was added prior to flow cytometry analysis on a Flow cytometer EPICS^R XL-MCL (Coulter, Co. Miami, FL.), with the System IITM Software and the percent positive cells was recorded. As a negative control, the cells were stained with isotype control (pure IgG) under the same conditions described above.

Immunoprecipitation of S-nitrosylated NF- κ B p50 (S-NO-p50).

The S-nitrosylation of NF- κ B p50 was analyzed by immunoprecipitation assay. The cells were grown in the presence and absence of DETANONOate (0, 500, and 1000 μ M) and then harvested and pelleted at 14,000 $\times$ g for 2 min. The resulting cell pellets were re-suspended and dissolved in 500 μ l ice-cold components of RIPA buffer. The supernatants were incubated overnight at 4°C on a shaking platform with 2% of rabbit anti-S nitrosylated proteins polyclonal Ab (Calbiochem, San Diego, CA) and were subsequently incubated with 30 μ l Immuno-Pure Plus Immobilized protein A (Lindmark et al., 1983) (Pierce, Rockford, IL) for 4h at 4°C on a shaking platform. The lysates were centrifuged for 1 min at 14,000 $\times$ g and the supernatants were discarded. The immuno-precipitates were washed twice with 1.0 ml of ice cold RIPA buffer prior to assay. The immuno-precipitates were resolved on a 12% SDS-PAGE gel and subsequently immunoblotted with anti-NF- κ B p50 polyclonal Ab (1:2000 dilution) (Active Motif, Carlsbad, CA). The immuno-staining was visualized by autoradiography.

Luciferase Bcl-x_L Promoter Reporter Assay.

The Bcl-x_L WT promoter-luciferase (Bcl-x WT promoter) reporter plasmid and the Bcl-x_L promoter missing the NF- κ B-binding sequence (Bcl-x κ B-promoter) have been previously characterized (Lee et al., 1999). PC-3 cells were transfected by electroporation using pulse at 250 V/975 μ F (BioRad), with 20% of Bcl-x WT promoter or Bcl-x κ B-promoter. After transfection, the cells were allowed to recover overnight and were cultured in 6-well plates. Cells were treated with the specific NF- κ B inhibitor Bay 11-7085 (2 or 3 μ M), NO donor DETANONOate (500 or 1000 μ M) or TNF- α (50 or 100 U/ml) for 18h. Cells were then harvested in lysis buffer and luciferase activity was measured according to the manufacture's protocol (BD Biosciences, Palo Alto, CA) using an analytical luminescence counter Monolith 2010. The assays were performed in triplicate.

Measurement of mitochondrial membrane depolarization

The mitochondria-specific dye 3,3'-dihexyloxacarbocyanine (DiOC₆) (Molecular Probes, Inc. Eugene, OR) was used to measure mitochondrial potential. PC-3 cells were grown in six-well plates and were treated with TRAIL (2.5 ng/ml) and/or DETANONOate (1000 μ M) simultaneously. After treatments, the cells were collected at 18 h. A total of 50 μ l of 40 μ M (DiOC₆) was loaded to stain the cells for 30 min immediately after

the cells were collected. The cells were detached by using PBS supplemented with 0.5 %M EDTA, washed twice in PBS, re-suspended in 1 ml of PBS, and analyzed by flow cytometry as reported (Ng et al., 2002).

Western Blot Analysis

PC-3 cells were cultured at a low FBS concentration (0.1%) 18 h prior to each treatment. After incubation, the cells were maintained in FBS-free medium (control), or treated with TRAIL (2.5 ng/ml), DETANONOate (1000 %M) or combination. The cells were then lysed at 4°C in RIPA buffer (50mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150mM NaCl), and supplemented with one tablet of protease inhibitor cocktail, Complete Mini Roche (Indianapolis, IN). Protein concentration was determined by a DC protein assay kit Bio-Rad (Hercules, CA). An aliquot of total protein lysate was diluted in an equal volume of 2XSDS sample buffer 6.2mM Tris (pH6.8), 2.3% SDS, 5% mercaptoethanol, 10% glycerol, and 0.02% bromphenol blue and boiled for 10 minutes. The cell lysates (40%g) were then electrophoresed on 12% SDS-PAGE gels (Bio-Rad) and were subjected to Western blot analysis as previously reported (Jazirehi et al., 2001). Levels of β -actin were used to normalize the protein expression. Relative concentrations were assessed by densitometric analysis of digitized autographic images, performed on a Macintosh computer (Apple Computer Inc., Cupertino, CA.) using the public domain NIH Image J Program (also available via the internet).

Isolation of cytosolic fraction and determination of cytochrome c and Smac/ DIABLO content

PC-3 cells were grown under the conditions explained for western blot. At the end of the incubation period the cells were recovered with 1XPBS/EDTA, washed with 1.0%PBS/0.1% BSA and resuspended in 2 volumes of homogenization buffer [20mM Hepes (pH:7.4), 10mM KCl, 1.5mM MgCl₂, 1mM sodium EDTA, 1mM sodium EGTA, 1mM DTT, one tablet of Complete Mini protease inhibitor cocktail in 250mM sucrose medium]. After 30 min on ice, the cells were disrupted by 40 strokes of a dounce glass homogenizer using a loose pestle (Bellco Glass, Inc., Vineland, NJ). The homogenate was centrifuged at 2500 % g at 4°C for 5min to remove nuclei and unbroken cells. The mitochondria were pelleted by spinning the homogenate at 16,000 % g at 4°C for 30 min. The supernatant was removed and filtered through 0.1%m Ultrafree MC filters (Millipore) to obtain the cytosolic fraction and was spun down at 16,000%g at 4°C for 15 min. The protein concentration of the supernatant was determined by the DC assay kit and was mixed with 2 % laemelli sample buffer and analyzed by SDS-PAGE for determination of cytochrome c and Smac/DIABLO contents in the cytosolic fraction as previously reported (Jazirehi et al., 2003).

Nuclear Extracts Preparation

Nuclear extracts preparations were done as previously described by our laboratory (Garban, et al., 2001b). Briefly, cells (10⁶) were harvested after treatment and washed twice with cold Dulbecco PBS (Cellgro, Herndon, VA). After washing, cells were lysed in 1 ml of NP40 lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM

NaCl, 3 mM MgCl₂, and 0.5% NP40) on ice for 5 min. Samples were centrifuged at 300 × g at 4°C for 5 min. The pellet was washed twice in NP40 buffer. Nuclei were then lysed in nuclear extraction buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and sonicated 10s at 4°C. Both buffers contained the complete protease inhibitor cocktail tablets from Roche (Indianapolis, IN). The protein concentration was determined using the Bio-Rad protein assay. The nuclear proteins were frozen at -80°C.

EMSA

Nuclear proteins (5 µg) were mixed for 30 min at room temperature with Biotin-labeled oligonucleotide probe NF-κB using EMSA Kit Panomics™ (Panomics, Inc. Redwood City, CA) following the manufacturer's instructions (Vega et al., 2003). 10 µl was subjected to denaturing 5% polyacrylamide gel electrophoresis for 90 min in TBE buffer (Bio-Rad Laboratories) and transferred to Nylon membrane Hybond-N+ (Amersham Pharmacia Biotech, Germany) using the Trans-Blot® SD semi-dry Transfer cell System (Bio-Rad, Hercules, CA). The membranes were transferred to a UV Crosslinker FB-UVXL-1000 Fisher technology (Fisher Scientific, NY) for 3 min. The detection was made following the manufacturer's instructions. The membranes were then exposed using Hyperfilm ECL (Amersham Pharmacia Biotech). The oligonucleotide sequences for NF-κB 5'-AGTTGAGGGGACTT TCCCAGGC-3' (Harada et al., 1994). Relative concentrations were assessed by densitometric analysis as mentioned above.

Isobologram Analysis for determination of synergy

To establish whether the cytotoxic effect of the TRAIL/NO combination was more than additive, isobolograms were constructed from treatments combining TRAIL at various concentrations (2.5, 5 and 10 ng/ml) with the NO donor DETANONOate (500 and 1000 µM) as described (Berenbaum, 1978). Combination yielding a cytotoxicity of 30 ± 5% were graphed as a percentage of the concentration of single agent alone that produced this amount of cytotoxicity. Analysis was performed on the basis of the dose-response curves using active caspase-3 analysis for LNCaP, DU145, CL-1 and PC-3 cells treated with TRAIL alone or NO donor alone and the combination for 18h.

Statistical Analysis

The experimental values were expressed as the mean ± SD for the number of separate experiments indicated in each case. One-way ANOVA was used to compare variance within and among different groups. When necessary, Student's *t* test was used for comparison between two groups. Significant differences were considered for probabilities > 5% (*p* > 0.05).

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FIGURE LEGENDS

Figure 1. DETANONOate sensitizes CaP cell lines to TRAIL-mediated apoptosis.

(A) The CaP cell lines DU145, CL-1 and PC-3 were grown in FBS-free medium and LNCaP cells were grown in medium with 1% FBS. The cell lines were treated with different concentrations of TRAIL (0, 2.5, 5 ng/ml) in the presence or absence of DETANONOate (1000 μ M) for 18 h at 37 $^{\circ}$ in a 5% CO₂ incubator. Fixed and permeabilized cells were stained with anti-active-caspase-3-FITC antibody and analyzed by flow cytometry as described in Materials and Methods. The findings reveal that DETANONOate sensitizes the CaP cell lines to TRAIL-mediated apoptosis. The data are the mean of three independent experiments. * p < 0.05, ** p < 0.02, *** p < .004.

(B) This figure establishes synergy as determined by isobologram analysis.

(C) PC-3 cells were grown in FBS-free medium and were treated with TRAIL (5 ng/ml) in the presence or absence of different concentrations of DETANONOate (100, 500, 1000 μ M) for 18 h and analyzed for apoptosis. Significant sensitization was observed at DETANONOate concentrations of 500 μ M and 1000 μ M.

(D) The PC-3 cells were treated with DETANONOate (1000 μ M), TRAIL (2.5 ng/ml), and combination and viable cell recovery was examined microscopically by trypan blue dye exclusion at 24 h. The data show that all agents inhibited cell proliferation.

Figure 2. NF- κ B is involved in TRAIL-mediated apoptosis in PC-3 cells.

(A) **Inhibition of NF- κ B activity.** Nuclear extracts from PC-3 cells grown in FBS-free medium were treated or left untreated with DETANONOate (500 or 1000 μ M) (top panel), or treated with different concentrations of the specific NF- κ B inhibitor Bay 11-7085 (0, 0.5, 1, 2 and 3 μ M) (bottom panel), and were analyzed by EMSA to assess NF- κ B DNA-binding activity. Relative NF- κ B binding activity was determined by densitometry analysis. The findings demonstrate that treatment of PC-3 cells with DETANONOate results in inhibition of NF- κ B activity.

(B) **Immunoprecipitation of S-nitrosylated NF- κ B p50 (S-NO-p50) upon DETANONOate (500, and 1000 μ M-18 h) treatment.** Total cell lysates were used in an immuno-precipitation assay using protein A beads as described in Materials and Methods. S-nitrosylated proteins were precipitated and the membranes were immunoblotted with anti-NF- κ B p50 polyclonal antibody. The results demonstrate that p50 was S-nitrosylated. The findings are representative of 2 independent experiments.

(C) **Sensitization of PC-3 to TRAIL-apoptosis by inhibition of NF- κ B.** PC-3 cells were treated with TRAIL (2.5 and 5.0 ng/ml) in the presence or absence of various concentrations of Bay11-7085 and apoptosis was assessed. The findings demonstrated that Bay11-7085 sensitizes PC-3 cells to TRAIL-mediated apoptosis. * p < 0.05, ** p < 0.02, *** p < 0.002.

Figure 3. DETANONOate-mediated downregulation of Bcl-x_L expression and sensitization to TRAIL-mediated apoptosis.

(A) PC-3 cells were grown in serum-free medium and the cells were treated or not treated for 18 h with DETANONOate (1000 μ M), TRAIL (2.5 ng/ml), or the combination. Total cellular protein was extracted and separated by SDS-PAGE and transferred onto nitrocellulose membranes as described in Methods. DETANONOate selectively downregulated Bcl-x_L expression. Treatment of PC-3 with different concentrations of the NF- κ B inhibitor Bay11-7085 resulted in inhibition of Bcl-x_L expression

(B) PC-3 cells were treated with different concentrations of the Bcl-x_L inhibitor 2MAM-A3 for 5 h and then treated with TRAIL (2.5 μ g/ml) for 18 h and analyzed for apoptosis. The data show that 2MAM-A3 sensitizes PC-3 to TRAIL apoptosis. *p = 0.036, **p < 0.02.

Figure 4. Inhibition of Bcl-x_L transcription by DETANONOate.

A Bcl-x_L promoter fragment spanning -640 to -9 relative to the transcriptional start site (Bcl-x_L WT promoter) and another fragment missing the NF- κ B binding sequence (Bcl-x_L κ B-promoter) were cloned into the pGL2-Basic luciferase reporter vector (Lee et al., 1999). PC-3 cells were transfected with 20 μ g of the indicated reporter plasmid and then treated with the specific NF- κ B inhibitor Bay11-7085 (2 or 3 μ M), DETANONOate (500 or 1000 μ M) or TNF- α (50 or 100 U/ml). The samples were harvested 18 h after treatment and assessed for luciferase activity. The data show that DETANONOate inhibits Bcl-x_L transcription by inhibition of luciferase activity. The data are representative of 2 experiments. *p = 0.031, **p < 0.02.

Figure 5. Mitochondrial membrane depolarization, release of cytochrome c and Smac/DIABLO into the cytosol, and activation of caspases 8 and 9.

(A) **Mitochondrial membrane activation.** PC-3 cells were grown in FBS-free medium and treated or left untreated for 18 h with DETANONOate (1000 μ M), TRAIL (2.5 ng/ml) or the combination. The PC-3 cells were then stained with DiOC6 and then analyzed by flow cytometry. The findings demonstrate that DETANONOate, TRAIL, and the combination induce significant mitochondrial depolarization. The data represent the mean fluorescence intensity (MFI), and are the mean of three independent experiments. *p < 0.05, medium vs. cells treated.

(B) **Release of cytochrome c and Smac/DIABLO.** PC-3 cells were grown in FBS-free medium and were treated or left untreated for 18 h with DETANONOate (1000 μ M), TRAIL (2.5 ng/ml) or the combination. Total cellular protein was extracted from the culture. The purified fraction of cytosolic protein was separated by SDS-PAGE and transferred onto the nitrocellulose membrane as described in Materials and Methods. The membrane was stained with polyclonal anti-human cytochrome c antibody (top panel), or anti-Smac/DIABLO antibody (bottom panel). The blots represent one of two separate experiments. The data show that DETANONOate and TRAIL induce some release of both cytochrome c and Smac/DIABLO and the

combination releases higher levels. The relative cytochrome c and Smac/DIABLO expression was determined by densitometric analysis of the blot. * $p < 0.05$, ** $p < 0.03$, *** $p < 0.002$ medium vs. cells treated.

(C) Activation of caspase 8 and caspase 9. PC-3 cells were treated as described above. The activation of caspases 8 and 9 was determined by Western. There was some activation of caspase 8 by DETANONOate and some activation of caspase 9 by TRAIL. However, the combination resulted in significant activation of both caspases.

Figure 6. Two-signal model for sensitization of CaP cells to TRAIL-induced apoptosis by DETANONOate and TRAIL

This figure schematically demonstrates that treatment of PC-3 cells with DETANONOate and TRAIL results in apoptosis and synergy is achieved. The synergy is the result of complementation in which each agent activates partially the apoptotic pathway and the combination results in apoptosis. Signal 1 is provided by DETANONOate which partially inhibits NF- κ B activity and this leads to downregulation of Bcl-_{xL} transcription. In addition, DETANONOate also partially activates the mitochondria and release of modest amounts of cytochrome c and Smac/DIABLO into the cytosol in the absence of downstream activation of caspase 9. Signal 2 is provided by TRAIL which also partially activates the mitochondria with some release of cytochrome c and Smac/DIABLO in the absence of caspase 9 activation. However, the combination treatment results in significant activation of the mitochondria and release of high levels of cytochrome c and Smac/DIABLO, activation of caspase 9 and 3, resulting in apoptosis. The two-signal model is corroborated by the use of specific inhibitors in which inhibition of NF- κ B by Bay11-7085 was sufficient to sensitize the CaP cells to TRAIL-induced apoptosis concomitant with downregulation of Bcl-_{xL} expression. The role of Bcl-_{xL} in the regulation of TRAIL apoptosis was corroborated by the use of the chemical inhibitor 2MAM-A3 which also sensitized the cells to apoptosis.

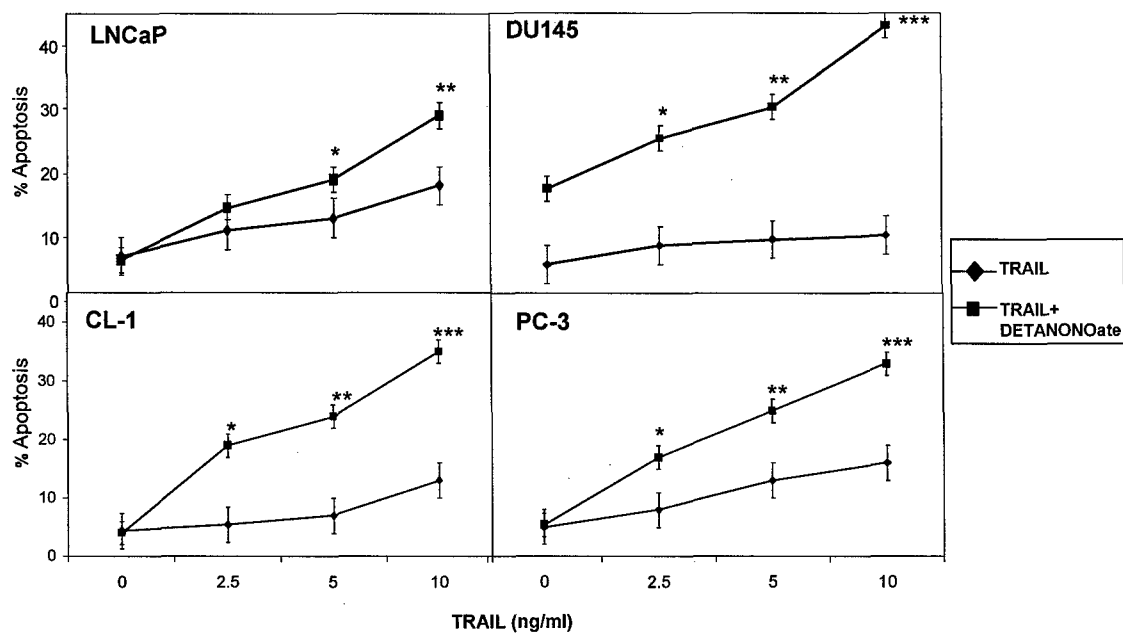


Figure 1(A)

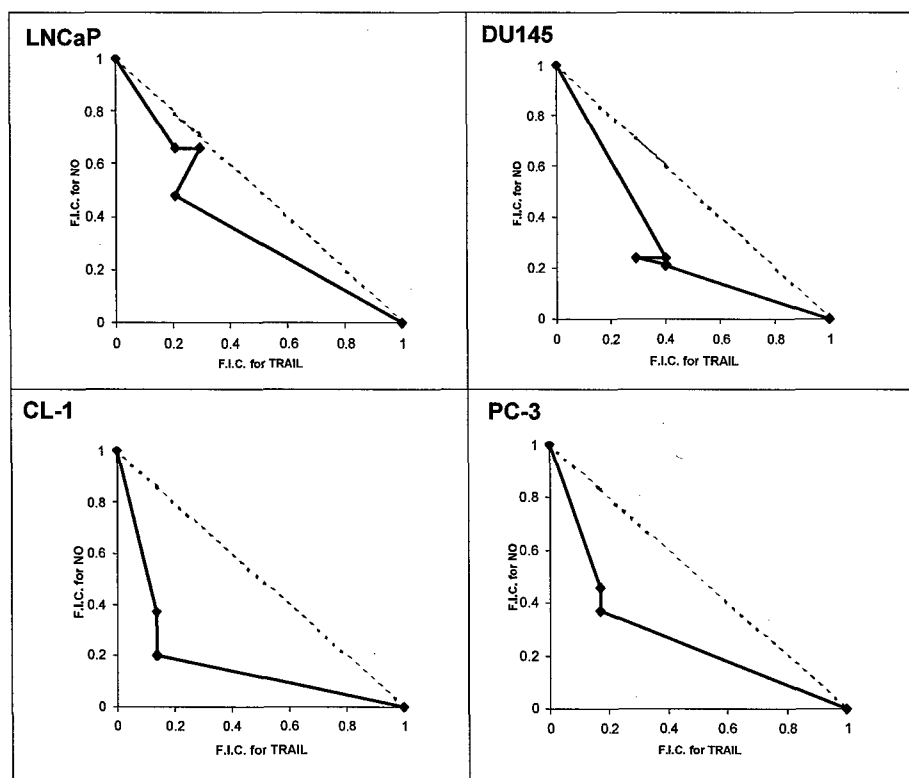


Figure 1(B)

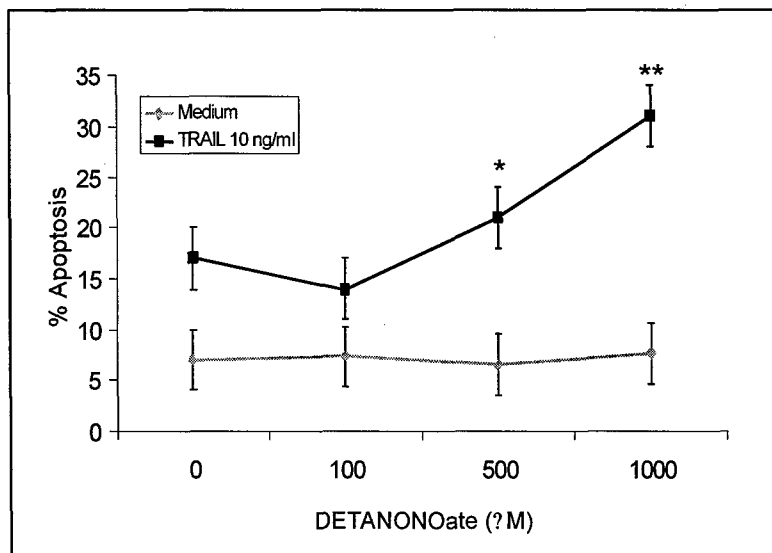


Figure 1(C)

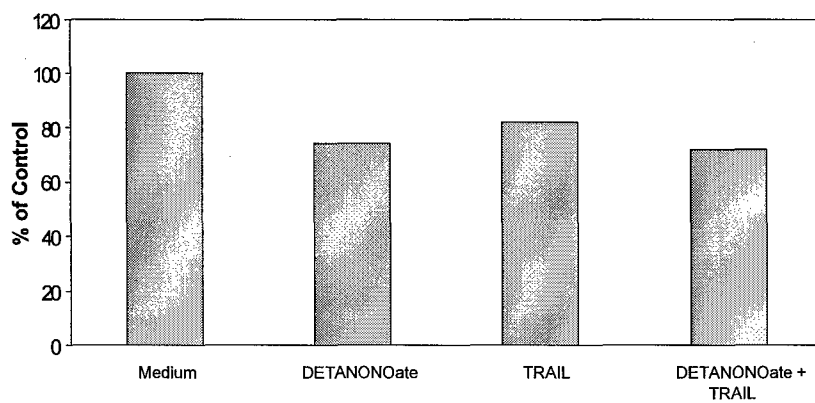


Figure 1(d)

Table I. DHT sensitizes LNCaP to TRAIL-mediated apoptosis

DHT (nM)	TRAIL (ng/ml)		
	0	5	10
0	5.1 ± 1	12 ± 2.1	17 ± 3.8
10	6.6 ± 0.9	18 ± 1.1	24 ± 5.1*
20	6.9 ± 1.1	23 ± 6.1*	30.6 ± 6.3**

Table 1. LNCaP cells were treated or left untreated with DHT (10 or 20 nM/ml) for 24 h and then treated with recombinant TRAIL (5 or 10 ng/ml) for 18 h. The cells were harvested and apoptosis was determined for active caspase-3 staining by flow. The data show that DHT sensitizes LNCaP to TRAIL-mediated apoptosis. The data represent the mean of two independent experiments. *p<0.04, **p<0.02 compared with the cells treated with DHT alone.

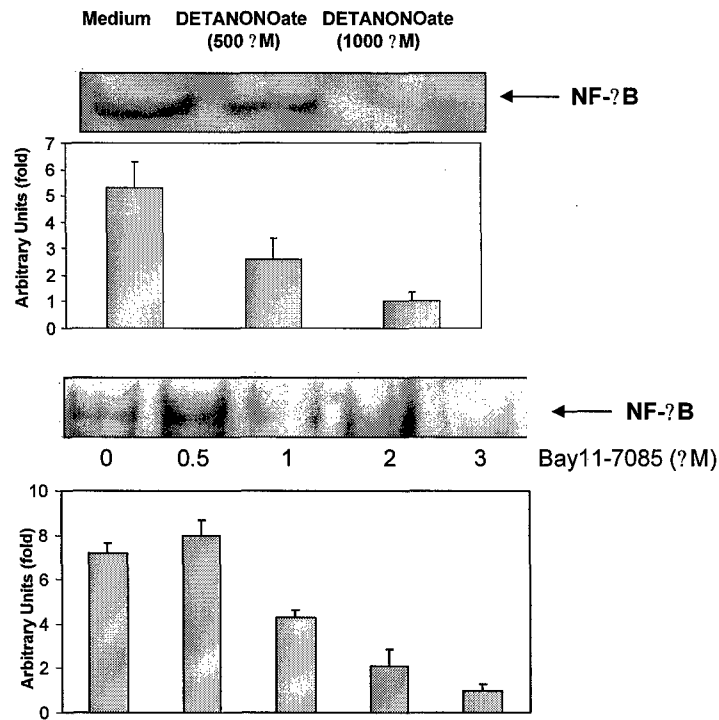


Figure 2 (A)

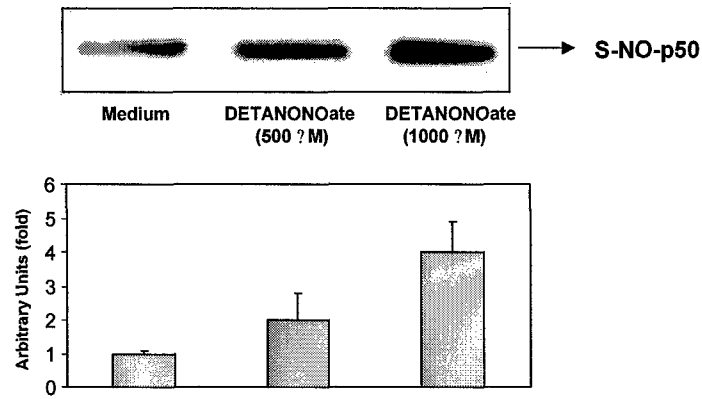


Figure 2 (B)

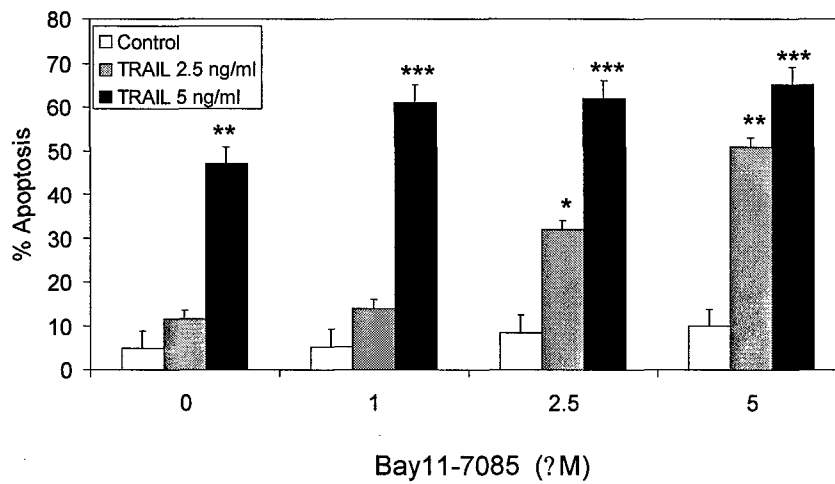


Figure 2 (C)

