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Introduction: Preliminary studies pointed to the ability for IFN-g to enhance sensitivity and/or reverse resistance to Fas transactivation on prostate cancer cells. and work during the past 2 years illustrated the ability of IFN-γ to restore or enhance sensitivity to Fas transactivation in vitro. Studies explored the usefulness of combining adenovirus mediated FasL expression with IFN-γ, the mechanism underlying IFN-g effects on Fas sensitivity in human cells lines and the deivatgion of a better vector to deliver FasL.
Results: In vitro and in vivo The combination of Ad.FasL and Ad.mIL-12-IFN-g resulted in powerful interactions to control growth. However, a staggered injection scheme was necesaary to prevent the negative effects of FasL on IL-12/IFN-g production. In the human prostate cancer cell lines, PC3 and LNCaP, studies with caspase substrates noted that the CPP32 family was activated and not the ICE family following exposure to IFN-γ and sFasL. Apoptosis was mediated through caspase 8. The enhancement of vthe delivery vector focused on vesicular stomatitis virus (VSV). Studies noted the ability of VSV to rapidly kill human and mouse prostate cancer cells but not normal prostate epithelial cells. In addition infected cells expressed high levels of FasL.
Conclusions: These studies validate the concept of exploiting Fas upregulation for FasL transactivation through manipulation of the host to produce IFN-γ through IL-12 gene therapy. The VSV vector is a powerful vector to kill prostate cancer cells and will be exploited further as a delivery vehicle for transgenes, such as FasL.

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Introduction

Fas is a potential target expressed on the cell surface of many tumor cells. The binding of its ligand, FasL, initiates an intracellular cascade resulting in apoptosis (1). This pathway was first identified within the lymphoid system where it is thought to act within the homeostatic mechanisms of T cells and other effectors. It is unknown why many tumors, including prostate, express Fas, but its use as a potential therapeutic target has generated interest. The idea of harnessing the Fas/FasL pathway as a vehicle for killing prostate cancer arose from studies looking at the growth suppression observed following injection of an adenovirus expressing IL12 (Ad.IL12) in an orthotopic mouse model of prostate cancer (RM-1) (2). Studies have noted significant local and systemic growth suppressive activities following a single intra-tumoral injection of Ad.mIL-12. Studies linked the ability of IL-12 to direct host T cells and NK cells to produce IFN-γ which in-turn increased tumor expression of Fas from 30 to 60-70% (2) Exposure of RM-1 cells to IFN-γ in vitro resulted in a dose-dependent killing as great as 30%. This effect could subsequently be blocked by adding anti-FasL IgG. Indicating interaction of constituitively low FasL expression (15%) and upregulated Fas expression (2). In-vivo blockade of Fas/FasL resulted in a modest reduction in local tumor killing . Interestingly, immunohistochemical studies showed that most of the surviving tumor cells were Fas+ and thus targets for FasL transactivation (2). Immunohistochemical studies have shown that human prostate cancers express Fas (3-5). Established human prostate cancer cell lines have also been found to express Fas., though many are generally resistant to agonist antibody Fas transactivation (6-8), The exposure of normally Fas resistant human prostate cancer cells (LNCaP and PC3) to IFN-γ for 24-48 hours restored Fas sensitivity to soluble FasL in vitro (9). Therefore the concept of adding vector mediated FasL to kill Fas+ cells was explored. Logical extension of this then led to combination of vector mediated IL-12 to stimulate host production of IFN-γ to up-regulate Fas/FasL to potentiate the tumoricidal effect of Fas and FasL.

Body

Specific Aim 1

1) Construction of an Adenovirus Expressing FasL.

The first priority for this proposal was the construction of an adenovirus expressing FasL. PCR amplification of FasL was performed, followed by ligation into an adenovirus backbone driven by the Rous Sarcoma Virus promoter. The pAd.RSV-FasL was co-transfected with pBHG10 in 293 cells by the calcium phosphate precipitation method to yield Ad.FasL. Large-scale production of vector was achieved following expansion in 293 cells and double cesium gradient ultracentrifugation purification. As discussed in the original application the problem to overcome with this process was the inhibition of

apoptosis of the 293 cells used to plaque recombinants and expand the vector, as they express high levels of FasL. We proposed using the caspase inhibitor, Z-VAD-FMK (20uM Enzyme System Products, Livermore, CA) to avoid this problem. However, we were unable to successfully plaque a sFasL, even with 100uM Z-VAD-FMK, presumably due its more potent ability. Therefore, studies proceeded with the membrane bound FasL due to these technical difficulties and the high likelihood that the soluble FasL construct will be excessively toxic.

2) <u>in Vitro Activity of Ad.FasL</u>

An intentionally low dose of IFNγ was used to minimize cytokine-mediated killing and to maximize the chance of witnessing cooperative actions. However, the addition of Ad.β-Gal resulted in a killing of ~30% (Figure 1A Selleck et al 2003 (10))To further understand this phenomenon we are exploring the possibility of Fas/FasL interactions via Fas upregulation from IFN-γ and FasL upregulation due to virus infection. Alone Ad.FasL demonstrated dose dependent killing in a fashion consistent with the transduction efficiency of RM-1 cells. Killing at the MOI (multiplicity of infection, ie the number of virus particles per cell) 25 which correlates to 25% was 30-35%. The combination of Ad.FasL with IFNγ induced and maintained maximum cell kill to between 60-70% at 4-fold lower vector dose, indicating synergistic killing (Figure 2, Selleck et al 2003 (10), IFNγ vs. Combination therapy p=0.035, Mann-Whitney). Killing of 60% of the cells with only 6% of cells expressing FasL would indicate the presence of a bystander effect as hypothesized.

3)<u>*In vivo* Gene Therapy</u>:

Dose escalations of Ad.FasL performed in mice given a standard dose of Ad.mIL12 ($1x10^9$ pfu) demonstrated a significant suppression of tumor growth at Ad.FasL doses greater than $1x10^9$ pfu. (Figure 3, Selleck et al 2003 (10), $1x10^8$ pfu vs. $1x10^9$ pfu, p=0.003, t-test) No additional benefit was evident from viral dosing greater than $1x10^9$ pfu. In fact higher Ad.FasL doses actually trended for larger tumors but this was not found to be statistically significant (p = 0.16, t-test).

Dose control experiments were performed to determine the relative effectiveness of the individual and the combined treatments with Ad.IL12 and Ad.FasL. Vector inoculation into tumor of C57BL6 mice with established prostatic tumors (RM-I) with Ad.FasL alone at dose of $1x10^9$ resulted in a 27%(+/-11%) decrease in tumor size from controls (Figure 3b, Selleck et al 2003 (10), p=0.04, t-test). Ad.mIL12 inoculation alone (dose $1x10^{10}$ pfu) into tumor resulted in a 66% (+/-7%) decrease in tumor size (p=0.001,t-test). Combined viral therapy with both Ad.mIL12 and Ad.FasL resulted in a 72% (+/-6%) decrease in tumor size (Ad.IL12 vs. Combination therapy, p=0.04, t-test). In addition to fully exploit remaining Fas+ cells an additional injection of FasL was performed on the third day post-Ad.FasL+Ad.mIL-12 injection. However, no improvement in growth suppression over a single Ad.FasL injection was noted.

In a survival study both a single and double injection of Ad.FasL improved survival over that of controls but surprisingly was inferior to Ad.mIL-12 (Figure 4A, Selleck et al 2003, (10), Ad.mIL-12: 22.8+/-.8 days vs combo Tx: 19.3+/-.6 days, p=0.0134, Mantel-Cox). We reasoned that perhaps combination therapy was inferior to due to the killing of Ad.mIL-12 transduced cells by FasL expression, thereby reducing both IL-12 and IFN-γ levels. This in turn may reduce the other abilities of IL-12 in this model, induction of cytotoxic neutrophils, T cells, and NK cells, and the upregulation of Fas through a reduction of IFN- γ (2). To test this hypothesis we injected Ad.mIL-12 into the contralateral lobe of the prostate which did not harbor a tumor with the injection of the tumor by Ad.FasL as before. In this assay as would be predicted injection of the contralateral lobe reduced the survival enhancing abilities of cytokine therapy, but when combined with intratumoral injection with Ad.FasL resulted in significantly enhanced tumor growth suppression (Figure 4B, Selleck et al 2003 (10), Ad.mIL-12 intratumoral versus Ad.mIL-12 contralateral + intratumoral FasL, p=0.027 Mantel-Cox).

Based on this study we then staggered the injections of Ad.mIL-12 and Ad.FasL – Ad.mIL-12 as per routine on day 6 and Ad.FasL on 9. The later time point would miss the peak of IL-12 noted in the serum of treated mice. In addition some addition mice received a 2nd injection on day 11. The addition of double injections of Ad.FasL resulted in significant enhancement of survival over Ad.mIL-12 alone (Figure 4C, Selleck et al 2003 (10); p=0.0003, Mantel-Cox) and doubled mean survival over no treatment alone. In addition by analysis of apoptotic activity this combination enhanced activity almost 3 fold over Ad.mIL-12 alone (0.0016, t-test) and more pronounced than Ad.FasL alone (p=0,03, t-test) (Figure 5, Selleck et al 2003 (10)).

5) Related Fas/FasL Work

The preliminary work for the grant had noted the ability of Ad.mIL-12-IFN-γ to influence the Fas/FasL pathway in the mouse prostate cancer model. This work was further expanded in vivo, underscored the strong influence of Fas/FasL interactions in mediating growth suppression of prostate cancer cells. We showed that serum levels of IFN-γ upregulated Fas expression on cells in disseminated lung lesions which then underwent apoptosis due high levels of intrinsic FasL in the lung. This may serve an important method to control the establishment and growth of metastases. This work was published in Human Gene Therapy (Sanford et al Hum Gene Ther 2001 (2)) and is attached. Furthermore, with partial support from this grant, parallel studies with Ad.mIL-12 and the Herpes Virus Simplex thymidine kinase and ganciclovir system demonstrated independent influences on Fas/FasL expression to enhance local cell kill. These studies demonstrated that HSV-tk+GCV itself could significantly increase cell expression of FasL, which in the presence of IFN-γ effects on Fas expression, resulted in a significant bystander effect. Indeed, this interaction allowed for maintenance of maximum cell kill a

very low vector doses. This work was published this year in Gene Therapy (Hall et al, Gene Therapy, 2002) and is attached.

Specific Aim 2

1. Effect of IFN-γ on the human cell lines PC3 and LNCaP.

Studies were performed demonstrating the ability of IFN-γ to influence Fas transactivation via 2 methods. The first involved using an agonist anti-Fas antibody and the counting of viable cells. The second explored the use of soluble FasL to lyse cells with or without IFN-γ. As demonstrated in Figure 1 of Selleck et al, by both assays IFN-γ either enhanced Fas sensitivity (PC3) or reversed Fas resistance (LNCaP).

2. Caspase Activation in PC3 and LNCaP Cells

Studies then addressed the caspase cascade following Fas transactivation as outlined in Specific Aim 2. The initial study focused on discriminating the caspase familiy activated by FasL in these, focusing on the ICE family (caspases 1, 4,& 5), the CPP32 family (caspases 3,6,7,8,9,and 10) and the ICH/Nedd2 subfamily (caspase 2) using specific fluorescent substrates. In both LNCaP and PC3 cells we found high amounts of DEVDase indicative of the CPP32 family following FasL exposure. In the FasL sensitive cell line PC3 the addition of IFN-γ increased levels of substrate indicating that cytokine pre-incubation enhanced the same pathway and did not re-route to another caspase pathway. Likewise, in LNCaPs there was only substrate in the presence of IFN-γ. To verify these results specific caspase inhibitors of each subfamily were used in the face of FasL. These studies noted that the CPP32 inhibitor, z-DEVD-FMK and not the ICE inhibitor, Ac-YVAD-CMK, blocked FasL-mediated cell lysis in both cell lines. Time course studies following Fas transactivation indicate that caspase 8 is the first caspase to be activated. This information is being prepared for publication.

Specific Aim 3.

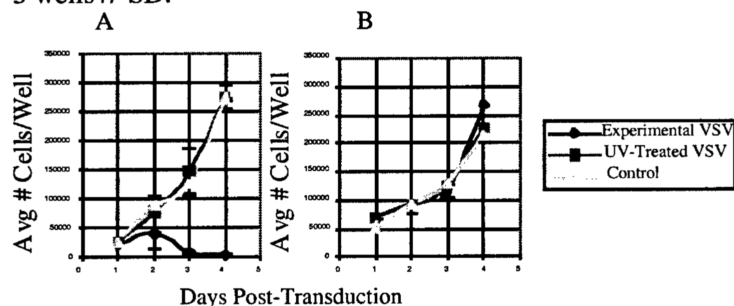
The original proposal would generate a prostrate specific adenovirus to restrict expression of FasL to prostate cells. However, in the intervening time since the submission of this grant it has become clear that there are limitations with replication incompetent adenoviral vectors. Therefore, we chose to explore a more novel and potentially more powerful vector to influence Fas/FasL interactions. In collaboration with colleagues within our Center for Gene Therapy and Molecular Medicine, we explored the use of a replication competent vector, Vesicular Stomatitis Virus (VSV). Vesicular stomatitis virus (VSV) is an enveloped, negative-sense RNA virus which expresses 5 genes. It displays several characteristics which make it an attractive candidate as a cytolytic and gene therapy vector for cancer (12). VSV has a favorable safety profile, as infections in humans are asymptomatic in most cases or result in a mild febrile illness, and do not have transformation capabilities. VSV has a broad host and cell infectivity range and can be produced at fairly high titer (10^9 pfu/ml). VSV replication is exquisitely sensitive to inhibitory effects of interferons (IFN), produced by the immune response against the virus (12). This limits replication and death in normal cells. However, most cancer cells display dysfunctional downstream responses to IFNs, so that VSV continues to replicate despite the presence of IFN (13, 14). Infected cells die within hours usually by apoptosis with completion of the

replication cycle within 6-8 hours (14). Experience with a variety of cancer lines have noted the ability of VSV to kill cells with a variety of mutations, such as p53, Ras and Myc (14). Limited experience in vivo notes that direct injection of tumors results in significant growth 13, 14). Vis a vis metastatis injection into a subcutaneous tumors results in growth control of an adjacent non-injected tumor (14). Furthermore, in vascular inoculation can likewise control the growth of an established subcutaneous tumor. Therefore, advantages of VSV as a cytolytic virus include: 1) VSV can be grown to high titers (up to 10^9 to 10^{10} pfu/ml), 2) VSV has a very short replication cycle (6-8 h in tumor cells) maximizing effect prior to a neutralizing immune response, and 3) its sensitivity to IFN maintains a suitable therapeutic window by bestowing activity to cancer cells and protecting normal infected cells.

The recent development of a "reverse genetics" system for negative-sense RNA viruses has made it possible to engineer the genome of VSV for the construction of novel gene therapy vectors (15). Recently, recombinant VSV vectors expressing the Herpes Simplex virus thymidine kinase (HSV-tk) suicide gene (rVSV-tk) or the cytokine interleukin 4 (IL-4) gene have been reported (16). Intratumoral injection of rVSV-tk with administration of ganciclovir (GCV) in s.c. established syngeneic breast or melanoma tumors in mice exhibited greater antitumoral effect than the wild-type virus. Therefore, the VSV vector can be genetically altered in the context of oncolytic VSV therapy appears to be more effective than the wild-type virus alone, both in the context of local injection and intravenous delivery against established metastases.

Preliminary Data

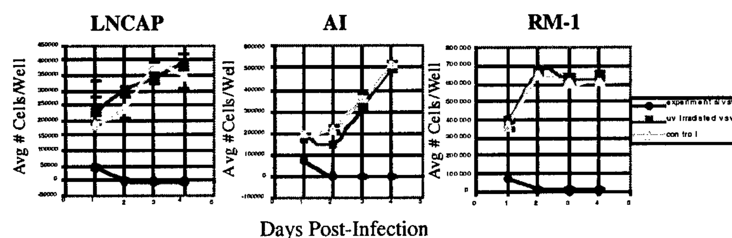
Figure 1. Effect of VSV on Growth of Prostate Epithelial Cells. Plated RWPE cells were exposed to either VSV-GFP, VSV-GFP exposed to UV light to inactivate the virus or buffer at dose of 1 virus per cell (MOI 1). Each treatment condition was further subdivided to PBS (A) or IFN-α (B) (100u/ml). Proliferation was ascertained by MTT assay daily for 4 days. Each data point represents the average of 3 wells+/-SD.



Preliminary studies explored the used of a recombinant VSV which expresses green fluorescent protein (GFP). The initial aim was to demonstrate that VSV replication could be inhibited by type I IFN. RWPE, a human prostate epithelial cell line (AR positive, expresses PSA from ATCC) was exposed to VSV-GFP alone or in the presence of IFN-a (100u/ml). Over the course of 2-3 days VSV killed virtually all RWPE cells, while UV-inactivated VSV and PBS had no inhibitory effect on growth (Figure 1A). However, the presence of IFN-_ cell growth resulted in identical growth patterns in all groups, consistent with the ability of IFN to inhibit VSV replication (Figure 1B).

The next studies addressed the effect of VSV on several prostate cancer cells lines: LNCaP, an AR-positive, androgen sensitive human prostate cancer cell line (ATCC), AI, an AR positive, androgen independent cell line derived from LNCaP cells (Obtained from Dr. Anna Ferrari, Mount Sinai School of Medicine, and RM-1, an AR positive, androgen independent mouse prostate cancer cell line derived from the mouse prostate reconstitution model (Obtained from Dr Timothy Thompson, Baylor College of Medicine). The object of these

Figure 2. Effect of VSV-GFP on Prostate cancer Cell Lines. Plated cells were exposed to VSV-GFP at a MOI of 1 in the presence of IFN-α (100u/ml). Proliferation was ascertained by MTT assay daily for 4 days. Each data point represents the average of 3 wells+/-SD.



studies was to prove that VSV would replicate in prostate cancer cell lines in the presence of IFN-_. Much like the experience with RWPE without IFN-_, VSV rapidly killed all 3 cell lines with maximum cell kill noted within 48 hours (Figure 2). Under the same conditions analysis of VSV-GFP infected prostate cancer cells by white light 8 hours following infection noted little change in cellular morphology compared to control treated cells. By UV light wells infected with VSV-GFP displayed green in only 2-3% of cells compared to no green fluorescence in control wells This would indicate that at a MOI of 1 as few as 2-3% of cells are initially infected with VSV. Analysis at 24 hours post-infection identified detached, rounded cells consistent with impending death and green fluorescence in virtually all cells, indicating the rapid replication and further infection of VSV-GFP in these cells. Since achieving high transduction efficieny with other gene therapy vectors has limited efficacy in vivo, studies then explored the ability of VSV-GFP to kill prostate cancer cells at 100 fold lower dose (MOI 0.01 or 1 vector to 1000 cells). Again, rapid cell kill was experienced within 48 hours of infection in all cell lines and virtually all cells expressing GFP within 24 hours (data not shown).