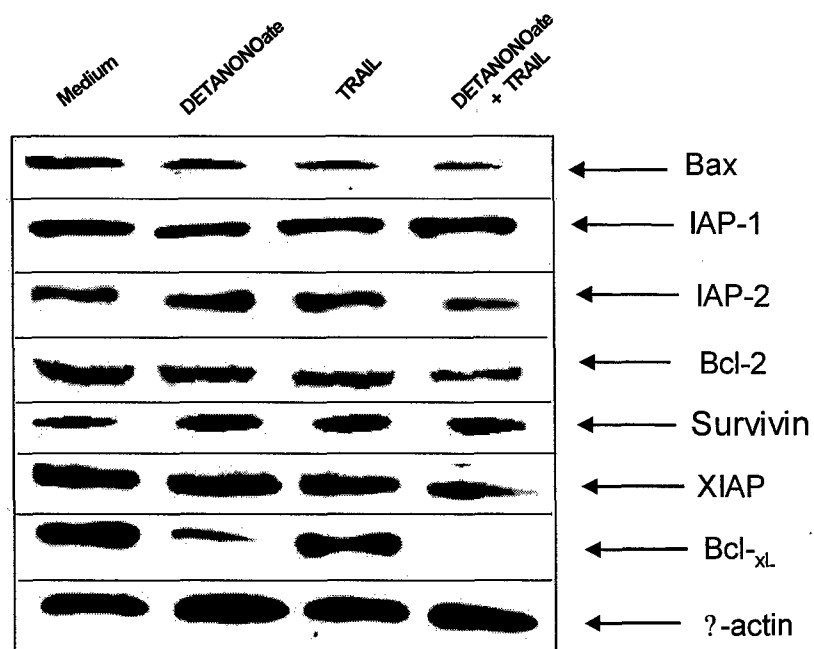


Figure 3 (A)

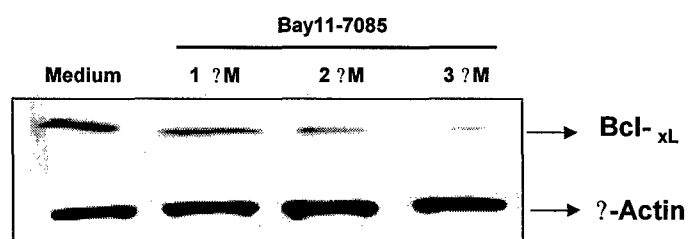


Figure 3 (B)

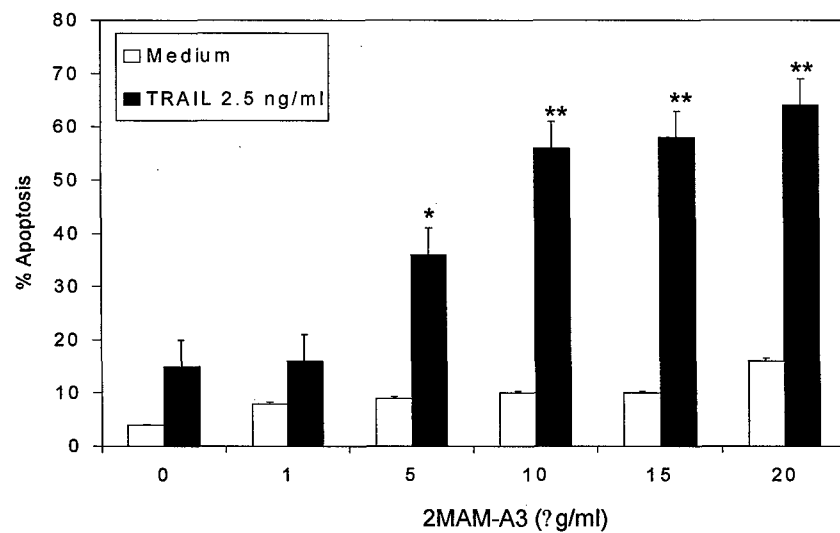
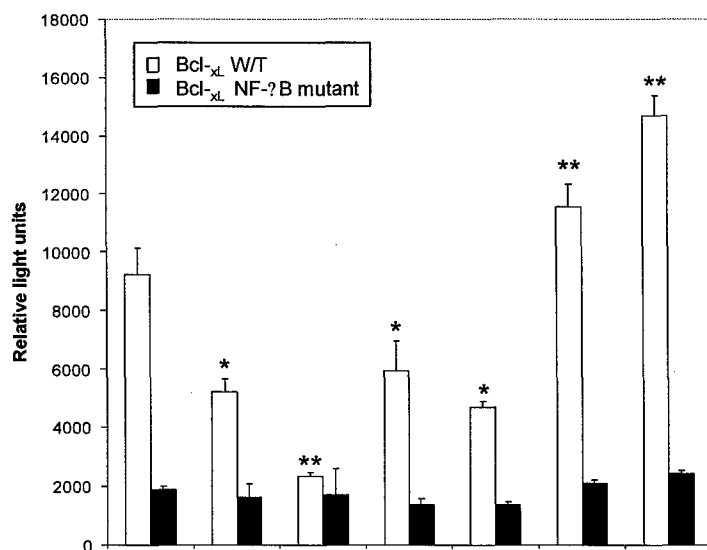


Figure 3 (C)



Bay11-7085 2 μM	—	+	—	—	—	—
Bay11-7085 3 μM	—	—	+	—	—	—
DETANONOate 500 μM	—	—	—	+	—	—
DETANONOate 1000 μM	—	—	—	—	+	—
TNFα 50 U/ml	—	—	—	—	—	+
TNFα 100 U/ml	—	—	—	—	—	—

Figure 4

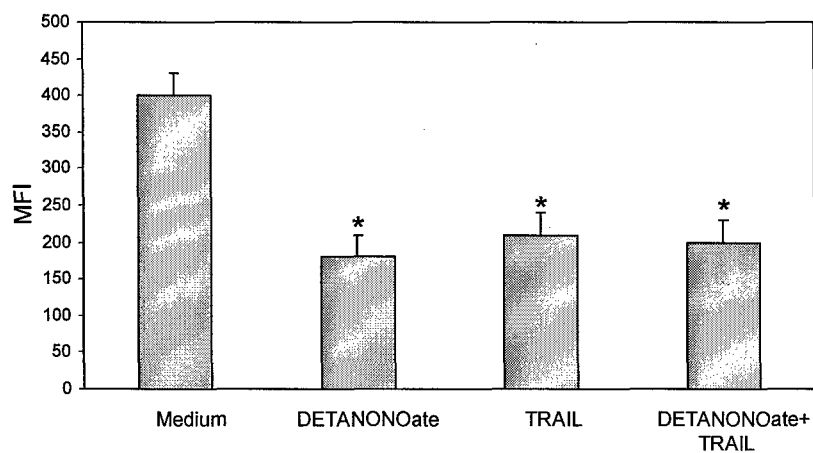


Figure 5 (A)

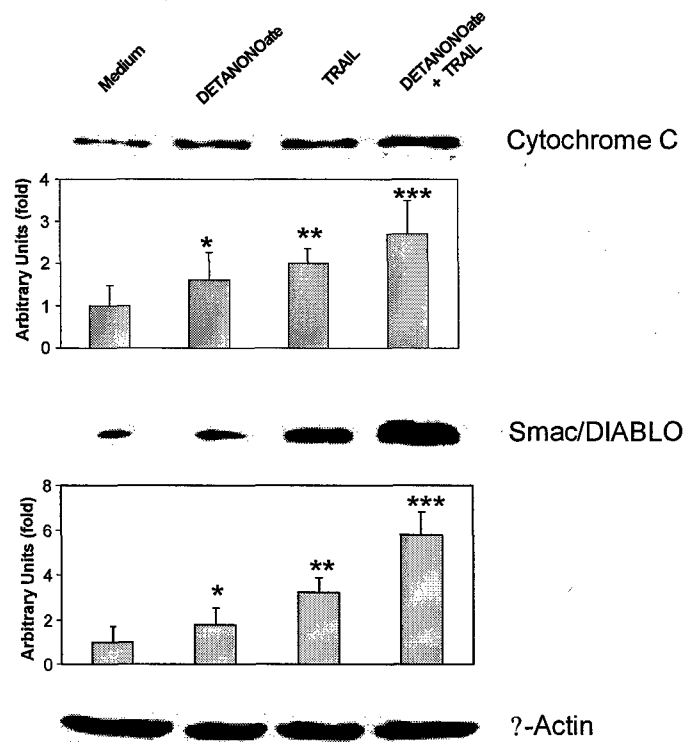


Figure 5 (B)

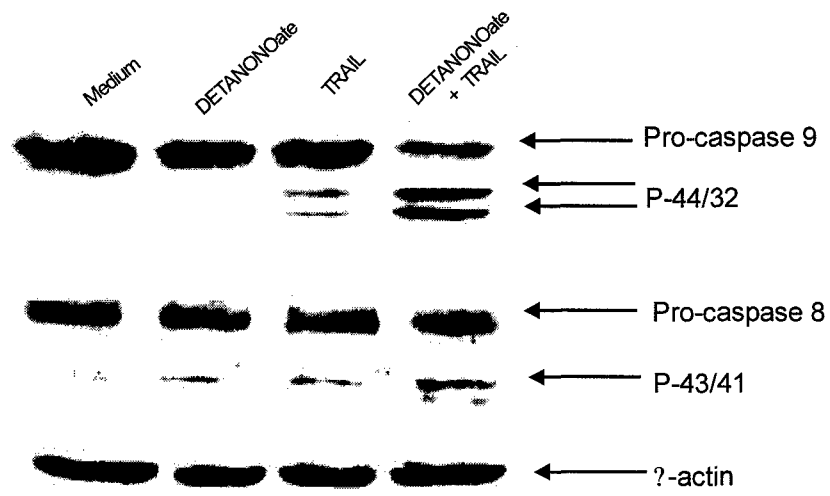


Figure 5 (C)

APPENDIX 4

The transcription repressor YY1 negatively regulates DR5 expression and controls cancer cells resistance to TRAIL-induced apoptosis: Reversal of resistance by inhibitors of YY1

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Running Title: **Negative regulation of DR5 transcription by YY1**

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Abbreviations

CaP: prostate cancer

DETANONOate: (Z)-1-[2-(2-aminoethyl)-N-(2-ammonio-ethyl)amino]diazen-1-ium-1, 2-diolate

DHMEQ- dehydroxymethylepoxyquinomicin

DHT: 5- α dihydrotestosterone

DR: death receptor

DTT: 1,4-dithiothreitol

EDTA: ethylenediaminetetraacetic acid

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

NF- κ B: nuclear factor κ B

NO: nitric oxide

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

RIPA: radioimmunoprecipitation assay (buffer)

SDS: sodium dodecyl sulfate

TPA: 12-O-tetradecanoylphorbolacetate

TRAIL: tumor necrosis factor-related apoptosis-inducing ligand.

GAPDH: glyceraldehydes-3-phosphate dehydrogenase

SiRNA: small interfering RNA.

ABSTRACT

Most tumors are resistant to TRAIL and need to be sensitized to undergo apoptosis. We have recently reported that TRAIL-resistant human prostate carcinoma cell lines can be sensitized by various NF- κ B inhibitors (Huerta-Yepez *et al.*, 2004), and sensitization correlated with upregulation of DR5 expression. We hypothesized that a gene product(s) regulated by NF- κ B with DR5 repressor activity may be responsible for the DR5 regulation. Inhibition of NF- κ B activity resulted in significant upregulation of DR5 expression and

sensitized prostate tumor cells to TRAIL-mediated apoptosis and synergy is achieved. Treatment of PC-3 cells with NO inhibited both NF- κ B and YY1 DNA-binding activity and also inhibited YY1 expression. Treatment of PC-3 cells with YY1 siRNA resulted in upregulation of DR5 expression and sensitization to TRAIL-induced apoptosis. The direct role of YY1 in the regulation of DR5 expression was examined in an DR5 luciferase reporter system (pDR5). Two constructs were generated, the pDR5/-605 construct with a deletion in the promoter region containing the putative YY1 DNA-binding region (-1224 to -605) and a construct pDR5-YY1 with a mutation of the YY1 DNA-binding site. Transfection of PC-3 cells with these two constructs resulted in significant (3-fold) augmentation of luciferase activity over baseline suggesting the repressor activity of YY1. The present findings demonstrate that YY1 negatively regulates DR5 transcription and expression and hence, regulates resistance to TRAIL-induced apoptosis. Inhibitors of YY1 expression and/or activity in combination with TRAIL may be useful in the treatment of TRAIL-resistant tumor cells.

INTRODUCTION

Conventional anti-tumor therapies consist primarily of chemotherapy, radiation, hormonal therapy and immunotherapy. Such treatments result in significant clinical responses. However, many patients experience relapses and the tumors become refractory to such therapeutics. Alternative therapies have been considered that include immunotherapy, both antibody and cell-mediated, with potential anti-tumor activity (Martinet *et al.*, 2002; Xu *et al.*, 2004; Senba *et al.*, 1998). Antibody-mediated therapies have been applied clinically in the treatment of lymphoid and non-lymphoid tumors (Blattman and Greenberg, 2004; Robak, 2004; Murillo *et al.*, 2003). There is considerable effort to generate anti-tumor CTL in an effort to overcome drug resistance (Chung *et al.*, 2004; Dermime *et al.*, 2004). Since cytotoxic lymphocytes and cytotoxic antibodies mediate their killing by various mechanisms, including the TNF- α family (TNF- α , Fas ligand and TRAIL) (Shresta *et al.*, 1998), several studies have also considered such soluble recombinant ligands or agonist antibodies for anti-tumor therapies (Shresta *et al.*, 1998). TNF- α and Fas ligand have been shown to be toxic to normal tissues, however, TRAIL is minimally toxic to normal tissues and is selectively toxic to transformed tumor cells (Walczak *et al.*, 1999; Ashkenazi and Dixit, 1999; Lawrence *et al.*, 2001). TRAIL is a type II transmembrane protein of the

TNF- α family (Wiley *et al.*, 1995) and forms homotrimers that bind three receptor molecules (Hymowitz *et al.*, 1999). Functional studies showed that this ligand triggers apoptosis in a variety of tumor cell lines but not most normal cells, implicating its potential therapeutic application in cancer treatment (Schmaltz *et al.*, 2002; Sayers *et al.*, 2003; Ashkenazi and Dixit, 1999). TRAIL induces apoptosis through interaction with its receptors. Four homologous human receptors for TRAIL have been identified, including: DR4, DR5, DCR1, DCR2 as well as a fifth soluble receptor called osteoprotegerin (OPG) (MacFarlane *et al.*, 1997; Walczak *et al.*, 1997; Pan *et al.*, 1997; Sheridan *et al.*, 1997). Both the death receptors DR4 and DR5 contain a conserved death domain (DD) motif and signal apoptosis. The other two receptors appear to act as decoys as they can inhibit TRAIL-induced apoptosis when overexpressed. Studies *in vitro* and *in vivo* have demonstrated that TRAIL exerts anti-tumor activity (Walczak *et al.*, 1999). Recently, agonist antibodies against TRAIL receptors DR4 and DR5 are being clinically tested in humans (Buchsbaum *et al.*, 2003). Although many tumors are sensitive to TRAIL-mediated apoptosis, the majority, however, are resistant. Resistance can be overcome by the use of sensitizing agents that modify the apoptosis signaling pathways, and thus facilitating the apoptotic effect of TRAIL. Several sensitizing agents have been reported by us as well as others in a variety of tumor cell models (Yamanaka *et al.*, 2000; Ng *et al.*, 2002; Jazirehi *et al.*, 2001; Huerta-Yepez *et al.*, 2004; Tillman *et al.*, 2003; Schmelz *et al.*, 2004). These studies revealed that the development of tumor cell resistance to TRAIL is multi-factorial and is dependent on the cell tumor system used.

Previous findings indicated that normal tissue resistance to TRAIL might have been due to the expression of decoy receptors DR1 and DR2 and these compete with the death receptors DR4 and DR5 (Sheridan *et al.*, 1997). However, further studies indicated that this paradigm is not generalized and that many tumor cells express both decoy and death receptors and that other mechanisms, like the downstream signaling events of the receptors, may be involved in the regulation of resistance and sensitivity to TRAIL (Aggarwal *et al.*, 2004; LeBlanc and Ashkenazi, 2003).

Several studies have revealed that several sensitizing agents upregulate DR5 and DR4 expression which correlated with sensitivity to TRAIL (Shigeno *et al.*, 2003; LaVallee *et al.*, 2003; Johnston *et al.*, 2003). However, the mechanisms by which the receptors are upregulated by these agents and the regulation of DR5

expression in TRAIL-resistant cells have not been studied. The regulation of DR5 expression has been reported by using a pDR5-reported system. In this study, the authors have demonstrated that SP1 is a major transcription factor that regulates DR5 expression (Yoshida *et al.*, 2001).

YY1 is a 414 amino acid Kruppel-related zinc transcription factor that binds to the CG (A/CC) CATNTT consensus DNA element located in promoters and enhancers of many cellular and virus genes. YY1 physically interacts with and recruits histone-acetyl-transferase, histone-deacetylase and histone-methyl-transferase enzymes to the chromatin and may thus direct histone-acetylation, deacetylation and methylation at YY1 activated or repressed promoters (Coull *et al.*, 2000). In previous findings, we have reported that Fas expression is negatively regulated by the transcription repressor Yin Yang 1 (YY1) through binding of YY1 to the silencer region of the Fas promoter (Garban and Bonavida, 2001). We thereby examined the DR5 promoter region and identified a putative YY1 binding site (-804 to -794 bp) (Yoshida *et al.*, 2001). Thus, we hypothesized that the upregulation of DR5 by certain sensitizing agents, such as the nitric oxide donor DETANONOate, to TRAIL-induced apoptosis (Huerta-Yepez *et al.*, 2004) may be due to inhibition of both NF- κ B and YY1 activities. This hypothesis was tested in this study and the followings were examined: (1) Does treatment of prostate cancer cells with DETANONOate sensitize the tumor cells to TRAIL-induced apoptosis? (2) Does DETANONOate treatment upregulate DR5 expression? (3) Does DETANONOate inhibit NF- κ B and YY1 DNA-binding activities? (4) Does YY1 negatively regulate DR5 transcription and expression as determined by a) treatment of tumor cells with YY1 siRNA and b) by deletion of a YY1 containing region in the DR5 promoter or mutation of the YY1 binding site in the promoter, and (5) does inhibition of YY1 activity by siRNA sensitize the cells TRAIL-induced apoptosis?

RESULTS

Mechanisms by which DETANONOate sensitize CaP cells to TRAIL-induced apoptosis

We have recently reported that the NO donor DETANONOate sensitizes tumor cells to TRAIL-induced apoptosis (Huerta-Yepez *et al.*, 2004), though the exact mechanism is not known. This study investigates the biochemical mechanism of sensitization of CaP cells to TRAIL apoptosis. Four CaP cell lines, namely, the androgen-dependent LNCaP and the androgen-independent PC-3, CL-1 and DU-145, were treated with pre-

determined optimal concentrations of TRAIL (5 ng/ml), DETANONOate (1000 μ M) or combination for 18 h. The cells were then analyzed for apoptosis by flow cytometry for the presence of activated caspase 3 as described in methods. The findings demonstrate that all 4 cell lines were relatively resistant to treatment with TRAIL or DETANONOate as single agents, whereas the combination resulted in significant potentiation of apoptosis in all 4 cell lines (Figure 1A). The potentiation of apoptosis was synergistic as determined by isobologram analysis (Figure 1B). The PC-3 cell line was selected as representative for the subsequent experiments.

A. Upregulation of DR5 expression by DETANONOate

Several reports demonstrated that sensitization of tumor cells to TRAIL-induced apoptosis by various agents correlated with the upregulation of DR5 expression (Wang and El-Deiry, 2003(a); Shigeno *et al.*, 2003; LaVallee *et al.*, 2003; Nakata *et al.*, 2004). Hence, we examined whether DETANONOate-induced sensitization to TRAIL-induced apoptosis also correlated with the upregulation of DR5 expression in CaP cells. PC-3 cells were treated with DETANONOate (1000 μ M for 18 h) and the cells were examined for DR5 expression. Treatment with DETANONOate significantly upregulated surface DR5 expression as determined by flow cytometry as there was a significant increase in the mean fluorescence intensity (MFI) (Figure 2A). RT-PCR analysis demonstrated a significant increase of DR5 transcription by DETANONOate compared to control GAPDH (Figure 2B Top Panel). In addition, DR5 total protein expression was significantly increased by DETANONOate as determined by western (Figure 2B Bottom panel). Altogether, these findings demonstrate that DETANONOate upregulates both surface and total DR5 protein expression.

B. Inhibition of NF- κ B activity and both YY1 activity and expression by DETANONOate

Our recent findings have demonstrated that NO inhibits NF- κ B activity in PC-3 cells (Huerta-Yepez *et al.*, 2004). The transcription repressor YY1 negatively regulates Fas expression through its binding to the silencer region of the Fas promoter (Garban and Bonavida, 2001). By analogy, we examined the DR5 promoter (Yoshida *et al.*, 2001) and detected a putative YY1 DNA-binding site. We postulated that inhibition of NF- κ B by DETANONOate will also inhibit YY1 repressor activity and will result in the upregulation of DR5. We analyzed the effect of DETANONOate on NF- κ B and YY1 DNA-binding activities. Treatment of PC-3 cells

with DETANONOate significantly inhibited NF- κ B DNA-binding activity as determined by EMSA. The inhibition by 1000 μ M DETANONOate was more significant than that by 500 μ M DETANONOate (Figure 3A). Similarly, DETANONOate inhibited YY1 DNA-binding activity and the inhibition was also dependent on the concentration of DETANONOate used (Figure 3B Top Panel). In addition, treatment with DETANONOate inhibited YY1 expression as determined by western (Figure 3B Bottom Panel). These findings demonstrate that DETANONOate inhibits both NF- κ B activity and YY1 DNA-binding activity and suggests that inhibition of YY1 by DETANONOate may be responsible, in part, for the observed (Figure 2) upregulation of DR5 expression.

REGULATION OF DR5 EXPRESSION BY YY1

The above finding suggested that YY1 may be involved in the negative regulation of DR5 transcription. Experiments were designed to directly demonstrate the role of YY1 in the regulation of DR5 expression and sensitivity to TRAIL-apoptosis. We first examined the effect of YY1 siRNA. Transfection of PC-3 cells with YY1 siRNA, but not with control siRNA, resulted in inhibition of YY1 transcription (Figure 4A), significant upregulation of DR5 surface expression (Figure 4B) and the cells were significantly more sensitive to TRAIL-induced apoptosis compared to cells transfected with siRNA negative control or untreated control cells (Figure 4C). These findings demonstrate that YY1 plays a role in both the negative regulation of DR5 expression and also in tumor cell resistance to TRAIL-induced apoptosis.

The direct role of YY1 in DR5 transcription was examined using a DR5 luciferase reporter system, pDR5 (Yoshida *et al.*, 2001). PC-3 cells transfected with the full-length pDR5 promoter showed baseline luciferase activity (Figure 5). In order to determine if YY1 repressor activity is involved in DR5 transcription, we used a pDR5 construct in which the putative YY1 DNA-binding region (-804 to -794 bp) was deleted (pDR5/-605) as described in methods. PC-3 cells transfected with pDR5/-605 resulted in upregulation (3 fold increase) of luciferase activity compared to cells transfected with pDR5 (Figure 5A) suggesting that the deleted YY1-conforming region was responsible for inhibition of transcription. To directly show that YY1 is responsible for the negative transcriptional regulation of DR5, we prepared a construct of the DR5 reporter system in which the YY1 DNA-binding site was mutated as described in methods. Cells transfected with the pDR5-YY1 mutant showed a significant increase in luciferase activity compared to cells transfected

with the wild type reporter pDR5 (Figure 5A) and the luciferase activity was comparable to cells transfected with pDR5/-605.

The role of NF- κ B in the regulation of DR5 expression via YY1 was corroborated by the use of the NF- κ B inhibitor DHMEQ (Ariga *et al.*, 2002). Treatment of PC-3 cells transfected with pDR5 with DHMEQ resulted in augmentation of luciferase activity compared to untreated cells (Figure 5B). The PC-3 cells transfected with pDR5- YY1 mutant showed significant augmentation of luciferase activity as compared to pDR5 transfected cells, and treatment with DHMEQ did not have any additional effect (Figure 5B). These findings suggest that DHMEQ inhibition of NF- κ B resulted in significant inhibition of YY1 and the regulation of DR5 by NF- κ B was primarily due to YY1 repressor activity. These findings demonstrate that YY1 negatively regulates DR5 transcription and that YY1 is the dominant transcription repressor factor in the pDR5/-605 construct.

Discussion

This study presents evidence for the first time for the role of the transcription repressor YY1 in the regulation of DR5 expression in tumor cells and its role in the resistance to TRAIL-mediated apoptosis. Prostate cancer cell lines, used as model system, are resistant to TRAIL-induced apoptosis. However, these cell lines become sensitive to TRAIL following treatment of the cells with inhibitors of YY1 expression and/or activity. Inhibition of YY1 resulted in the upregulation of DR5 expression and sensitization of the cells to TRAIL-induced apoptosis. Inhibition of YY1 by DETANONOate or by YY1 siRNA in PC-3 cells resulted in upregulation of DR5 expression and sensitization to TRAIL-induced apoptosis. The direct role of YY1 in the negative regulation of DR5 expression was demonstrated by using a pDR5 reporter system in which the YY1 binding site was either deleted or mutated and transfectants resulted in significant augmentation of luciferase activity over baseline of cells transfected with the full promoter. These findings suggest that tumor cells can escape killing by TRAIL via constitutive overexpression of YY1, which in turn negatively regulates DR5 expression and sensitivity to TRAIL. These findings also suggest that agents that can inhibit YY1 transcription, expression or activity may be suitable for their use in the treatment of TRAIL-resistant tumor cells when used in combination with TRAIL or an agonist DR5 antibody.

TRAIL selectively induces apoptosis in a variety of TRAIL sensitive tumor cells and it has been shown not to be cytotoxic to the majority of normal tissues (Ashkenazi *et al.*, 1999). Therefore, TRAIL and agonist antibody to DR5 are

currently being examined clinically *in vivo* as potential cancer therapeutics. The apoptotic anti-DR5 monoclonal antibody is a promising agent for cancer treatment (Ichikawa *et al.*, 2001; Ohtsuka *et al.*, 2003). These strategies are based on the expression of functional death receptors on cancer cells. However, many cancer cells are resistant to TRAIL due to dysregulation of the apoptotic signaling pathways. For example, resistance to TRAIL was shown in neuroblastoma cells to be due to the lack of expression of caspase 8 and caspase 10. In addition, there was dysregulation of signal adaptors and activation of inhibitory molecules (Eggert *et al.*, 2000). There was a correlation between levels of the cellular FLICE inhibitory protein (c-FLIPs), with sequence homology to caspases 8 and 10, and TRAIL resistance (Griffith *et al.*, 1998; Kim *et al.*, 2000). Bax inactivation in MMR deficient tumors caused resistance to TRAIL (Burns and El-Deiry, 2001; LeBlanc *et al.*, 2002). Levels of Smac/DIABLO also conferred resistance to TRAIL (Ng and Bonavida, 2002; Fulda *et al.*, 2002). Therefore, agents that can sensitize tumor cells to TRAIL apoptosis are sought. Overexpression of DR5 in TRAIL resistant tumor cells restores TRAIL sensitivity (Yeh *et al.*, 1998; Kuang *et al.*, 2000; Mitsiades *et al.*, 2001). DR5 expression in a number of JURKAT clones was highly correlated with sensitivity to TRAIL (Jang *et al.*, 2003). These findings provide evidence for a strategy to induce DR5 expression in order to enhance the susceptibility of cancer cells to TRAIL or anti-DR5 monoclonal antibody-induced apoptosis. Our findings here support this contention and demonstrate that inhibition of YY1 resulted in upregulation of DR5 expression and sensitization to TRAIL apoptosis.

It is not clear why certain tumors express low levels of DR5 and what regulates DR5 expression in cancer cells. The low expression of DR5 in tumor cells and its upregulation by inhibitors of NF- κ B prompted us to examine the possible role of transcription repressors under the regulation of NF- κ B that result in the upregulation of DR5 expression. Previous findings demonstrated that Fas expression was under the negative regulation of NF- κ B via a transcription repressor YY1 (Garban and Bonavida, 2001). We examined the possible role of YY1 in the negative regulation of DR5 following transfection of PC-3 with the DR5 promoter, which has a putative YY1 DNA-binding site. Our findings implicate the role of YY1 in the regulation of DR5 by various lines of evidence. We demonstrate that treatment of CaP cells with the NO donor DETANONOate, which we have reported to inhibit both NF- κ B and YY1 DNA-binding activity (Huerta-Yepez *et al.*, 2004; Hongo *et al.*, unpublished), resulted in upregulation of DR5 expression and sensitization of the cells to TRAIL-induced apoptosis. The direct role of YY1 in the negative regulation of DR5 was shown in experiments in which cells transfected with YY1 siRNA, but not with control siRNA, resulted in upregulation of DR5 and sensitization to TRAIL. Further, transfection of PC-3 cells with a luciferase DR5 reporter system in which a deletion in the promoter that contains the YY1 binding site (pDR5/-605) or direct mutation of the YY1 site (pDR5-YY1 mutant), resulted in significant augmentation of luciferase activity over baseline activity in cells transfected with the wildtype

reporter pDR5. DR5 transcription has been examined by Yoshida *et al.*, (2001) using a reporter system for human DR5 and demonstrated that transient transfection with several 5 prime deletion mutants identified the minimal promoter element spanning -198 to -116. Two SP1 sites were found responsible for the basal transcription activity of the DR5 gene promoter. Our results here demonstrate that DR5 expression can be negatively regulated by YY1. In addition to YY1, other mechanisms in the negative regulation of DR5 may also be involved. Recently, Nakata *et al.*, 2004 reported that histone deacetylase inhibitors upregulate DR5 expression. HDACs activated DR5 transcription through its promoter activation in a p53 independent manner. Also, HDAC inhibitors sensitized the cells to TRAIL-induced apoptosis. HDAC's upregulate transcription of certain genes through the inhibition of HPAG and subsequent changes in the chromatin structure (Kouzarides, 1999; Strahl and Allis, 2000).