Figure 3. Effect of VSV-GFP on Prostate Cancer Cell Growth at MOI of 0.01. Plated cells were exposed to VSV-GFP at a MOI of 0.01 in the presence of IFN-α (100u/ml). Proliferation was ascertained by MTT assay daily for 4 days. Each data point represents the average of 3 wells+/-SD.

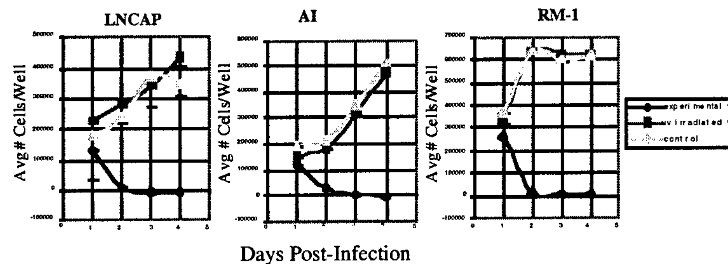
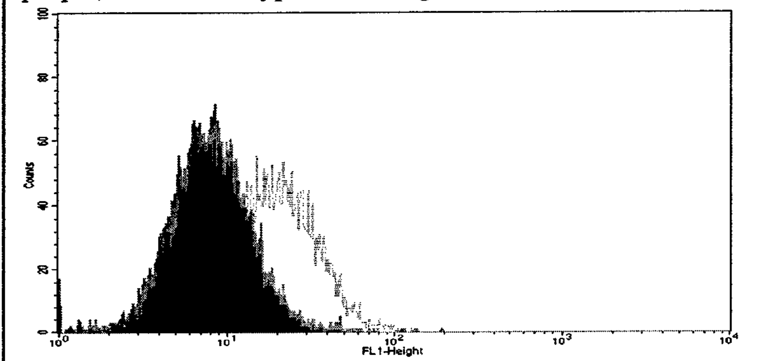


Figure 4. Effect of VSV on FasL Expression on Prostate Cancer Cells. Plated RM-1 cells were infected with VSV-GFP (purple & pink) or UV-inactivated VSV (green) at MOI 0.01 in the presence of z-FMK-VAD. Twenty-four hours later the cells were harvested and stained for FasL (pink & purple) or with isotype control (green).



The ability of VSV infection to influence Fas/FasL expression was explored following exposure in RM-1 cells. Since cells are killed so quickly a caspase inhibitor, z-VAD-fmk (20um) was added to the media immediately after infection to prevent apoptotic cell death (the method of VSV-mediated death). One day later cells were harvested and stained for Fas/FasL. By FACS analysis FasL expression was significantly enhanced following infection with active VSV (Figure 4) while there was little effect on Fas (data not shown).

In summary these data demonstrate the profound ability of VSV to rapidly kill prostate cancer cell lines, even at doses of 1 virus per 1000 cells, while sparing normal prostate cells under the same conditions. In addition VSV infection induces FasL expression, which could be further exploited in combination with IL-12/IFN-g. The generation of a second generation VSV expressing IL-12 is one of the goals of a recently submitted grant to the DOD Prostate Progam (PC 031123).

Key Research Accomplishments

1) production of replication incompetent adenovirus expressing FasL

2) demonstration of synergistic killing in vitro and in vitro

3) demonstration of enhancement/reversal of Fas resistance in human prostate cancer cells lines by IFN-γ, mediated through caspase 8.
4) demonstration of marked killing of prostate cancer cells but not benign prostate epithelial cells by VSV.

Reportable Outcomes:

Sanford MA, Yan Y, Canfield SE , Hassen W, Atkinson G, Chen SH, and Hall SJ. Independent Contributions of Gr-1+ Leukocytes and Fas/FasL Interactions to Induce Apoptosis Following IL-12 Gene Therapy in a Mouse Model of Prostate Cancer. Hum Gene Ther 12: 1485-1498, 2001.

Hall SJ, Canfield SE, Yan Y, Hassen W, and Chen S-H. A Novel Bystander Effect Involving Tumor Cell Derived Fas and FasL Interactions Following Ad.HSV-tk and Ad.mIL-12 Gene Therapies in Experimental Prostate Cancer. Gene Ther. 9: 511-517, 2000.

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CONCLUSIONS

Manipualtion of Fas/FasL expression by prostate cancers can result in powerful local and systemic growth effects through gene therapy techniques. IFN-g can reverse Fas resistance in human cell lines, though the nature of Fas activation in human tumors is unknown, The VSV vector is an attractive more powerful and restricted vector which can exploit Fas/FasL interactions in addition to its already impressive anti-cancer characteristics.

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HUMAN GENE THERAPY 12:1485–1498 (August 10, 2001)
Mary Ann Liebert, Inc.

APPENDIX 1

Independent Contributions of GR-1$^+$ Leukocytes and Fas/FasL Interactions to Induce Apoptosis Following Interleukin-12 Gene Therapy in a Metastatic Model of Prostate Cancer

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ABSTRACT

In a mouse model of prostate cancer, adenovirus-mediated interleukin-12 (Ad.mIL-12) gene therapy resulted in significant growth inhibition of both the injected primary tumor and synchronous metastases. Within 2 days of vector injection, two distinct patterns of apoptosis were detected within the primary tumor, the inhibition of which with a caspase inhibitor substantially negated growth suppression. The dominant pattern displayed localized sheets of apoptotic cells in close association with necrosis containing polymorphic neutrophils (PMNs). Depletion of PMNs resulted in the loss of this pattern of apoptosis and reduced growth suppression. A second major wave of growth suppression within the primary tumor was mediated by an immune response. Natural killer (NK) cell activity was detected within tumor-infiltrating lymphocytes (TIL) by the eighth day post-vector injection, the depletion of which resulted in a significant loss of survival enhancement. A more modest role for T cells was identified, which in the absence of documented cytotoxic T lymphocyte (CTL) activity may be related to a significant reduction in interferon-γ (IFN-γ) levels found in mice depleted of T cells, thereby reducing the secondary influences of IFN-γ. However, depletion of NK cells or T cells had no discernible negative effect on IL-12-mediated anti-metastatic activity. Attention focused on the role of IFN-γ, observed following Ad.mIL-12 therapy, to mediate the diffuse pattern of apoptosis seen in the primary and metastatic lesions. *In vitro* studies noted the ability of IFN-γ to up-regulate tumor cell expression of Fas and FasL to mediate apoptosis, whereas *in vivo* blockage of Fas/FasL interactions with soluble Fas resulted in a modest reduction in primary tumor growth suppression but complete abrogation within metastatic lesions.

OVERVIEW SUMMARY

The mechanisms underlying adenovirus-mediated interleukin-12 (Ad.mIL-12) gene therapy to support local and systemic growth suppression were explored in a mouse model of prostate cancer. Studies noted two phases of growth inhibition, apoptosis during days 2–6 post-vector injection and an immune response after day 8 post-vector injection. Likewise, apoptosis occurred in two distinct patterns, inhibition of which by a caspase inhibitor significantly reduced growth suppression in both the orthotopic and metastatic sites. The dominant pattern noted only in the primary tumor was associated with polymorphic neutrophils (PMN), which if depleted, resulted in a significant loss of apoptotic activity and negation of local growth control. The later phase of growth suppression was mediated by natural killer (NK) and T cell responses. Although enhancement of animal survival correlated most closely with an NK response, abrogation of T cell activity had a less profound effect and in the absence of detectable cytotoxic T cell (CTL) activity loss of activity may be due to significantly less production of interferon-γ (IFN-γ) in these animals. A second pattern of diffuse apoptosis was noted within primary tumors between areas of necrosis and was the sole pattern observed in metastases. Studies demonstrated the ability of IFN-γ following IL-12 therapy to upregulate Fas initiating Fas/FasL interactions to control metastatic growth.

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INTRODUCTION

INTERLEUKIN-12 (IL-12) is a multifunctional cytokine that mediates a range of functions which may be useful in cancer treatment. Primarily, it directs Th1 differentiation of immune responses and enhances the proliferation and cytotoxicity of T cells and natural killer (NK) cells, dependent to a large degree on production of interferon-γ (IFN-γ) by these effectors to maintain activity (Zeh *et al.*, 1993; Nastala *et al.*, 1994; Hendrzak and Brunda, 1995). On the basis of these influences, interest in using IL-12 treatment for cancer has focused on inducing antitumor immunity. In numerous cancer models, IL-12 treatment, either as recombinant protein or through a variety of gene therapy approaches, has demonstrated active growth inhibition of solitary tumors and may result in systemic growth suppression through inhibition of experimental metastases and/or resistance to challenge tumor cell injections (Brunda *et al.*, 1993; Tahara *et al.*, 1995; Bramson et al., 1996; Colombo *et al.*, 1996; Rakhmilevich *et al.*, 1996; Tan *et al.*, 1996; Cui *et al.*,1997; Coughlin *et al.*, 1998; Nanni *et al.*, 1998; Siders *et al.*, 1998; Nasu *et al.*, 1999; Hirschowitz *et al.*, 1999; Mendiratta *et al.*, 1999; Pham-Nguyen *et al.*, 1999; Cavallo *et al.*, 1999). Assays for T cells, NK cells, and NKT cells have validated individual and, in some instances, cooperative roles for these immune effectors in growth suppression. A further major component of tumor growth inhibition has been attributed to the ability of IFN-γ to mediate anti-angiogenic activity through expression of inhibitors such as IFN-γ-inducible protein-10 (Voest *et al.*, 1995; Sgadari *et al.*, 1996; Coughlin *et al.*, 1998). Secondary, less well understood mechanisms have also been proposed as potential mechanisms underlying IL-12 therapy. Treated tumors contain polymorphic neutrophils (PMNs) and macrophages that may influence tumor growth suppression (Tsung *et al.*, 1997; Cavallo *et al.*, 1999; Mendiratta *et al*, 1999; Nasu *et al.*, 1999). IL-12 therapy may result in antiangiogenesis through down-regulation of proangiogenic substances such as vascular endothelial growth factor (VEGF) and matrix metalloproteinases (Dias *et al.*, 1998) or result in local cytotoxicity through tumor cell production of substances such as nitric oxide (Yu *et al.*, 1996). Last, IL-12 may directly alter cell motility and invasiveness which may impact on spontaneous metastatic behavior as a method of inhibiting metastases (Hiscox *et al.*, 1995). Yet, a comprehensive understanding of potential interactions or temporal relationships or degree of influence of these various mechanisms is lacking. Furthermore, it is unclear how these varying mechanisms correlate with the proximity to the site of gene transduction.

With approximately 180,000 diagnoses and 32,000 deaths attributed to prostate cancer in 2000 in the United States, this disease remains a serious public health issue (Greenlee *et al.*, 2000). Patients diagnosed with clinically localized disease may be offered definitive therapy in the form of radical surgery or radiation therapy. However, long-term freedom of disease as measured by a rising serum prostate-specific antigen (PSA) notes recurrence rates approaching 50% in higher-risk patients as defined by an initial PSA > 10 or presence of higher-grade disease (gleason score ≥ 7) on prostate biopsy (Catalona *et al.*, 1999). Similar results may be experienced with radiation therapy indicating the need for neo-adjuvant or adjuvant therapies in these patients to target both locally advanced primary and unrecognized microscopic metastatic disease. RM-1 is a mouse prostate cancer cell line established from a primary prostate tumor derived in the mouse prostate reconstitution model system (Thompson *et al.*, 1989) and has been a useful tool for preclinical studies exploring gene therapy approaches to prostate cancer therapy. In this model, a single injection of adenovirus-mediated (Ad.m)IL-12 resulted in significant growth inhibition of both a primary tumor and pre-established metastases. Different mechanisms were responsible for growth suppression relating to chronology and proximity to IL-12 gene expression. Within the primary tumor several mechanisms sustaining growth suppression were defined, dominated within the first 2 days of vector injection by apoptosis due to an influx of GR-1$^+$ leukocytes. Long-term growth suppression was mediated by a NK response noted within tumor infiltrating lymphocytes (TIL) induced 8 days post-vector injection and to a lesser extent a T cell response. Less significant within the injected tumor, but mediating the growth suppression within metastatic lesions, were the tumor cell-directed Fas/FasL interactions that were secondary to the ability of IL-12-directed host production of IFN-γ to up-regulate both Fas and FasL expression on RM-1 cells, leading to apoptosis.

MATERIALS and METHODS

Cell lines

The RM-1 mouse prostate cancer cell line was established from a ras + myc-induced primary tumor, derived in the mouse prostate reconstitution model in C57BL/6 mice (obtained from Dr. Timothy Thompson, Baylor College of Medicine, Houston, TX; Thompson *et al.*, 1989; Baley *et al.*, 1995). RM-1 is androgen insensitive, susceptible to CTL lysis, exhibits low levels of MHC class I molecule expression that are up-regulated in response to IFN-γ, and only mildly immunogenic (Baley *et al.*, 1995; Kawakita *et al.*, 1997; Nasu *et al.*, 1999). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 10 m*M* HEPES, penicillin (100 U/ml), and streptomycin (100 mg/ml), passaged by trypsinization and maintained with routine media changes. YAC-1 is a NK-sensitive mouse lymphoma cell line (ATCC, MD), maintained in RPMI-1640 with 10% FBS. Materials for cell culture were obtained from Sigma Chemical Company (St. Louis, and MO).

Adenoviral vectors

A replication-incompetent, E1-deleted adenovirus expressing mouse IL-12 was constructed as previously described (Caruso *et al.*, 1996). The p40 and p35 mIL-12 subunits, connected by an internal ribosomal entry site, were placed under the control of the Rous sarcoma virus (RSV) promoter. To construct a replication incompetent adenovirus expressing soluble Fas (sFas) immunoglobulin (Ig), the extracellular domain of Fas was amplified by PCR and ligated to the Fc region of mouse IgG_{2a}. The sFas-Ig cDNA was placed in the adenovirus backbone driven by the RSV promoter. The pAd.RSV-sFasIg was co-transfected with pBHG10 in 293 cells by the calcium phosphate precipitation method to yield Ad.sFasIg. Preliminary characterization noted the presence of both Fas and mouse IgG in MCA-26 cells (mouse colon cancer cells) transfected with Ad.sFasIg by fluorescence-activated cell sorting (FACS) anal-

ysis (anti-FasIgG and anti-mouseIgG, Pharmingen). DL-312, an E1 region-deleted Ad without transgene, was used a vector control. Large-scale production of vector was achieved following expansion in 293 cells and double cesium gradient ultracentrifugation purification. The viral titer was ascertained in 293 cells in a plaque assay and expressed as plaque-forming units (PFU) per milliliter.

In vivo *gene therapy*

Orthotopic tumors were induced as previously described (Hall *et al.*, 1997). Briefly, tumors were induced by an injection of 7500 RM-1 cells into the dorsolateral prostates of C57/BL6 mice (Jackson Laboratories) or NKT knockout mice (C57/BL6 background Vα 14 NKT knockouts, obtained from Dr. Masaru Taniguchi, Chiba, Japan) (Cui *et al.*, 1997). Vector injections were performed on the 6th day post-incoulation when tumors essentially filled the lobe injected. To evaluate the induction of systemic antitumor activity against pre-established metastasis, a synchronous metastatic model was used, as previously described (Hall *et al.*, 1998). Briefly, lung tumors were induced by a tail vein injection of RM-1 cells on the same day as primary tumor induction, leaving sufficient time for establishment of microscopic lesions prior to vector injection of the primary prostate tumor (day 6). At sacrifice the lungs were removed and placed in Bouin's solution for several hours prior to transfer into 70% ethanol. Lung metastases were then quantified by counting visible lesions with a dissecting microscope. All animals were used in accordance with established protocols approved by the institutional animal use committee.

Immunohistochemistry

Tissues from both primary prostate tumors and lung were either formalin fixed (10%), followed by paraffin embedding for sectioning or snap frozen in O.C.T. compound (Tissue-Tek, Sakura Finetek, Torrance CA) for frozen sectioning. After sectioning, formalin-fixed tissues were stained with hematoxylin and eosin for routine histopathologic screening. Presence of apoptotic activity was achieved by TUNEL assay as per manufacturer instructions (Apop Tag, Oncor, Gaithersburg, MD). The number of positive bodies were counted at 400× by viewing across the long and short axis of tissue sections and expressed as number of bodies per high-power field (HPF). Immunohistochemistry to determine expression or presence of Fas (hamster anti-mouse IgG; Pharmingen, San Diego, CA) and FasL (goat anti-rat IgG; Santa Cruz Biotechnologies, Ca) was performed on frozen tissue sections.

Inhibition of apoptosis in vivo

Inhibition of apoptosis was achieved using the general caspase inhibitor, Z-Val-Ala-Asp (Ome)-fluoromethyl ketone (z-VAD-FMK, Enzyme Systems Products, Livermore, CA). *In vitro* z-VAD-FMK was added to plated cells at a dose of 20 μM at the time of IFN-γ or phosphate-buffered saline (PBS) exposure. Viable cells as per trypan blue exclusion were counted 24 hr later. *In vivo* z-VAD-FMK was administered to mice in twice daily i.p. injections of 200 μg, beginning the day of virus injection through the 8 days prior to sacrifice, as previously reported (Hotchkiss *et al.*, 1999; Piguet *et al.*, 1999).