Several inducers of DR5 have been reported. For example, p53 has been reported to trans-activate DR5 gene expression (Wu *et al.*, 1997, 1999; Takimoto and El-Deiry, 2000). In addition, genotoxic agents like doxorubicin, etoposide, gamma radiation also induce expression of DR5 in a p53-dependent or independent manner (Sheikh *et al.*, 1998; Gibson *et al.*, 2000; Gong and Almasan, 2000; Nagane *et al.*, 2000; Wen *et al.*, 2000). Preliminary findings demonstrate that CDDP-induced upregulation of DR5 expression in CaP cells is due, in part, to inhibition of YY1 activity (Baritaki *et al.*, unpublished). Dexamethasone and interferon gamma induce apoptosis and DR5 expression in cell lines with mutant p53 (Meng and El-Deiry, 2001). Other agents such as sulindac-sulfide (Huang *et al.*, 2001; Tang *et al.*, 2002; He *et al.*, 2002) and 2-methoxy-estradiol (LaVallee *et al.*, 2003) have been reported to be strong inducers of DR5. However, the mechanisms of these inducers are poorly understood. It will be of interest to determine whether YY1 is implicated in some of these above studies.

The negative regulation of DR5 by YY1 reported here is reminiscent of other studies in which YY1 negatively regulates gene transcription. Recently, Sui *et al.*, 2004 reported that loss of YY1 resulted in a significant increase in the level p53. The augmentation of p53 by ablation of YY1 resulted in the induction of p53 ubiquitination *in vivo*. In that study, the function of YY1 is independent of its transcription activity. YY1 activates and represses transcription by the interaction with cellular transcription factors, namely TVP, TAF, TF2B and SP1 (Austen *et al.*, 1997; Seto *et al.*, 1993; Lee *et al.*, 1993; Chiang and Roeder, 1995; Usheva and Shenk, 1994).

The NF- κ B transcription factor family consists of several structurally-related proteins such as c-Rel, Rel-A, Rel-B, p50/p105, p52/p100 which form homo or heterodimers with each other and regulate the expression of a number of genes (Barkett and Gilmore, 1999). Shigeno *et al.*, (2003) show that the hut-7 cells exhibited binding activity of NF- κ B composed of a p50 homodimer without any stimulation and TRAIL-induced inhibition of NF- κ B binding by Rel-A/p50

heterodimers. Rel-A/p50 appears to play a role in resistance to TRAIL (Ravi *et al.*, 2001). Interferon- γ pre-treatment inhibits Rel-A/p50 NF- κ B in cells sensitized to TRAIL-induced apoptosis. The mechanism of interferon-induced upregulation of DR5 and inhibition of c-Rel-A-p50 NF- κ B activity was not examined at the transcription level. It is possible that c-Rel-A-p50 regulates YY1 expression and its inhibition by interferon- γ or by DETANONOate, shown to inhibit p50, may explain the upregulation of DR5. The role of NF- κ B in the regulation of DR5 expression and TRAIL-induced apoptosis was reported by Ravi *et al.*, (2001). These studies show that NF- κ B induced the expression of both death receptors DR4 and DR5. The c-Rel subunit of NF- κ B transcription factor induces expression of DR4 and DR5. Conversely, a trans-dominant mutant of the inhibitory protein I κ B- α , or a transactivation deficient mutant of c-Rel reduces expression of either death receptor. These studies, however, did not address directly the role of NF- κ B in the transcription regulation of DR5.

The role of DETANONOate in the inhibition of NF- κ B and sensitization to TRAIL-apoptosis is in agreement with studies by Chawla-Sarkar *et al.*, (2003) who showed that the NO donor nitrosylcobalmin (NO-Cbl) sensitized tumor cells to TRAIL-induced apoptosis. NO-Cbl inhibits IKK activity by decreasing phosphorylation of I κ B- α and inhibition of NF- κ B DNA-binding activity and confirmed by transfection of an NF- κ B-driven luciferase reporter system. Further, NO-Cbl was shown to increase expression of DR5 and DR5 mRNA (Bauer *et al.*, 2002). In this study, the regulation of DR4 and DR5 transcription by NF- κ B was not shown and may be through inactivation of YY1 as reported here. Yoshida *et al.*, (2001) reported the promoter structure and transcription initiation sites of the TRAIL receptor DR5. The nuclear factor-kappaB (NF- κ B binding sites) lies between +385 and +394 in intron 1. It is possible that NF- κ B activates DR5 expression via these binding sites in intron 1. In our present findings we demonstrate that NF- κ B inhibition by DHMEQ affects only the YY1 deletion mutant using a reporter system not containing intron 1. A recent study (Nakata *et al.*, 2004) demonstrates that histone deacetylase inhibitors (HDCAI) upregulate DR5 expression and sensitize cells to TRAIL apoptosis.

When compared to non-transformed cells, cancer cells are more sensitive to TRAIL-induced apoptosis following exposure to TRAIL treatment (Ashkenazi *et al.*, 1999). Apoptosis induction in response to most DNA-damaging drugs usually requires the function of the tumor suppressor p53, which engages primarily the intrinsic type of the apoptotic signaling pathway. However, many tumor cells exhibit inactivated and/or mutated p53 and thus resist chemotherapy (Gasco and Crook, 2003; Soussi, 2003). TRAIL induces apoptosis in cancer cells regardless of the p53 status, and thus can reverse resistance to chemotherapy (Wang and El-Deiry, 2003(b)). The combination of chemotherapy with TRAIL

has been found to be effective in killing cancer cells with wildtype p53, presumably through the induction of DR5 expression (Nagane *et al.*, 2001; Wang and El-Deiry, 2003(a)). *In vitro*, prior exposure of Bax deficient cells to topoisomerase inhibitors such as CPT-11 and VP-16 restores TRAIL sensitivity mainly by upregulation DR5. Knocking down p53 targets, DR5 and Bak, which are most likely involved in sensitizing Bax deficient human cancer cells to TRAIL with small molecules such as siRNA, showed that silencing DR5 in Bax deficient cells significantly inhibited TRAIL sensitivity. P53-dependent upregulation of DR5 contributed significantly to restoration of TRAIL sensitivity in Bax deficient cells upon DNA damage. These studies suggest the usefulness to identify small molecules that can reverse TRAIL resistance in cancer cells containing mitochondrial apoptotic defects as well as p53 mutations. Targeting of DR5 in cancer cells might be a useful therapeutic strategy. The agents so far available to upregulate DR5 expression are largely those that activate p53. Efforts to identify agents to upregulate DR5 expression independently of p53 may be useful in TRAIL-based cancer therapies. In this study, we have identified YY1 as a factor that negatively regulates DR5 expression independent of p53 since the PC-3 tumor cells are deficient in p53 and inhibition of YY1 sensitizes the cells to TRAIL-induced apoptosis by chemicals such as DETANONOate, siRNA or inhibition of its transcription (e.g. NF- κ B inhibitors) (See Figure 6- schematic diagram).

We have recently reported that prostate cancer tissues overexpress YY1 compared to normal prostate epithelial cells. This was accomplished by immunohistochemistry using human prostate cancer tissue microarrays (Seligson *et al.*, unpublished). Based on the present findings, we suggest that overexpression of YY1 in cancer tissues may negatively regulate the expression of the TNF- α family and thus, governs tumor cells' resistance to host immune effector cells and/or immunotherapy. In addition, YY1 overexpression may be implicated in the pathogenesis of human cancer. The present findings suggest that agents that upregulate DR5 expression, such as targeting YY1 to inhibit its expression and/or activity, and in combination with other therapies should result in the reversal of tumor cell resistance to TRAIL-induced apoptosis.

Materials and methods

Cells and culture conditions

The human androgen-independent PC-3 and DU145 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The androgen-dependent LNCaP and the androgen-independent CL-1 (LNCaP-derived) (Tso *et al.*, 2000) cell lines were kindly provided by Dr Arie Belldegrun at

UCLA. Cells were maintained as a monolayer in 80 mm² plates in RPMI 1640 (Life Technologies, Bethesda, MD, USA), supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Life Technologies) (to ensure the absence of complement), 1% (v/v) penicillin (100 U/ml), 1% (v/v) streptomycin (100 U/ml), 1% (v/v) L-glutamine, 1% (v/v) pyruvate, and 1% nonessential amino acids (Invitrogen Life Technologies, Carlsbad, CA, USA). FBS was charcoal-stripped to maintain CL-1 cells in an androgen-free medium. The LNCaP cell medium was supplemented with 0.1 nmol/l R1881 methyltrienolone (New Life Science Products, Boston, MA, USA). The cell cultures were incubated at 37°C and 5% carbon dioxide.

Reagents

The anti-DR5 and anti- β -actin monoclonal antibodies were purchased from Biosource International (Camarillo, CA, USA) and from Calbiochem (San Francisco, CA, USA), respectively. The human recombinant TRAIL was obtained from PeproTech Inc. (Rocky Hills, NJ, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-active caspase 3 and FITC-conjugated IgG were purchased from PharMingen (San Diego, CA, USA). The DETANONOate was obtained from Alexis (San Diego, CA, USA). The SureSilencingTM siRNA kit was purchased from SuperArray Bioscience Corporation (Frederick, MD, USA). The QuickChange Site-Directed Mutagenesis kit was obtained from Stratagene (La Jolla, CA, USA). DHMEQ was derived from Dr. Umezawa, Keio University, Tokyo, Japan (Ariga *et al.*, 2002).

Plasmid construction.

The pDR5 WT promoter luciferase (pDR5 promoter) reporter plasmid and the pDR5 promoter with the 5'-deletion mutant -605 that includes the YY1 binding site (pDR5/-605) have been previously characterized (Yoshida, *et al.*, 2001). The pDR5 plasmid missing the YY1 binding sequence (pDR5-YY1 mutant) was generated by using the QuikChange site-directed mutagenesis method (Stratagene). The mutagenesis reaction contained the pDR5 plasmid as a template DNA and two complementary oligonucleotides, each containing the desired mutation surrounded by 15 bp of flanking sequence on both the 5' and the 3' sides. A PCR-based method used the complementary primers pDR5-yy1 F (5'-TGT CATG TACTGGGACTACAGGCC-3') and

pDR5-yy1 R (5'-GGGAGGCTGAGGTGGGAGTATCTGC-3'). The PCRs contained 125 ng of each primer, 1X PFU buffer [20 mM Tris-HCl (pH 8.8), 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 100 µg of bovine serum albumin/ml, 0.1% Triton X-100], a 2.5 mM concentration of each deoxynucleoside triphosphate, and *Pfu* polymerase. Cycling conditions were 95°C for 3 min, followed by 30 cycles of 95°C for 45 sec, 69°C for 1 min, and 72°C for 11 min. PCR products were purified by QIAquick PCR purification kit QIAGEN Inc (Valencia, CA, USA).

Luciferase DR5 promoter reporter assay

PC-3 cells were transfected by electroporation using pulses at 250 V/975 µF (Bio-Rad), with 20 µg of pDR5 promoter, pDR5-YY1 mutant or pDR5/-605. After transfection, the cells were allowed to recover overnight and were cultured in six-well plates. Cells were treated or left untreated with the NO donor DETANONOate (1000 µM) for 18 h. Cells were then harvested in 1X lysis buffer and luciferase activity was measured according to the manufacturer's protocol (BD Biosciences, Palo Alto, CA, USA) using an analytical luminescence counter Monolith 2010. The assays were performed in triplicate. Data were normalized by protein concentration using Bio-Rad protein assay.

Cell treatments

Log-phase prostate carcinoma cell lines cells were seeded into six-well plates at approximately 6X10⁴ cells/ml and grown in 1 ml of medium as described above in 5% FBS for 24 h to approximately 70% confluence. The DU145, CL-1, and PC-3 cells were synchronized by treatment with 1% FBS for 18 h prior to each experiment. The treatment of LNCAP cells was in a medium with 1% of serum and the treatments of DU145, CL1, and PC-3 were in serum-free conditions. For experiments to measure TRAIL-mediated apoptosis by DETANONOate, the cells were treated with TRAIL, DETANONOate, or the combination for 18 h.

FLOW CYTOMETRY

To examine the expression of DR5 on the surface of PC-3 cells, flow cytometric analysis was performed. PC-3 cells were detached with PBS-EDTA (1 mM), washed with PBS, resuspended in PBS containing 10% of human normal serum and incubated for 1 h at room temperature. Cells were washed with PBS containing 0.5% BSA and resuspended in PBS- 0.5 % BSA and incubated with the anti-DR5 monoclonal antibody (Mab) at room temperature for 45 min. Cells were washed twice with PBS-0.5% BSA. PE-conjugated goat anti-mouse IgG Caltag (Burlingame, CA, USA) was added and incubated at room temperature for 30 min. Cells were washed again in PBS-0.5% BSA and fixed with 1% paraformaldehyde. Flow-cytometric analysis was performed using EPICSR XL-MCL (Coulter, Co. Miami, FL, USA), with the System II™ Software and the mean fluorescence intensity was recorded.

Determination of apoptosis

After each treatment, the adherent cells and the floating cells were recovered by centrifugation at 1800 rpm for 8 min. Afterwards, the cells were washed once with ice-cold 1X phosphate-buffered saline (PBS) and were resuspended in 100 μ l of the cytofix/cytoperm solution (PharMigen, San Diego, CA, USA) for 20 min. Thereafter, the samples were washed twice with ice-cold 1X perm/wash buffer solution (PharMingen) and were stained with FITC-labeled anti-active caspase 3 mAb for 30 min (light protected). The samples were subsequently washed once with 1X perm/wash buffer solution and 250 ml of 1X PBS were added prior to flow cytometry analysis on a flow cytometer EPICSR XL-MCL (Coulter, Co. Miami, FL, USA), with the System II™ Software and the percent positive cells was recorded. As a negative control, the cells were stained with isotype control (pure IgG) under the same conditions described above.

Western blot analysis

PC-3 cells were cultured at a low FBS concentration (1%) 18 h prior to each treatment. After incubation, the cells were maintained in FBS-free medium (control), or treated with DETANONOate (1000 mM). The cells were then lysed at 4°C in RIPA buffer (50mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150mM NaCl), and supplemented with one tablet of protease inhibitor cocktail, Complete Mini

Roche (Indianapolis, IN, USA). Protein concentration was determined by a DC protein assay kit (Bio-Rad, Hercules, CA, USA). An aliquot of total protein lysate was diluted in an equal volume of 2 X SDS sample buffer, 6.2mM Tris (pH 6.8), 2.3% SDS, 5% mercaptoethanol, 10% glycerol, and 0.02% bromophenol blue and boiled for 5 min. The cell lysates (40 μ g) were then electrophoresed on 12% SDS-PAGE gels (Bio-Rad) and were subjected to Western blot analysis as previously reported (Jazirehi et al., 2001). Levels of β -actin were used to normalize the protein expression. Relative concentrations were assessed by densitometric analysis of digitized autographic images, performed on a Macintosh computer (Apple Computer Inc., Cupertino, CA, USA) using the public domain NIH Image J Program (also available via the internet).

Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA of PC-3 cells was extracted and purified from 1×10^6 cells for each experimental condition by a single-step monophasic solution of phenol and guanidine isothiocyanate-chloroform using Trizol® reagent (Life Technologies, Inc.). 3 μ g of total RNA was reverse-transcribed to first strand cDNA for 1 h at 42°C with SuperScript™ II reverse transcriptase (Life Technologies, Inc) in a 20 μ L reaction and performed per the manufacturer's specifications using random primers. Amplification of 1/10 of these cDNA by PCR was performed using the gene-specific primers of DR5. Internal control for equal cDNA loading in each reaction was assessed using the following gene specific glyceraldehydes-3-phosphate dehydrogenase (GAPDH). PCR amplification of each DNA sequence was carried out by the "Hot Start" method using Titanium Taq® polymerase (Clontech) with the following one-step thermal cycling incubation: 95°C/30 s, 68°C/1 min for 30 (DR5) or 25 (GAPDH) cycles, with a final extension at 68°C/3 min. The number of cycles was established based on preliminary titration of the relative amount of amplified product for each gene representing the linear phase of amplification process. The amplified products were resolved on 1.5% agarose gel electrophoresis and their relative concentrations were assessed by densitometric analysis of digitized ethidium bromide-stained image, performed on a Macintosh computer (Apple Computer Inc., Cupertino, CA.) using the public domain NIH Image J Program (available on the internet).

Nuclear extracts preparation

Nuclear extract preparations were carried out as previously (Garban and Bonavida, 2001). Briefly, cells (10^6) were harvested after treatment and washed twice with cold Dulbecco PBS (Cellgro, Herndon, VA, USA). After washing, cells were lysed in 1ml of NP-40 lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40) on ice for 5 min. Samples were centrifuged at 300 g at 4°C for 5 min. The pellet was washed twice in NP-40 buffer. Nuclei were then lysed in nuclear extraction buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and sonicated for 10 s at 4°C. Both buffers contained the complete protease inhibitor cocktail tablets from Roche (Indianapolis, IN, USA). The protein concentration was determined using the Bio-Rad protein assay. The nuclear proteins were frozen at -80°C.

EMSA

Nuclear proteins (5 µg) were mixed for 30 min at room temperature with Biotin-labeled oligonucleotide probe NF-κB or YY1 using EMSA Kit Panomicst (Panomics Inc., Redwood City, CA, USA) following the manufacturer's instructions and as described previously (Vega et al., 2004). 10 µl was subjected to 5% polyacrylamide gel electrophoresis for 90 min in TBE buffer (Bio-Rad Laboratories) and transferred to Nylon membrane Hybond-Np (Amersham Pharmacia Biotech, Germany) using the Trans-Blots SD semi-dry Transfer cell System (Bio-Rad, Hercules, CA, USA). The membranes were transferred to a UV Crosslinker FB-UVXL-1000 Fisher technology (Fisher Scientific, NY, USA) for 3 min. The detection was carried out as per the manufacturer's instructions, after the membranes were exposed using Hyperfilm ECL (Amersham Pharmacia Biotech). The Relative concentrations were assessed by densitometric analysis as mentioned above.

siRNA Transfections

PC-3 cells were cultured in 1 ml of RPMI medium supplemented with 5% FBS. Transfections were performed by using lipofectamine 2000 CD Reagent supplied by Invitrogen (Life Technologies, Carlsbad, CA, USA) and the SureSilencingTM siRNA kit supplied by SuperArray Bioscience Corporation (Fredrick, MD)

according to the manufacturers' instructions. Briefly, 3 μ l of YY1 siRNA or a negative control of siRNA solution were incubated with 4 μ l of the transfection reagent in serum-free RPMI medium 1640 for 25 min to facilitate complex formation. The resulting mixture was added to PC-3 cells cultured in a 24-well plate with 1 ml of medium. To determine the extracellular expression of the DR5 receptor, the cells were harvested 36 hours after transfection and stained with anti-DR5 monoclonal antibody for 30 min then anti-mouse IgG-PE for 20 min. The expression was then analyzed by flow cytometry. To determine the PC-3 sensitization to TRAIL-mediated apoptosis, 24 h after transfection the cells were treated for 18 hrs with TRAIL (1 and 2.5 ng/ml) and fixed and permeabilized for anti-active caspase-3-FITC antibody staining. The cells were then analyzed by flow cytometry under the same conditions described above.

Isobologram analysis for determination of synergy

To establish whether the cytotoxic effect of the TRAIL/NO combination was more than additive, isobolograms were constructed from treatments combining TRAIL at various concentrations (2.5, 5, and 10 ng/ml) with the NO donor DETANONOate (500 and 1000 mM) as described (Berenbaum, 1978). Combinations yielding a cytotoxicity of 30-75% were graphed as a percentage of the concentration of single agent alone that produced this amount of cytotoxicity. Analysis was performed on the basis of the dose-response curves using active caspase 3 analysis for LNCaP, DU145, CL-1, and PC-3 cells treated with TRAIL alone or NO donor alone and the combination for 18 h.

Statistical analysis

The experimental values were expressed as the mean $\pm$ s.d. for the number of separate experiments indicated in each case. One-way ANOVA was used to compare variance within and among different groups. When necessary, Student's t-test was used for comparison between two groups. Significant differences were considered for probabilities $\leq$ 5% ($P \leq 0.05$).

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Figure Legends

Figure 1. DETANONOate sensitizes CaP cell lines to TRAIL-mediated apoptosis. (A) The CaP cell lines LNCaP, DU145, CL-1, and PC-3 were treated with TRAIL (5 ng/ml) in the presence or absence of DETANONOate (1000 μ M) for 18 h. Fixed and permeabilized cells were stained with anti-active-caspase-3-FITC antibody and analyzed by flow cytometry as described in methods. The findings reveal that DETANONOate sensitizes the CaP cell lines to TRAIL-mediated apoptosis. The data are the mean of three independent experiments. * $p < 0.05$, ** $p < 0.02$. (B) This figure establishes synergy as determined by isobologram analysis.

Figure 2. DETANONOate induces upregulation of DR5 expression. (A) Upregulation of DR5 surface expression by DETANONOate. PC-3 cells were treated with 1000 μ M DETANONOate for 18 h and surface expression was performed with anti-DR5 mAb as described in methods. The data represent the observed mean fluorescence intensity (MFI) and are the mean of three independent experiments. * $P < 0.05$, medium vs cells treated. (B) Top panel: Upregulation of mRNA expression of DR5 in PC-3 cells by DETANONOate. Untreated or cells treated with 1000 μ M of DETANONOate for 18 hr were used to isolate total RNA and a semi-quantitative RT-PCR reaction was performed for detection of DR5 transcripts. The amplification of GAPDH was performed as a positive control. The data show that DETANONOate upregulates DR5 mRNA levels. Bottom panel: Upregulation of DR5 protein by western. PC-3 cells were treated or left untreated with 1000 μ M of DETANONOate for 18 hr. Total cellular protein was extracted and separated by SDS-PAGE and transferred onto nitrocellulose membrane as described in methods. The membrane was stained with anti-DR5 mAb. Levels

of β -actin were used to normalize the protein expression. The blots represent one of three separate experiments. Densitometric analysis was performed. The data show that DETANONOate upregulates DR5 protein expression.

Figure 3. DETANONOate inhibits NF- κ B and YY1 DNA-binding activities and inhibits YY1 expression. Nuclear extracts from PC-3 cells were treated or left untreated with DETANONOate (500 or 1000 μ M) and then were analyzed by EMSA to assess NF- κ B DNA-binding activity (Figure 3A) or YY1 DNA-binding activity (Figure 3B Top panel). Relative NF- κ B and YY1 DNA-binding activity was determined by densitometry analysis. YY1 protein expression was determined by using PC-3 cells treated with DETANONOate (500 or 1000 μ M) for 18 hr. The membrane was stained with polyclonal anti-human YY1 antibody. The blots represent one of two separate experiments (Figure 3B Bottom panel). The findings demonstrate that treatment of PC-3 cells with DETANONOate results in inhibition of NF- κ B and YY1 DNA-binding activity and also inhibition of YY1 protein expression.

Figure 4. Specific inhibition of YY1 expression induces up-regulation of DR5 expression and sensitizes PC-3 cells to TRAIL-mediated apoptosis. The PC-3 cells were transfected using the SureSilencingTM siRNA for YY1 or siRNA negative control. (A) RT-PCR for YY1 was performed and the data show inhibition of YY1 transcription by siRNA YY1. (B) The surface expression of DR5 was determined by flow cytometry analysis as described in methods. The data represent the mean fluorescence intensity (MFI) and are the mean of three independent experiments. * $P < 0.05$, medium vs cells transfected with siRNA YY1. (C) After transfection, the cells were treated or left untreated with different concentrations of TRAIL (1 or 2.5 ng/ml) for 18 h. Fixed and permeabilized PC-3 cells were stained with FITC-labeled anti-active-caspase-3 and then analyzed by flow cytometry as described in methods. The data are the mean of three independent experiments. * $p < 0.05$. The findings reveal that YY1 negatively regulates DR5 expression and inhibition of YY1 sensitizes PC-3 cells to TRAIL-mediated apoptosis.

Figure 5. YY1 negatively regulates DR5 transcription. The *SacI-NcoI* fragment of the 5'-flanking region of the DR5 promoter (pDR5) was subcloned into the *SacI-NcoI* site of pGVB2 luciferase assay vector (Toyo ink, Tokyo, Japan). The pDR5 promoter with the 5'-deletion mutant -605 that includes the YY1 binding site (pDR5/-605) was generated with deletion kits (Takara, Tokyo, Japan) (Yoshida, et al, 2001). The pDR5 plasmids missing the YY1 binding sequence (pDR5-YY1 mutant) was generated by using the QuikChange site-directed mutagenesis method as described in Materials and Methods. (A) The PC-3 cells were transfected with 10 μ g of pDR5, pDR5-YY1 mutant or pDR5/-605 by electroporation as described in methods. 36 h after transfection the cells were harvested and the luciferase activity was determined. The data show that the PC-3 cells transfected with either the pDR5-YY1 mutant or the pDR5/-605 show a significant increase of luciferase activity (3 fold). (B) PC-3 cells were transfected with 10 μ g of pDR5, pDR5 YY1 mutant or pDR5/-605 by electroporation as described in methods. 24 hr after transfection the cells were treated or not treated with DHMEQ (2 μ g/ml) for 18 hr. The cells were harvested and luciferase activity was determined. The data show that the NF- κ B inhibitor DHMEQ augmented luciferase activity in both constructs and suggests that NF- κ B regulates DR5 in YY1. The data represent the % of control and are the mean of two independent experiments.

Figure 6. Two-signal model for sensitization of CaP cells to TRAIL-induced apoptosis by DETANONOate and TRAIL. This figure schematically demonstrates that treatment of PC-3 cells with NF- κ B or YY1 inhibitor and TRAIL results in apoptosis and synergy is achieved. The synergy is the result of complementation in which each agent activates partially the apoptotic pathway and the combination results in apoptosis. Signal 1 is provided by the inhibitor, which partially inhibits NF- κ B and YY1 DNA-binding activity. Inhibition of YY1 transcription diminishes its repressor activity in the DR5 promoter, and this results in the upregulation of DR5 transcription. Signal II is provided by TRAIL and combination of inhibitors and TRAIL results in apoptosis and synergy.

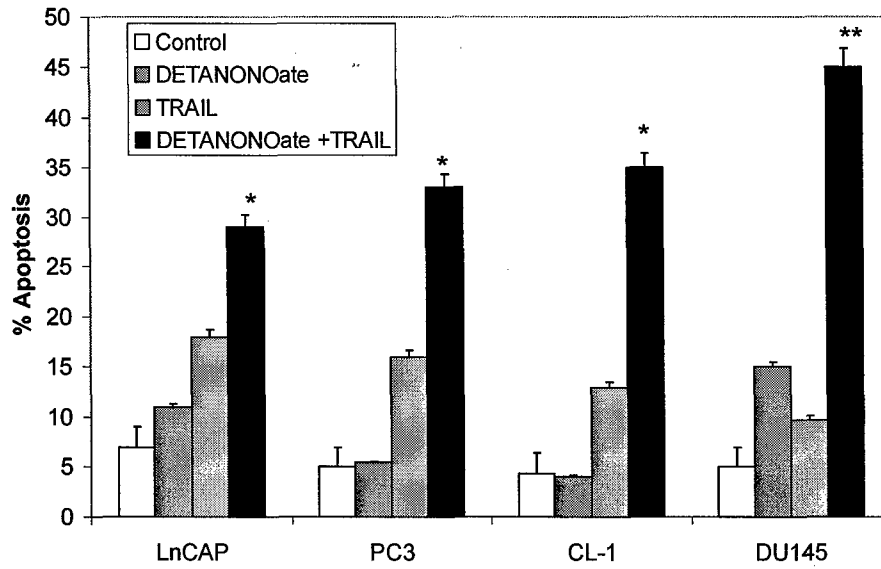


Figure 1(A)

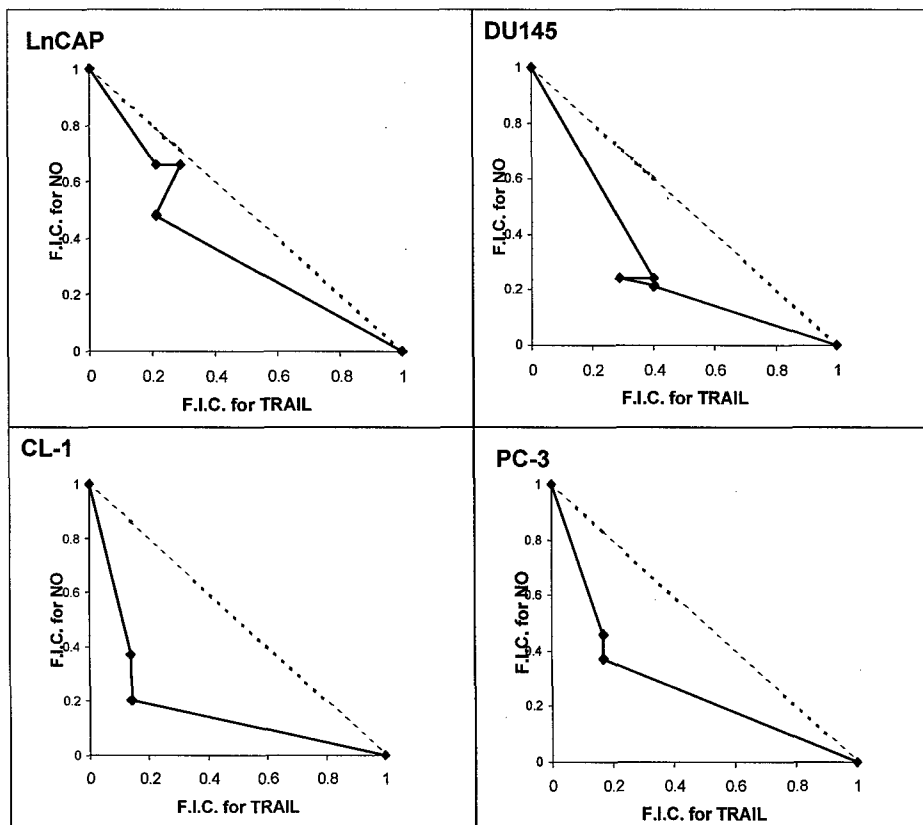


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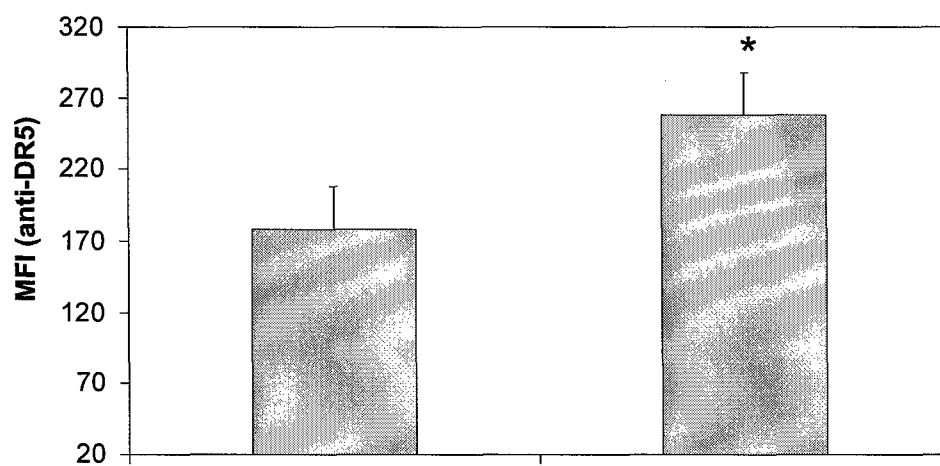


Figure 2(A)

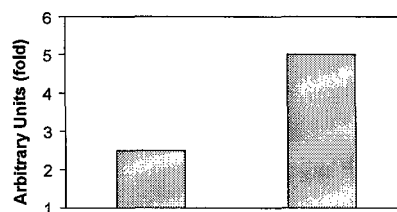
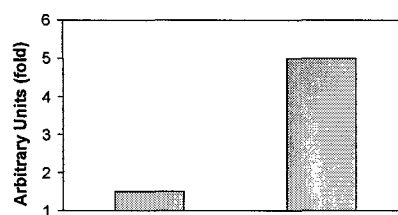
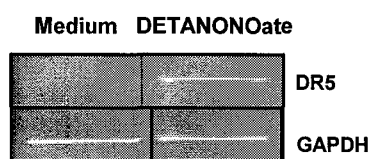


Figure 2(B)

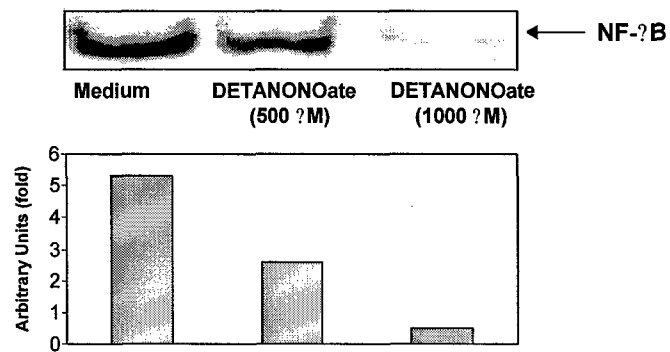


Figure 3(A)

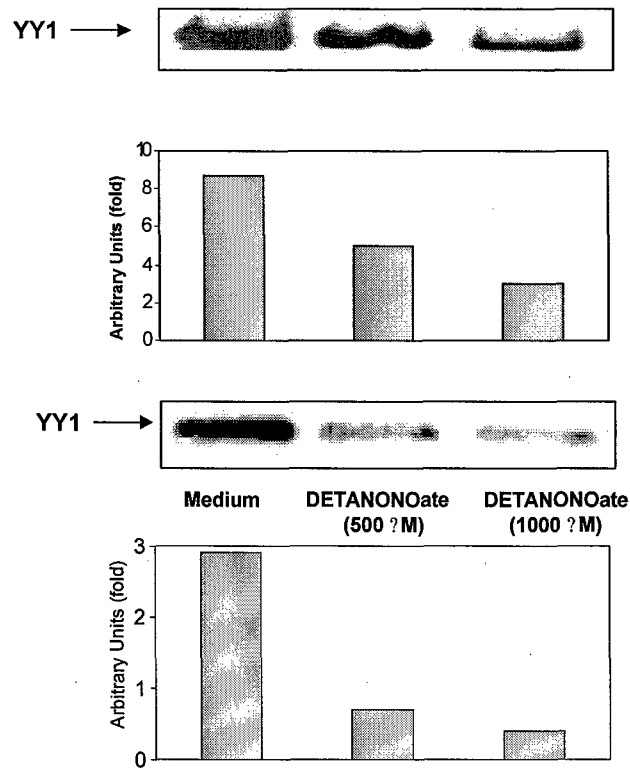


Figure 3(B)

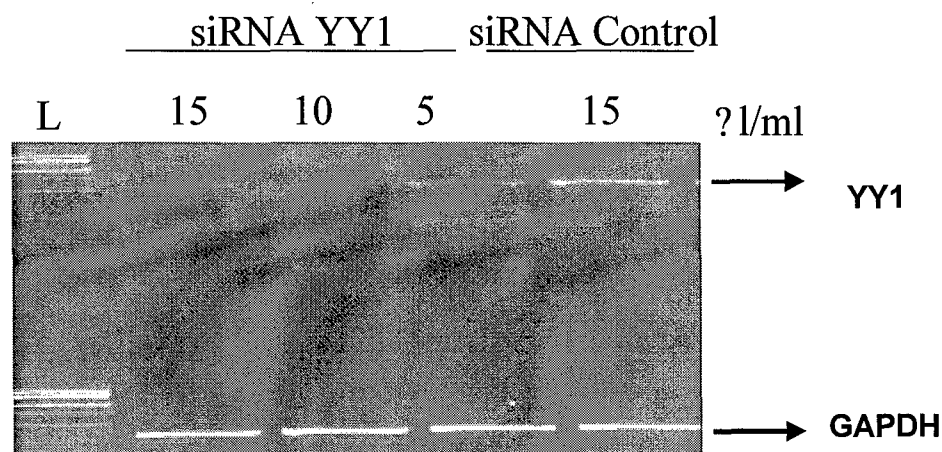


Figure 4(A)

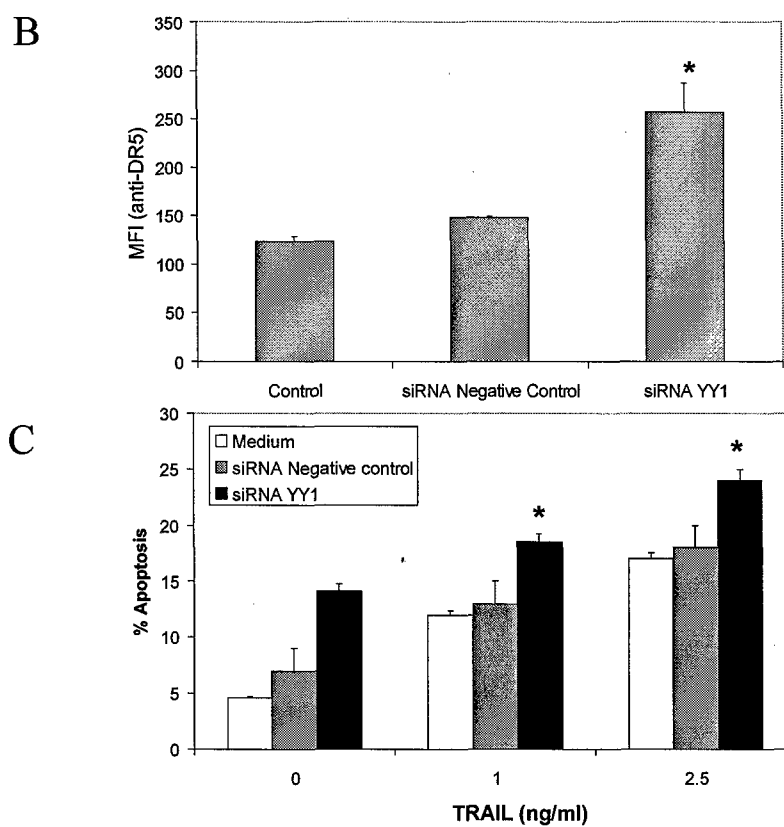


Figure 4

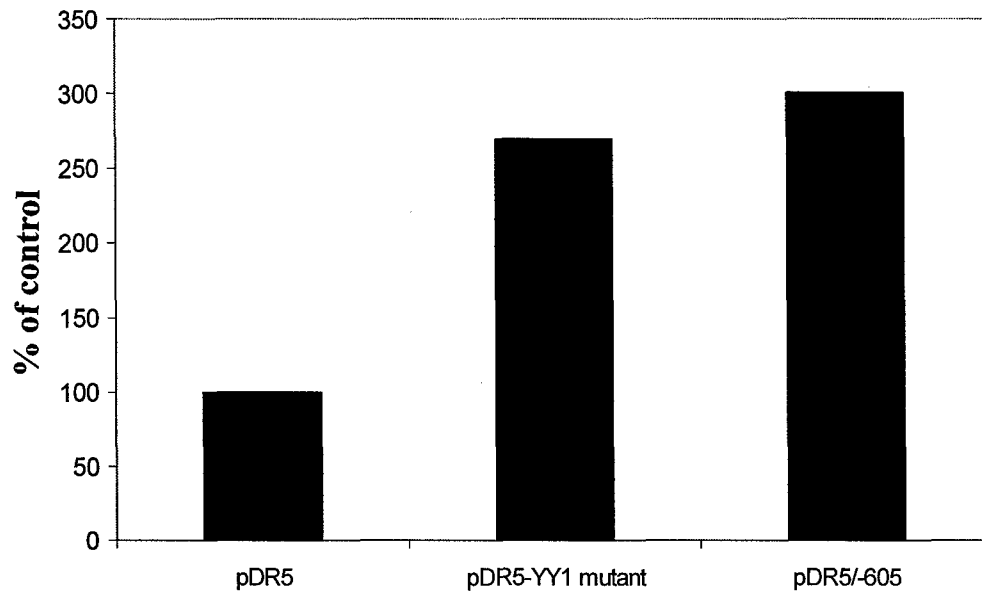


Figure 5(A)

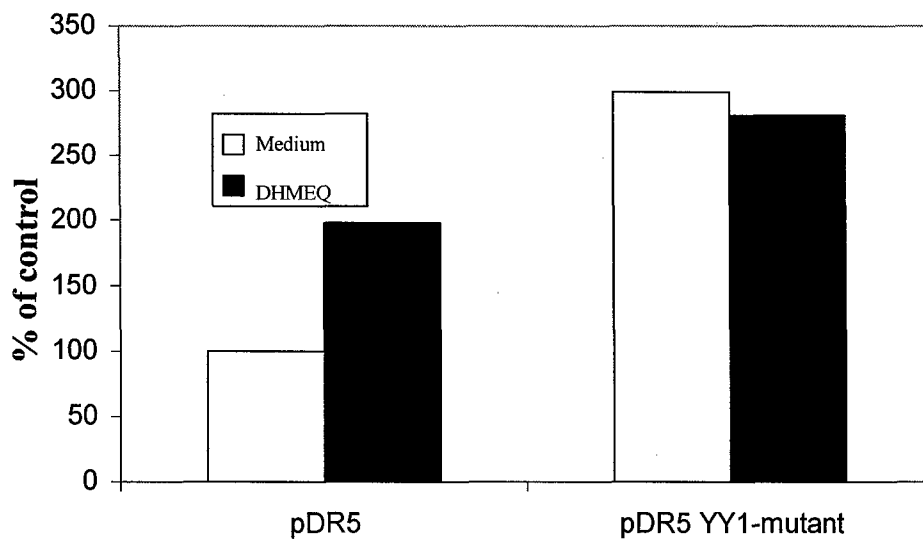
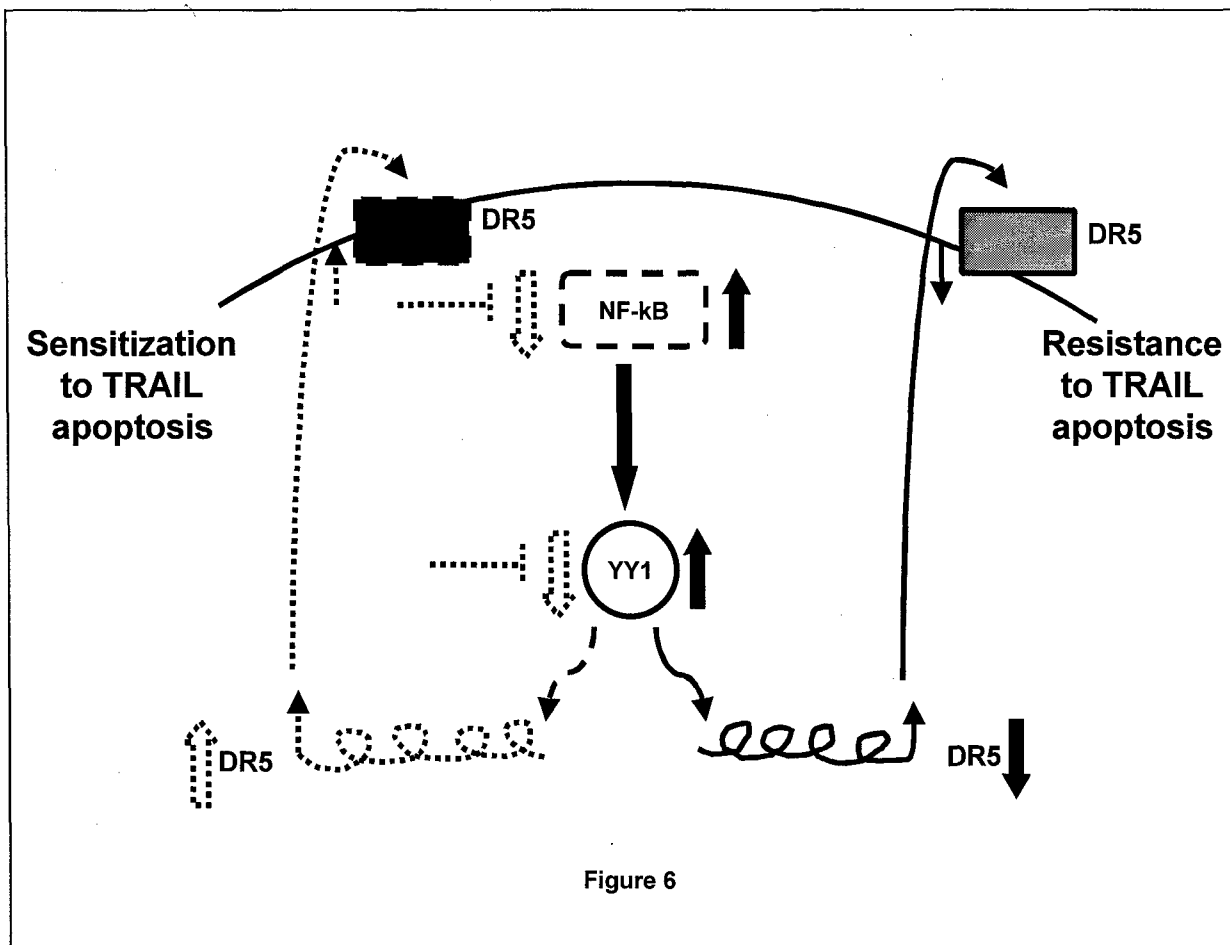


Figure 5(B)



APPENDIX 5

Control/Tracking Number : 05-AB-32737-ASCO

Activity :Abstract Submission

Current Date/Time : 12/10/2004 5:01:02 PM

Sensitization of prostate cancer cells to TRAIL-induced apoptosis by CDDP: Involvement of NF- κ B, YY1 and RKIP in upregulation of DR5 expression.

Short Title:

Mechanisms of CDDP-induced DR5

M. Neshat*, S. Baritaki*, S. Huerta-Yepez*, A. Katsman, T. Delgado, K. Umezawa, T. Sakai, K. C. Yeung, K. C. Yeung, D. Chatterjee, B. Bonavida; University of California, Los Angeles, Los Angeles, CA; University of California at Los Angeles, Los Angeles, CA; Keio University, Yokohama, Japan; Kyoto Prefectural University of Medicine, Kyoto, Japan; Medical College of Ohio, Toledo, OH; Rhode Island Hospital, Providence, RI

Background:

Treatment of the androgen-independent, p53- PC-3 prostate cancer cells with CDDP or the NO donor, DETANONOate, resulted in inhibition of NF- κ B activity, upregulation of DR5 expression and sensitization to TRAIL-induced apoptosis. CDDP-dependent mechanisms of regulation of DR5 were examined.

Methods:

Apoptosis was assessed by PI staining and by activated caspase-3; DNA-binding activity by EMSA; transcription by luciferase reporter systems; and protein expression by flow cytometry and western analysis.

Results:

Preliminary findings demonstrated that inhibition of NF- κ B activity by CDDP and DETANONOate, as measured by NF- κ B reporters, was corroborated in PC-3 cells. DHMEQ, an inhibitor of NF- κ B nuclear translocation, paralleled CDDP and DETANONOate effects and resulted in upregulation of DR5 expression and sensitization to TRAIL-induced apoptosis. DR5 promoter activity was augmented by deletion of the -1224 to -605 region (Yoshida et al., FEBS Lett., 2001, 507: 381-5), or direct mutation of YY1 binding motif at -800 to -796. DR5 upregulation and sensitization to TRAIL was further demonstrated by YY1 siRNA. RKIP has been shown to regulate the level of activity of NF- κ B, MAPK and GPCR-dependent pathways. Forced RKIP overexpression inhibited CDDP-dependent DR5 induction, suggesting that CDDP-induced response is the outcome of positive and negative regulatory mechanisms.

Conclusions:

The findings reveal for the first time that CDDP- mediated sensitization of tumor cells to TRAIL-induced apoptosis results in inhibition of NF- κ B-dependent transcription of YY1. Inhibition of YY1 upregulates DR5 expression and sensitizes the tumor cells to TRAIL-induced apoptosis. The findings identify NF- κ B, YY1 and RKIP as new targets for therapeutic intervention for reversal of tumor cells resistance to TRAIL.

[* These authors contributed equally to this work]

Nitric oxide decreases the transcription repressor activity of Yin-Yang 1 (YY1) via S-nitrosylation: Role in the immunosensitization of tumor cells to apoptosis

Fumiya Hongo, Hermes Garban, Sara Huerta-Yepes, Mario Vega, Ali Jazirehi, Yoichi Mizutani, Benjamin Bonavida.

University of California, Los Angeles, Los Angeles, CA and Kyoto Prefectural University of Medicine, Kyoto, Japan.

Yin-Yang 1 (YY1) is a transcription factor that may activate or repress gene expression. We have reported the upregulation of the TNF receptor family members by nitric oxide (NO) resulting in the sensitization of tumor cells (e.g. ovarian, prostate, lymphoma) to TNF family-mediated apoptosis. The sensitization by NO was suggested to be mediated in part to S-nitrosylation of the transcription repressor YY1 and consequently, the inhibition of its DNA-binding activity in the silencer region of the receptor promoter. In this study, we examined the direct S-nitrosylation of YY1 using the human prostate cancer cell line, PC-3, as model. Culture cells were incubated for 18 h in the presence of various concentrations of the NO donor DETANONOate (500 μ M, 1000 μ M) that sensitized PC-3 to Fas ligand- and TRAIL-mediated apoptosis. Subsequently, we analyzed for S-nitrosylation of YY1 by various methods. Using immunohistochemistry, we found that general S-nitrosylation of proteins was increased after treatment with NO. Noteworthy, significant constitutive S-nitrosylated proteins were detected. Further, using double immunofluorescence staining microscopy, we colocalized the presence of S-nitrosylated proteins and YY1. In addition, a specific significant increase in YY1-SNO protein was determined in culture cells exposed to NO by immunoprecipitation with anti-Cys SNO antibody followed by immunodetection of YY1. These findings were corroborated by demonstrating that immunoprecipitation of NO-treated PC-3 cells by anti-SNO antibody revealed that YY1 was S-nitrosylated using the method by Miles et al (Meth Enzym., 268:105, 1996). These findings altogether reveal a novel mechanism of YY1 regulation by nitric oxide showing direct S-nitrosylation of this transcription repressor, the consequent decrease in DNA-binding activity and derepression of gene transcription. This work was supported by a grant from the Department of Defense (DAMD 17-02-1-0023), the UCLA SPORE in Prostate Cancer (P50 CA92131-01A1), the Jonsson Comprehensive Cancer Center (AJ), UC-MEXUS (SH-Y), and a grant from Fogarty (SH-Y, HG, MV).

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APPENDIX 7

Nitrosylation of the Transcription Repressor Yin-Yang 1 (YY1) Mediates Upregulation of Fas Expression in Cancer Cells: Nitric Oxide (NO)-Induced Sensitization to Fas-Mediated Apoptosis^{*†}

Running Title: S-nitrosylation of YY1 by NO

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Keywords: Yin Yang 1 (YY1), NF- κ B, S-nitrosylation, Fas expression, Fas-induced apoptosis.

Abstract

Treatment of several prostate cancer (CaP) cell lines (PC3, CL1, DU145) with the nitric-oxide donor, DETA NONOate, upregulated Fas expression and sensitized CaP cells to Fas-induced apoptosis. This study examined the mechanism by which NO regulates Fas expression and tumor cell sensitivity to CH-11-induced apoptosis. Treatment of CaP cells with DETA NONOate inhibited the constitutive NF- κ B and YY1 DNA-binding activity as assessed by EMSA. Treatment of PC3 cells with DETA NONOate resulted in the S-nitrosylation of p50 and inhibition of NF- κ B activity and consequently, inhibition of YY1 expression. In addition, treatment with DETA NONOate resulted in the S-nitrosylation of YY1 as demonstrated by two methods. The first is the DAN-based method in which cell lysates were immunoprecipitated with anti-YY1 antibody and the NO released was determined quantitatively by fluorometry. The second method consisted of immunoprecipitation of the lysates

by anti-SNO cysteine antibody and the immunoprecipitate immuno-blotted with anti-YY1 antibody. These findings were corroborated by immunohistochemistry using dual color immunofluorescence. The direct role of YY1 in the negative regulation of Fas expression was demonstrated by transfection of cells with YY1 siRNA. The transfectants showed up-regulation of Fas expression as well as they were sensitized to CH-11-induced apoptosis. Altogether, these findings reveal that NO inhibits YY1 DNA-binding activity through S-nitrosylation and subsequently Fas expression is upregulated and the cells are sensitized to CH-11-induced apoptosis.

INTRODUCTION

Nitric-oxide (NO) is a versatile signaling molecule that has been shown to play a variety of physiological functions in mammals (1). NO is a water soluble free radical gas that readily diffuses through membranes and reacts with heme containing proteins and other effector molecules. NO is produced in biological systems from arginine by the enzyme nitric-oxide synthase (NOS). Three types of NOS have been detected and include the eNOS (endothelial nitric-oxide synthase), nNOS (neuronal nitric-oxide synthase), and iNOS (inducible nitric-oxide synthase). Nitric oxide is known to induce zinc (Zn^{2+}) release from the-zinc storing protein, metallothionein, and to induce Zn^{2+} release within the nuclei and cytoplasm of cells. These findings suggest that zinc finger proteins may be primary targets of NO induced stress (2). More recently, it has been proposed that S-nitrosylation of cysteine-thiols may constitute a major route of NO trafficking through which NO-related bioactivity is affected, serving as a ubiquitous post-translational modification that regulates dynamically a broad functional spectrum of proteins (3-5). However, the analysis of protein S-nitrosylation *in situ*, originated with endogenous NOS activity, has been impeded by substantial technical barriers and there is little direct evidence for cellular protein S-nitrosylation that can be ascribed specifically to the activity of any NOS isoform (5, 6-8). Protein S-nitrosylation is not enough to survive the rigors of denaturing SDS-polyacrylamide gel electrophoresis, and thus anti-S-nitroso cysteine antibodies may be of little use in the context of proteomics investigations. However, they have been generated and applied in immunohistochemistry studies (9). It has been proposed that immuno-staining with S-nitro-s-cysteine antibody reveals the localization of S-nitrosylated proteins such as S-nitrosylated antigens were detected in fixed trypanosomes (10). Basal and stimulated S-nitrosylated proteins were detected in multiple cell types and proteins (11). In addition, S-nitrosylated proteins

• were detected in a murine model of inhaled NO therapy (12). Recently, the S-nitrosylation of metalloproteinases was detected by peptide mass fingerprinting analysis of modified thiol groups of the cysteine residues (13). S-nitrosylated channel proteins were also detected by electro-physiology (14).

Yin-Yang 1 (YY1) is a zinc finger transcription factor involved in the positive and negative regulation of many mammalian genes (15). Recently, we have demonstrated that NO treatment of tumor cell lines resulted in the up-regulation of Fas expression via the specific inactivation of the transcription repressor YY1 DNA binding activity to the silencer region of the Fas promoter (16). We hypothesized that NO reacts with YY1 resulting in the S-nitrosylation of the protein and subsequent inhibition of its DNA-binding activity, and thus resulting in the up-regulation of Fas expression and sensitivity of the cells to Fas-induced apoptosis.

The present study was undertaken to test the above hypothesis and the followings were investigated: (1) Does NO treatment result in S-nitrosylation of YY1? (2) Is the S-nitrosylation of YY1 responsible for its inhibition of DNA-binding activity? and (3) Is YY1 inhibition responsible for the upregulation of Fas expression and sensitization to Fas-induced apoptosis?

Materials and methods

Cell lines

The prostate carcinoma (CaP) cell lines PC-3 and DU145 were obtained from the American Type Culture Collection (Manassas, VA). The CL-1 (LnCaP-derived) cell line was kindly provided by Dr. Arie Belldegrun at UCLA (17). The cell cultures were maintained as monolayers on plastic dishes and incubated at 37°C and 5% carbon dioxide in RPMI 1640 Life Technologies (Bethesda, MD), supplemented with 5% heat-inactivated FBS (to ensure the absence of complement), 1% (v/v) penicillin (100 U/ml), 1% (v/v) streptomycin (100 U/ml), 1% (v/v) l-glutamine, 1% (v/v) pyruvate, and 1% nonessential amino acids Life Technologies. For every experimental condition, the cells were cultured in 0.1% FBS, 18 h prior to treatments.

Reagents

The monoclonal antibody (mAb) against YY1 was obtained from Santa Cruz (California, USA) and the mAb against Fas, UB2, for surface expression was obtained from MBL (Nagoya, Japan). The mAb against β -actin was obtained from Calbiochem (San Francisco, CA). The polyclonal antibody anti-YY1 was obtained from Active Motif (Carlsbad, CA) and the polyclonal anti-Fas antibody, for western, was obtained from Santa Cruz. Anti-S-nitrosocysteine antibody was obtained from Calbiochem (San Diego, CA). The Fas ligand cytotoxic agonist monoclonal antibody, CH-11, was obtained from MBL (Nagoya, Japan). FITC-conjugated anti-active caspase-3 and FITC-conjugated IgG were obtained from PharMingen (San Diego, CA). The inhibitor of NF- κ B Bay11-1075 (specific inhibitor of I κ B phosphorylation) (18) was obtained from Calbiochem (San Francisco, CA). The DETA NONOate was obtained from Alexis (San Diego, CA).