In vivo *cytotoxic assays*

TIL were extracted for *in vitro* analysis as previously described (Hall *et al.*, 1998). At designated time points, excised primary tumors from treatment and control groups were pooled and mechanically lysed between glass sides in Hanks' balanced salt solution (HBSS) with 1% fetal calf serum (FCS). Tumor debris was removed by serial gravity precipitations. The resulting single-cell suspension was then treated with red cell lysis buffer (Sigma Chemical Co,) and, following precipitation, passed through cotton wool pipettes. After washing and resuspension, viable lymphocytes, as determined by exclusion of trypan blue, were counted and exposed at 37°C to chromium-51 (^{51}Cr)-loaded targets (150 μCi/5 × 10^6 cells) for 4 hr at various effector-to-target ratios in triplicate. The percentage of specific lysis was calculated as (experimental release − spontaneous release)/(maximal release − spontaneous release) from gamma counter measurements of supernatant harvested by the Skatron system (Skatron Instruments Inc, VA). Mechanically lysed splenocytes were purified by double-density centrifugation (Lympholyte-M solution, Cedarlane Labs, CA) and prepared for cytotoxic T lymphocyte (CTL) assay by priming with low-dose mIL-2 (20 U/ml, Pharmingen, San Diego, CA) in the presence of lethally irradiated RM-1 cells for 5 days. Primed splenocytes were then collected, counted, and exposed for 4 hr at varying effector to target ratios with the measurement of radioactivity as detailed above.

Antibody-mediated in vivo *lymphocyte depletions*

Specific lymphocyte populations were depleted *in vivo* through i.p. injections of the appropriate antibodies beginning 1 day prior to Ad.mIL-12 injection, as previously described (Hall *et al.*, 1998). Inactivation of CD4/CD8 T cells was accomplished with purified ascites from hybridoma GK 1.5 (anti-CD4 T-cells) (ATCC, Rockville, MD) and from hybridoma 2.43 (anti-CD8 T cells) (ATCC, Rockville, MD). NKs were inactivated with anti-asialo GM1 IgG (Wako Chemicals, Reston, Va), and controls with rabbit IgG (Dako Corp, Carpinteria, Ca) and rat IgG (Accurate Chemical & Scientific Corp, Westbury, NY). GR-1 leukocytes were depleted with anti-Ly-6 IgG (Pharmingen, San Diego, Ca) (Cavallo *et al.*, 1999; Mendritta *et al.*, 1999). Anti-NK and control antibody injections were given every other day, whereas anti-CD4, anti-CD8, and Anti-Ly-6 IgGs were given every third day as determined previously. In each case, the end point was loss of >90% of each subset from spleens of treated tumor naive mice.

Measurement of serum cytokines

Blood was drawn from animals at designated time points via tail vein. After coagulation at room temperature serum from each group was pooled and frozen at −70°C. Serum cytokine determination was achieved with commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN).

In vitro *actions of IL-12 and IFN-γ on RM-1 cells*

The potential for IL-12 or IFN-γ to mediate toxicity *in vitro* was assayed by adding recombinant mIL-12 (2–200 ng/ml, Biosource International) or mIFN-γ (50–200 U/ml, Pharmingen) to media of plated RM-1 cells. Cells were harvested 48 hr

later and viable cells, as determined by trypan blue exclusion (0.4%, Sigma Chemical Company, St. Louis, MO), were counted. The ability of IFN-γ to impact of Fas and/or FasL was addressed through FACS analysis of stained cells. RM-1 cells *in vitro* were randomized to exposure to 100 U/ml of mIFN-γ or PBS for 24 hr. Cells were harvested, fixed in 0.1% paraformaldehyde, and stained with fluorescein isothiocyanate (FITC)-labeled anti-Fas IgG (hamster anti-mouse Fas IgG, Pharmingen), FITC-labeled anti-FasL (hamster antimouse FasL IgG, Pharmingen) or FITC-labeled hamster IgG. Cells were processed through FACS scan to ascertain the percentage and intensity of Fas/FasL staining. Mediation of the growth inhibitory activity of IFN-γ through the Fas/FasL pathway in RM-1 cells was addressed through *in vitro* growth assays. RM-1 cells were exposed to PBS or escalating doses of IFN-γ for 24 hr. Each treatment group had been randomized to receive 10 $\mu$of g/ml anti-FasL IgG (Pharmingen) or hamster anti-mouse IgG (Pharmingen). In parallel studies, blockage of Fas/FasL was achieved by using conditioned media from RM-1 cells transfected with Ad.sFasIg at a MOI of 50 (transduction of 50% of cells) for 48 hr. This media was added at the time of PBS or IFN-γ exposure. Likewise, blockage of apoptosis was achieved by adding z-VAD-FMK (20 μM) at the time of PBS or IFN-γ. Viable cells were counted 24 hr later.

Blockage of Fas/FasL pathway in vivo

To block Fas/FasL interactions *in vivo* tumor-bearing mice were injected with DL312 or Ad.sFasIg twice. Mice were randomized to 1×10^8 PFU to either vector by tail vein 2 days prior to vector injection of the primary tumor (day 4 post-tumor cell inoculation), followed by a second injection at a dose of 1×10^9 PFU directly into the primary tumor with/without Ad.IL-12. Mice were sacrificed on day 14 for evaluation of primary tumor wet weight and the number of lung metastases.

RESULTS

In vivo *gene therapy*

To determine the *in vivo* therapeutic dose of Ad.mIL-12, a dose escalation experiment was performed in the synchronous metastasis model. Established prostate tumors were injected in half- log increments from 5×10^8 to 1×10^{10} PFU of Ad.mIL-12. A total of 1×10^{10} PFU was the maximum dose tested given volume constraints at higher dosing that would result in gross spillage of vector into the peritoneal cavity. All animals were sacrificed at 14 days post-tumor cell inoculation, with all groups appearing healthy through the 8 days post-Ad.mIL-12 injection. By wet weight measurements, there was a dose-dependent inhibition of primary tumor growth (Fig. 1A), whereas reductions in the number of lung metastases were only encountered at higher doses of Ad.mIL-12. (Fig. 1B). A vector dose controlled study comparing the two higher doses was performed in the synchronous metastasis model. By 8 days, post-vector injection of 5×10^9 and 1×10^{10} PFU Ad.mIL-12 resulted in tumors which were 75 ± 3% and 83 ± 2.6%, respectively, smaller than controls, but this difference only approached statistical significance ($p = 0.053$, *t*-test) (Fig. 1C). Although both doses inhibited lung metastases, 32 ± 6% and 58 ± 5% compared to control, the higher dose was clearly superior ($p = 0.035$, *t*-test, 5×10^9 PFU versus 1×10^{10} PFU) (Fig. 1D). Likewise, a survival study in mice bearing only primary tumors was performed with both doses of Ad.mIL-12. Again, the higher dose was superior with a mean survival of 26.1 ± 1.7 days for treated mice compared to 16.5 ± 0.35 days for control tumors (Fig. 1E, $p < 0.0001$, Mantel-Cox Log Rank test).

Induction of apoptosis by Ad.mIL-12

By the second day post-Ad.mIL-12 injection, treated primary tumors contained areas of necrosis dominated by a marked influx of polymorphic neutrophils (PMNs) but also containing lymphocytes, eosinophils, and macrophages. TUNEL assay noted the induction of apoptosis at a mean level six times higher than control tumors ($p < 0.0001$, *t*-test) (Fig. 2A) in two distinct patterns. The first was notable for localized sheets of apoptotic cells in close association with the necrotic areas with ~100 apoptotic bodies per HPF (Fig. 3A–C). The second pattern was a diffuse but modest increase in apoptosis, approximately two times higher than background. By the fourth day post-vector injection the inflammatory infiltrate and accompanying sheets of apoptotic cells had resolved. The more diffuse pattern of apoptosis continued at approximately two times control (Fig. 2A) ($p = 0.004$, *t*-test). By the sixth day post-vector injection, no differences in apoptosis were apparent between IL-12-treated and control tumors. Histopathology of lung lesions during the 6 days post-vector injection failed to demonstrate the type or degree of inflammation seen in the primary tumor. However, TUNEL assay of lung lesions noted a doubling of apoptosis with Ad.mIL-12 therapy compared to controls ($p =$ o.0006, *t*-test) (Figs. 2B and 3D,E).

The functional significance of apoptosis in Ad.mIL-12-mediated growth suppression was addressed using the caspase inhibitor, z-FMK-VAD, in the synchronous metastasis model. Analysis of tumor tissue by TUNEL assay 2 days following injection with Ad.mIL-12 and receiving z-FMK-VAD noted a reduction in apoptotic activity from six times control levels to ~1.5x higher than control levels (5.17 ± 1.17 apoptotic bodies for Ad.mIL-12+z-FMK-VAD versus 3.18 ± 0.71 for control and 18.68 ± 4.5 for Ad.mIL-12). This decrease in apoptotic activity significantly reduced the ability of Ad.mIL-12 to mediate growth suppression, both within the primary tumor and the lung lesions (Fig. 4). However, the degree of influence was different within the primary and metastatic lesions. By 8 days post-vector injection primary tumors treated with Ad.mIL-12 and z-FMK-VAD were 36% larger than Ad.mIL-12-treated tumors as compared to control tumors ($p = 0.0013$, *t*-test) but remained significantly smaller than control tumors ($p = 0.012$, *t*-test). In contrast inhibition of apoptosis resulted in abrogation of IL-12-mediated growth effects on the average number of lung lesions ($p = 0.42$, *t*-test, control versus Ad.mIL-12+z-FMK-VAD).

Histopathology noted a relatively high proportion of PMNs within the areas of necrosis associated with apoptosis. Examination of tumors treated with z-FMK-VAD at day 2 noted the continued presence of clustered PMNs within Ad.mIL-12 treated tumors without the associated sheets of apoptotic cells, although there were scattered more localized areas of PMNs and apoptosis. To explore directly the role of these cells in the induction of apoptosis and resulting growth inhibition, IL-12

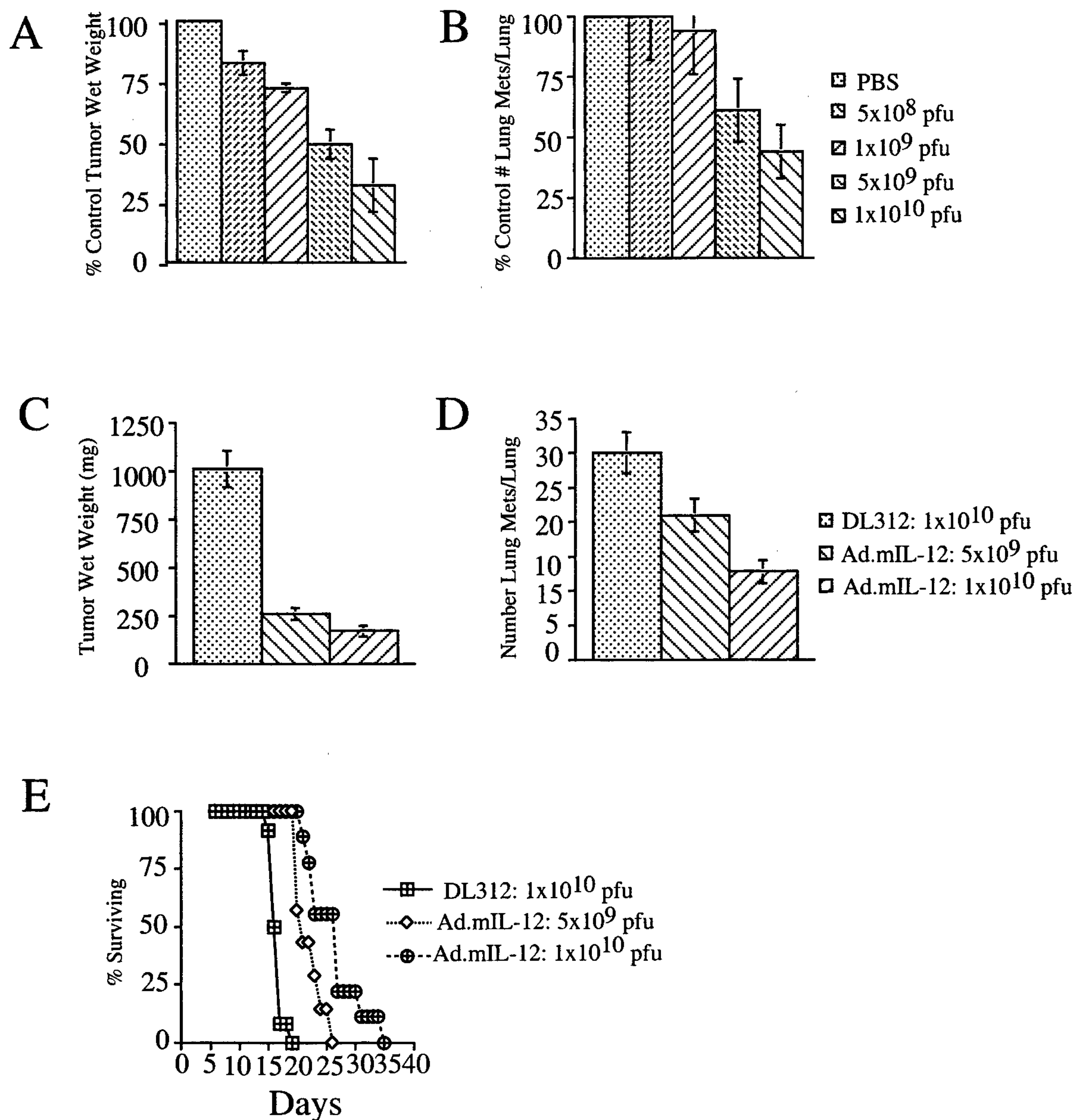

FIG. 1. Growth-suppressive activities of Ad.mIL-12 *in vivo*. (**A** and **B**). Dose escalation study. Established primary tumors in the synchronous metastasis model were injected with escalating doses of Ad.mIL-12 or PBS. All mice were sacrificed 8 days later and the wet weight of the primary tumor (**A**) and the number of lung metastases per lung recorded (**B**) and compared to control (buffer only). (**C** and **D**) Dose control study. Established tumors were injected with Ad.IL-12 or control vector at 1×10^{10} PFU. All mice were sacrificed 8 days later for recording of primary tumor wet weight (**C**) or average number of lung metastases per lung (**D**). (**E**) Survival study. Mice with primary prostate tumors only were injected with Ad.mIL-12 or control vector at 1×10^{10} PFU. Time of death was recorded to generate a survival curve. For **A–D**, each bar represents the average ±SE. (For **A** and **B**, $n = 4$ per group; for **C** and **D**, $n = 12$ mice per group; for **E**, $n = 8$ mice per group.)

gene therapy was performed in mice depleted of GR-1^+ cells. By day 2 post-vector injection, histopathology noted the absence of significant necrosis in tumors from Gr-1- Ad.mIL-12 mice, whereas TUNEL assay noted only the diffuse pattern of apoptosis and a lowering of average apoptotic activity (Fig. 5A). The reduction in apoptotic activity in GR-1^- tumors correlated with the abrogation of IL-12-mediated growth suppression of primary tumors; these tumors were on average were more than three times larger than Ad.mIL-12-treated tumors and approached the size of control tumors (Ad.mIL-12+isotype IgG versus Ad.mIL-12+anti-Gr-1 IgG, $p = 0.03$; Control tumors versus Ad.mIL-12+anti-Gr-1 IgG, $p = 0.1$, *t*-test)(Fig. 5B). In contrast, no detrimental effect was noted with regard systemic growth suppression (Fig. 5C).

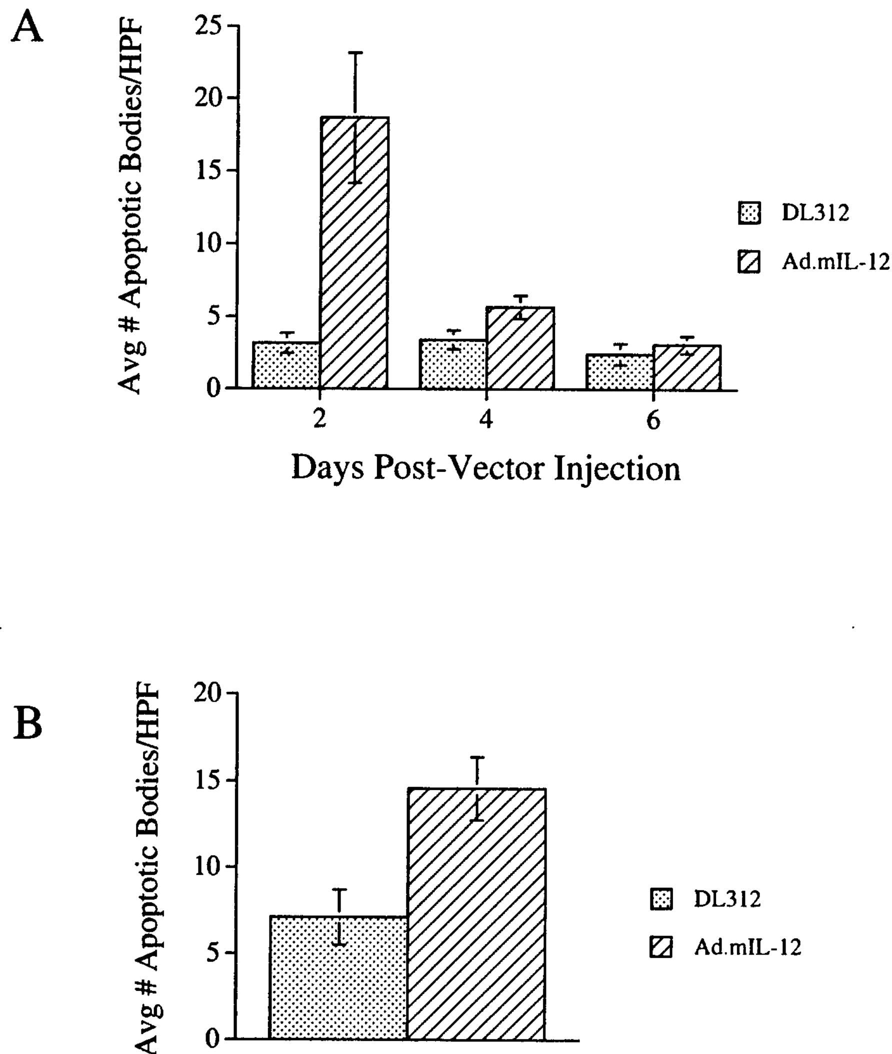

FIG. 2. Apoptosis following Ad.mIL-12. (**A**) Serial assay of apoptosis in primary tumors. Vector control and Ad.mIL-12-treated tumors were prepared for TUNEL assay on days 2, 4, and 6 post vector injection. The number of apoptotic cells per HPF (400×) were counted across the long and short axis of each tissue section and reported as the average number of apoptotic cells per HPF ± SD. (n = 9 for each condition.) (**B**) Assay for apoptosis in lung lesions. Cross sections of lungs from control and Ad.mIL-12-treated animals were prepared for TUNEL assay from day 6 and handled as described above. This day was chosen to ensure adequate size of lesions for accurate counting of apoptotic bodies. (n = 9 for each condition.)