Determination of apoptosis

After each treatment, the adherent cells were recovered by centrifugation at 1800 rpm for 8 min. Afterwards, the cells were washed once with ice cold 1XPBS and were resuspended in 100 μ l of the cytofix/cytoperm solution (PharMingen, San Diego, CA) for 20 min. Thereafter, the samples were washed twice with ice cold 1Xperm/wash buffer solution (PharMingen) and were stained with FITC-labeled anti-active-caspase-3 mAb for

30 min (light protected). The samples were subsequently washed once with 1Xperm/wash buffer solution and 250 μ l of 1XPBS was added prior to flow cytometry analysis on a Flow cytometer EPICS^R XL-MCL (Coulter, Co. Miami, FL.), with the System IITM Software and the percent positive cells was recorded. As a negative control, the cells were stained with an isotype control (pure IgG) under the same conditions described above. Synergy was determined by isobologram analysis (19).

Fas expression

Surface Fas Ag expression on the tumor cells was determined by flowcytometry as previously reported (20).

Western blot analysis

PC-3 cells were cultured at a low serum concentration (0.1%) 18 h prior to each treatment. After incubation, the cells were maintained in serum-free medium (control), or treated with DETANONOate (500 μ M, 1000 μ M). The cells were then lysed at 4°C in RIPA buffer {50mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150mM NaCl}, and supplemented with one tablet of protease inhibitor cocktail (Complete Mini Roche, Indianapolis, IN). Protein concentration was determined by a DC protein assay kit Bio-Rad (Hercules, CA). An aliquot of total protein lysate was diluted in an equal volume of 2XSDS sample buffer 6.2mM Tris (pH6.8), 2.3% SDS, 5% mecraptoethanol, 10% glycerol, and 0.02% bromphenol blue and boiled for 10 minutes. The cell lysates (40 μ g) were then electrophoresed on 12% SDS-PAGE gels (Bio-Rad) and were subjected to Western blot analysis as previously reported (21). Levels of β -actin were used to normalize the protein expression. Relative concentrations were assessed by densitometric analysis of digitized autographic images, performed on a Macintosh computer (Apple Computer Inc., Cupertino, CA.) using the public domain NIH Image J Program (available on the internet).

Preparation of Nuclear Extracts

Nuclear extract preparations were done as previously described by our laboratory (16). Briefly, cells (10^6) were harvested after treatment and washed twice with cold Dulbeco PBS (Cellgro). After washing the cells were

lysed in 1 ml of NP40 lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM MgCl₂, and 0.5% NP40) on ice for 5 min. Samples were centrifuged at 300 X g at 4°C for 5 min. The pellet was washed twice in NP40 buffer. Nuclei were then lysed in nuclear extraction buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and sonicated 10 s at 4°C. The protein concentration was determined using the Bio-Rad protein assay. The nuclear proteins were frozen at -80°C. Both buffers contained the complete protease inhibitor cocktail tablets from Roche (Indianapolis, IN).

EMSA

Nuclear proteins (5 µg) were mixed for 30 min at room temperature with Biotin-labeled oligonucleotide probes for NF- κ B and YY1 using the EMSA Kit PanomicsTM (Panomics, Inc. Redwood City, CA) as described previously and following the manufacturer's instructions (22). 10 µl were subjected to denaturing 5% polyacrylamide gel electrophoresis for 90 min in TBE buffer (Bio-Rad Laboratories) and transferred to Nylon membrane Hybond-N+ (Amersham Pharmacia Biotech, Germany) using the Trans-Blot^{SD} semi-dry Transfer cell System (Bio-Rad, Hercules, CA). The membranes were transferred to a UV Crosslinker FB-UVXL-1000 Fisher technology (Fisher Scientific, NY) for 3 min. The detection was made following the manufacturer's instructions. The membranes were then exposed using Hyperfilm ECL (Amersham Pharmacia Biotech). Relative concentrations were assessed by densitometric analysis.

Immunostaining of S-nitrosylated YY1

The PC-3 cells were incubated with or without treatment and fixed with 4% paraformaldehyde with 0.2% Triron-X100. After blocking (5% Goat serum, 3% BSA and 0.2% Triton X-100 in PBS), the cells were incubated with anti S-nitrosocysteine antibody (over night at 4 °C) After incubation, the cells were treated with an FITC conjugated anti-rabbit antibody (Jackson Immunoresearch PA). For YY1 detection, the cells were treated with murine anti-YY1 antibody and developed with (Mouse) rhodamine Red-X conjugated anti-mouse antibody (Jackson Immunoresearch PA).

Detection of S-nitrosylated YY1

PC-3 cells grown in the presence and absence of DETA NONoate were harvested and pelleted at 14,000 $\times$ g for 2 min. The resulting cell pellets were resuspended and dissolved in 500 μ l ice-cold RIPA buffer. The supernatants were incubated overnight at 4°C on a shaking platform with 2 μ g of rabbit anti-YY1 Ab (Geneka,) and were subsequently incubated with 30 μ l Immuno-Pure Plus Immobilized protein A (Pierce, Rockford, IL) for 4 h at 4°C on a shaking platform (23). The cells were centrifuged for 1 min at 14,000 $\times$ g, the supernatants were discarded and the immunoprecipitates were washed twice with 1.0 μ l of ice cold RIPA buffer prior to assay. The detection of S-nitrosylated YY1 was performed as described by Park *et al.*, (24) and by Haendeler, *et al.*, (25). The pellet was resuspended in 500 μ l of PBS. After addition of 100 μ M HgCl₂ and 100 μ M 2,3-diamino-naphthalene (DAN), then samples were incubated in the dark at room temperature for 30 min and 1 M NaOH was added. The generated fluorescent triazole from the reaction of 2,3-diamino-naphthalene with the NO released from S-nitrosylated YY1 was measured using an excitation wavelength of 375 nm and an emission wavelength of 450 nm in a fluorometer (Perkin Elmer Applied Biosystems, Foster City, CA). Immunoprecipitates using a control IgG served as negative control. We detected S-nitrosylated YY1 by immunoblot by anti-YY1 antibody after the lysates were immunoprecipitated by anti-S-nitrosocysteine antibody.

siRNA Transfections

PC-3 cells were cultured in 1 ml of RPMI medium supplemented with 5% FBS. Transfections were performed using lipofectamine 2000 CD Reagent supplied by Invitrogen (Carlsbad, CA) and the SureSilencingTM siRNA kit supplied by SuperArray Bioscience Corporation (Frederick, MD) according to the manufacturers' instructions. Briefly, 3 μ l of YY1 siRNA or a negative control of siRNA solution were incubated with 4 μ l of the transfection reagent in serum-free RPMI medium 1640 for 25 min to facilitate complex formation. The resulting mixture was added to PC-3 cells cultured in a 24-well plate with 1 ml of medium. To determine the extracellular expression of the Fas receptor, the cells were harvested 36 hours after transfection and stained with anti-Fas monoclonal antibody for 30 min then anti-mouse IgG-PE for 20 min. The expression was then

analyzed by flow cytometry. To determine the PC-3 sensitization to CH-11 mediated apoptosis, 24 h after transfection, the cells were treated for 18 h with CH-11 (5, 10 ng/ml) and fixed and permeabilized for anti-active caspase-3-FITC antibody staining. The cells were then analyzed by flow cytometry.

STATISTICAL ANALYSIS

All of the values are expressed as a mean $\pm$ SE. Each value is the mean of at least three separate experiments in each group. One-way ANOVA was used to compare variance within and among different groups. When necessary, Student's *t* test was used for comparison between two groups. A p-value of less than 0.05 was designated as being statistically significant.

RESULTS

Sensitization by NO of CaP cell lines to CH-11-induced apoptosis

The CaP cell lines PC3, DU-145 and CL-1 were treated with 1000uM of the NO donor DETA NONOate for 18h and with different concentrations of CH-11. The cells were then examined for induction of apoptosis by flow for the presence of activated caspase 3 as described in methods. All of the cell lines were resistant to CH-11 treatment alone but were all sensitized to apoptosis by DETA NONOate and the degree of apoptosis was a function of the CH-11 concentration used. The potentiation of apoptosis was synergistic as determined by isobologram analysis (Figure 1).

We have previously reported that DETA NONOate treatment of ovarian carcinoma cells resulted in sensitization of the cells to CH-11-induced apoptosis via inhibition of NF- κ B (16). We examined the effect of DETA NONOate treatment of PC3 cells on NF- κ B activity and found that, indeed, DETA NONOate inhibited NF- κ B activity as assessed by EMSA (Figure 2A). The inhibition followed an inverse relationship with the concentration of nitric-oxide donor used.

We have also reported that DETA NONOate mediated sensitization of ovarian carcinoma cells to CH-11-induced apoptosis was the result of DETA NONOate -mediated inhibition of the transcription repressor YY1, that negatively regulates Fas expression via its binding to the silencer region of the Fas promoter (16).

Treatment of PC3 cells with DETA NONOate resulted in inhibition of YY1 activity as assessed by EMSA and the inhibition was a function of the concentration of DETA NONOate used (Figure 2B). In addition, DETA NONOate inhibited the expression of YY1 as determined by flow (Figure 2C) and by western (Figure 2D).

These results demonstrate that DETA NONOate sensitized the CaP cells to CH-11 induced apoptosis and was concomitant with inhibition of NF- κ B activity and YY1 expression and activity.

S-nitrosylation of p50 and YY1 by DETA NONOate

It has been reported that DETA NONOate inhibits NF- κ B by interference in the phosphorylation and deregulation of I κ B- α (26). We show here that treatment of PC3 with DETA NONOate resulted in S-nitrosylation of p50 as detected by western and thus inhibition of NF- κ B activity (Figure 3A). There was increased S-nitrosylation of p50 with 1000 μ M than with 500 μ M DETA NONOate.

YY1 is a zinc finger protein with 4 cysteines and is highly reactive to NO. We tested the effect of DETA NONOate on YY1 and its S-nitrosylation by two methods. The first method consisted of immunoprecipitation of all proteins that are S-nitrosylated by anti-S-NO antibody and followed by western for the detection of YY1. The findings in Figure 3b (top panel) reveal that YY1 was significantly S-nitrosylated by DETA NONOate compared to untreated cells. The second method examined the release of NO from immunoprecipitated YY1 from PC3 cells by the DAN-based assay. The findings in Figure 3B (bottom panel) reveal that there was significant S-nitrosylation of YY1 following treatment with DETA NONOate and the extent of S-nitrosylation was a function of the concentration of DETA NONOate used. The S-nitrosylation of YY1 by DETA NONOate was corroborated by fluorescence immunohistochemistry whereby green fluorescence for anti-YY1 antibody and red fluorescences for anti-s-nitrocysteine antibody revealed a yellow fluorescence by convergence (Figure 3 C). In addition, the findings reveal that there was constitutive S-nitrosylated YY1 in the PC3 cells.

Role of YY1 in the regulation of Fas expression and sensitization to CH-11-induced apoptosis

The role of YY1 in the regulation of Fas expression was examined in PC3 cells treated with DETA NONOate. Treatment of PC-3 with DETA NONOate resulted in upregulation of surface Fas expression as determined by flow cytometry (Figure 4A) and for total Fas protein by western (Figure 4B). These findings

demonstrate that there was a correlation between inhibition of YY1 by DETA NONOate and upregulation of Fas expression. The direct demonstration of the role of YY1 in the regulation of Fas expression and sensitization to CH-11-induced apoptosis was examined in cells transfected with YY1 siRNA. The transfected cells showed significant upregulation of Fas expression compared to both untreated or cells transfected with siRNA negative control (Figure 5A). Further, transfection with YY1 siRNA resulted in the sensitization of PC-3 cells to CH-11-induced apoptosis (Figure 5B). Transfection with siRNA control was similar to untreated control cells. These findings demonstrate that inhibition of YY1 by S-nitrosylation results in the inactivation of the transcription repressor activity of YY1 on the Fas promoter and this results in the upregulation of Fas expression and concomitantly sensitization to CH-11-induced apoptosis.

DISCUSSION

The present study provides for the first time direct evidence that S-nitrosylation of YY1 in CaP cell lines following treatment with the NO donor DETA NONOate results in the upregulation of Fas expression. S-nitrosylation of YY1 was demonstrated by three different methods, namely the DAN method, immunoprecipitation and western and by double immunofluorescence. YY1 negatively regulates Fas transcription through its interaction with the silencer region of the Fas promoter (16). The direct role of YY1 in the negative regulation of Fas expression and its inhibition by DETA NONOate was demonstrated in cells transfected with YY1 siRNA that resulted in upregulation of Fas expression. The negative regulation of Fas expression by YY1 correlated with the resistance to CH-11-induced apoptosis. Hence, treatment by DETA NONOate sensitized the CaP cells to CH-11-induced apoptosis. Further, treatment with YY1 siRNA resulted in the sensitization of CaP cells to CH-11-induced apoptosis. Altogether, these findings demonstrate that S-nitrosylation of YY1 upregulates Fas expression and sensitizes CaP cells to CH-11-induced apoptosis. These findings suggest that Fas expression in tumor cells may be downregulated through overexpression of YY1 and can lead to resistance to immune-mediated apoptosis. Thus, regulation of the level of YY1 in tumor cells may provide a new therapeutic strategy to potentiate tumor directed immunotherapy.

Our previous findings have demonstrated that treatment of ovarian tumor cells with IFN- γ , which induces iNOS expression, or with NO donors such as DETA NONOate resulted in upregulation of Fas expression and sensitization to CH-11-induced apoptosis (20). Further studies demonstrated that NO-mediated upregulation of Fas expression was the result of inhibition of the transcription repressor YY1 which negatively regulates Fas expression through its binding to the silencer region of the Fas promoter (16). We confirm here that treatment of CaP cell lines with DETA NONOate resulted in upregulation of Fas expression and sensitization to CH-11-induced apoptosis. However, the mechanism by which NO inhibits YY1 expression and activity was not known. We hypothesized that NO may chemically modify YY1 through S-nitrosylation. YY1 is a zinc finger transcription factor that is involved in the negative regulation of many mammalian genes (15). The most prevalent DNA-binding motif of several transcription factors is the zinc finger structure (27). NO is known to interfere with the DNA-binding activity of many zinc finger transcription factors via S-nitrosylation of cysteine thiol groups and subsequent S-nitrothiol formation (28,29). The yeast zinc finger transcription factor, LAC9, was the first transcription factor reported to be inhibited by NO (30). Since YY1 is a transcription factor that can both activate and repress gene expression, its S-nitrosylation by NO may result in either upregulation of gene transcription or inhibition of gene transcription, respectively. For instance, it has been reported that NO activates the TNF- α gene transcription by S-nitrosylation of the repressor transcription factor SB1 and thus disrupts SB1 DNA-binding activity (31).

In this study, we demonstrate directly that NO biochemically interacts with YY1 and results in its S-nitrosylation and consequently inhibition of its DNA-binding activity. The direct demonstration of S-nitrosylation of YY1 was found following the use of the DAN assay for NO release following immunoprecipitation of YY1 as has been reported by others for other proteins (32,33). The DAN method was used to show that NO negatively regulates c-Jun N-terminal kinase/ stress-activated protein kinase (24) and of thioredoxin by S-nitrosylation (25). The DAN method for S-nitrosylation of YY1 was confirmed by immunoprecipitation and Western (Figure 3B). Protein S-nitrosylation is not stable enough to survive the rigors of denaturing SDS-PAGE. Hence, anti S-nitrocysteine antibody was applied for immunohistochemistry and could not be applied for immunoblots. We detected S-nitrosylated YY1 by Western blot using anti-YY1 antibody

following immuno-precipitation with S-nitrocysteine antibody. The biochemical detection of S-nitrosylated YY1 described above was confirmed by immunohistochemistry. We demonstrate the co-localization of S-nitrosylated proteins and YY1 by double immuno-fluorescence (Figure 3C). Other studies with immunohistochemistry reported the S-nitrosylation of antigens in fixed trypanosomes (10). Basal and stimulated proteins which were S-nitrosylated were detected in multiple cell types and tissues (11). In this study, we also demonstrate that S-nitrosylated proteins and S-nitrosylated YY1 are detected in normal untreated tumor cells suggesting that endogenous NO production may be an important regulator of protein expression and activity. Like protein phosphorylation, protein S-nitrosylation could potentially represent a fundamental mechanism for transcriptional control of protein activity and cellular function (5,34).

In this study, we also show that NO inhibits YY1 expression. Recent studies suggest YY1 may be, in part, under the control of NF- κ B (Unpublished). Further, it has been reported that NO inhibits NF- κ B activity (35) by S-nitrosylation of p50 which inhibits NF- κ B DNA-binding activity (36) and this was confirmed in this study (Figure 3A). NF- κ B is a transcription factor that displays redox-sensitive DNA-binding (37,38). The redox-sensitivity is confirmed by a single cysteine residue within the DNA-binding site (39). These cysteines are potential sites for post translational modification by S-nitrosylation (35).

Inhibition of NF- κ B activity and YY1 expression and activity by NO resulted in the upregulation of Fas expression. Previous findings from our laboratory have demonstrated that upregulation of Fas expression by NO resulted from inhibition of the transcription repressor YY1 DNA-binding activity onto the silencer region of the Fas promoter (16). In this study, we demonstrate directly the role of YY1 in the upregulation of Fas by using cells transfected with YY1 siRNA and demonstrate that the transfectants show significant upregulation of Fas expression (Figure 5A).

We have demonstrated that treatment of CaP cell lines with DETA NONOate sensitized these cells to CH-11-induced apoptosis. These findings are in contrast to those reported by Mannick *et al.*, (40) who demonstrated that basal NOS activity in human leukocytes inhibits Fas-induced apoptosis via an cGMP-independent mechanism. They also show that inhibition of iNOS activity significantly increased Fas-induced apoptosis. Further, they reported that NOS expression does not alter Fas expression. It has been recognized that

NO or related molecules can exert pro or anti apoptotic effects depending on the cell types and stimulus (41-43). It has been shown that NO inhibits apoptosis through S-nitrosylation and inactivation of caspases (42, 44-45). Brune *et al.* (43) used the NO donor SNAP in their studies and demonstrated that NO attenuates the processing of caspases in addition to inhibiting their activity. NO interferes with the apoptosome formation and cell death execution. Our findings showing NO-induced sensitization to Fas apoptosis is compatible with other studies on the role of NO in apoptosis (41,46-47). We have recently reported that NO activates the mitochondria resulting in the release in the cytoplasm of modest amounts of cytochrome c and Smac/DIABLO in the absence of activation of the apoptosome. However, combination with TRAIL resulted in the activation of the apoptosome and synergy in apoptosis was achieved (48).

In conclusion, our findings indicate that NO donors upregulate Fas expression and can sensitize tumor cells to Fas-induced apoptosis through inhibition of YY1 activity (See schematic diagram in Figure 6). We have recently found that YY1 is overexpressed in human prostate cancer arrays compared to normal tissues (49). Overexpression of YY1 may regulate resistance and inhibits tumor cell destruction by the host immune system and may lead to tumor progression. Therapeutic interventions that can inhibit YY1 activity may restore sensitivity of the tumor cells to host immune cells and/or to immunotherapeutic interventions.

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Footnotes

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Figure Legends

Figure 1: Sensitization of CaP cell lines by DETA NONOate to CH-11-induced apoptosis.

The three cell lines PC3, CL1, and DU145 were treated with DETANONOate (1000 uM) and various concentrations of CH-11. These cells were examined for apoptosis as described in methods. The findings demonstrate that all three lines were significantly sensitized to CH-11-induced apoptosis and the extent of apoptosis was a function of the CH-11 concentration used (Figure 1-Top). The sensitization by DETA NONOate to CH-11-induced apoptosis was synergistic as determined by isobologram analysis (Figure 1-Bottom). $*p<0.05$ $**p<0.02$

Figure 2: Inhibition of NF- κ B DNA-binding activity and YY1 DNA-binding activity and expression by DETA NONOate

The PC3 cell line was treated with two concentrations of DETA NONOate, 500 uM and 1000 uM, and the cells were harvested and both nuclear lysates and total cell lysates were prepared for analysis. The nuclear lysates were examined for NF- κ B and YY1 DNA-binding activity by EMSA as described in methods. The findings demonstrate that DETA NONOate inhibits both NF- κ B (Figure 2A) and YY1 DNA-binding activity (Figure 2B) and DETA NONOate and concentration of 1000 uM was more inhibitory than DETANONOate at 500 uM. The inhibition of YY1 expression by DETANONOate was examined by flow cytometry and western. The findings demonstrate that there was significant inhibition of YY1 expression by DETA NONOate as determined by flow (Figure 2C) ($*p<0.05$) and by western (Figure 2D).

Figure 3: Treatment of PC3 cells with DETA NONOate results in the S-nitrosylation of NF- κ B P50 and YY1

PC3 cells were treated with two concentrations of DETA NONOate, 500 uM and 1000 uM, and the cells were treated for immuno-precipitation using anti-S-nitrocysteine-antibody and then immunoblotted with anti-P50 antibody (Figure 3A) or anti-YY1 (Figure 3B Top). The findings demonstrate that both P50 and YY1 were S-nitrosylated by DETA NONOate and there was more S-nitrosylated protein following treatment with 1000 uM DETA NONOate compared to 500 uM DETA NONOate. The S-nitrosylation of YY1 was further corroborated by the DAN method as described in methods. Clearly, treatment with DETA NONOate resulted in

significant fluorescence activity compared to medium control and there was more fluorescence released by 1000 μ M DETA NONOate compared to 500 μ M DETA NONOate (Figure 3B Bottom).

The biochemical determination of S-nitrosylated YY1 was corroborated by immunohistochemistry using double-immuno-fluorescence. PC3 cells were cultured on Culver slides in the presence of DETA NONOate (500 μ M). The cells were then treated with anti-YY1 antibody and determined with a secondary antibody with red fluorescence. The cells were then treated with S-nitrocysteine-antibody and secondary antibody with green fluorescence. The findings demonstrate that treatment of the cells with DETA NONOate results in significant red fluorescence. Likewise treatment with S-nitrocysteine results in significant green fluorescence compared to untreated cells. Co-localization of the merger resulted in significant yellow fluorescence compared to untreated cells. These findings demonstrate that treatment with DETA NONOate results in upregulation of YY1 S-nitrosylation above baseline (Figure 3C).

Figure 4: Regulation of Fas expression on PC3 cells by treatment with DETA NONOate and the role of YY1 in the regulation of Fas expression

PC3 cells were treated with two concentrations of DETA NONOate and following incubation, they were examined for the expression of Fas by flow cytometry and by Western. The findings demonstrate that treatment with DETA NONOate significantly upregulated the expression of Fas compared to untreated cells when examined by flow cytometry for surface expression (Figure 4A) and for total Fas expression as determined by Western (Figure 4B).

Figure 5: The direct role of YY1 in the negative regulation of Fas expression and sensitivity to Fas-induced apoptosis

The direct role of YY1 in the regulation of Fas expression was done using YY1 siRNA transfection of PC3 cells. The results demonstrate that treatment with YY1 siRNA resulted in significant upregulation of Fas expression compared to cells treated with control siRNA or untreated cells (Figure 5A). In addition, inhibition by YY1 siRNA resulted in significant potentiation of CH-11-induced apoptosis compared to untreated cells or cells treated with negative control of siRNA. The potentiation of apoptosis by YY1 siRNA was a function of the CH-11 concentration used (Figure 5B)

**p<0.05 **p<0.02*

Figure 6: Schematic diagram illustrating the role of YY1 S-nitrosylation in the regulation of Fas expression and sensitization to Fas-induced apoptosis

In the left hand of the figure, the tumor cells express constitutive YY1. YY1 activity negatively regulates the expression of Fas via its repressor activity on the silencer region of the Fas promoter and regulates tumor cell sensitivity to CH-11-induced apoptosis (Garban and Bonavida, 2001). The cells also remain resistant to CH-11-induced apoptosis. In the right hand side of the figure, treatment of the tumor cells with the nitric oxide donor DETA NONOate results in the downregulation of YY1 transcription and YY1 DNA-binding activity. DETA NONOate directly modifies YY1 DNA-binding activity via S-nitrosylation and inhibits its repressor activity on the Fas promoter and results in the upregulation of Fas expression. These effects correlate with the sensitization of the tumor cells to Fas-induced apoptosis.

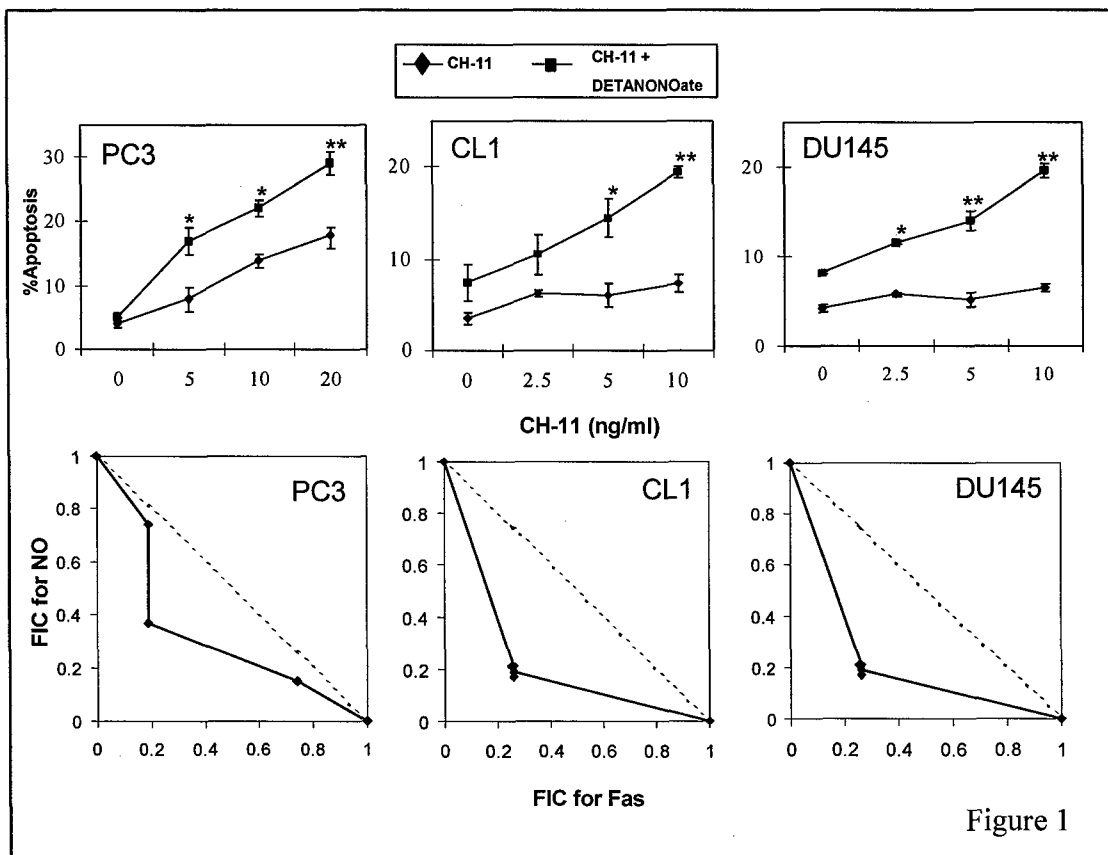


Figure 1

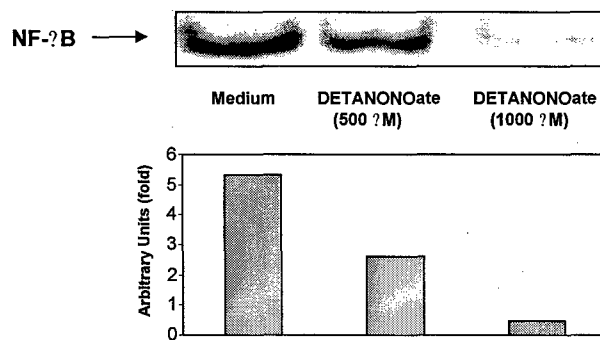


Figure 2(A)

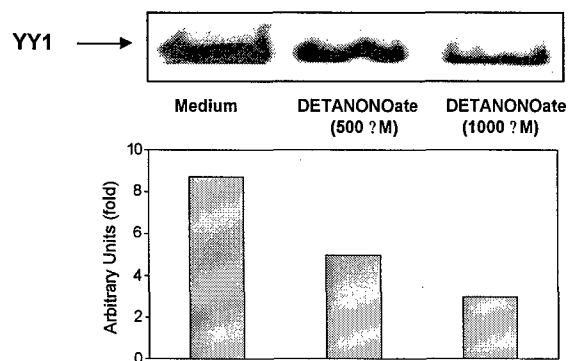


Figure 2(B)