Immunological assays

The induction of an immune response was studied first by identifying the presence of antitumor lymphocytes (TIL) harvested sequentially from treated and control primary tumors in a ^{51}Cr release assay. No discernible lytic activity was noted until the eighth day post vector injection, at which time both RM-1 and YAC-1 cells were lysed by TIL from Ad.mIL-12 treated tumors, implying the induction of a NK response (Fig. 6A). The *in vivo* role of NKs in growth suppression was addressed through antibody-directed depletion of specific candidate lymphocyte populations during Ad.mIL-12 therapy in two independent experiments with distinct end points. In the first study, mice in the synchronous metastasis model were sacrificed at 8 days post-vector to study influences on primary and systemic growth suppression in this time period. Abrogation of NK activity had no effect on either local or systemic growth suppression. However, negation of CD4 and CD8 T cells reduced IL-12 effects, because resulting primary tumors were ~20% larger than those treated with IL-12 in intact animals (p = 0.0014, versus Ad.mIL-12 + control IgG, t-test), but there was no influence on the number of lung metastases (Fig. 6 B,C).

In the second series of experiments, the longer-term role of an immune response was addressed in a survival study. Loss of NK cells had a profound detrimental effect on Ad.mIL-12-mediated survival, lowering mean survival from 24 ± 1.2 days to 18 ± .4 days, although remaining an improvement over control animals, 18 ± 0.4 days versus 16 ± 0.3 days (p = 0.012, Mantel-Cox Log Rank) (Fig. 6D). Likewise, loss of T cells had a negative effect but not to the degree of NK loss, 21.2 ± .2 days

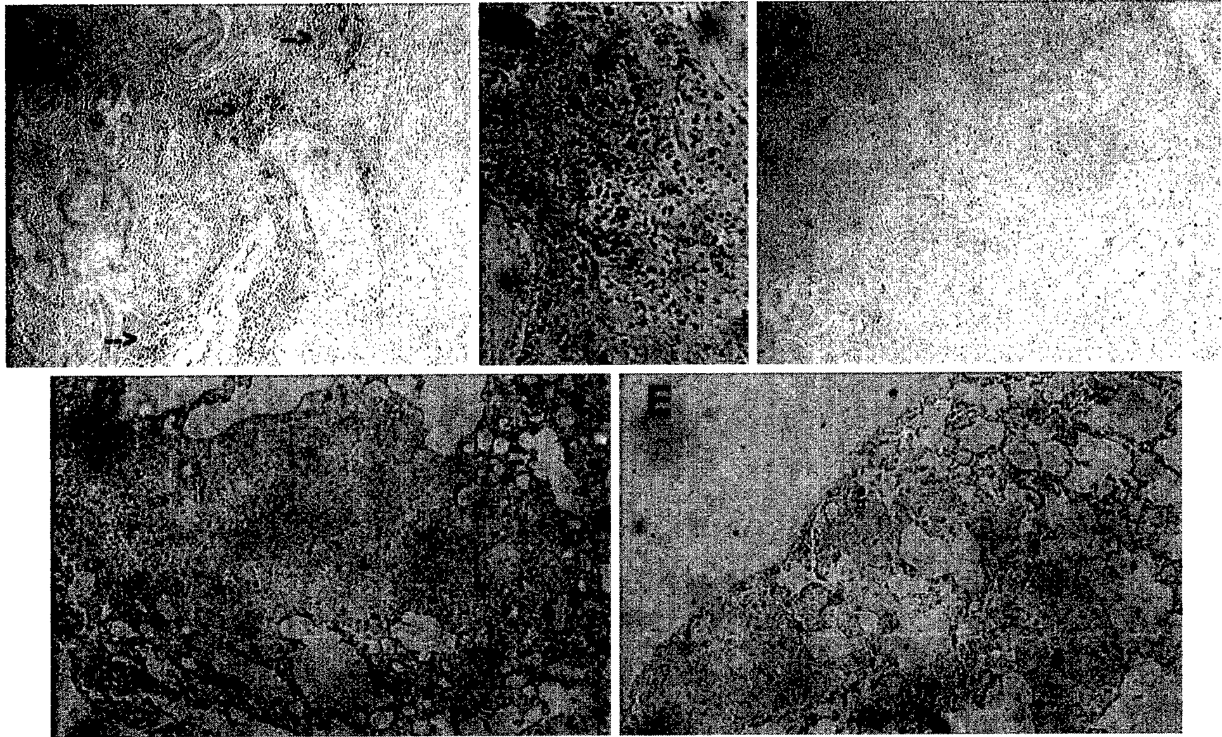

FIG. 3. TUNEL assay following Ad.mIL-12 therapy. (**A–C**) Photomicrographs of primary tumors harvested 2 days post vector injection. (**A**) Localized areas of apoptosis marked by arrows of tumor cells between normal prostate glands. These areas correlate to necrosis with high numbers of PMNs by H&E. (100×). (**B**) High-power view (400×) of a representative area of apoptosis. (**C**) Matched control tumor noting absence of localized areas of apoptosis in background of diffuse apoptotoic activity (100×). (**D** and **E**) Photomicrograph of lung lesions from Ad.IL-12-treated (**D**) and control (**E**) mice sacrificed day 6 post-vector, noting difference in numbers of positive cells. (Original magnification, 100×.)

versus 24.2 ± 1.2 days (p = 0.03, Mantel-Cox Log Rank). No CTL activity could be demonstrated within splenocytes from the same animals 10 days post-vector injection (data not shown). Because NKT cells have been identified as an important mediator of IL-12-mediated growth suppressive activities (Cui *et al.*, 1997), a survival study with Ad.mIL-12 gene therapy was performed in NKT knockout mice to subcategorize NK activity. Mean survival for IL-12-treated NKT mice was 26.8 ± 3.8 days versus 25.4 ± 2.3 days for IL-12-treated wild-type mice in a parallel experiment (p = 0.33, Mantel-Cox Log Rank).

Role of IFN-γ and the Fas/FasL pathway

Studies thus far have noted that both GR-1-mediated apoptosis and an immune response are important in controlling growth of the injected primary tumor, but have not elucidated the basis for antimetastatic activity. The role for IL-12-induced

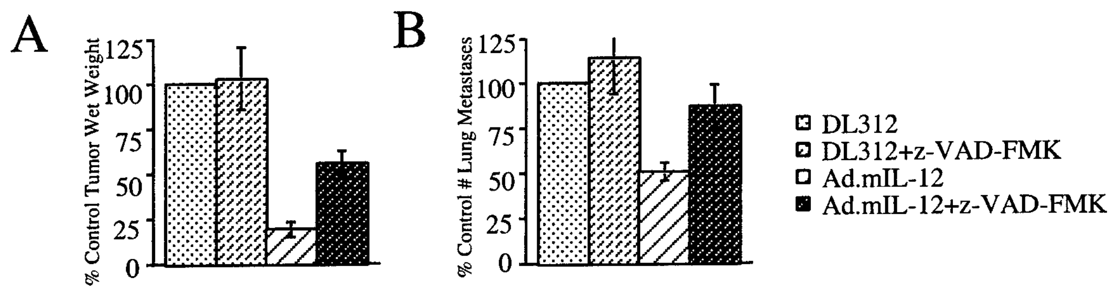

FIG. 4. Effect of apoptosis inhibition on Ad.mIL-12-mediated growth suppression. To ascertain the functional significance of apoptosis, the general caspase inhibitor, z-VAD-FMK (200 μg), was injected i.p. twice a day beginning on the day of vector injection (control vector or Ad.mIL-12). All mice were sacrificed on the eighth day post-vector injection for assay of primary tumor wet weight (**A**) and average number of lung metastases per lung (**B**). Each bar represents the average ±SD. (n = 5 for each group.)

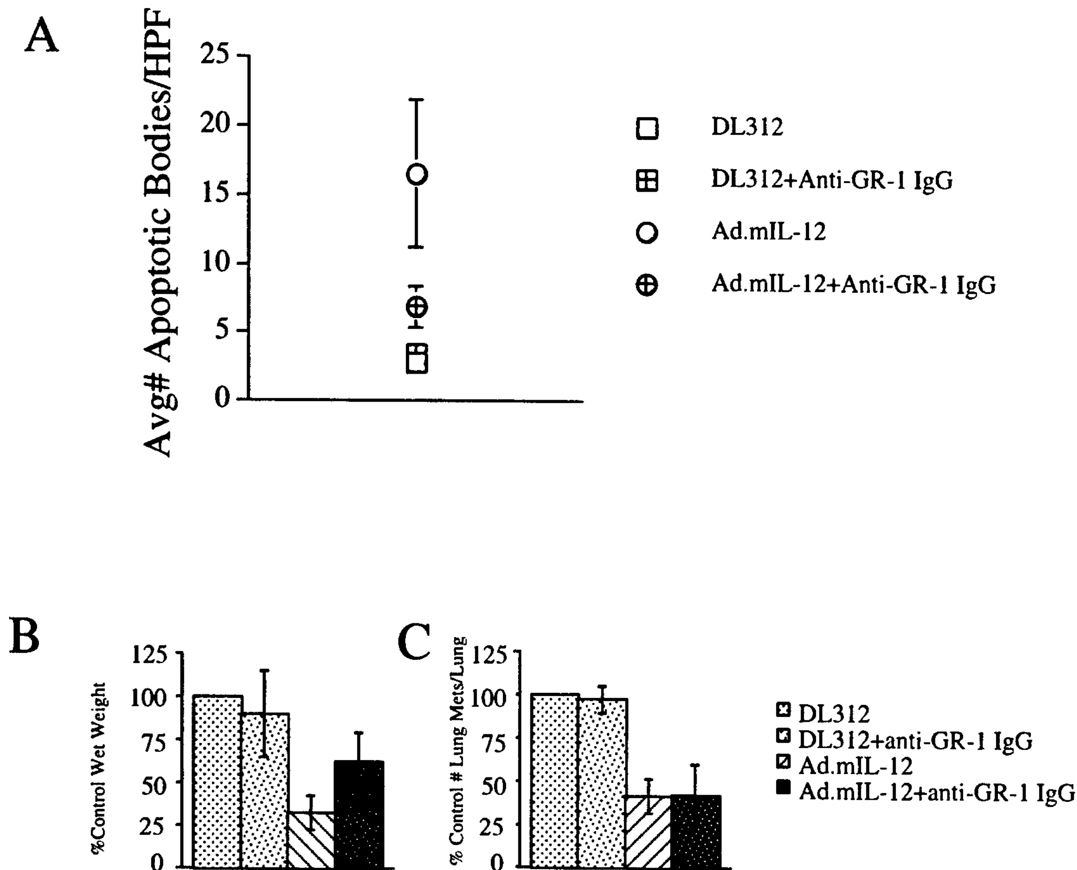

FIG. 5. Effect of GR-1 leukocyte depletion on Ad.mIL-12 therapy. (**A**) Differences in average number of apoptotic bodies per HPF under different treatment conditions. Tumors were harvested on day 2 post-vector injection. (**B**) Beginning the day prior to vector injection mice were divided to receive i.p. injections of anti-GR-1 antibody or control antibody every third day until all animals were sacrificed on the eighth day post-vector injection. Assay of efficacy was determined through primary tumor wet weight and average number of lung metastases per lung. Each bar represents the average ±SD. (n = 5 for each group.)

IFN-γ expression in directing antimetastatic effect was addressed. ELISA measurement of serum noted 2 peaks IL-12, the first at day 2 and the second on day 8 post-vector injection (Fig. 7A). Likewise, 2 peaks of IFN-γ were detected on day 4 and day 11 post-vector injection (Fig. 7B). Measurements of serum levels of IFN-γ on the eighth day post-vector inject in intact mice versus CD4/CD8 T cell- and NK-depleted mice demonstrated an average 35% reduction in T cell-depleted mice (p = 0.0097, compared to Ad.mIL-12 + control IgG, *t*-test, whereas an average 15% reduction was noted in NK-depleted mice, although this was not statistically significant (p = 0.64, compared to Ad.mIL-12 + control IgG, *t*-test). The potential cytotoxicity of either IL-12 or IFN-γ was tested by adding recombinant cytokines to RM-1 cells *in vitro*. Doses of IL-12 up to 100x greater than that measured *in vivo* (200 ng/ml) had no growth-suppressive effect on RM-1 cells (data not shown). However, exposure to IFN-γ (200 U/ml; 1000x lower than IFN-γ measured in serum of treated mice) resulted in a 40% reduction in the number of RM-1 cells (Fig. 8A). Fas is a transmembrane receptor within the tumor necrosis factor (TNF) superfamily. which initiates apoptosis when activated by its ligand, FasL, with further sensitization following IFN-γ exposure (Weller *et al.*, 1994). Therefore, the role of host production of IFN-γ in response to Ad.mIL-12 and its ability to mediate apoptosis through the Fas/FasL pathway was addressed. *In vitro* a 24-hr exposure to IFN-γ resulted in an increase in cell-surface expression of Fas from 15–20% to 40–55% (Fig. 8B), whereas expression of FasL changed only slightly (10% to 20–25%). Blocking FasL with an antiFasL IgG blunted the IFN-γ dose-dependent killing of RM-1 (Fig. 8A). In addition, co-exposure of RM-1 to IFN-γ in the presence of Ad.sFasIg-conditioned media or z-VAD-FMK negated the inhibitory effects of cytokine treatment (data not shown).

In vivo immunohistochemistry noted a change in the pattern of Fas staining as early as 2 days following Ad.mIL-12 injection compared to control tumors (Fig. 9), whereas staining for FasL was limited to occasional infiltrating lymphocytes in IL-12 treated tumors. Ad.sFasIg was utilized to block Fas/FasL interactions *in vivo*. Use of Ad.sFasIg in animals treated with Ad.mIL-12 resulted in a limited negative impact on growth suppression within the primary tumor with lesions being ~10% larger than Ad.mIL-12 alone as compared to control (p = 0.046, *t*-test) (Fig. 8C). However, Ad.mIL-12-directed systemic growth suppression was completely negated with the addition of Ad.sFasIg, indicating the role of Fas/FasL interactions underlying the inhibition of lung metastasis development.

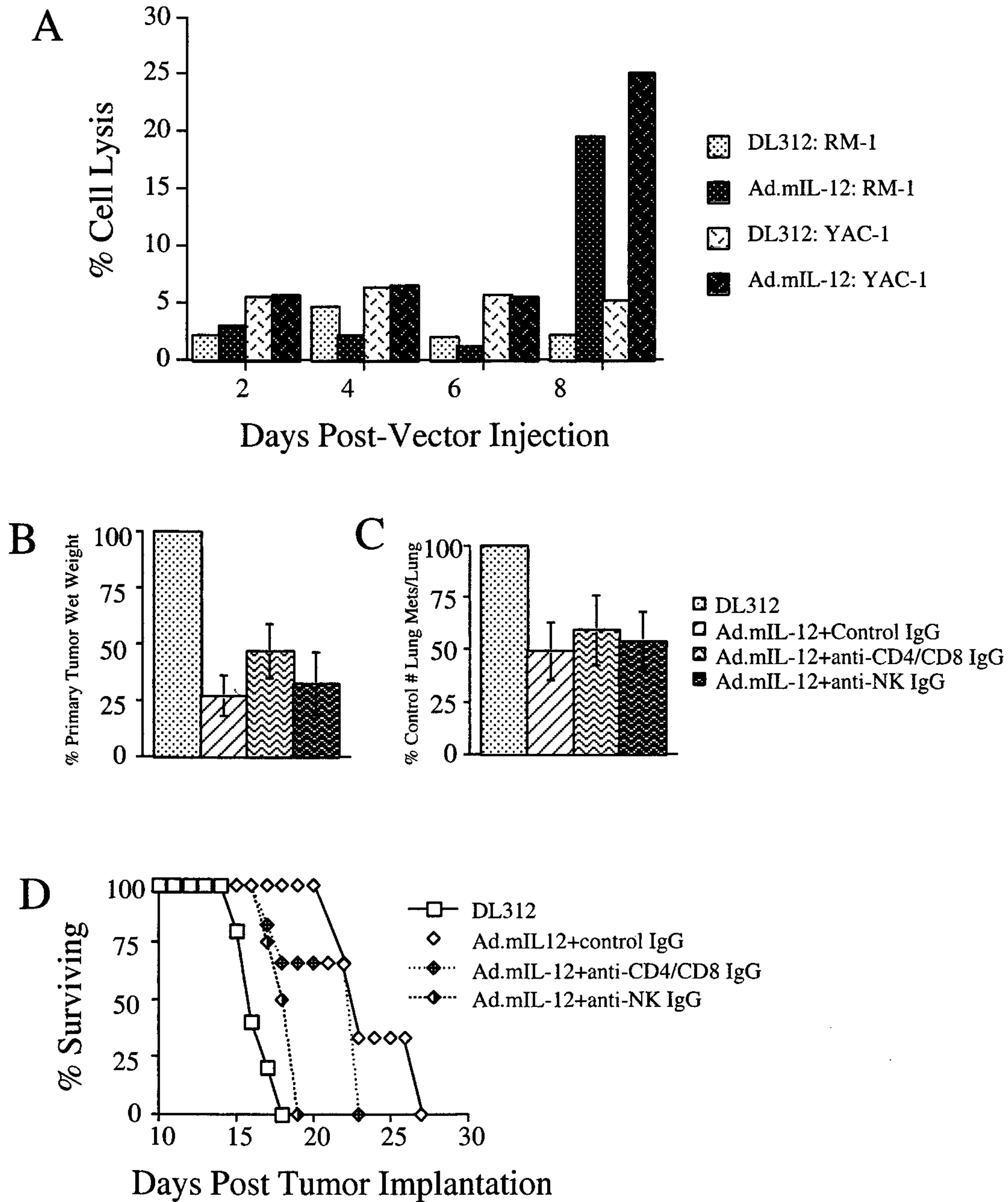

FIG. 6. Immune response post-Ad.mIL-12 therapy. (**A**) Time course induction of TIL in a ^{51}Cr release assay. Fresh TIL were harvested days 2, 4, 6, and 8 from control and Ad.mIL-12-treated primary tumors and exposed *in vitro* to ^{51}Cr-loaded RM-1 and Yac-1 cells. Each bar represents the E:T ratio of 100:1 and is the average of triplicate wells. (**B** and **C**) Effect of T- and NK-cell depletion on Ad.mIL-12-mediated growth suppression. Mice in the synchronous metastasis model were randomized to receive i.p. injections beginning the day before vector injection and continuing as outlined in Materials and Methods for 8 days post injection. All mice were sacrificed at day 8 post-vector injection for analysis of primary tumor wet weight (**B**) and the average number of lung metastases per lung (**C**) ±SD on each case. (n = 8 for each group.) (**D**) Effect of lymphocyte depletion of survival. Detrimental effects of lymphocyte depletion on longer-term growth suppression was determined in mice with primary tumors only randomized to receive the appropriate antibodies as in **A** and **B**, but continued for the duration of a survival experiment. (n = 5 in each group.)

DISCUSSION

Use of Ad.mIL-12 gene therapy in the RM-1 tumor model results in growth suppression both within the transduced tumor and in pre-established tumor lesions remote from IL-12-expressing tissues in a dose-dependent manner, which translates into enhanced survival. Analysis of serum noted 2 peaks of both IL-12 and IFN-γ. Analysis of the underlying basis for these activities in the primary notes two phases of growth suppression as manifest by apoptosis within the first 2 days post-IL-12 ther-

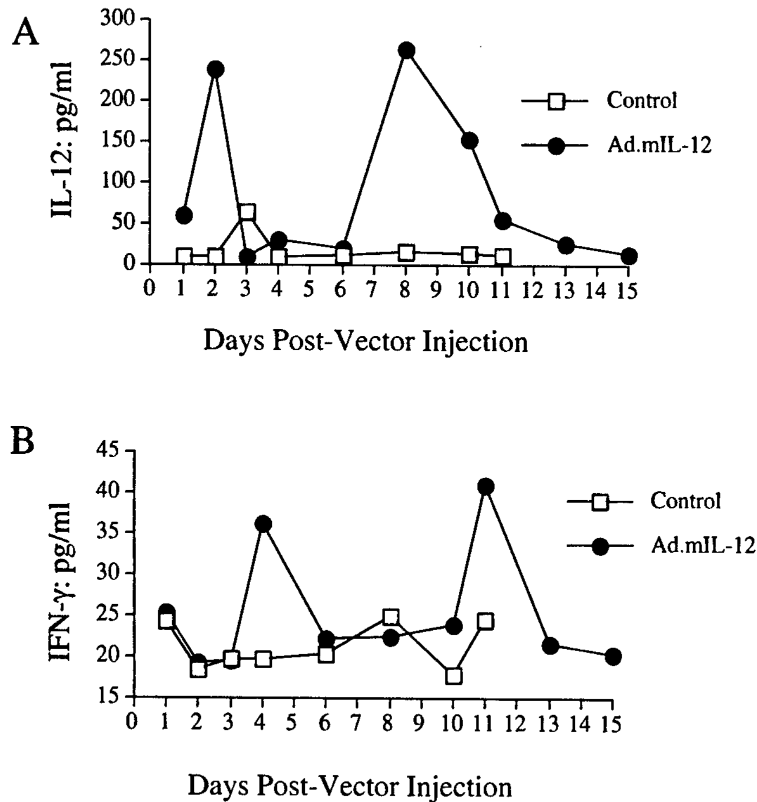

FIG. 7. ELISA serum measurements of IL-12 and IFN-γ. Serum from tumor-bearing mice treated with Ad.mIL-12 or control vector was assayed for IL-12 (**A**) and IFN-γ (**B**) by ELISA. Each data point represents the average of pooled blood samples for each group on each day.

apy followed by an induction of an immune response after 8 days post-vector injection. Apoptosis has been noted in two prior reports as a potential mediator of growth suppression following local IL-12 gene expression (Dias *et al.*, 1998; Wang *et al.*, 1999), in one case as early as 24 hr (Dias *et al.*, 1998). However, the mechanism underlying this activity was not explored. In the present study serial assay of primary tumors noted the presence of apoptosis within 48 hr of vector injection which had dissipated by the sixth day in two distinct patterns. The dominant pattern demonstrated localized but high levels of apoptosis in close proximity to an associated inflammatory infiltrate containing a high percentage of PMNs and eosinophils. The second pattern of apoptotic activity was diffuse and responsible for a more modest increase in cell death. Inhibition of the lung lesions was associated with the diffuse pattern of apoptosis without an inflammatory infiltrate. Use of the general caspase inhibitor, z-VAD-FMK, demonstrated the functional importance of apoptosis in both local and remote growth suppression induced by Ad.mIL-12 therapy.

Although roles in tumor regression have focused on T cell, NKs, and NKTs as major cellular effectors of IL-12-mediated growth suppression, mostly descriptive roles for PMNs have thus far been determined. Indeed, each of these cell types may be recruited into IL-12-expressing benign or malignant tissues (Allavena *et al.*, 1994; Tsung *et al.*, 1997; Bussolati *et al.*, 1998; Ha *et al.*, 1998; Papp *et al.*, 2000). In IL-12-treated SCID mice depleted of NKs, PMNs and macrophages surrounding regressing tumor nodules were thought to mediate growth suppression (Siders *et al.*, 1998). More direct evidence has noted that depletion of PMNs prior to IL-12 therapy has a negative impact on efficacy, but the mechanism through which these activities were mediated was not pursued further (Cavallo *et al.*, 1999; Mendiratta *et al.*, 1999). In the present study, PMNs and, to a lesser extent, eosinophils were localized to sites of profoundly high levels of apoptosis. Depletion of GR-1$^+$ cells resulted in a loss of apoptotic activity which correlated to a significant negative impact of local growth suppression to a point where on average IL-12-treated tumors were not statistically different in size than control tumors. Therefore, one would postulate that the PMNs are recruited to the area by locally high levels of IL-12 leading to concentrated but very high levels of apoptosis. Because PMNs were present in tumors treated with z-FMK-VAD, with some higher density in some breakthrough areas of apoptosis, it would appear that the PMNs are not present as a result of apoptosis but are directing it. The ability of PMNs to destroy tumor tissue such as following FasL transduction has been documented in the literature, but it is unclear through what mechanism this occurs and whether this is tumor specific or involves random tissue destruction in an area of stimulation (Arai *et al.*, 1997; Seino *et al.*, 1997). Certainly, the present study demonstrates that PMN-mediated killing may be directed through induction of apoptosis, which is a rapid and powerful response; however, it is localized, as evidenced by the lack of impact on lung metastases. However, rapid and intense apoptosis leading to areas of necrosis may set the stage to provide tumor antigen necessary to induce an immune response.

The role of eosinophils in the observed tumor killing is more uncertain. Eosinophils have been noted to mediate antitumor activity following IL-4 therapy (Tepper *et al.*, 1992), although others have reported that PMNs are actually responsible for this

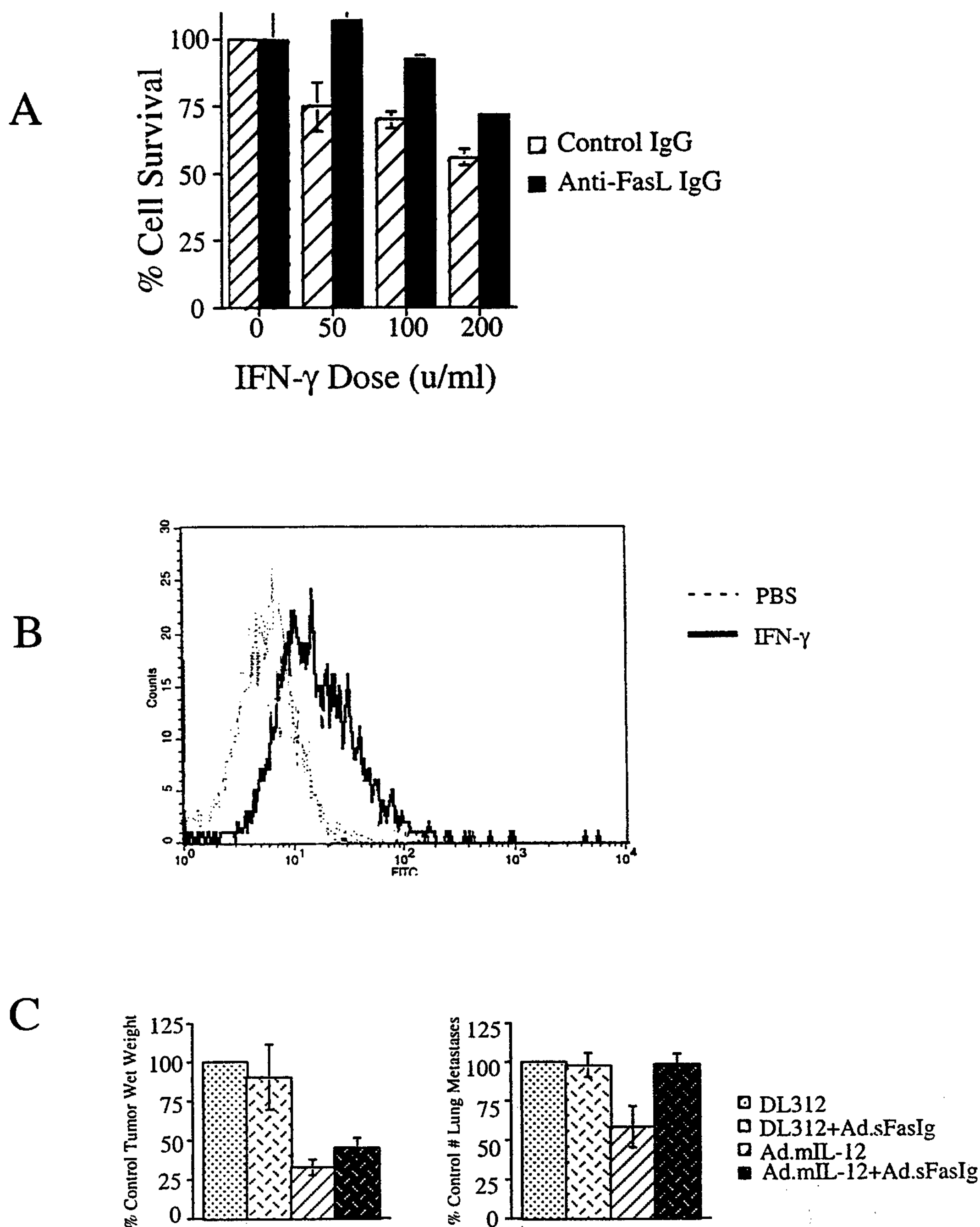

FIG. 8. Fas/FasL Interactions. (**A**) *In vitro* IFN-γ toxicity. Plated RM-1 cells were exposed to escalating doses of IFN-γ with or without anti-FasL antibody. Twenty-four hours later, viable cells as assessed by trypan blue exclusion were counted. Each bar represents the average of triplicate wells ± SD. (**B**) FACS analysis of Fas expression following IFN-γ exposure. Plated RM-1 cells were exposed to IFN-γ or PBS. Twenty-four hours later, cells were prepared for staining with anti-Fas or control antibodies and FACS analysis. (**C**) *In vivo* effects of blocking Fas/FasL. Tumor-bearing mice were randomized to receive tail vein injections of control vector or Ad.sFasIg 2 days prior to tumor vector injection at which time primary tumors were injected with control vector or Ad.mIL-12 ± Ad.sFasIg or DL312. All mice were sacrificed 8 days post-vector injection with assay of efficacy determined through average primary tumor wet weight and average number of lung metastases per lung ± SD. ($n = 7$ for each group.)

growth suppression (Noffz *et al.*, 1998). But studies focusing on asthma and responses to parasitic infection have noted the ability of IL-12 to inhibit esoinophilia (Gavett *et al.*, 1995; Mehlotra *et al.*, 1998; Schwarze *et al.*, 1998). Therefore, it is unclear if eosinophils are present but are functionally inactive, or in the case of malignancy IL-12 acts differently on eosinophils to mediate growth suppression. Further studies will be necessary to understand reasons for eosinophil recruitment and address a potential role of eosinophils in IL-12 therapy.

It is clear that the induction of apoptosis is an important as-

FIG. 9. Fas immunohistochemistry. Frozen sections of tumor tissue harvested day 2 post-vector injection and stained for either Fas. Distinct staining pattern for Fas is noted in Ad.mIL-12 treated tissue (**A**) compared to control (**B**), as evidenced by more distinctive "haloing" of cellular membrane. (Original magnification, 400×.)

pect of Ad.mIL-12 therapy; however, the presence of prolonged survival enhancement in this aggressive model of prostate cancer appeared not to be solely related to this mode of killing. NK activity was noted within TIL by 8 days post-Ad.IL-12 therapy, depletion of which within the same time period had little effect on either local or systemic growth suppression, but in the long term exhibited marked importance in mediating enhanced survival through growth suppression of the primary tumor. This would indicate that the NK response is most active in the time period following the eighth day post-vector injection. T cells too were important in the activities of Ad.IL-12 therapy, but not to the degree that NKs were. Although no CTL activity could be detected within splenocytes, depletion of T cells resulted in a significant but modest negative impact on both primary tumor wet weight within the 8- day period post-vector injection and survival. This finding may reflect direct T cell killing of tumor cells, which may be more efficient due to upregulation of Fas by IFN-γ on target tumor cells. Furthermore, the second peak of IL-12 and IFN-γ would indicate the induction of an immune response. However, the significant reduction in IFN-γ levels within the second peak in the absence of T cells may be negatively influencing secondary consequences of IFN-γ, such as reductions in NK activity or in FasL/Fas interactions. Indeed, both local and systemic effects of Ad.mIL-12 in this model were dose dependent, with significant differences documented from doses differing by 50%. Therefore, a 35% reduction in IFN-γ in mice depleted of T cells itself may be responsible for reductions in efficacy in this scenario. Interpretations of the functional significance of changes in outcome of IL-12 therapy performed in antibody-depleted or nude mice may not denote only direct responsibility but may also be related to the secondary functions of IFN-γ.

Both GR-1-mediated apoptosis and the NK/T cell immune response played a critical role in controlling local tumor growth, but neither had any role in the inhibition of lung metastases. Use of z-VAD-FMK had implicated apoptosis as important in mediating the anti-metastatic activity with the TUNEL assay documenting a diffuse pattern of apoptosis approximately two times above background in the lung lesions, similar to that noted in the primary tumor interspersed between necrotic areas. The exploration of the Fas/FasL pathway as the basis for inducing apoptosis centered on several lines of evidence. RM-1 cells constituitively express low levels of both Fas and FasL; *in vitro* exposure to IFN-γ results in increased surface expression of both surface markers, Fas > FasL. Indeed, *in vitro* growth suppression mediated by IFN-γ at doses 1000x lower than that measured in serum of treated mice could be blunted by blockage of FasL, indicating the ability of IFN-γ to mediate tumor cell fratricide. The use of z-VAD-FMK indicated that apoptosis was the method of death. *In vivo* interruption of this pathway with Ad.sFas-Ig had a limited influence on growth suppression within the primary tumor but resulted in a near loss of systemic antitumor activity against the established lung lesions. The difference in the degree of inhibition between the two sites is most likely related to the lack of FasL in the prostate and the presence of FasL in lung tissue. Indeed, immunohistochemistry failed to demonstrate the presence of intrinsic FasL within the prostate, confirming published observations (Sasaki *et al.*, 1998). Conversely, intrinsic FasL$^+$ lung cells which will transactivate Fas$^+$ tumor cells has been reported as a mechanism for decreased melanoma metastases for Fas$^+$ tumor cells versus Fas$^-$ tumor cells (Owen-Schaub *et al.*, 1998) to add additional negative pressure on Fas$^+$ tumor cells in the metastatic lesions. In the present, study baseline apoptosis in control lung lesions was double that of control orthotopic prostate tumors, perhaps indicative of this phenomenon against even baseline Fas expression of RM-1 cells. A potential further source of FasL would be host T cells and NKs; yet depletion of these subsets had no detrimental effect on antimetastatic activity. A secondary point lies in the obvious differential in tumor sizes at the time of therapy (macroscopic versus microscopic), which may be further supported through the loss of systemic activity following Ad.mIL-12 therapy, with higher metastatic tumor burdens achieved by injecting more RM-1 cells via the tail vein to overwhelm this process (unpublished observation).

In conclusion in this synchronous metastatic model of

prostate cancer growth control of the injected primary tumor is mediated primarily by GR-1$^+$ lymphocyte-directed apoptosis and an NK response with lesser roles for T cells and Fas/FasL interactions, whereas anti-metastatic activity is mediated by apoptosis secondary to Fas/FasL interactions.