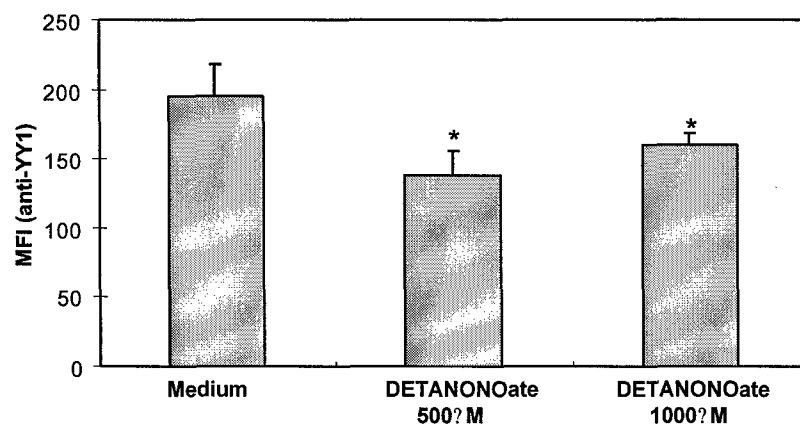


Figure 2(C)

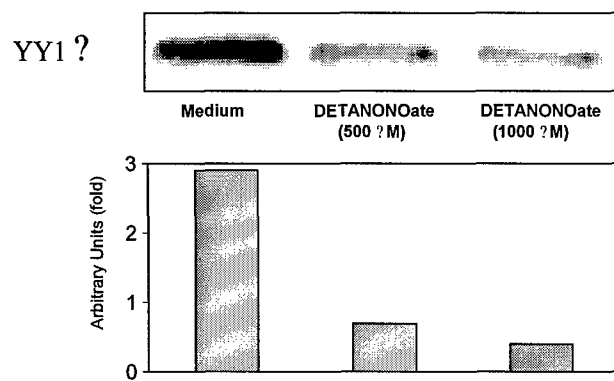


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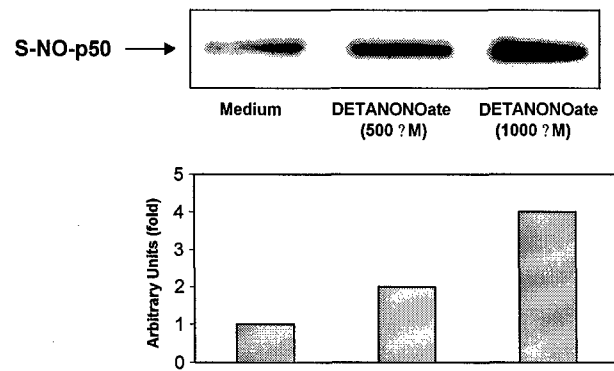


Figure 3(A)

S-NO-YY1

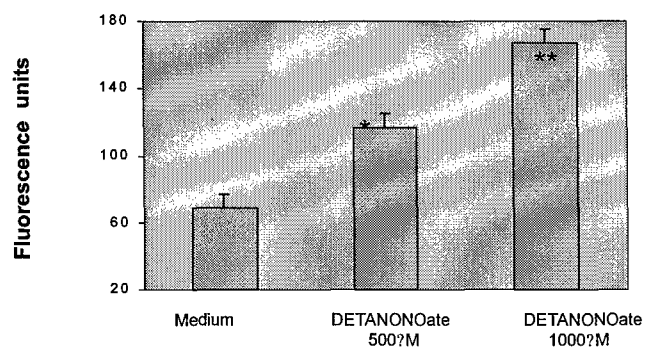
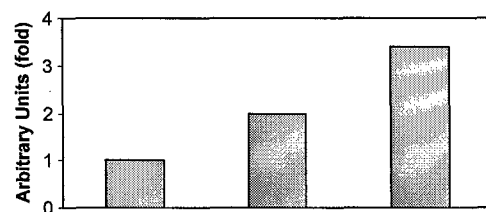
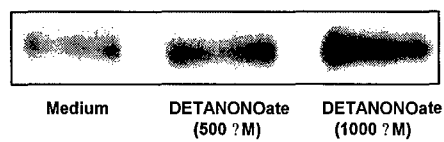
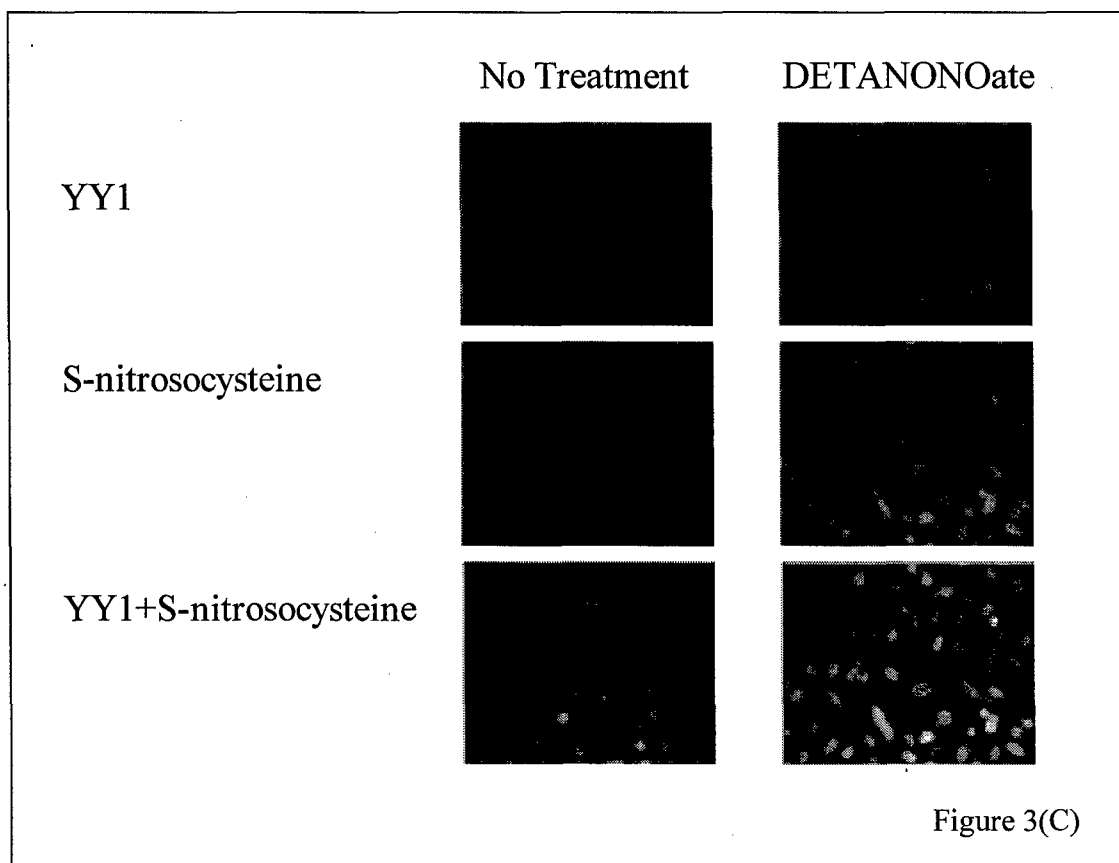


Figure 3(B)



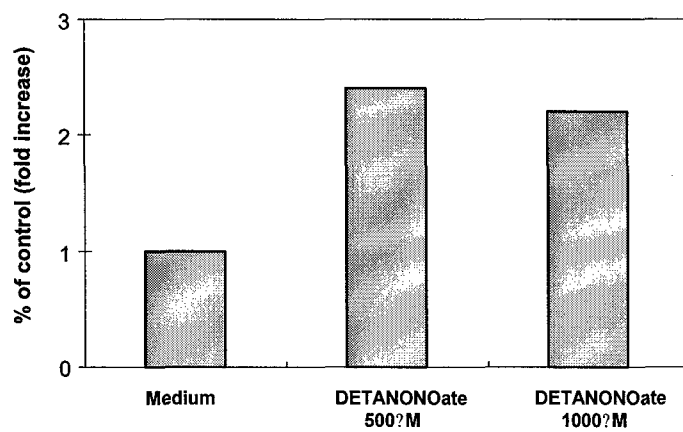


Figure 4(A)

Fas ?

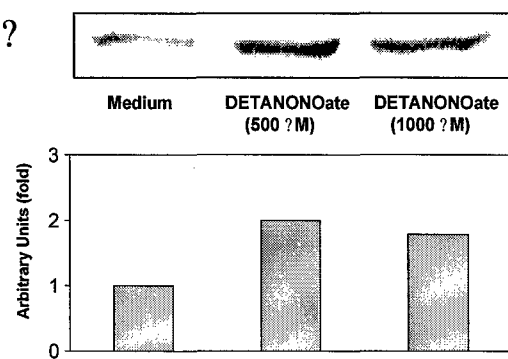


Figure 4(B)

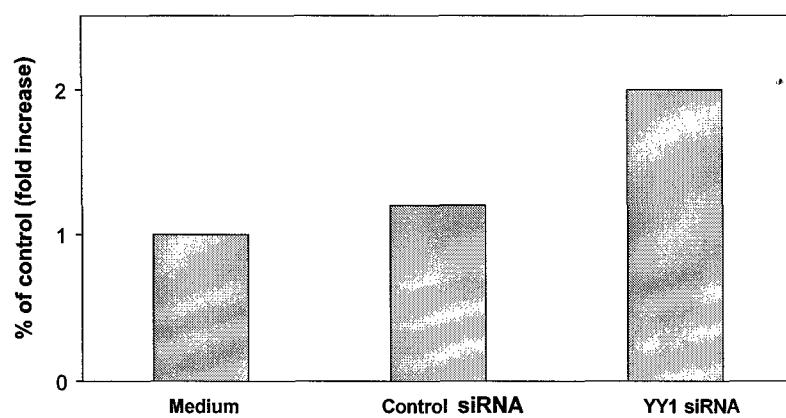


Figure 5(A)

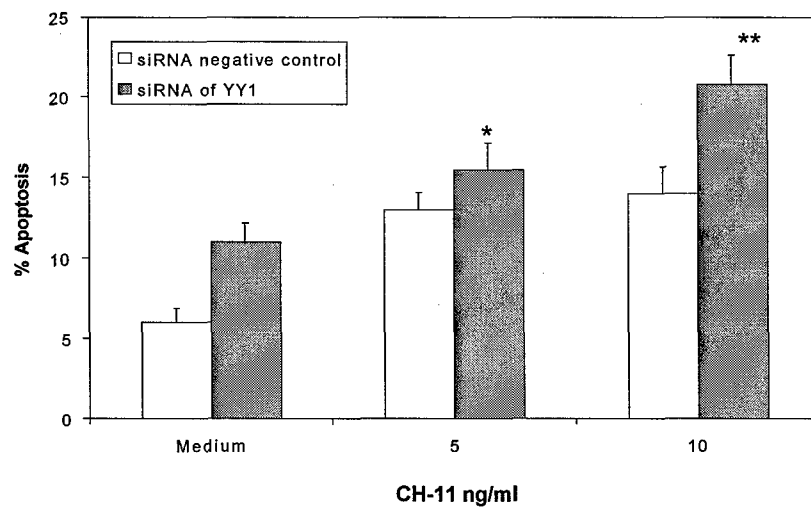


Figure 5(B)

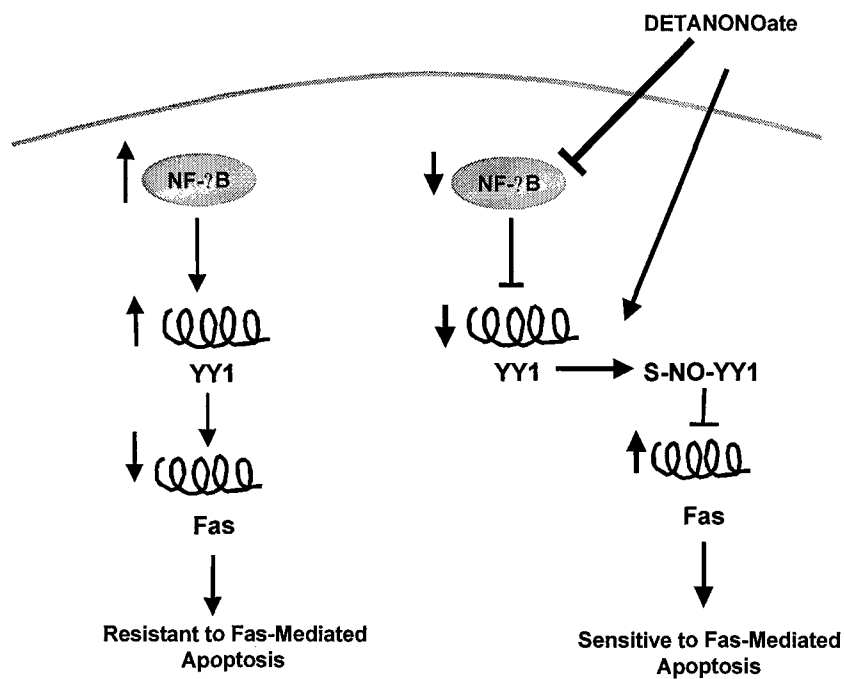


Figure 6

APPENDIX 8

Role of TNF- α autocrine/paracrine loop in the activation of NF- κ B and YY1 in the regulation of tumor cell resistance to Fas-induced apoptosis.

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Running title: NF- κ B-mediated regulation of YY1 expression and activity

Key words: Yin-Yang 1, NF- κ B, TNF- α , prostate cancer, YY1 transcription

ABBREVIATIONS USED

NO: nitric oxide

sTNF-R1: soluble tumor necrosis factor receptor 1

TNF- α : tumor necrosis factor alpha

YY1: Yin-Yang 1

EMSO: Electrophoretic mobility-shift assays

siRNA: Small-interfering RNA

NF- κ B: Nuclear factor kappa B

PE: Phycoerythrin

FITC: Fluorescein Isothiocyanate

PAGE: Polyacrylamide gel electrophoresis

FBS: Fetal bovine serum

ABSTRACT

Tumor-derived factors have been implicated in the regulation of tumor cells' sensitivity to apoptotic stimuli. This study investigated the role of tumor-derived TNF- α in the regulation of cell sensitivity to Fas-induced apoptosis. We hypothesized that TNF- α , through an autocrine/paracrine loop, activates NF- κ B activity and results in the upregulation of the transcription repressor Yin Yang 1 (YY1). In turn, YY1 regulates tumor resistance to Fas-induced apoptosis through the negative regulation of Fas expression. Hence, inhibition of YY1 should sensitize cells to Fas-induced apoptosis. This hypothesis was tested in a prostate cancer cell line, PC-3, which synthesizes and secretes TNF- α and expresses constitutive active NF- κ B. Treatment of PC-3 cells cultured under serum-free conditions with TNF- α (10 units) resulted in increased NF- κ B and YY1 DNA-binding activity, upregulation of YY1 expression and downregulation of Fas expression. In contrast, blocking the activity of secreted TNF- α with soluble sTNF-R1 resulted in significant inhibition of both NF- κ B and YY1 DNA-binding activities, downregulation of YY1 expression, upregulation of Fas expression and sensitization to Fas (CH-11)-induced apoptosis. The regulation of YY1 expression and activity by NF- κ B was demonstrated by using a GFP reporter system whereby deletion of the YY1 tandem binding sites in the promoter enhanced significantly GFP expression. The role of NF- κ B in the regulation of YY1 was corroborated by treatment of PC-3 cells with the NF- κ B inhibitor Bay 11-7085 which resulted in inhibition of both NF- κ B activity and YY1 expression and CH-11 activity. The role of NF- κ B/YY1 in the negative regulation of Fas expression and sensitivity to CH-11 apoptosis in PC-3 was demonstrated by treatment with the inhibitors sTNF-R1 or Bay 11-7085 and resulted in upregulation of Fas and sensitization to CH-11-induced apoptosis. The direct role of YY1 in the regulation of PC-3 resistance to Fas was shown in using cells transfected with siRNA YY1 and the transfectants exhibited upregulation of Fas expression and were sensitized to CH-11-induced apoptosis. These findings demonstrate that the TNF- α autocrine-paracrine loop is involved in the constitutive activation of NF- κ B and YY1 leading to inhibition of Fas expression and resistance to Fas-induced apoptosis. Inhibition of TNF- α synthesis/ secretion, NF- κ B activity, or YY1 can reverse tumor cell resistance to Fas-mediated apoptosis.

INTRODUCTION

Cytotoxic lymphocytes kill target cells by various mechanisms including perforin/granzymes and the TNF superfamily that kills primarily by apoptosis (1, 2). Tumors that develop anti-apoptotic mechanisms to chemotherapeutic drugs/radiation-mediated apoptosis can also develop cross-resistance to immune cytotoxic lymphocytes (3, 4). The molecular mechanisms that govern anti-apoptotic resistance in cancer cells are numerous and vary from tumor to tumor. Our recent findings revealed a novel mechanism of tumor cell resistance to immune-mediated apoptosis. For example, we have shown that resistance to Fas-mediated apoptosis of human ovarian and prostate cancer cells is in part due to the repressor activity of the transcription factor Yin Yang 1 (YY1)(5). YY1 negatively regulates Fas expression and sensitivity to Fas-mediated apoptosis and inhibition of YY1 DNA-binding activity resulted in up-regulation of Fas expression and sensitization of tumor cells to Fas-mediated apoptosis (6).

YY1 is a 414-amino acid KRUPPEL-related zinc finger transcription factor that binds to the CG (A/C) CATNTT consensus DNA element located in promoters and enhancers of many cellular and viral genes (Usheva and Shenk, 1994; Hyde-DeRuyscher *et al.*, 1995; Shi *et al.*, 1997; Austen *et al.*, 1997). YY1 is a transcription factor that can act as a transcriptional repressor, activator, or initiator element binding protein (Shi *et al.*, 1997; Thomas and Seto, 1999). The transcription activity of YY1 can be regulated by viral onco-proteins such as adeno-virus E1A (Shi *et al.*, 1991). The transcription factor YY1 has been identified as a potential repressor factor in the human interferon- γ gene (Ye *et al.*, 1994(a); 1996(a)), the GMCSF promoter (Ye *et al.*, 1994(b); Ye *et al.*, 1996(b)) and the IL-3 gene promoter (Ye *et al.*, 1999). YY1 also regulates p53 dependent transcription (Yakovlena *et al.*, 2004).

The transcription factor NF- κ B is an important regulator of cells' ability to undergo apoptosis. NF- κ B coordinates the expression of many genes involved in the regulation of inflammation, the immune response, cell proliferation and apoptosis. In its anti-apoptotic capacity, NF- κ B attenuates TNF- α -induced apoptosis through upregulation of anti-apoptotic gene products (Karin *et al.*, Nature Review. 2:301, 2002; Ivanov *et al.*, Oncogene. 22: 3152, 2002). Positive regulation of Fas transcription has been shown to depend on NF- κ B (Chan

et al., Mol. Cell. Biol. 19: 2098, 1999; Ivanov and Ronai, 2000. Oncogene. 19: 3003). However, negative regulation of Fas expression may also take place indirectly via a transcription repressor like YY1.

Computer-based transcription search (TESS) analyses of the promoter region of the YY1 gene revealed the presence of 4 NF- κ B putative binding sites clustered within the promoter of YY1 (-227bp from transcription site). Tumor cells in general exhibit constitutively active NF- κ B which might regulate YY1 expression and activity. We hypothesized that the constitutive activation of NF- κ B in some tumors may be due to the autocrine-paracrine loop of tumor derived factors such as TNF- α , IL1- α , IL-6 (REF). We also hypothesized that the activation of YY1 by NF- κ B may be involved in the negative regulation of Fas expression and sensitivity of the cells to Fas-induced apoptosis. Changes in Fas expression and activity have been reported in many types of tumors (Hug, 1997, Biol. Chem. 378: 1405; Shui MS *et al.*, 1999, Am. J. Path. 154: 1785; Bullani *et al.*, 2002, Melanoma Res. 12: 2631). Above hypotheses, linking the autocrine-paracrine loop of TNF- α with the regulation of NF- κ B and YY1 and their role in the regulation of Fas, were investigated in this study. We examined the prostate cancer cell line PC-3 which has been reported to secrete TNF- α and expresses constitutive NF- κ B activity (8) as a model system. The followings were investigated: 1) The role of endogenously-secreted TNF- α in PC-3 in the regulation of NF- κ B and YY1 activity via an autocrine-paracrine loop 2) The role of NF- κ B in the regulation of YY1 expression and activity 3) The role of NF- κ B and YY1 in the regulation of Fas expression and 4) The direct role of YY1 in the negative regulation of Fas expression and resistance to Fas-induced apoptosis.

MATERIALS AND METHODS

Cell Culture and Reagents

The human androgen-independent PC-3 cell line is a metastatic bone-derived prostatic adenocarcinoma. PC-3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). PC-3 cells express low surface Fas and are resistant to Fas ligand (9). SW480 and SW620 cell lines were derived from a colon carcinoma of the same individual with the latter being from an advanced-stage, metastatic tumor (10, 11). While SW480 expresses high levels of surface Fas receptor and is sensitive to Fas-mediated apoptosis, SW620

expresses low levels of surface Fas receptor and is resistant to Fas-induced apoptosis. K562 is known to be Fas-resistant and Raji a Fas-sensitive lymphoma line (12).

The cell cultures were maintained as monolayers on plastic dishes. All the cells were maintained at 37°C and 5% carbon dioxide in RPMI 1640 (Life Technologies Bethesda, MD), supplemented with 10% heat-inactivated FBS, 1% (v/v) penicillin (100 U/ml), 1% (v/v) streptomycin (100 U/ml), 1% (v/v) L-glutamine, 1% (v/v) pyruvate, and 1% nonessential amino acids (Life Technologies). For every experimental condition, the cells were cultured in 1% FBS, 18 h prior to treatments.

The human recombinant TNF- α and human recombinant soluble TNFRI were obtained from PeproTech, Inc (Rocky Hills, NJ). The cytotoxic anti-Fas monoclonal antibody (IgM, clone CH-11) and the surface-staining monoclonal antibody (IgG₁, clone UB2) were purchased from Biomedical Co. (Thousand Oaks, CA). The rabbit anti-YY1 polyclonal antibody was obtained from Geneka (Montreal, Canada). FITC-conjugated anti-active caspase-3 and FITC-conjugated IgG were purchased from PharMingen (San Diego, CA). The inhibitor of NF- κ B (Bay-specific inhibitor of I κ B γ phosphorylation) was obtained from Calbiochem (San Francisco, CA).

Cytokine Treatment

Log phase PC-3 cells were used to seed six-well plates at approximately 5×10^5 cells/ml and the cells were grown in 2 ml of medium as described above in 10% FBS for 24 h to approximately 70% confluence. The cells were synchronized and treated with 1% FBS for 18 h prior to treatment with TNF- α (10 U/ml) in serum-free RPMI medium for 24 h. Untreated cultured PC-3 cells in serum-free RPMI medium were used as a control for basal expression levels in the absence of exogenous cytokine.

Reporter system and site directed mutagenesis

The human Ornithine Decarboxylase Antizyme 1 (ODA1) minimal promoter (13) containing 201 bp upstream of the translation initiation site that includes an unique wild type responsive site (cgccatttgcca) for the transcription repressor Yin Yang 1 (YY1) was amplified by PCR using the forward primer 5'-CGG GCG CGA CTT TTT TTC CCG GC-3' and the reverse primer 5'-CCG GCC GCT GGG GTC CGA AAC CAG-3'.

Genomic DNA extracted from cultured PC3 cells was used as template. PCR amplifications were conducted using the Advantage-HF2 system (Clontech, Palo Alto, CA) following the manufacturer's recommendations. The gel-purified amplicon was ligated to the green fluorescent protein (GFP)-based pGlow-TOPO[®] reporter vector (Invitrogen, Carlsbad, CA) and further screened and sequenced in order to confirm fidelity and orientation of the construct (pGlow-OAZmp/WT-YY1). We further generated one more construct where the YY1 cis-acting element (cgttgttttgcga) was mutated using the GeneTailor[™] Site-Directed Mutagenesis System (Invitrogen) following the manufacturer's recommendations. We confirmed the mutated reporter construct (pGlow-OAZmp/Mu-YY1) by automated sequencing. GFP-based reporter activity from transfected cells with these constructs, was analyzed by direct fluorescence emission at 510 nm using excitation at 395 nm in a Fluorometer (Perkin Elmer Applied Biosystems, Foster City, CA).

Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For each of the cell lines, total RNA was extracted and purified from 1×10^6 cells for each experimental condition by a single-step monophasic solution of phenol and guanidine isothiocyanate-chloroform using Trizol[®] reagent (Life Technologies, Inc.). 3 μ g of total RNA was reverse-transcribed to first strand cDNA for 1 h at 42°C with SuperScript[™] II reverse transcriptase (Life Technologies, Inc) in a 20 μ L reaction and performed per the manufacturer's specifications using random primers. Amplification of 1/10 of these cDNA by PCR was performed using the following gene-specific primers: YY1 (forward) (5'-GAA AAC ATC TGC ACA CCC ACG GTC C-3'), YY1 (reverse)(5'-GTC CTC CTG TTG GGA CCA CAC-3'), and Fas (forward)(5'-ATG CTG GGC ATC TGG ACC CT-3'). Internal control for equal cDNA loading in each reaction was assessed using the following gene specific glyceraldehydes-3-phosphate dehydrogenase (GAPDH) primers: GAPDH (forward)(5'-GAA CAT CAT CCC TGC CTC TAC TG-3') and GAPDH (reverse)(5'-GTT GCT GTA GCC AAA TTC GTT G-3'). PCR amplification of each DNA sequence was carried out by the "Hot Start" method using Titanium Taq[®] polymerase (Clontech) with the following one-step thermal cycling incubation: 95°C/30 s, 68°C/1 min for 30 (Fas and YY1) or 25 (GAPDH) cycles, with a final extension at 68°C/3 min. The number of cycles was established based on preliminary titration of the relative amount of amplified product for each gene representing the linear phase of amplification process. The amplified products were resolved on 1.5%

agarose gel electrophoresis and their relative concentrations were assessed by densitometric analysis of digitized ethidium bromide-stained image, performed on a Macintosh computer (Apple Computer Inc., Cupertino, CA.) using the public domain NIH Image J Program (available on the internet).

Western Blot Analysis

PC-3 cells were cultured at a low serum concentration (0.1%) 18 h prior to each treatment. After incubation, the cells were maintained in serum free medium (control), or treated with TNF- α (1, 10, and 100 U/ml-24 h). The cells were then lysed at 4°C in RIPA buffer {50mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150mM NaCl}, and supplemented with one tablet of protease inhibitor cocktail, Complete Mini Roche (Indianapolis, IN). Protein concentration was determined by a DC protein assay kit Bio-Rad (Hercules, CA). An aliquot of total protein lysate was diluted in an equal volume of 2XSDS sample buffer 6.2mM Tris (pH6.8), 2.3% SDS, 5% mercaptoethanol, 10% glycerol, and 0.02% bromophenol blue and boiled for 10 minutes. The cell lysates (40 μ g) were then electrophoresed on 12% SDS-PAGE gels (Bio-Rad) and were subjected to Western blot analysis as previously reported (14). Levels of β -actin were used to normalize the YY1 expression. Relative concentrations were assessed by densitometric analysis of digitized autographic images, performed on a Macintosh computer (Apple Computer Inc., Cupertino, CA.) using the public domain NIH Image J Program (available on the internet).

NUCLEAR EXTRACTS PREPARATION

Nuclear extracts preparation were done as previously described by our laboratory (Garban and Bonavida, 2001). Briefly, cells (10⁶) were harvested after treatment and washed twice with cold Dulbecco PBS (Cellgro). After washing, cells were lysed in 1 ml of NP40 lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM MgCl₂, and 0.5% NP40) on ice for 5 min. Samples were centrifuged at 300 g at 4°C for 5 min. The pellet was washed twice in NP40 buffer. Nuclei were then lysed in nuclear extraction buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and sonicated 10 s at 4°C. The protein concentration was determined using the Bio-Rad

protein assay. The nuclear proteins were frozen at -80° C. Both buffers contained the complete protease inhibitor cocktail tablets from Roche.

EMSA

Nuclear proteins (5 μ g) were mixed for 30 min at room temperature with Biotin-labeled oligonucleotide probe NF- κ B and YY1 using EMSA Kit PanomicsTM (Panomics, Inc. Redwood City, CA) following the manufacture's instructions. 10 μ l of the reaction was subjected to denaturing 5% polyacrylamide gel electrophoresis for 90 min in TBE buffer (Bio-Rad Laboratories) and transferred to Nylon membrane Hybond-N+ (Amersham Pharmacia Biotech, Germany) using the Trans-Blot^{SD} semi-dry Transfer cell System (Bio-Rad, Hercules, CA)). The blotted membranes were transferred to a UV Crosslinker FB-UVXL-1000 Fisher technology (Fisher Scientific, NY) for 3 min. The detection was made following the manufacture's instructions. The membranes were then exposed using Hyperfilm ECL (Amersham Pharmacia Biotech). The oligonucleotide consensus sequences for NF- κ B are as described: 5'-AGTTGAGGGGACTT TCCCAGGC-3' for YY1: 5'-CGCTCCGCGGCCATCTTGGCGGCTGGT-3'. Relative concentrations were assessed by densitometric analysis as mentioned above.