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APPENDIX 2

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RESEARCH ARTICLE

A novel bystander effect involving tumor cell-derived Fas and FasL interactions following Ad.HSV-tk and Ad.mIL-12 gene therapies in experimental prostate cancer

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To enhance the NK population induced by Herpes Simplex virus thymidine kinase (HSV-tk) gene transduction and ganciclovir (GCV) treatment, adenovirus-mediated (Ad) expression of IL-12 was added to Ad.HSV-tk + GCV as combination gene therapy. This approach resulted in improved local and systemic growth suppression in a metastatic model of mouse prostate cancer (RM-1). In vitro assay of tumor infiltrating lymphocytes noted superior lysis of both RM-1 and Yac-1 targets with combination therapy, but in vivo depletion of NK cells only negatively impacted on systemic growth inhibition. TUNEL assay of primary tumors noted induction of apoptosis between two and four times higher than controls lasting for 6–8 days post-vector injection. After demonstrating that Ad.HSV-tk/GCV and Ad.mIL-12-induced IFN-γ independently up-regulated expression of FasL and Fas, respectively, studies examined tumor cell-mediated death through Fas/FasL-induced apoptosis as a mechanism of primary tumor growth suppression. In vitro, combination therapy at low vector doses resulted in synergistic growth suppression, which could be negated by the addition of anti-FasL antibody. In vivo co-inoculation of an adenovirus expressing soluble Fas resulted in combination therapy-treated tumors, which were three times larger than expected, and a reduction in apoptosis to baseline levels. In FasL knockout mice, combination therapy maintained the superior results experienced in wild-type mice, indicating that tumor cell, not host cell FasL, was responsible for Fas transactivation. Therefore, the combination of Ad.HSV-tk/GCV + Ad.mIL-12 results in enhanced local growth control via apoptosis due to tumor cell expression of Fas and FasL and improved anti-metastatic activity secondary to a strong NK response.
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Keywords: *prostate cancer; gene therapy; Fas; FasL; HSV-tk; IL-12*

Introduction

Pro-drug activation gene therapy using Herpes Simplex virus thymidine kinase (HSV-tk) gene transduction in combination with ganciclovir (GCV) is under investigation as a potential cancer therapy. Intrinsic to its ability to control growth in a variety of tumor models has been the observation that anti-tumor immunity is induced, as manifest by inhibition of challenge injections or synchronously growing tumors.[1–5] While the steps involved in the initiation of an immune response following HSV-tk/GCV remain unclear, efforts to enhance this response have involved the empiric addition of cytokines, such as IL-2, to stimulate cytotoxic T cells and GM-CSF to enhance antigen presentation. In some tumor models such combinations have yielded superior results,[6–9] though in some models no improvement over HSV-tk/GCV was noted.[10] In an orthotopic mouse model of prostate cancer adenovirus-mediated (Ad.) HSV-tk/GCV therapy resulted in the induction of natural killer cells (NK) within tumor infiltrating lymphocytes.[11] Performance of gene therapy in mice lacking NKs modestly reduced the impact of Ad.HSV-tk/GCV within the injected primary tumor and completely abrogated systemic growth suppression of pre-established metastatic deposits.[11]

The strategy of combining HSV-tk with IL-12 gene therapy was conceived to exploit the ability of IL-12 to stimulate NK proliferation and cytotoxicity,[12] to thereby further the innate immune response induced by Ad.HSV-tk + GCV. Parallel studies had demonstrated the individual growth suppressive ability of adenovirus-mediated IL-12 gene therapy (Ad.IL-12) to result in growth inhibition of both the injected tumor and pre-established metastases in a dose-dependent manner.[13] High-dose IL-12 therapy mediated growth suppression within the primary tumor through induction of innate (neutrophils and NKs) and acquired immunity (T cells), while anti-metastatic activity was mediated through up-regulation of Fas thereby enhancing sensitivity to FasL transactivation by lung epithelial cells.[13] The combination of Ad.HSV-tk and

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Ad.IL-12 illustrated the superior benefits of combination therapy to result in primary tumors which were 85–90% smaller than control tumors and a 75% reduction in the number of pre-established lung metastases.[14] Since prior work had noted the ability of both therapies to independently direct systemic growth suppression,[11,13] the strength of systemic anti-tumor activity mediated by combination therapy was tested by increasing the metastatic tumor burden to a point where neither Ad.HSV-tk/GCV nor Ad.IL-12 had a significant effect. In this setting, combination gene therapy continued to suppress the number of lung metastasis by 50%.[14]

In the present report, the underlying mechanisms of combination therapy are elucidated. Ad.HSV-tk/GCV + Ad.IL-12 enhanced NK activity, but performance of therapy in the absence of NK cells negatively impacted only on systemic growth suppression. TUNEL analysis noted the induction and maintenance of apoptosis by combination therapy. The resulting studies noted the independent ability of HSV-tk/GCV and IL-12-induced IFN-γ to up-regulate tumor cell expression of FasL and Fas, respectively. Blockage of Fas/FasL interactions both *in vitro* and *in vivo* significantly blunted the effects of combination therapy. Performance of combination therapy in FasL knockout mice maintained the superior results of combination therapy to that experienced in wild-type mice, indicating that tumor cell, not host cell, FasL was responsible for Fas transactivation.

Results

Assay of TIL harvested post-vector injection noted lytic activity in the Ad.HSV-tk/GCV, Ad.mIL-12 and Ad.HSV-tk/GCV + Ad.mIL-12 groups (Figure 1a and b). Induction of NK activity was noted initially in both groups treated with Ad.HSV-tk/GCV, peaking on day 4 post-vector injection and demonstrating superior activity for combination therapy ($P = 0.006$, t test, Ad.HSV-tk + GCV *versus* Ad.HSV-tk + GCV + Ad.mIL-12). As noted by past-experience,[13] Ad.mIL-12 therapy induced NK activity by day 8. TIL from both Ad.HSV-tk/GCV and combination gene therapy lysed RM-1s in a similar pattern, peaking on day 6 post-vector injection which again illustrated the superiority of combination gene therapy ($P = 0.0054$, t test, Ad.HSV-tk + GCV *versus* Ad.HSV-tk + GCV + Ad.mIL-12). The functional significance of this presumed NK response was explored by performing combination gene therapy in mice depleted of CD4 and CD8 T cells or NKs in the pre-established metastasis model. Assay over the first 8 days post-vector injection failed to demonstrate a negative impact with regard to primary tumor growth suppression following removal of T cells and NKs. In each instance tumors remained over five times smaller than control tumors ($P < 0.0001$ for all conditions of combination therapy *versus* control, t test) (Figure 1c). However, suppression of pre-established metastases was completely lost in mice depleted of NK cells ($P = 0.85$ *versus* control, t test), while the 50% reduction seen with combination treatment continued in mice depleted of T cells ($P = 0.0001$ *versus* control, t test) (Figure 1d).

Serial assay for apoptosis noted enhanced activity in tumors treated with combination gene therapy, lasting for 8 days post-vector injection (Figure 2). By the 2nd day post-vector, all three treatment groups resulted in increased apoptosis manifest by an eight times higher level for combination therapy compared with control, though appearing to be additive of the independent effects of Ad.mIL-12 and Ad.HSV-tk/GCV ($P = 0.005$, combination therapy *versus* control; $P = 0.04$, combination therapy *versus* Ad.mIL-12; $P = 0.13$, combination therapy *versus* Ad.HSV-tk/GCV, t test). In succeeding days apoptosis relating to Ad.mIL-12 resolved to control levels, while Ad.HSV-tk/GCV remained at approximately twice control and combination at four times control, ($P < 0.05$ combination therapy *versus* control for days 4–8; $P = 0.005$, combination therapy *versus* Ad.HSV-tk/GCV for days 4 and 6, t test).

Studies with Ad.mIL-12 therapy alone in the RM-1 model noted measurable serum levels of IFN-γ, which increased tumor cell expression of Fas both *in vitro* and *in vivo* (Figure 3).[13,15] In contrast IFN-γ had little effect on FasL expression (Figure 3). Likewise, escalating doses of IL-12, 100× higher than that measured in the serum of Ad.mIL-12-treated animals,[13] had no effect on Fas/FasL expression (Figure 3). Since HSV-tk/GCV has been reported to influence Fas and FasL expression in other tumor models,[16,17] the influence of Ad.HSV-tk/GCV was ascertained in RM-1 cells. FACS analysis of Ad.HSV-tk infected cells 24 h after exposure to GCV noted marked expression of FasL to approximately 50%. This correlated with the transduction efficiency of 40–50% at the vector dose used.[18] Ad.HSV-tk/GCV had little effect on Fas expression (Figure 3). Exposure to vector or GCV alone likewise had minimal influence on Fas/FasL expression (data not shown). By 48 h after GCV therapy, the number of surviving cells was reduced to 10–15%, with Fas/FasL resembling that of control (data not shown). A hypothesis was thus generated that the superior tumor control was due to the ability of IFN-γ, produced in response to Ad.mIL-12, to enhance Fas expression and the ability of Ad.HSV-tk/GCV to enhance FasL, resulting in Fas transactivation and apoptosis within the primary tumor.

In vitro cell kill from Ad.HSV-tk/GCV at MOI of 25 is approximately 80% (Figure 4a). The addition of 25 U/ml IFN-γ, a dose without growth suppressive activity itself, added no additional benefit at this vector dose. However, at progressively lower MOI the killing ability of Ad.HSV-tk/GCV lessened in a dose-dependent manner, but the addition of IFN-γ maintained maximum cell kill to the MOI of 6.25 ($P = 0.026$, Ad.HSV-tk + GCV + IFN-γ *versus* AdHSV-tk + GCV, Mann–Whitney). The addition of anti-FasL IgG at the time of GCV exposure blocked this effect such that the level of cell survival was identical to that of Ad.HSV-tk/GCV alone at all MOI tested (Ad.HSV-tk/GCV + IFN-γ+ anti-FasL IgG *versus* Ad.HSV-tk/GCV $P = 0.63$, Mann–Whitney; Ad.HSV-tk/GCV + IFN-γ+ anti-FasL IgG *versus* Ad.HSV-tk/GCV + IFN-γ, $P = 0.0015$, Mann–Whitney) (Figure 4b).

In vivo blockage of Fas/FasL interactions was achieved by co-inoculation with Ad.sFasIg at the time of Ad.HSV-tk + AdmIL-12 injection. Use of this vector significantly reduced the growth suppression of combination therapy such that tumors were three times larger than expected ($P < 0.0001$, combination therapy *versus* combination therapy + Ad.sFasIg) (Figure 4c). In addition, blockage of Fas/FasL interactions was associated with a loss of apoptosis generated by combination therapy. By day 8 post-vector apoptosis in combination therapy treated tumors was lowered to that of controls: 1.73 ± 0.22 apop-

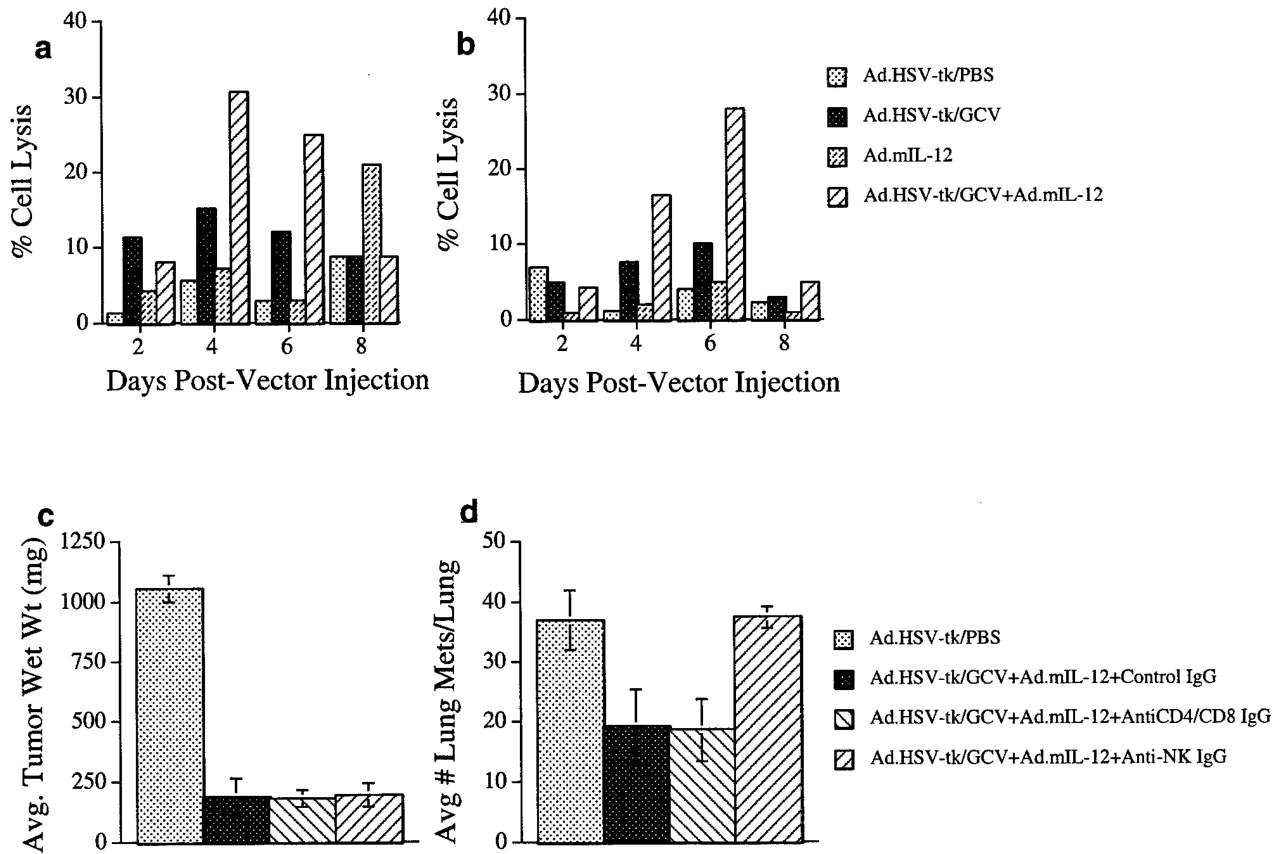

Figure 1 *NK induction following Ad.HSV-tk/GCV + Ad.mIL-12 gene therapy. (a and b) Fresh TIL were harvested 2, 4, 6 and 8 days after vector injection from Ad.HSV-tk/PBS, Ad.HSV-tk/GCV, Ad.mIL-12 and Ad.HSV-tk/GCV + Ad.mIL-12 treated tumors and exposed to 51Cr-labeled targets, YAC-1 (a) and RM-1 (b) cells. Each time point represents the effector:target ratio of 100:1 and the average of triplicate wells ± s.d. (c and d) Tumor-bearing mice were randomized to receive i.p. injections of anti-NK IgG, anti-CD4 IgG + antiCD8 IgG or control IgG 1 day prior to vector injection and continued as outlined in the text to deplete each population. Eight days after vector injection mice were killed and the tumor wet weight recorded (c) and the lung metastases counted. (d) Each bar represents the average of recorded data ± s.d.*

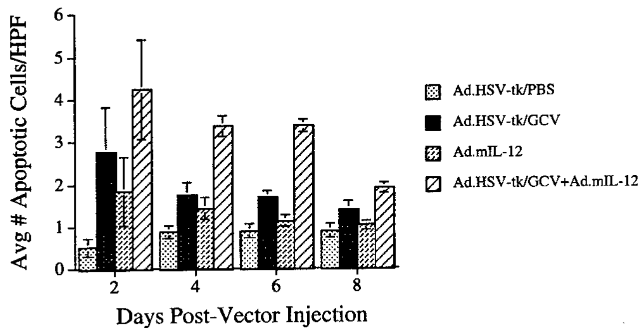

Figure 2 *Apoptosis following Ad.HSV-tk/GCV + Ad.mIL-12 gene therapy. Primary tumors from each treatment group were prepared for TUNEL assay on days 2, 4, 6 and 8 after vector injection. For each treatment condition, four tumors were examined and the number of apoptotic cells per HPF (×400) was counted across the long and short axis of each tumor. The number of apoptotic cells per HPF for each tumor was then averaged for each condition and time point and graphed ±s.d. The number of HPFs counted per tumor ranged from 5 at day 2 to 14 at day 8.*

totic bodies/HPF for combination gene therapy, 0.89 ± 0.22 apoptotic bodies per HPF for control, and 0.89 ± 0.17 apoptotic bodies per HPF for combination gene therapy + Ad.sFasIg ($P = 0.005$, t test; combination gene therapy + Ad.sFasIg *versus* control, $P = 0.98$, t test). To verify that the source of FasL was indeed tumor cells and not host sources, these studies were repeated in FasL knockout mice (Figure 4d). The marked growth suppression of Ad.HSV-tk/GCV + Ad.mIL-12 continued to the same degree as in wild-type mice with reversal only in the presence of Ad.sFasIg.