Caspase-3 Activity

PC-3 cells were grown in a six-well plate at a low serum concentration (0.1%) 18 h prior to each treatment. After incubation, the cells were maintained in serum free medium (control), or treated with TNF- α (10 U/ml-24 h), CH-11 antibody (30 ng/ml-12 h) or a combination of TNF- α and CH-11 antibody. Some samples were treated and some were left untreated with recombinant soluble TNFRI (0.3 μ g/ml). At the end of the incubation period, the cells were washed once with ice cold 1XPBS and were resuspended in 200 of the cytofix/cytoperm solution (PharMigen, San Diego, CA) for 20 min. Thereafter, the samples were washed twice with ice cold 1Xperm/wash buffer solution (PharMigen) and were stained with FITC-labeled anti-active-caspase-3 mAb for 30 min (light protected). The samples were subsequently washed once with 1Xperm/wash

buffer solution and 200 μ l of 1XPBS was added prior to flow cytometry analysis (Coulter). As a negative control, the cells were stained with isotype control (pure IgG) under the same conditions described above.

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Statistical Analysis.

The experimental values were expressed as the mean $\pm$ SEM for the number of separate experiments indicated in each case. One-way ANOVA was used to compare variance within and among different groups. When necessary, Student's *t* test was used for comparison between two groups. Significant differences were considered for probabilities $\leq$ 5% ($p \leq 0.05$).

RESULTS

Endogenous and exogenous TNF- α regulate YY1 gene expression and indirectly control Fas gene expression

We have previously reported that PC-3 cells secrete TNF- α (8) and PC-3 express TNF-RI (15). Thus, we selected the PC-3 prostate carcinoma cell line for investigation of underlying mechanisms of TNF- α -mediated regulation of YY1 expression and activity. Since TNF- α activates NF- κ B and NF- κ B may be regulating YY1 expression, we investigated the role of TNF- α on the transcriptional regulation of YY1 in the PC-3 cell line. PC-3 cells were treated with TNF- α and YY1 expression was measured by flow cytometry (Figure 1A). YY1 expression dropped when PC-3 cells were cultured in the absence of serum, suggesting that soluble factors present in the serum and derived from the tumor may be involved in YY1 expression. However, serum-free PC-3 cultured in the presence of TNF- α (10U/ml), resulted in significant upregulation of YY1 expression determined by flow (Figure 1A). This was confirmed by Western blot analysis whereby TNF- α up-regulated the expression of YY1 in a concentration-dependent manner (Figure 1B). Also, we examined the relative mRNA expression of YY1 in PC-3 cells cultured for 18 h under serum-free conditions in the presence or absence of TNF- α (10U/ml). Semiquantitative RT-PCR reflected a significant increase in the level of YY1 mRNA expression upon treatment with TNF- α (data not shown). We have previously reported that YY1

activity negatively regulates Fas expression (Garban and Bonavida, 2001), hence, we investigated the role of TNF- α on the transcriptional regulation of Fas in the PC-3 cell line. PC-3 cells were either left untreated or treated with TNF- α (10 U/ml) for 24 h under serum free conditions. Upon treatment with TNF- α , Fas expression dropped significantly (Figure 1C), suggesting that activation of YY1 by TNF- α negatively regulates Fas expression.

Based on the findings above with exogenous TNF- α , we then examined whether endogenous TNF- α was involved in the regulation of YY1 expression in the PC-3 cells by an autocrine-paracrine loop. Upon treatment with recombinant sTNFR1 (0.1, 0.3 μ g/ml), to block TNF- α -TNFR interaction, PC-3 cells showed significantly lower YY1 expression compared to untreated cells and the inhibition was a function of the sTNFR1 concentration used (Figure 1D). Altogether, the above findings demonstrate that TNF- α activates YY1 and inhibits Fas expression in PC-3 cells. These findings were corroborated physiologically by showing that endogenously synthesized and secreted TNF- α by PC-3 cells, via an autocrine-paracrine loop, is involved in the upregulation of YY1 expression, and YY1 negatively regulates Fas expression in PC-3 cells.

Regulation of YY1 by TNF- α

1. TNF- α regulates YY1 expression via activation of NF- κ B

The YY1 core promoter contains a significant cluster of NF- κ B responsive elements (Figure 2A). TNF- α has been shown to stimulate anti-apoptotic responses by activation of NF- κ B in tumor cells (15, 16). Therefore, we have postulated that TNF- α may upregulate YY1 expression through activation of NF- κ B and accordingly, inhibition of NF- κ B may, in turn, downregulate YY1 expression. PC-3 cells were treated with different non-toxic concentrations of the NF- κ B inhibitor Bay 11-7085 (0, 1, 2 or 3 μ M/ml) for 1 h and the cells were then cultured in the presence or absence of TNF- α (10 U/ml) in serum free conditions and YY1 expression was examined. YY1 expression was significantly inhibited by Bay 11-7085 and the inhibition was a function of the Bay 11-7085 concentration used (Figure 2B). The observed TNF- α -mediated upregulation of YY1 expression was also significantly inhibited by the NF- κ B inhibitor Bay 11-7085 in a concentration-dependent manner (Figure 2B). These findings suggest that NF- κ B upregulates YY1 expression and corroborates the findings observed with sTNFR1 (Figure 1D) which inhibited YY1 expression.

2. TNF- α upregulates YY1 DNA-binding activity via activation of NF- κ B

The above findings demonstrated that both TNF- α and NF- κ B regulate YY1 expression. We examined

the effect of TNF- α on the DNA binding activity of NF- κ B and YY1 in PC-3 cells by EMSA. Nuclear extracts from PC-3 cells grown in serum free medium and treated with TNF- α (10 U/ml) for 24 h showed augmented NF- κ B (Figure 3A Top panel) and YY1 (Figure 3A Bottom panel) (lane 3) DNA-binding activity compared to both untreated serum free (lane 2) and serum containing controls (lane 1). In comparison with PC-3 cultured with 10% FSS, PC-3 cultured in serum-free medium showed less NF- κ B and YY1 DNA-binding activity, consistent with the role of serum and tumor-derived factors that regulate these activities. The specificity of the YY1 DNA-binding reaction was determined by competition assays performed with a 10-fold excess of unlabeled YY1 oligonucleotide (data not shown). The role of NF- κ B in the regulation of YY1 activity was corroborated by the use of the NF- κ B inhibitor Bay 11-7085. Treatment of PC-3 cells with Bay 11-7085 inhibited both NF- κ B (Figure 3B Top panel) and YY1 DNA-binding (Figure 3B Bottom panel) activity and the inhibition was a function of the inhibitor concentration used. These results demonstrate that TNF- α activates both NF- κ B and YY1 DNA-binding activities.

The role of the autocrine/paracrine loop of TNF- α secreted by PC-3 cells and its effect on the activation of NF- κ B and YY1 DNA-binding activities were analyzed. PC-3 cells were treated with sTNF-R1 (0.3 and 0.6 μ g/ml) for 18 h and nuclear lysates were prepared. Treatment with sTNF-R1 significantly inhibited NF- κ B (Figure 3C Top panel) and YY1 (Figure 3C Bottom panel) DNA-binding activity. These findings complement the above findings on the regulation of YY1 expression by TNF- α (Figure 1D) and reveal that, TNF- α through the autocrine-paracrine effect of TNF- α , upregulates both NF- κ B and YY1 DNA-binding activities.

3. Regulation of YY1 transcription NF- κ B

The above findings demonstrated a positive correlation between NF- κ B activity and YY1 expression and activity. To determine whether NF- κ B is involved in the regulation of YY1 transcription, transient transfection assays were performed. PC-3 Cells were transfected with either the pGlow-OAZmp/WT-YY1 or pGlow-OAZmp/Mu-YY1 reporter plasmids. Twenty-four hours after transfection, the cells were treated with sTNFR-1 (1 and 2 μ g/ml) or with the specific inhibitor of NF- κ B, Bay11-7085 (3 μ M). The baseline activity of the transfectants with WT-YY1 (lane 3) was minimal and significantly reduced compared to the MU-YY1 (lane 6) (Figure 4) suggesting that YY1 negatively regulates OA activity. Both sTNFR-1 treatment (Figure 4A) and Bay11-7085 (Figure 4B) induced significant augmentation of GFP activity in the WT-YY1 transfectants

suggesting that inhibition of NF- κ B and consequently YY1 activities relieved the YY1 repressor activity. In contrast, these inhibitors had no effect on the Mu-YY1 transfectants. These results demonstrate that NF- κ B regulates YY1 activity and inhibition of NF- κ B results in inhibition of YY1 and activity.

TNF- α -Dependent Activation of NF- κ B Protects Human Cancer Cells Against Fas-Mediated Apoptosis via upregulation of YY1 activity

Since TNF- α upregulates YY1 activity and YY1 activity negatively regulates Fas expression, we expected that TNF- α will confer resistance to CH-11-mediated apoptosis. Treatment of PC-3 cells cultured in the presence of 10% FCS with the Fas-ligand-agonist antibody CH-11 resulted in moderate apoptosis as measured by flow cytometry, specific activation of caspase 3 and PARP cleavage (data not shown) (REF). PC-3 cells were grown under serum-free conditions in the presence or absence of TNF- α for 12 h and then treated with CH-11 antibody (30 ng/ml) for 12 h. Treatment with CH-11 antibody alone resulted in significant apoptosis. However, treatment with both TNF- α and CH-11 reduced significant sensitivity of PC-3 to CH-11-mediated apoptosis (Figure 5A Top panel). In order to investigate the specific effect of endogenous TNF- α in the resistance of PC-3 cells to Fas-mediated apoptosis, the constitutively secreted TNF- α -mediated-signaling was blocked by sTNF-R1. Treatment of PC-3 cells with sTNF-R1 sensitized the cells to CH-11 apoptosis (Figure 5A Top panel). These above findings were confirmed by the use of the NF- κ B inhibitor Bay11-7085 since TNF- α induces NF- κ B activation. Treatment with Bay11-7085 significantly sensitized PC-3 cells to CH-11-mediated apoptosis (Figure 5A Bottom panel). These findings revealed that TNF- α regulates the resistance of PC-3 to Fas-mediated apoptosis through the activation of NF- κ B and consequently through the activation of the transcriptional repressor YY1.

The direct role of YY1 in the regulation of Fas expression and sensitivity to CH-11-induced apoptosis was corroborated in PC-3 transfected with YY1 siRNA. Transfection with siRNA resulted in significant upregulation of Fas expression compared to cells transfected with siRNA negative control or non-transfected cells (Figure 5B). The siRNA transfected cells show significant potentiation of CH-11-induced apoptosis compared to controls (Figure 5C). These findings implicate directly the role of YY1, via TNF- α dependent NF- κ B activity, in the regulation of Fas expression and sensitivity to Fas-induced apoptosis.

Fas/YY1 gene expression correlates with tumor cell sensitivity to Fas-mediated apoptosis

The above findings were all done in PC-3 cells and established the relationship between YY1 activity and sensitivity to Fas. The correlation between YY1 expression and sensitivity to Fas was examined in other tumor cell lines. We examined by semi-quantitative RT-PCR the transcription profile of YY1 in five human tumor cell lines that have been shown to exhibit a wide range of sensitivity to Fas-mediated apoptosis. The cell lines were arranged in decreasing order of their sensitivity to Fas: Raji and SW480 being the most sensitive, followed by PC-3 and SW620 which are moderately resistant, and K562 which cannot be sensitized to Fas-mediated apoptosis. All of the tested cell lines expressed YY1. The Fas/YY1 transcription ratios were used to assess whether YY1 expression correlates with Fas resistance. An inverse correlation was found with Fas sensitivity in the tested cell lines (Figure 5D). The Fas sensitive Raji and SW480 cell lines exhibited a Fas/YY1 ratio greater than one, while the Fas-resistant PC-3, SW620, and K562 cell lines exhibited Fas/YY1 ratios less than one. Thus, the Fas/YY1 ratios appear to predict sensitivity to Fas-mediated apoptosis.

DISCUSSION

Evidence is presented which demonstrates that the autocrine-paracrine loop mediated by TNF- α in PC-3 cells regulates the cell's resistance to Fas-induced apoptosis. Endogenously secreted TNF- α regulates in large part the constitutive activation of NF- κ B activity. The role of NF- κ B in the negative regulation of Fas expression and resistance to Fas apoptosis was found to be due to NF- κ B-induced activation of the transcription

repressor YY1. Both endogenous and exogenous TNF- α via NF- κ B activation, resulted in the upregulation of the expression and activity of YY1 and consequently downregulation of Fas expression. The role of NF- κ B in the regulation of the YY1 repressor activity was corroborated using a reporter system with a deletion of YY1 binding sites in the promoter which augmented baseline luciferase activity. This finding was further confirmed by the use of the NF- κ B inhibitor Bay 11-7085 which resulted in downregulation of YY1 and upregulation of Fas. Several lines of evidence support the direct role of YY1, via NF- κ B activation in the regulation of Fas expression and resistance to Fas apoptosis. Treatment of PC-3 cells with TNF- α upregulated YY1 expression and activity and downregulated Fas expression. In contrast, inhibition of the TNF- α -mediated activation of NF- κ B and YY1 resulted in upregulation of Fas expression and sensitization to CH-11 apoptosis. Similar findings were obtained with inhibitors of NF- κ B. Further, treatment with siRNA YY1 resulted in upregulation of Fas expression and sensitization to CH-11-induced apoptosis. Altogether, these findings provide for the first time, evidence of the role of tumor-derived TNF- α autocrine-paracrine loop in the negative regulation of Fas expression and sensitivity to Fas apoptosis via activation of the transcription factors NF- κ B and YY1.

Several reports have demonstrated that tumor cells sensitize and secrete various cytokines and growth factors that play an important role in cell survival and cell growth via autocrine/paracrine loops (REF). Likewise, such factors are also encountered by the tumor cells *in vivo* in their microenvironment (REF). In addition, it has been reported that cytokines secreted by the tumor cells also regulate the sensitivity and resistance of tumor cells to various cytotoxic stimuli (REF). These may be due in part to the stimulation of cell survival pathways and antiapoptotic mechanisms and/or inhibition of proapoptotic-regulatory gene products (REF). In this study, we have examined the role of tumor-derived TNF- α autocrine-paracrine loop for its role in the regulation of cells resistance to Fas-induced apoptosis. The findings revealed that secreted TNF- α from the tumor cells in an autocrine-paracrine fashion activates NF- κ B activity and in turn, NF- κ B upregulates the expression and activity of YY1. YY1 activation negatively activates Fas expression via its activity on the silencer region on the Fas promoter and to Fas-induced apoptosis (see schematic diagram in Figure 6).

TNF- α is a potent activator of NF- κ B and NF- κ B has been shown to regulate cell survival and numerous genes that are anti-apoptotic (REF). We show that inhibition of endogenous TNF- α secreted by PC-3

cells by neutralization of the autocrine-paracrine loop by sTNF-R1 and inhibited significantly NF- κ B activity. These findings suggested that in PC-3 cells the observed constitutive NF- κ B activity is due to endogenous TNF- α -TNF receptor signaling. Hence, a loop is involved in which TNF- α activates NF- κ B and activation of NF- κ B induces TNF- α and thus the cells are maintained with NF- κ B that mediates survival and anti-apoptosis.

The role of NF- κ B in Fas expression and resistance to CH-11 apoptosis was demonstrated by neutralizing secreted TNF- α by sTNF-R1 which resulted in upregulation of Fas and sensitization to CH-11 apoptosis. These findings were corroborated by blocking NF- κ B activity with a chemical inhibitor, Bay 11-7085. Previous findings demonstrated that YY1 negatively regulates Fas expression and sensitivity to Fas-induced apoptosis (REF). The YY1 promoter has consensus NF- κ B binding sites and suggests that NF- κ B regulates YY1 transcription. We show that inhibition of NF- κ B resulted in inhibition of YY1 expression and DNA-binding activity. There was a good correlation between NF- κ B activity and both YY1 expression and activity.

Previous findings demonstrated that NF- κ B regulates the survival of cells and also regulates the transcription of anti-apoptotic gene products. Inhibition of NF- κ B results in the sensitization of cells to various apoptotic stimuli (17). This study provides a new insight into the mechanism of NF- κ B regulation of survival and resistance to Fas apoptosis. Evidence presented here demonstrates that TNF- α -mediated activation of NF- κ B results in the upregulation of YY1 expression and augmented YY1 DNA-binding activity. Treatment of PC3 cells with TNF- α resulted in the activation of NF- κ B and consequently, the expression of YY1 was upregulated. This NF- κ B-mediated effect was inhibited by cells treated with the NF- κ B inhibitor Bay11-7085. The endogenous YY1 expression in PC3 cells is shown to be regulated in part by constitutive NF- κ B activity which is activated by TNF- α in an autocrine/paracrine manner. Thus, blocking the autocrine/paracrine loop of TNF- α -TNF-R1 by sTNFR1 resulted in inhibition of YY1 expression and activity. Noteworthy, we observed the presence of a correlation between YY1 expression and YY1 DNA-binding. When YY1 expression was up-regulated, YY1 DNA-binding activity was also up-regulated and the reverse was also true. Since YY1 expression in PC3 cells was found to be in large part localized in the nucleus (data not shown), its DNA-binding

activity may be based on its abundance. For instance, it has been reported that PC-3 expresses high constitutive NF- κ B activity compared to normal cells and NF- κ B is primarily localized in the nucleus and high levels correlated with higher activity (27). The role of NF- κ B in the regulation of the repressor activity of YY1 was demonstrated in a reporter system whereby the YY1 binding sites were deleted from the promoter, and this resulted in upregulation of luciferase activity. In addition, inhibition of NF- κ B by Bay 11-7085 inhibited the repressor activity of YY1. The direct role of YY1 in the regulation of Fas was supported by the use of transfectants with siRNA-YY1, whereby the transfectants showed upregulation of Fas and sensitivity to CH-11-induced apoptosis. Previous findings demonstrated that YY1 negatively regulates Fas expression and sensitivity to Fas apoptosis in PC3 cells. This was the result of the transcription repression activity of YY1 in the silencer region of the Fas promoter (5).

Ivanov and Ronai (2000) reported that p38 negatively regulates the expression of Fas via inhibition of NF- κ B transcriptional activity in melanoma. Inhibition of NF- κ B activity correlated with significant downregulation of Fas expression and UV-induced apoptosis. The Fas promoter contains 3 NF- κ B sites (Chan *et al.*, 1999) and inhibition of p38 resulted in significant increase in Fas reporter luciferase activity. Our findings are not consistent with these findings, and the differences may reflect differences in the tumor system used and the apoptotic stimulus. It is also possible that the counteracting regulation of Fas by NF- κ B and YY1 may be due to a balance. In our system, it is possible that YY1 overexpression is dominant over NF- κ B, whereas it is not in the melanoma cell line studied.

A large number of genes have been found to be potentially regulated by YY1 and a large number have been claimed to interact with YY1 (19-23). However, little is known about the transcriptional regulation of YY1 itself. Patten *et al* (24) reported that IL-1- β increases the abundance of YY1 in cardiac myocytes. Santiago *et al* (25) demonstrated that YY1 is activated in rat vascular smooth muscle cells shortly after injury and this was due to endogenous FGF-2 mRNA, protein and DNA binding and transcription activity of YY1 that was increased 3-fold by FGF-2. Also FGF-1 has been shown to regulate YY1 expression in NIH 3T3 cells (26). The present findings demonstrate the role of NF- κ B and stimuli that activate NF- κ B like TNF- α in the regulation of YY1 activity.

The relationship between YY1 expression and Fas sensitivity was corroborated in several cell lines whether it was an inverse correlation between YY1 and Fas. Thus, based on our present findings demonstrating that TNF- α -induced NF- κ B activation regulates in part YY1 expression and activity, we expected that Fas expression will be regulated by TNF- α and NF- κ B. Indeed, treatment of PC3 cells with TNF- α inhibited PC3-mediated apoptosis by the Fas ligand agonist antibody CH-11. In contrast, blocking TNF- α autocrine/paracrine loop-mediated activation of NF- κ B by sTNFR1 resulted in sensitization to Fas. These findings corroborate the role of NF- κ B in the regulation of Fas via YY1 activity. The direct role of YY1 in the regulation of Fas expression and sensitization to Fas was shown by transfection of PC-3 cells with siRNA YY1. The transfected cells showed upregulation of Fas and sensitization to CH-11. NF- κ B also has been reported to regulate Fas directly in T cells (29).

Although YY1 is generally regarded as an ubiquitous protein expressed in many different tissues and cell types (6), YY1 is differentially regulated in different cell types. For example, expression of YY1 mRNA in NIH3T3 cells has been shown to be affected by cell density and growth factors such as IFG-1 (26). YY1 is also downregulated in F9 cells following long-term treatment with retinoic acid (30). Levels of YY1 activity also change during myoblast differentiation and during aging (31). We have found strong nuclear YY1 immunostaining in several cancer cell lines (AD10, SW620, SW480, and PC-3) (data not shown). Further, recent studies in our laboratory have demonstrated by immunostaining, using tissue arrays, upregulation of YY1 expression in prostate cancer and YY1 was found to be a prognostic factor (Seligson *et al.*, 2004). Based on our findings, we suggest that overexpression of YY1 may be detrimental in the response of tumor cells to Fas-induced and to immunotherapy stimuli. The present findings established one mechanism of resistance to tumor cells to Fas via the autocrine-paracrine TNF- α loop and the constitutive activation of NF- κ B and YY1. Figure 6 schematically describes the effect mediated by TNF- α and also establishes various targets whose modifications may reverse resistance to immune-mediated stimuli.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Correlation between Fas expression and YY1 expression in tumor cell lines

A. Fas/YY1 ratios of gene expression and sensitivity to Fas-mediated apoptosis in five human tumor cell lines

The cell lines were synchronized and then cultured in RPMI 10% FSS, as described in Materials and Methods. Total RNA was extracted and RT-PCR was used to examine the basal levels of YY1 and Fas expression. All the samples were normalized against GAPDH. The ratios of Fas/YY1 were calculated and are shown. Furthermore, the cell lines were tested for sensitivity to Fas-mediated apoptosis using the CH11 anti-Fas antibody. The data show that there is a correlation between the low levels of YY1 (high Fas /YY1 ratio) and sensitivity to Fas-mediated apoptosis.

B. YY1 and Fas expression in PC-3 cells

PC-3 cells were grown in RPMI 10% FSS, serum free medium alone or serum free medium with TNF- γ (10 U/ml) as described in Materials and Methods. Fixed and permeabilized PC-3 cells were stained with anti-YY1 antibody (B) and goat-anti-rabbit-PE and then analyzed by flow cytometry. The data are presented as mean fluorescence intensity and the mean of three independent experiments * $p < 0.05$, serum-free vs. cells treated with TNF- γ .

C. TNF- γ - dependent YY1 expression in PC-3 cells

PC-3 cells were grown in serum free medium, treated (lane 1) or left untreated for 24 h with 0.1, 1, and 10 U/ml of TNF- γ (lanes 2, 3 and 4). Total cellular protein was extracted from the culture. Total protein was separated by SDS-PAGE and transferred onto the nitrocellulose membrane as described in Materials and Methods. The membrane was stained with the polyclonal antibody to human YY1. The relative YY1 expression was determined by densitometric analysis of the blots. The blots represent one of two separate experiments. The data show that TNF- γ up-regulates the expression of YY1. * $p < 0.05$, serum free vs. cells treated with TNF- γ .

D. Surface Fas expression in PC-3 cells

PC-3 cells were treated as described above in (B). The cells were stained for surface expression using anti-Fas monoclonal antibody as described. The data are provided as mean fluorescence intensity (MFI) and the means of three independent experiments * $p < 0.05$ serum-free versus treated cells with TNF- γ .

E. Endogenous TNF- γ is involved in the regulation of YY1 expression in PC-3 cells

PC-3 cells were grown in serum free medium and then treated or left untreated for 24 h with recombinant soluble TNF-R1 (0.1, 0.3 μ g/ml). Fixed and permeabilized PC-3 cells were stained with anti-YY1 antibody and analyzed by flow cytometry. Clearly, treatment of PC-3 with sTNF-R1 inhibited the expression of YY1

significantly ($p < 0.05$), demonstrating that blocking secreted TNF- α interaction with cell surface TNF-R 1 on PC3 by sTNF-R1 resulted in inhibition of TNF- α -mediated signaling and YY1 expression. The data are the mean of two independent experiments * $p < 0.05$, serum free vs. cells treated with sTNF-R1.

Figure 2. NF- κ B mediates TNF- α dependent expression of YY1

NF- κ B responsive elements in the YY1 core promoter

Sequence analysis of the YY1 proximal core promoter revealed the presence of four putative cis-acting responsive elements for NF- κ B.

NF- κ B mediates TNF- α dependent expression of YY1. PC-3 cells were treated with different concentrations of the NF- κ B inhibitor Bay11-7085 (0, 1, 2 or 3 μ M/ml) for 1 h. PC-3 cells were then treated or left untreated for 24 h with TNF- α (10 U/ml) in serum free conditions. Fixed and permeabilized PC-3 cells were stained with anti-YY1 antibody and analyzed by flow cytometry. The data are presented as mean fluorescence intensity, and the mean of two independent experiments. The findings reveal that Bay11-7085 inhibits constitutive and TNF- α dependent YY1 expression. * $p < 0.05$, ** $p < 0.05$, for serum free in the presence and absence of Bay11-7085 and serum free plus TNF- α 10 U/ml in the presence or absence of Bay11-7085 respectively.

Figure 3. Regulation of YY1 DNA-Binding activity by NF- κ B

A. TNF- α augments NF- κ B and YY1 DNA binding activities

Nuclear extracts from PC-3 cells grown in RPMI 10% FCS, or serum free medium treated or left untreated with TNF- α (10U/ml) were analyzed using EMSA to assess the specific NF- κ B (upper panel) and YY1 (bottom panel) DNA-binding activity. Relative NF- κ B and YY1 DNA-binding activity was determined by densitometric analysis. The findings reveal that TNF- α treatment of PC-3 cells results in augmenting both NF- κ B and YY1 DNA binding activities.

B. The specific NF- κ B inhibitor Bay11-7085 inhibits both NF- κ B and DNA-binding activities

Nuclear extracts from PC-3 cells grown in serum-free medium treated or left untreated with Bay 11-7085 (0.5, 1, 2 and 3 μ g) were analyzed using EMSA to assess the specific NF- κ B (top panel) and YY1 (bottom panel) DNA binding activity. Relative NF- κ B and YY1 DNA binding activity was determined by densitometric analysis. The findings reveal that inhibition of NF- κ B results in inhibition of both NF- κ B and YY1 DNA binding activity.

C. sTNF-R1 inhibits both NF- κ B and YY1 DNA binding activities

Nuclear extracts from PC-3 cells grown in serum-free medium treated or left untreated with recombinant sTNF-R1 (0.3, 0.6 μ g/ml) were analyzed using EMSA to assess the specific NF- κ B (top panel) and YY1 (bottom panel) DNA binding activity. Relative NF- κ B and YY1 DNA binding activity was determined by densitometric analysis. The data show that TNF/TNF-R interaction inhibited by sTNF-R1 results in inhibition of TNF- α -mediated signaling for NF- κ B activity and subsequently inhibition of NF- κ B and YY1 DNA-binding activity.

Figure 4. The suppressor activity of YY1 is modulated via TNF- α / NF- κ B pathway

100 bp fragment of enzyme OAZ promoter relative to the transcriptional start site (OAZ-YY1 W/T promoter) and another fragment missing the YY1-binding sequence (OAZ-YY1-mutant) were cloned into the pGlow-TOPO GFP reporter vector. PC-3 cells were transfected with 20 μ g of the indicated reporter plasmid and then treated with (A) sTNFR1 (1 or 2 μ g/ml) or (B) with the specific NF- κ B inhibitor Bay11-7085 (3 μ M). Samples were harvested 18 h after treatment and assessed for GFP activity. sTNF-R1 and Bay 11-7085 inhibits the YY1 repressor activity. * $p < 0.05$

Figure 5. TNF- α protects PC-3 sensitivity to CH-11 apoptosis via NF- κ B activation

Top Panel: C-3 cells were cultured in serum-free medium and were left untreated or treated with TNF- α (10 U/ml) in the presence or absence of recombinant sTNFR1 (0.3 μ g/ml) for 12 h. PC-3 cells were then treated or left untreated with CH-11 antibody (30 ng/ml) for 12 h. Fixed and permeabilized PC-3 cells were stained with anti-active caspase-3-FITC antibody and analyzed by flow cytometry as described in Materials and Methods. The data are calculated as percentage of control cells cultured in serum-free medium. The findings reveal that TNF- α protects PC-3 from CH-11-induced apoptosis * $p < 0.05$ compared to cells treated with CH-11.

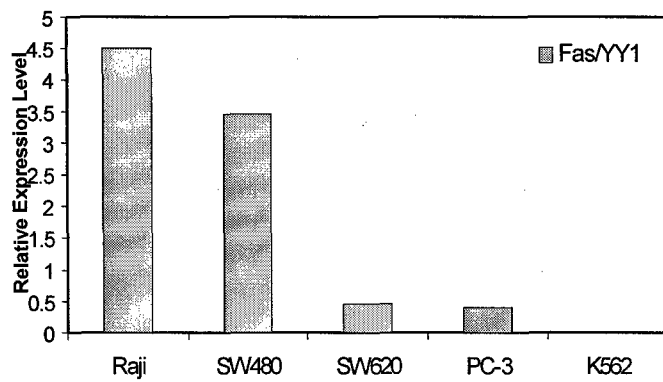
Bottom Panel: PC-3 cells were treated the same as aforementioned in (A) except that Bay 11-7085 (2um/ml/1 h) was used. The findings reveal that inhibition of NF- κ B sensitizes the cells to CH-11-induced apoptosis *p=0.05 compared to cells treated with CH-11 alone.

Figure 6. Schematic diagram of the mechanism by which TNF- α regulates YY1 expression and activity via NF- κ B and the regulation by YY1 of Fas expression and sensitivity to Fas apoptosis.

- A.** Binding of TNF- α (endogenous by autocrine-paracrine loop or exogenous) to TNF-R1 activates NF- κ B which in turn activates the expression of the TNF- α and YY1 genes. As a result, YY1 binds to the silencer region of the Fas promoter and blocks (Garban and Bonavida, 2001) Fas expression leading to downregulation of Fas expression and resistance of cells to Fas-mediated apoptosis.
- B.** Addition of soluble TNF-R1 or Bay11-7085 to the cells will inhibit constitutive NF- κ B activity and as a result, YY1 expression is down-regulated and Fas expression is increased and the cells become sensitized to Fas-mediated apoptosis.

Figure 1

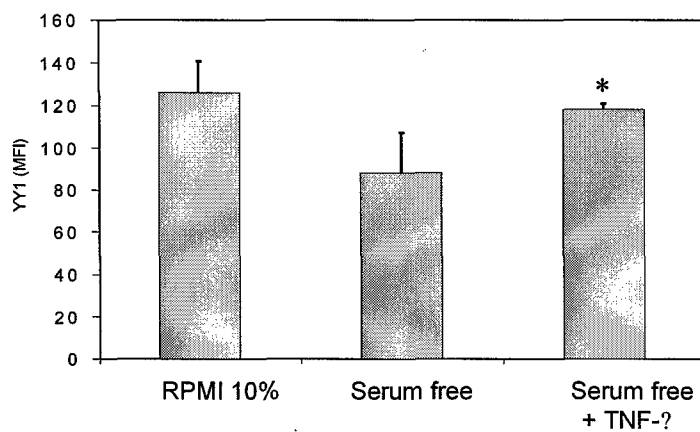
A



Sensitivity to Fas-Mediated apoptosis



B



C

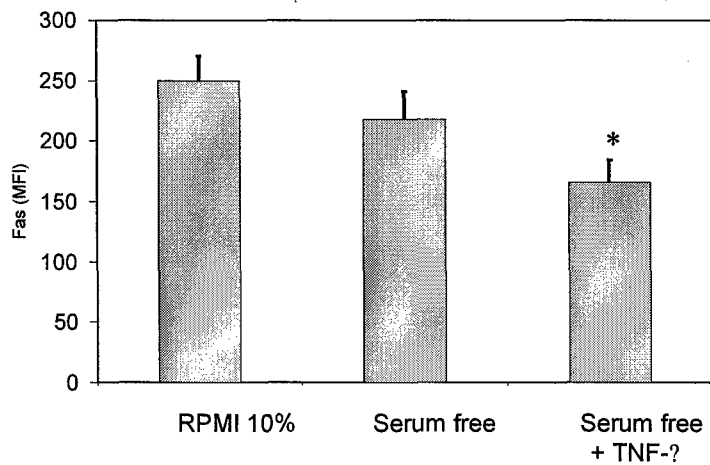
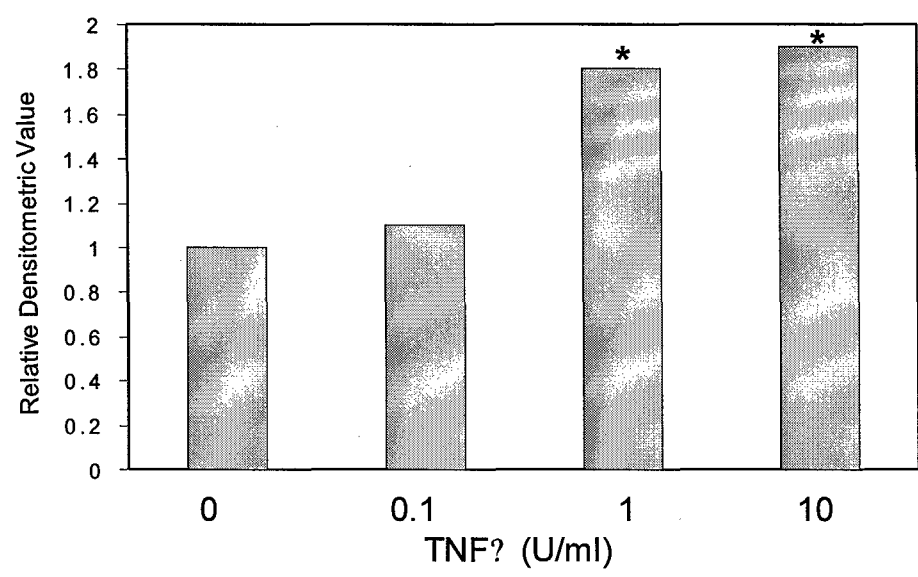


Figure 2

YY1



β -Actin



Figure 3

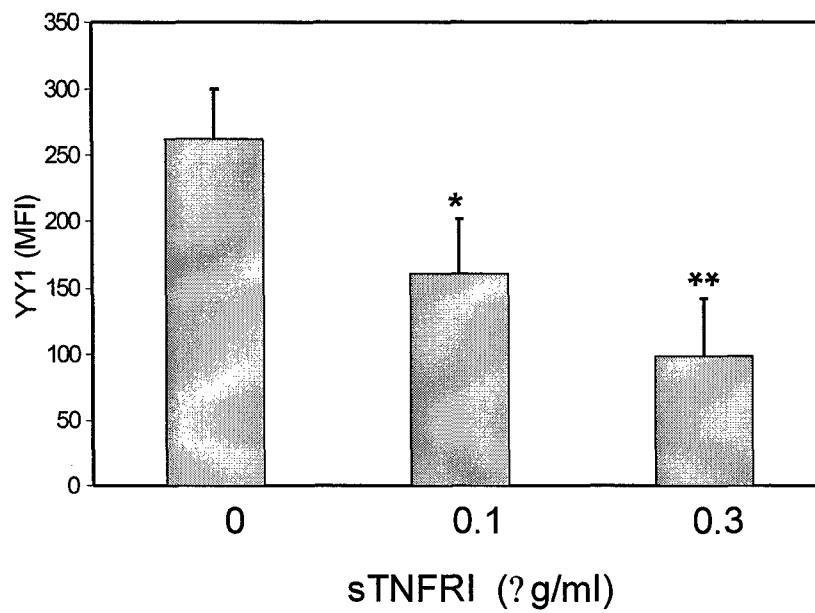


Figure 4A

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Figure 4B

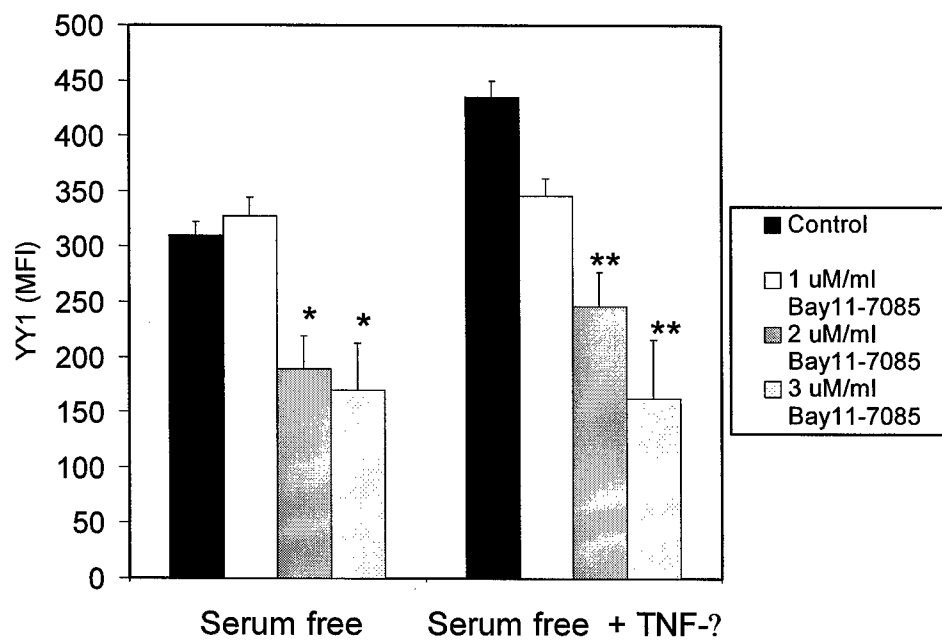


Figure 4C

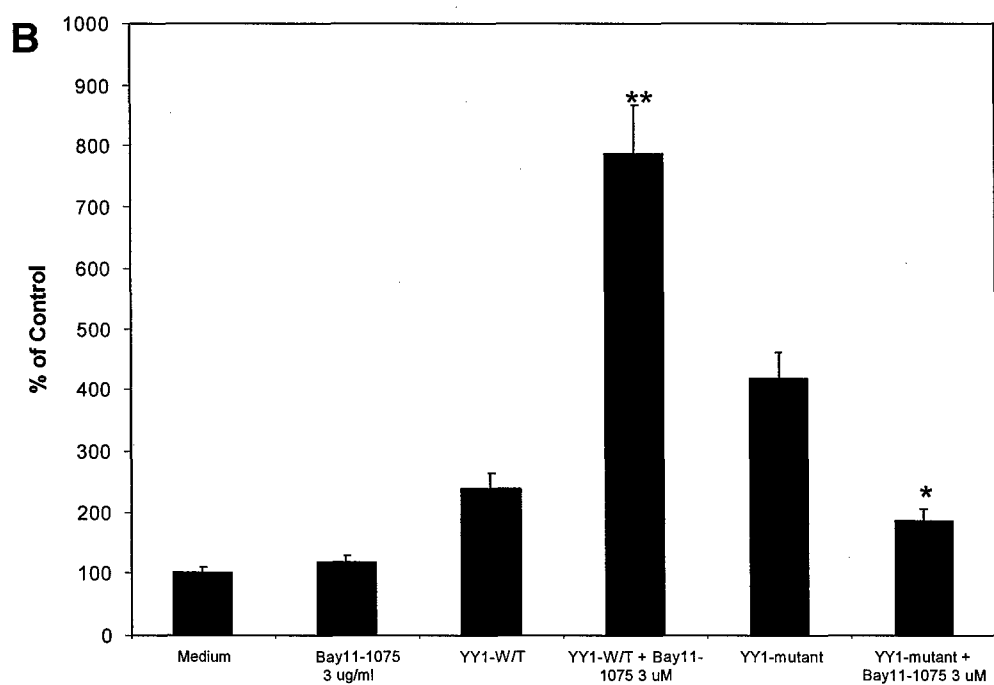
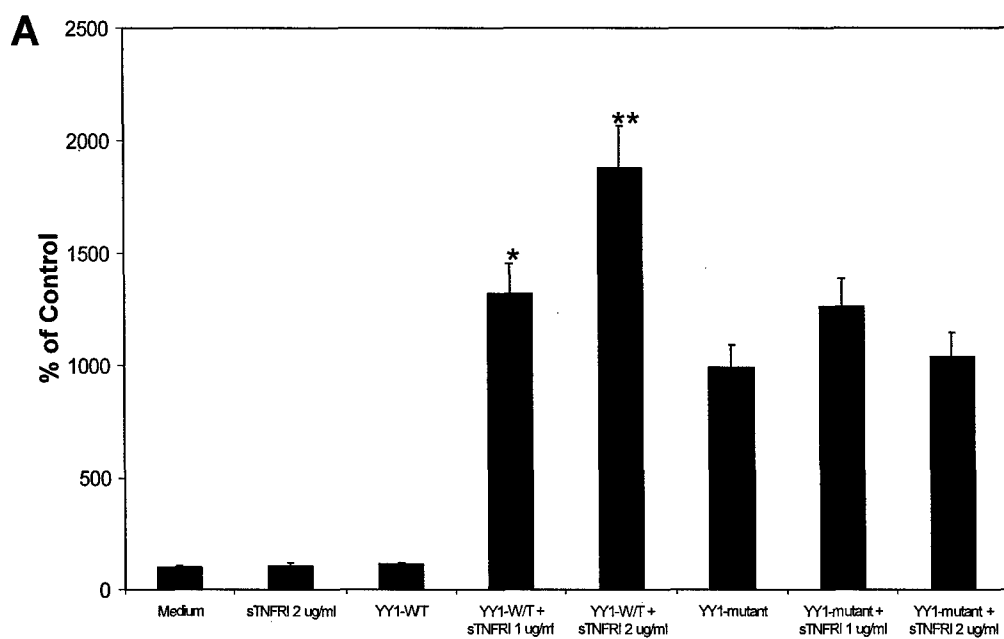


Figure 5A

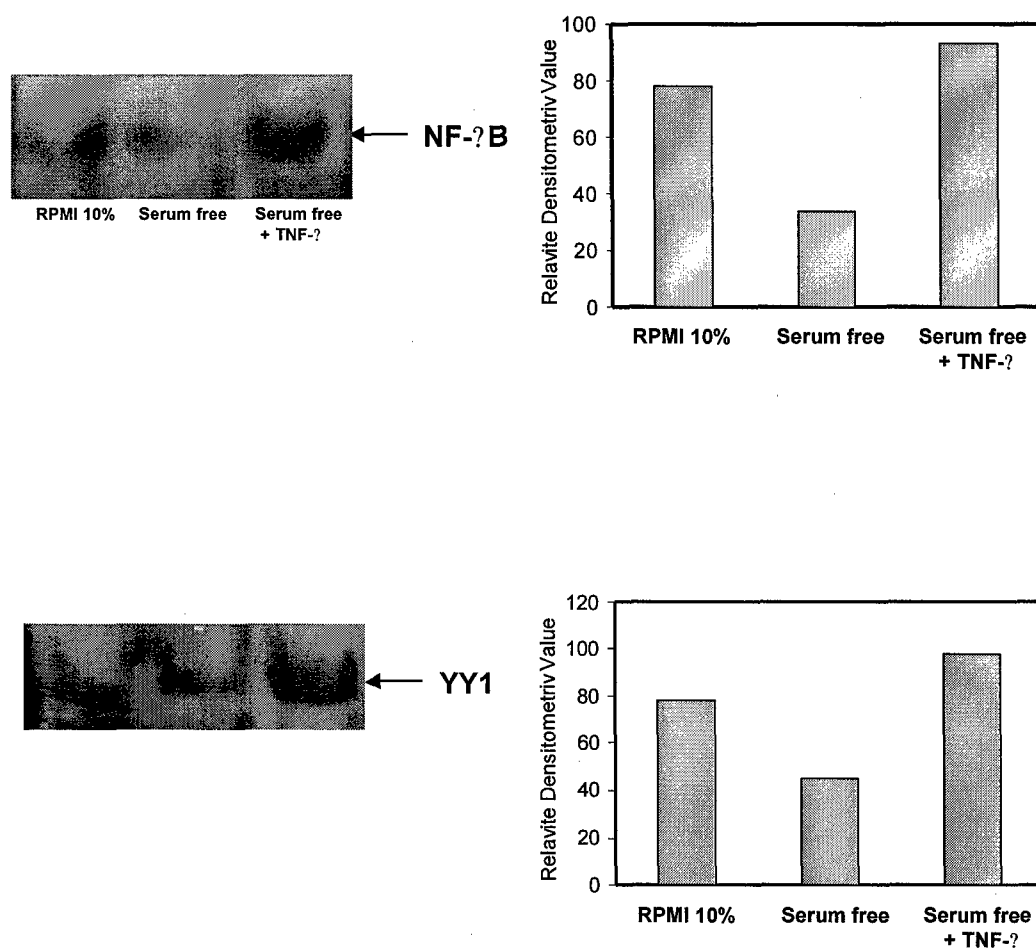


Figure 5B

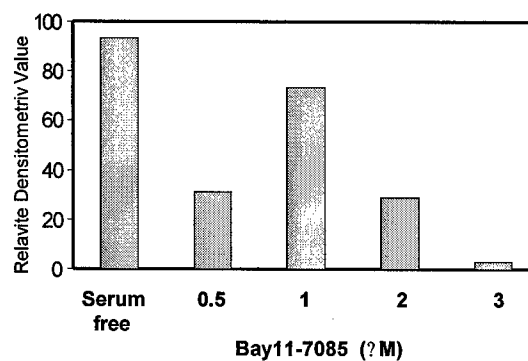
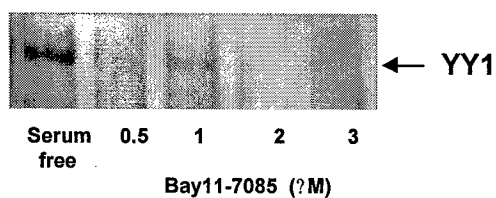
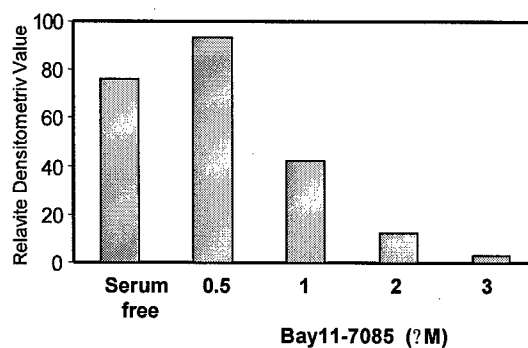
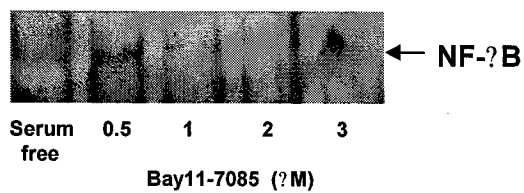


Figure 5C

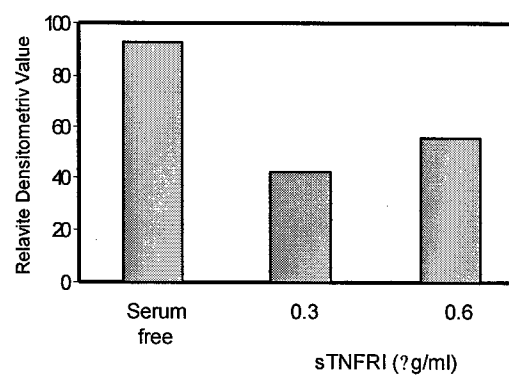
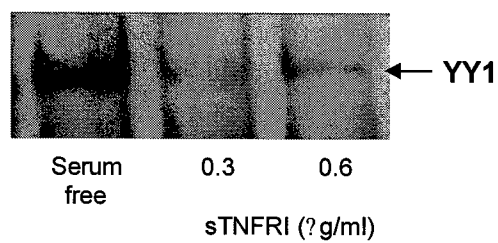
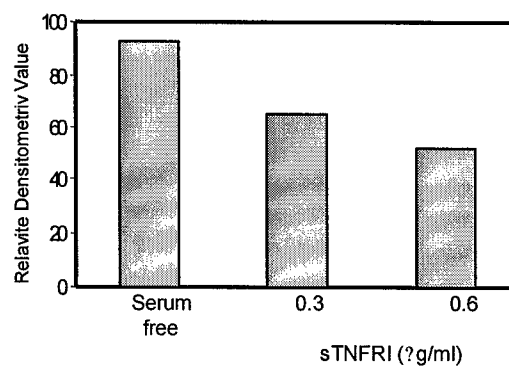
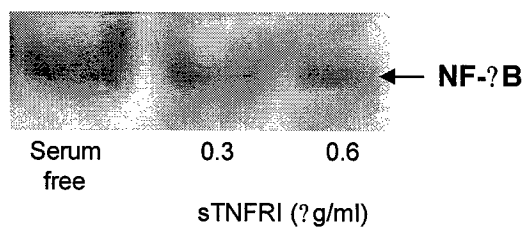
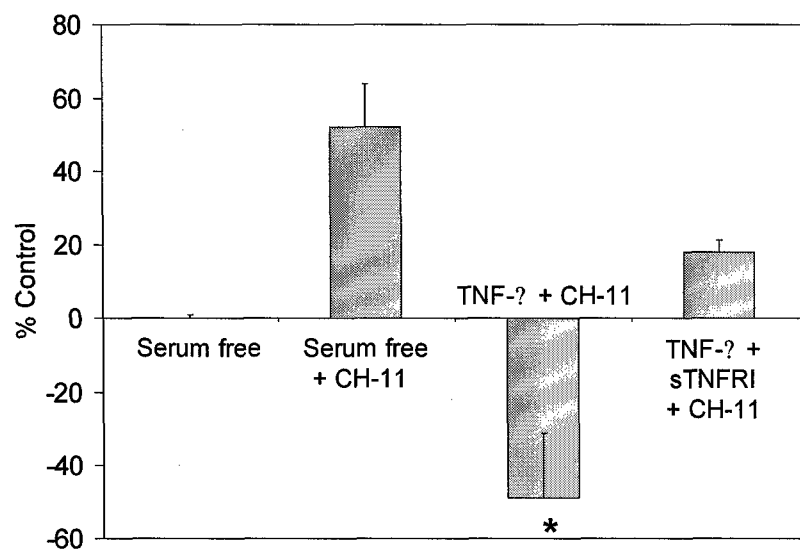


Figure 6

A



B

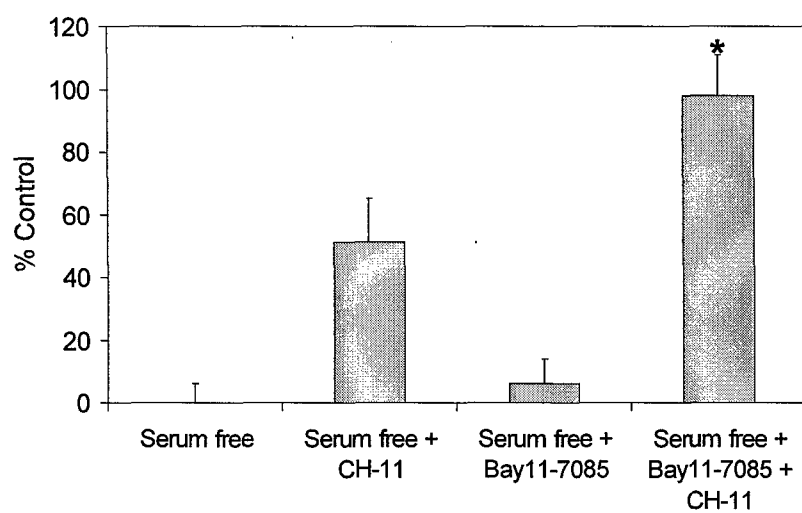
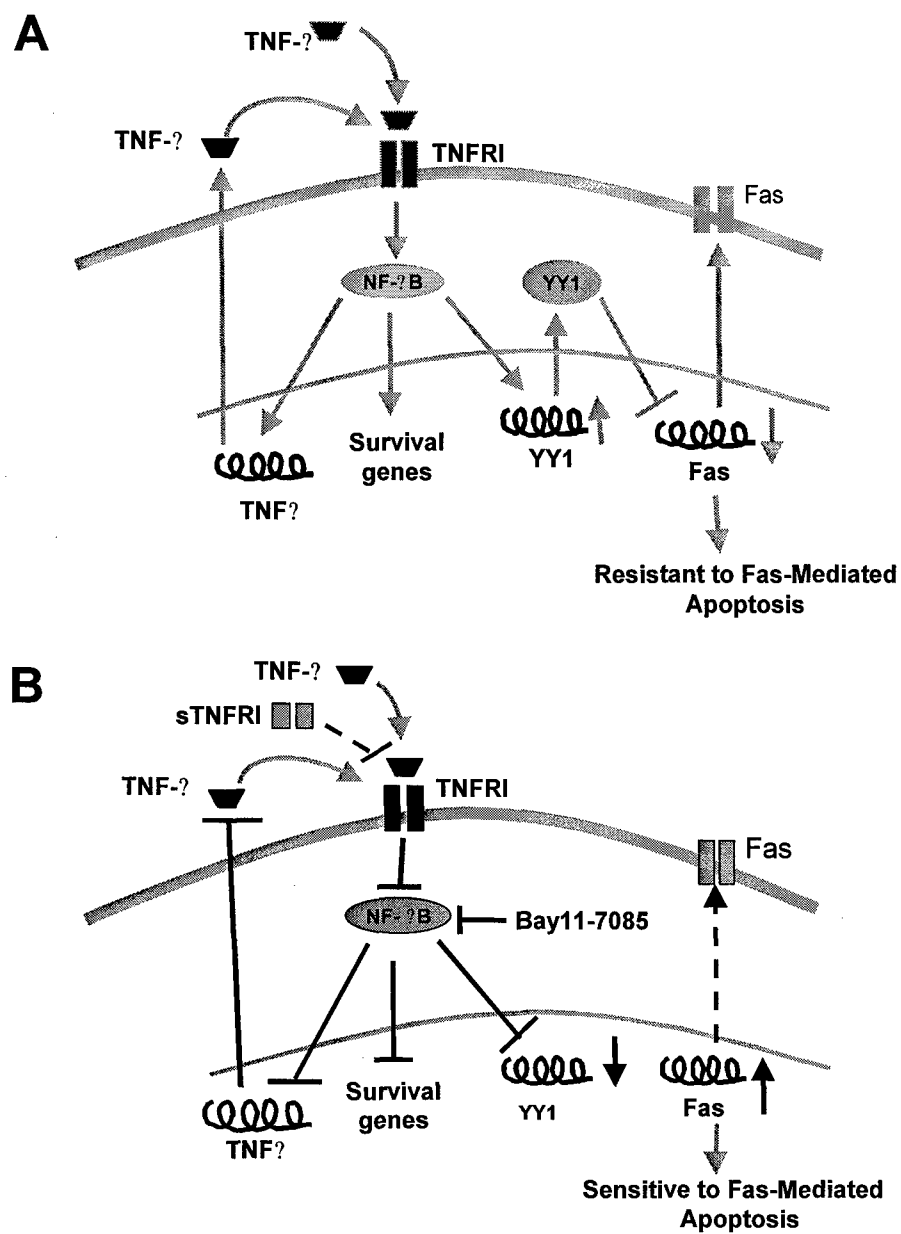


Figure 7



Abstract Number: 4826

Regulation of prostate cancer sensitivity to apoptosis by CDDP by downregulation of XIAP and Bcl-x_L expression via inhibition of constitutive NF- κ B activity

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Prostate cancer patients develop chemoresistance following initial treatment. Several studies have been addressed to determine the underlying mechanisms of resistance and included the development of the MDR phenotype, dysregulation of the apoptotic signaling pathways, etc. We and others have demonstrated in CaP cell lines that NF- κ B is constitutively activated and regulates survival and growth of the tumor cells. We have demonstrated that PC-3 and DU-145 synthesize and secrete cytokines such as TNF- α and IL-6 that regulate NF- κ B activity via an autocrine paracrine pathway. Further, these cytokines were found to regulate drug resistance. Therefore, we hypothesized that NF- κ B may regulate drugs-induced apoptosis via dysregulation of the apoptotic signaling pathways and hence inhibition of NF- κ B may chemosensitize the cells. The objective of this study was to test this hypothesis and delineate the gene products that are transcriptionally regulated by NF- κ B and contribute to chemoresistance. We demonstrate that treatment of PC-3 cells with NF- κ B inhibitors sensitized the cells to CDDP-induced apoptosis and synergy was achieved. Further, inhibition of NF- κ B by a nitric oxide donor (DETANONOate), via the S-nitrosylation of p50, also resulted in chemosensitization. The effect of NF- κ B on the apoptosis signaling pathways, as determined by western, revealed that there was selective downregulation of the anti-apoptotic XIAP and Bcl-x_L gene products following inhibition of NF- κ B. The role of these proteins in chemosensitization was corroborated by the use of specific inhibitors. These findings suggest that tumor cells develop a self-contained mechanism to resist drug-induced apoptosis by tumor-derived autocrine/paracrine factors which activate NF- κ B and secure cell growth and survival. Further, the present findings suggest that NF- κ B, XIAP and Bcl-x_L are targets for intervention in the reversal of drug resistance. Supported by a grant from the Department of Defense (US Army DAMD 17-02-1-0023), Jonsson Comprehensive Cancer Center (MV, AJ), UCLA SPORE in Prostate Cancer (P50 CA92131-01A1), Fogarty (SH-Y, MV, HG), and UC MEXUS (SH-Y).

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