Discussion

A variety of gene therapy strategies for the treatment of cancer have been developed, generally through one of two basic methods: local growth suppression via direct inoculation of tumor suppressor or pro-drug activation genes (ie p53 or HSV-tk) or systemic growth inhibition following local gene expression (ie antiangiogenesis gene therapy or gene-modified immunotherapy). A major limitation of *in situ* approaches is the low efficiency of vector uptake following direct injection, yielding it difficult to establish uniform cure rates. Attempts to bypass this problem have utilized intricate injection schemes, replication-competent vectors, and 'bystander effects', whereby more cells are killed than are transduced. The combination of Ad.HSV-tk/GCV and Ad.mIL-12 was chosen to exploit the ability of IL-12 to enhance proliferation and cytotoxicty of the NK response induced by HSV-tk + GCV in this model to improve both local and systemic

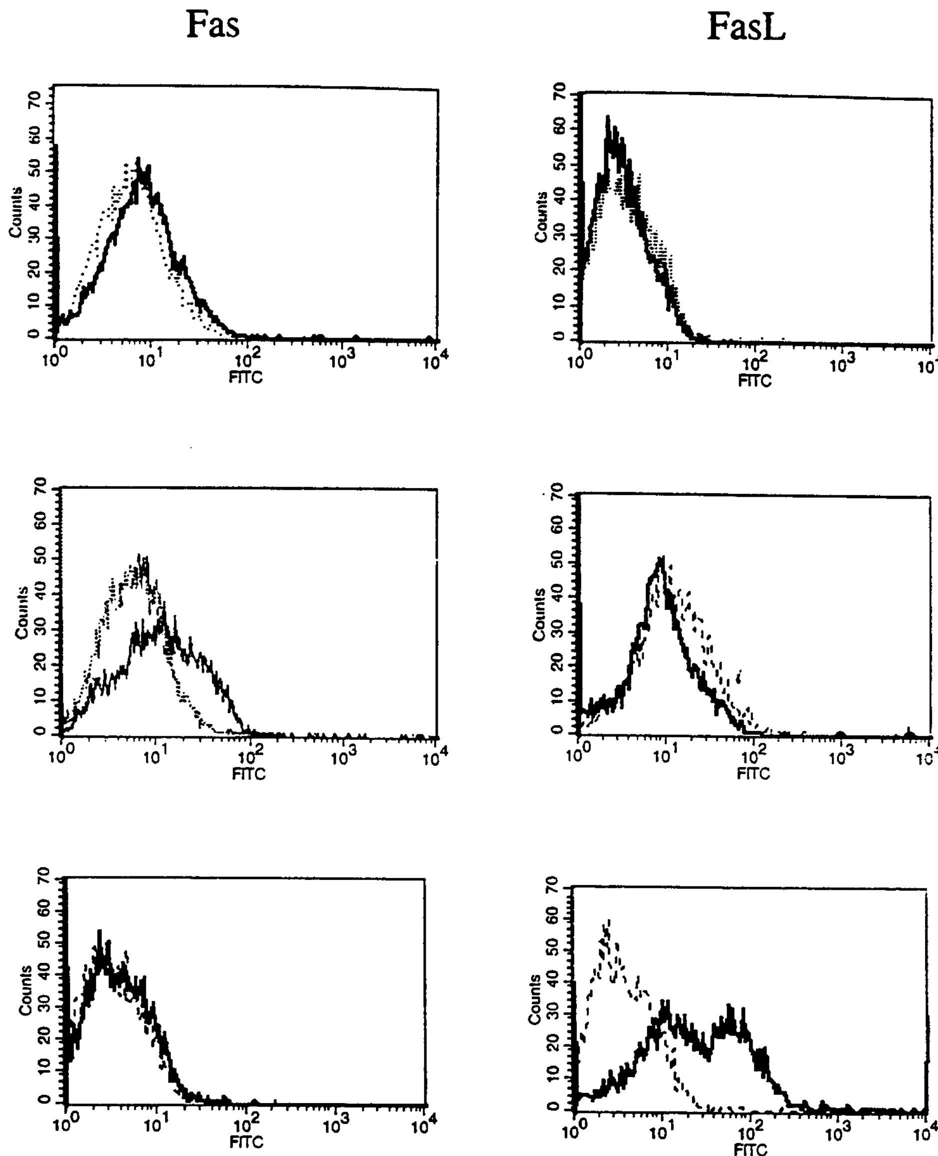

Figure 3 FACS analysis of Fas/FasL expression following (a) IL-12, (b) IFN-γ, and (c) HSV-tk/GCV therapy. Plated RM-1 cells were exposed to 25 U/ml IFN-γ, 200 ng/ml IL-12, 50 MOI of Ad.HSV-tk or PBS. For (a) and (b), cells were prepared for immunostaining 24 h later with anti-Fas, anti-FasL, or control antibodies and FACS analysis. Cells exposed to Ad.HSV-tk were randomized to GCV or PBS treatments the following day and prepared for immunohistochemistry 24 h after GCV/PBS. For each graph the dotted line represents exposure to PBS and solid line the treatment condition.

growth suppression. Studies of TIL from combination therapy-treated mice indicated an enhancement of NK activity from Ad.HSV-tk/GCV alone by adding Ad.mIL-12. The *in vivo* relevance of this population was validated by a near complete loss of growth inhibition of pre-established metastatic lesions in the absence of NKs. Indeed, at the metastatic tumor burden tested the individual therapies had no effect on pre-established metastases,[14] indicating the strong systemic growth suppressive capabilities of this NK population.

While the control of disseminated lesions was dependent on NKs, the enhanced growth control of the injected tumor was not directly related to an immune response. Studies noted the independent ability of IFN-γ and HSV-tk/GCV to influence Fas/FasL. RM-1 cells express both Fas and FasL (Fas approximately 25–30% and FasL approximately 10–15%); IFN-γat doses well below that measured in serum of Ad.mIL-12-treated animals to upregulated Fas *in vitro* consistent with the literature.[13,15] *In vivo* increased Fas expression could be seen in Ad.mIL-12 injected tumors as early as 2 days post-vector, lasting to at least the 8th day (Ref. 13 and unpublished observations). In contrast high doses (up to 200 ng/ml) of recombinant IL-12 resulted in neither cytotoxicity[13] nor changes in Fas/FasL expression. In parallel, it has been shown that HSV-tk + GCV therapy may also influence Fas/FasL expression.[16,17] In a human neuroblastoma cell line HSV-tk/GCV resulted in apoptosis via FasL-independent aggregation of upregulated Fas.[17] In four different cancer cell lines HSV-tk/GCV increased expression of both Fas and FasL which mediated some degree of bystander killing, as documented by a reduction in HSV-tk/GCV effects by blockage of Fas/FasL interactions.[17] In three of four cases, Fas expression was more pronounced

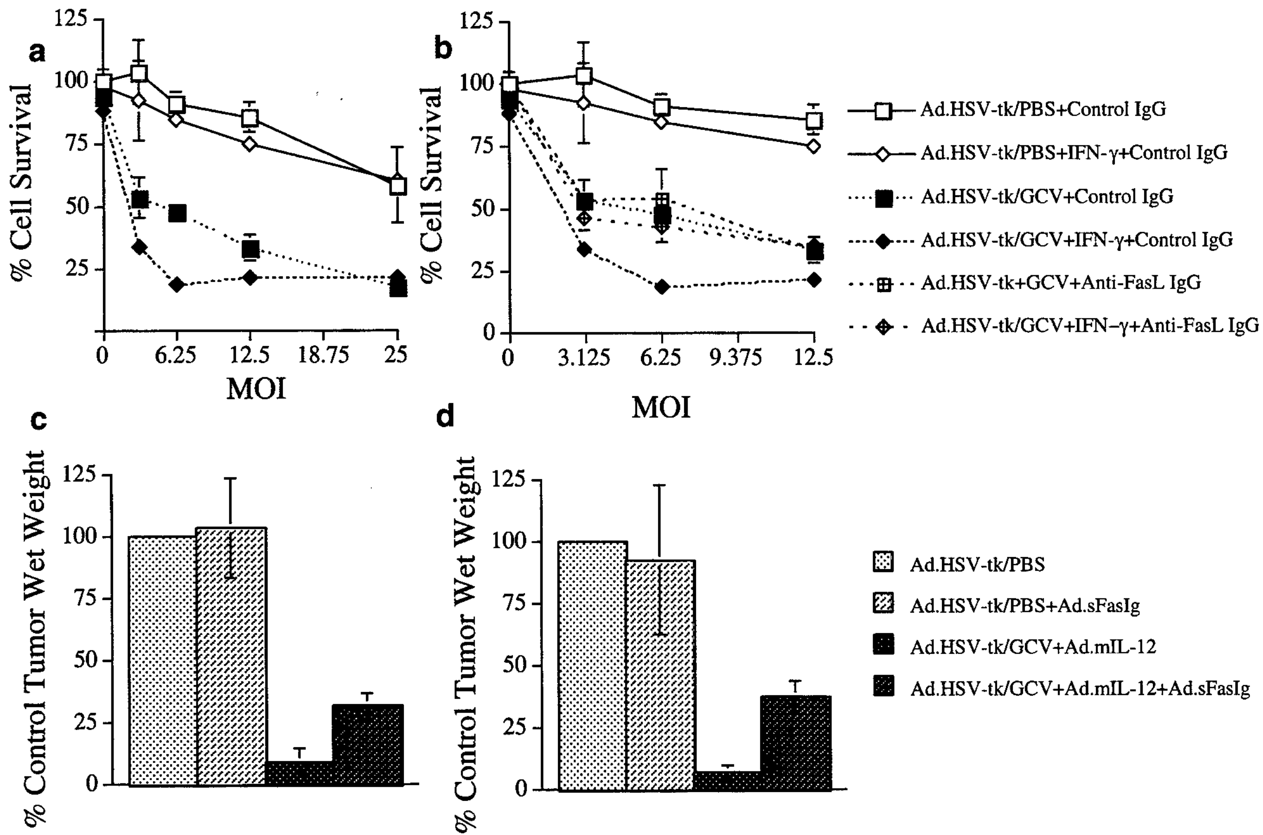

Figure 4 *Fas/FasL interactions following combination therapy. (a) Plated RM-1 cells were exposed to escalating MOI of Ad.HSV-tk for randomization to PBS, PBS + IFN-γ (50 U/ml), GCV or GCV + IFN-γ. Viable cells were counted the following day. Each data point represents the average of triplicate wells ± s.d. (b) The same groups as (a) were included, to which randomization included anti-FasL IgG or control IgG. Viable cells were counted the following day. Each data point represents the average of triplicate wells ± s.d. (c and d) Primary tumors in wild-type (c) or FasL knockout mice (d) were injected with Ad.HSV-tk or Ad.HSV-tk + Ad.mIL-12 divided for co-inoculation with DL312 or Ad.sFasIg. The following day PBS or GCV as indicated was injected as outlined in the text. The wet weight of the primary tumor was recorded 8 days after injection. Each bar represents the average wet weight per group ± s.d.*

than FasL. In RM-1 cells Ad.HSV-tk + GCV more profoundly affected FasL with only a small increase in Fas expression. This effect was most pronounced 24 h after GCV exposure, a time defined by a large population of dying cells. Assay at 48 h noted only 10–15% surviving cells with Fas.FasL expression pattern identical to controls. The addition of anti-FasL antibody did not negatively impact on Ad.HSV-tk/GCV cytotoxicity, indicating, that Fas/FasL interactions do not play a role in a HSV-tk/GCV bystander effect in this cell line.

In vitro the addition of IFN-γ, at a dose which itself does not impact on RM-1 cell growth, to Ad.HSV-tk + GCV, maintained maximum cell kill at a four-fold lower vector dose, indicating the presence of strong cooperative activities. The co-administration of an anti-FasL antibody reverses this phenomenon, clearly defining a role for Fas/FasL activities in this enhanced killing. Likewise, *in vivo* blockage of Fas/FasL results in a significant loss of growth suppression and apoptosis within the primary tumor, demonstrating that a proportion of enhancement due to combination therapy is related to Fas/FasL interactions. The correlation between loss of growth suppression and the decrease in apoptosis noted following use of Ad.sFasIg would suggest that apoptosis through Fas transactivation is the mechanism of cell death. While the hypothesis had placed the source of FasL as tumor cells infected with Ad.HSV-tk and exposed to GCV, FasL could be derived from host sources *in vivo* such as immune cells (NK and/or T cells) or within prostate tissues.[19] Therefore, to further delineate the source of FasL, combination gene therapy was performed in FasL KO mice. As noted in wild-type mice, growth suppression with Ad.HSV-tk/GCV + Ad.mIL-12 resulted in a nearly 90% size differential with loss of activity achieved with the addition of Ad.sFasIg. Therefore, a tumor cell-mediated bystander effect is defined through Ad.HSV-tk/GCV and Ad.mIL-12-IFN-γ independent induction of Fas and FasL expression to result in further killing through Fas transactivation. Both *in vitro* and *in vivo* studies illustrate the power of this phenomenon in combination gene therapy and characterize an additional approach to more efficiently kill cancer cells within the confines of an injected tumor.

Materials and methods

Cell lines

The RM-1 cell line (passage 14–17) is a well-characterized mouse prostate cancer cell line, established from a ras/myc-induced primary tumor derived in the mouse prostate reconstitution (MPR) model system in C57BL/6

mice (obtained from Dr TC Thompson, Baylor College of Medicine, Houston, TX, USA).[20,21] Cells used in the reported studies were grown in DMEM with 10% FBS and penicillin (100 units/ml) and streptomycin (100 mg/ml). YAC-1 is a NK cell-sensitive mouse lymphoma cell line, maintained in RPMI 1640 with 10% FBS. Routine passaging was carried out using cell dissociation media. All reagents pertaining to cell culture were obtained from Sigma (Sigma Chemical, St Louis, MO, USA).

Adenovirus vectors

Replication-defective E1 deleted recombinant adenovirus vectors expressing HSV-tk, mouse IL-12 (mIL-12), and sFasIg, all under control of the Rous sarcoma virus promoter (RSV), were constructed as previously described.[13,22,23] The Ad.sFasIg construct links the extracellular domain of Fas to the Fc region of mouse IgG2a. DL312 is an E1-deleted adenovirus without a transgene and was used as vector control. Virus titer for *in vivo* use was determined by plaque assay in 293 cells and expressed as plaque-forming units (p.f.u.) following expansion and double cesium gradient ultracentrifugation purification.

In vivo gene therapy

Orthotopic primary tumors and synchronous metastases were established as previously described.[11] Briefly, at the same sitting 7500 RM-1 cells were injected into the dorsolateral prostate and 7500 cells were injected via the dorsolateral tail vein. Primary tumors were injected with vector 6 days later, at which time the lungs contain microscopic metastases. The therapeutic dose of Ad.HSV-tk had been previously established at 5×10^8 p.f.u.,[5] while Ad.mIL-12 was 3.3×10^9 p.f.u. Mice were killed 14 days after tumor cell inoculation. The primary tumors were removed, the bladder and seminal vesicles excised, and a wet weight obtained for each tumor. The lungs were likewise removed, placed in Bouin's solution, and changed to 70% ethanol 1 h later. Visible lung metastases were counted with the aid of a dissecting microscope.

In vitro cytotoxic assays

At designated time-points, mice were killed, and the primary prostate tumors removed. Tumor infiltrating lymphocytes were extracted and prepared for chromium (^{51}Cr) release assay, as previously described.[11] Briefly, pooled tumors from each treatment group were mechanically lysed and serially purified by gravity precipitation to remove tumor debris. Following treatment with red cell lysis buffer (Sigma) and passage through cotton wool pipettes, remaining viable lymphocytes were counted and exposed to ^{51}Cr-loaded targets at various E:T ratios. The percentage lysis was calculated as (experimental release − spontaneous)/(maximal release − spontaneous release) from the gamma counter measurements of supernatant harvested by the Skatron system (Skatron Instruments, Sterling, VA, USA).

In vivo depletions

Mice were depleted of specific lymphocyte populations by i.p. injections of antibodies beginning 1 day before Ad./HSV-tk + Ad.mIL-12 injection on a schedule as previously reported:[11] CD4 T cells with purified ascites from hybridoma GK 1.5 (American Type Culture Collection, Rockville, MD, USA), CD8 T cells with purified ascites from hybridoma 2.43 (American Type Culture Collection), NK cells with anti-AsialoGM1 IgG (Wako Chemicals, Reston, VA, USA), and controls with rabbit IgG (DAKO, Carpinteria, CA, USA) and rat IgG (Accurate Chemical and Scientific, Westbury, NY, USA). Control mice were given a total of 20 μg of IgG to equal that given to CD4/CD8 mice. All mice were killed on day 14 with the removal of primary tumor and lungs with appropriate examination carried out as outlined above.

Apoptosis assay

Tumors representing each treatment arm, Ad.HSV-tk/PBS, Ad.HSV-tk/GCV, Ad.mIL-12, and Ad.HSV-tk/GCV + Ad.mIL-12, were harvested every other day beginning on day 2 after vector injection to 8 days after vector injection. In addition, tumors from mice treated with Ad.sFasIg alone or with combination therapy were killed 14 days after tumor inoculation and were similarly prepared. After fixation in formalin, parrafin embedding, and sectioning, tissue samples were prepared for TUNEL assay, according to the maunfacturer's instructions (Trevigne Industries, Gaithersburg, MD, USA). Individual tumor sections were scanned along the long and short axes at high power (×400) with a light microscope. The number of apoptotic bodies was counted and reported as average number of bodies per high power field.

FACS assay of Fas/FasL expression

Plated RM-1 cells were exposed to either mouse IFN-γ (100 U/ml, Pharmingen, San Diego, CA, USA), mouse IL-12 (1-200 ng/ml, Pharmingen) or Ad.HSV-tk at a multiplicity of infection (MOI: number of p.f.u. per cell) of 50. This vector dose correlates with approximately 50% cell transduction.[18] Twenty-four hours following the addition of IFN-γ, cells were removed with cell dissociation medium and prepared for immunohistochemistry. Cells exposed to vector were randomized 24 h later to saline (PBS) or GCV (10 μg/ml, Roche Laboratories, Nutley, NJ, USA). The following day, single cell suspensions were randomized to exposure to anti-FasL IgG, anti-Fas IgG, or iso-control IgG (Pharmingen) for 45 min on ice. Following washing in PBS, cells were exposed to PE-labeled anti-hamster IgG (Pharmingen) for 30 min followed by FACS analysis.

In vitro gene therapy

Plated RM-1 cells were exposed to escalating MOI of Ad.HSV-tk for 1 h. Twenty-four hours later, wells were randomized to receive PBS, GCV (10 μg/ml), mouse IFN-γ (25 U/ml) or GCV + IFN-γ. Disassociated viable cells as per trypan blue exclusion assay were counted the following day. Blockage of FasL/Fas interactions was achieved by adding anti-mouse FasL IgG or isotype control IgG (Pharmingen) to designated wells at the same time as the addition of PBS/GCV/IFN-γ.

Blockage of Fas/FasL interactions in vivo

Interference of Fas/FasL transactivation *in vivo* was achieved by co-inoculation with Ad.sFasIg, as previously described.[13] In the synchronous metastasis model on day 6, primary tumors were injected with either 1×10^9 p.f.u. DL312 or Ad.sFasIg, followed by randomization for treatment with combination gene therapy or control vector. Mice were killed on the 8th day after vector injection and the effects of gene therapy measured in both the primary

tumor and lungs as described earlier. Further evaluation of Fas/FasL activities were carried out by repeating the same experiments in FasL knockout mice (Jackson Laboratory, Bar Harbor, ME, USA).

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IFN-γ Sensitization of Prostate Cancer Cells to Fas-Mediated Death: A Gene Therapy Approach

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While human prostate cancers and cell lines express Fas, most of these cell lines are resistant to Fas-mediated death. In the present studies we addressed the ability of IFN-γ to influence Fas-mediated cell death in prostate cancer cells. *In vitro* exposure of the human cell lines LNCaP and PC3 and the mouse cell line RM-1 to agonist anti-Fas antibody and/or soluble Fas ligand resulted in killing of only PC3 cells. However, preincubation with IFN-γ resulted in synergistic killing in all three cell lines. *In vitro* treatment of RM-1 with a replication-incompetent adenovirus expressing mouse FasL (Ad.FasL) resulted in maximal cell kill near 40%, which correlated with baseline Fas expression. The addition of IFN-γ enhanced cell kill to a degree consistent with the resulting higher levels of Fas and maintained synergistic killing at very low doses of vector. Co-inoculation of orthotopic RM-1 primary tumors with Ad.mFasL and an adenovirus expressing mouse IL-12 (Ad.mIL-12) to drive host production of IFN-γ negated the survival advantage of Ad.mIL-12 alone. However, the staggered injection of Ad.mIL-12 and Ad.FasL achieved almost threefold higher levels of apoptosis in primary tumor tissue and doubled median survival. Therefore, IFN-γ is capable of bestowing increased sensitivity to Fas-mediated cell death in prostate cancer cells and, in a gene therapy approach, may define a powerful tool to treat prostate cancers.

Key Words: prostate cancer, gene therapy, Fas, FasL, IFN-γ, IL-12

INTRODUCTION

Gene therapy offers the ability to kill tumor cells through a variety of distinct strategies including tumor suppressor gene therapy, prodrug activation gene therapy, and immunomodulatory gene therapy [1]. The success of *in situ* gene therapy relies on the transduction of sufficient tumor cells with transgene to achieve tumor control; this has been a particularly difficult practical hurdle to overcome *in vivo*. In many instances successful approaches have exploited a bystander effect—the ability to kill more cells than were transduced with transgene. In general these strategies achieve this phenomenon by increasing exposure of surrounding, nontransduced tumor cells to cytotoxic by-products, either through a cell-mediated process such as with HSV-tk plus ganciclovir or through local diffusion such as with cytosine deaminase plus 5-fluorocytosine [1]. Targeting specific markers unique to cancer cells would attain more specific and efficient killing. Indeed, immunotherapy employs inherent characteristics of tumor-specific antigens to mark cancer cells for death. In a similar vein the redirection of vectors to cancer cell-specific membrane-bound molecules would allow for restrictive transduction. Yet to date, few such candidate targets have been identified.

Fas is a potential target expressed on the cell surface of many tumor cells. The binding of its ligand, FasL, initiates an intracellular cascade resulting in apoptosis [2]. It is unknown why many tumors, including prostate, express Fas, but its use as a therapeutic target has generated interest. The idea of exploiting the Fas/FasL pathway as a vehicle for killing prostate cancer cells arose from studies exploring the mechanisms of local and systemic growth suppression observed following injection of an adenovirus expressing IL-12 (Ad.mIL-12) in an orthotopic mouse model of prostate cancer [3]. Studies linked the ability of IL-12 to direct host production of measurable serum levels of IFN-γ, which in turn significantly increased tumor cell expression of Fas but not FasL. *In vitro* IFN-γ resulted in a dose-dependent cell death due to the interaction of low constitutive FasL expression and high Fas expression [3]. *In vivo* blockade of Fas/FasL interactions resulted in a modest reduction in primary tumor killing, a site of in-

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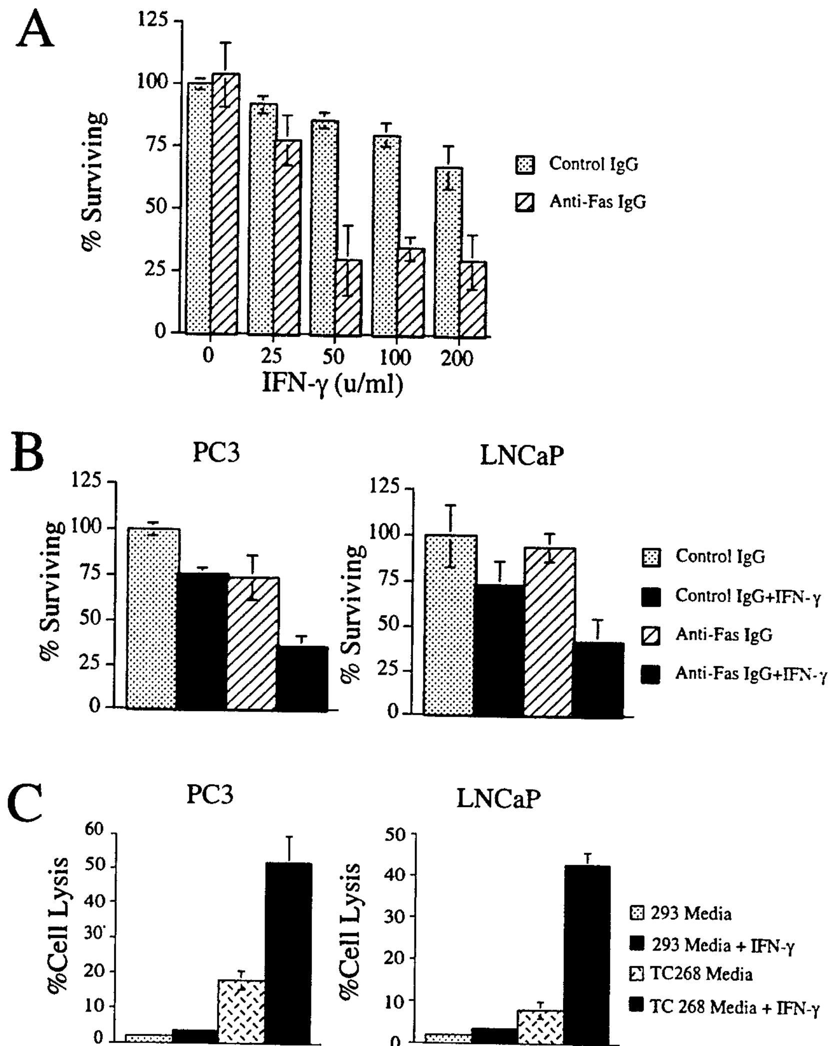

FIG. 1. Fas transactivation of prostate cancer cells *in vitro*. (A and B) Plated RM-1, PC3, and LNCaP cells were exposed to either escalating doses of mouse IFN-γ (A; RM-1) or a fixed dose of 100 u/ml human IFN-γ (B; PC3 and LNCaP) or to PBS. Twenty-four hours later each treatment condition was randomized to receive either anti-Fas IgG or isotype control IgG (10 μg/well). One day later viable cells as determined by trypan blue exclusion were counted. Each bar represents the average of triplicate wells ± standard deviation (SD). For RM-1 cells at 50 u/ml IFN-γ control IgG versus anti-Fas IgG, $P = 0.0016$ (*t* test). For PC3 cells control IgG + IFN-γ or anti-Fas IgG alone versus IFN-γ + anti-Fas IgG, $P = 0.0025$ (*t* test). For LNCaP cells control IgG + IFN-γ versus anti-Fas IgG + IFN-γ, $P = 0.03$ (*t* test). (C) Plated PC3 and LNCaP cells were exposed to 100 u/ml IFN-γ or PBS and the following day loaded with ^{51}Cr. Cells were then plated in 96-well plates in 50 μl medium to which 50 μl concentrated medium from either 293 or TC 268 was added. Medium was harvested and radioactivity measured 18 h later. Each bar represents the average of triplicate wells ± SD. For PC3 and LNCaP cells TC268 medium + IFN-γ versus TC268 medium, $P = 0.0074$ and $P < 0.0001$, respectively (*t* test).

herently low FasL expression, and a significant loss in suppression of metastatic lesions in the lung, a site of high FasL expression [3]. By immunohistochemistry, however, most of the surviving primary tumor cells remained Fas$^+$ and thus targets for FasL transactivation. Therefore, a hypothesis was generated that in the face of high Fas expression relatively lower levels of FasL expression, as would be achieved by *in situ* gene therapy, may mediate higher levels of growth suppression due to the ability of a single FasL-expressing cell to kill many surrounding Fas-expressing cells.

Immunohistochemical studies have shown that human prostate cancers express Fas [4–7]. Established human prostate cancer cell lines have also been found to express Fas, though most are generally resistant to Fas transactivation by agonist antibody [4,5,8]. Initial studies noted the ability of IFN-γ to enhance or restore sensitivity to Fas transactivation in prostate cancer cells. Alone, a replication-incompetent adenovirus expressing FasL resulted in modest killing, reflective of baseline expression of Fas. In the presence of IFN-γ-mediated high Fas expression, markedly enhanced killing was experienced *in vitro*, even at low vector doses. In an aggressive orthotopic model of prostate cancer a staggered dosing of Ad.mIL-12 and Ad.FasL could result in significant increases in apoptosis and a doubling of mean survival.

RESULTS

Effects of IFN-γ on Fas-mediated Toxicity *In vitro*

We first explored the effect of IFN-γ on Fas-mediated cytotoxicity in three different prostate cancer cell lines. Agonist anti-Fas antibody alone demonstrated little killing of RM-1 cells, but the addition of a minimum dose of 50 u/ml IFN-γ resulted in significant killing, leaving a surviving fraction of 30 ± 14% (Fig. 1A). Higher doses of IFN-γ did not further increase anti-Fas IgG-mediated killing. For PC3 cells, known to be sensitive to Fas-mediated

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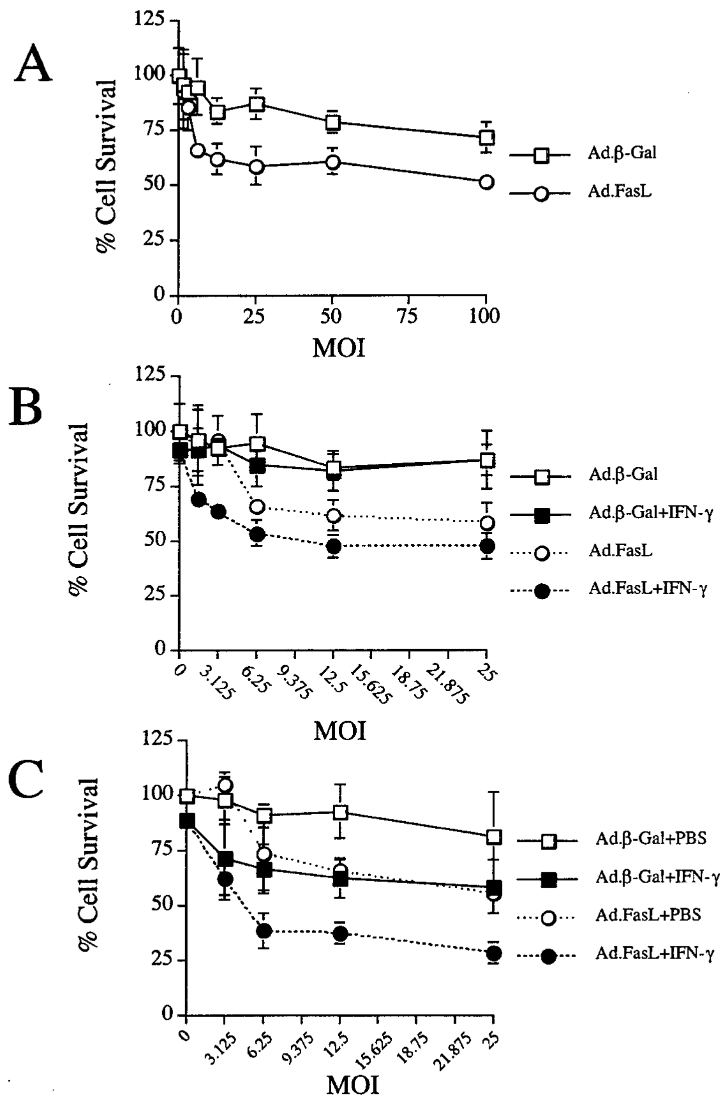

FIG. 2. Effects of Ad.FasL on RM-1 cells *in vitro*. (A) Plated RM-1 cells were transduced at escalating m.o.i. for 1 h and the following day viable cells were counted. Each data point represents the average of triplicate wells ± SD. (B and C) Plated RM-1 cells were randomized to exposure to PBS or 25 or 50 u/ml mouse IFN-γ. The next day each treatment condition was randomized for transduction with escalating m.o.i. of Ad.β-Gal or Ad.FasL. Twenty-four hours later viable cells were counted. Each data point represents the average of triplicate wells ± SD. Ad.FasL versus Ad.FasL + IFN-γ—50 u/ml, P = 0.035, Mann–Whitney; 25 u/ml, P = 0.014, Mann–Whitney.

death [4,8], both IFN-γ and anti-Fas IgG resulted in cell kill, 24 ± 3% and 25 ± 12%, respectively (Fig. 1B). The combination of IFN-γ and anti-Fas IgG resulted in a surviving fraction of 36 ± 6%. For LNCaP cells, known to be resistant to Fas-mediated death [4,8], anti-Fas IgG alone had no effect but in combination IFN-γ resulted in a surviving fraction of 42 ± 13% (Fig. 1B). We obtained similar results using soluble human FasL (sFasL) instead of agonist anti-Fas IgG with PC3 and LNCaP cells, noting the marked enhancement of cell lysis by preexposure to IFN-γ prior to Fas transactivation (Fig. 1C). By this assay Jurkat cells were lysed 93 ± 3% by TC268 medium versus 5 ± 2.3% for 293 medium (data not shown).

We then addressed the effects of IFN-γ on Fas expression as analyzed by FACS. Prior experience with RM-1 cells had shown a baseline expression of 30%, which following exposure to 50 u/ml IFN-γ resulted in increased expression of Fas to ~60–70% [3,16]. FACS analysis of PC3 and LNCaP cells demonstrated baseline expression of Fas at 77 and 95%, respectively. Preincubation of both cell lines with IFN-γ at 100 u/ml did not increase Fas expression (data not shown).

In vitro activity of Ad.FasL. Alone, Ad.FasL demonstrated dose-dependent killing to a maximum of near 40% (Fig. 2A). At multiplicities of infection (m.o.i.) greater than 12.5 there was no further killing from this level, which correlates with the baseline expression of Fas [3]. We addressed the effects of IFN-γ at two doses, the higher of which enhanced the toxicity of control vector (Fig. 2C). The combination of Ad.FasL and IFN-γ at both doses resulted in enhanced cell kill over Ad.FasL alone. The maximum cell kill of combination therapy correlates with the dose effect of IFN-γ on Fas expression following cyto-

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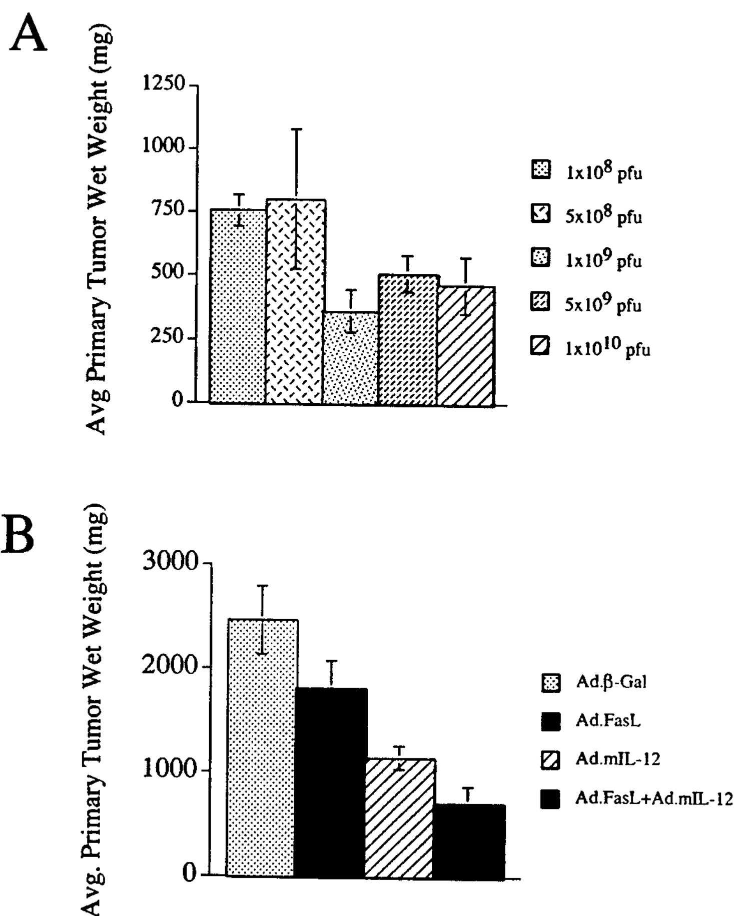

FIG. 3. Effects of Ad.FasL and Ad.mIL-12 on orthotopic tumor size. (A) Established RM-1 tumors were injected with Ad.mIL-12 and escalating doses of Ad.FasL on day 6 post-tumor cell inoculation. All mice were sacrificed on day 14 and the wet weight of each tumor was recorded. Each bar represents the average ± SD (n = 5 for each group). 5×10^8 pfu vs 1×10^9 pfu, $P = 0.003$, and 1×10^9 pfu vs 1×10^{10} pfu, $P = 0.16$ (t test). (B) Established RM-1 tumors were randomized for injection with Ad.β-Gal, Ad-.FasL, Ad.mIL-12, or Ad.FasL + Ad.mIL-12 on day 6. All mice were sacrificed on day 14 and the wet weight of each tumor was recorded. Each bar represents the average ± SD (n = 6 per group). Ad.FasL versus Ad.β-Gal, $P = 0.04$; Ad.mIL-12 versus Ad.β-Gal, $P = 0.001$; Ad.mIL12 versus combination therapy, $P = 0.04$ (t test).

kine exposure [3]. Importantly, reducing Ad.FasL dose maintained more than additive effects through to m.o.i. of 1.5 (Fig. 2B), indicating the presence of synergistic killing.

In vivo gene therapy. Initial studies focused on identifying the therapeutic dose of Ad.FasL in combination with a fixed dose of Ad.mIL-12. The dose of Ad.mIL-12 chosen, 1×10^9 plaque-forming units (pfu), was 10-fold lower than the therapeutic dose of Ad.mIL-12 alone in the RM-1 model [3]. Combination therapy demonstrated a significant suppression of tumor growth with Ad.FasL doses greater than 5×10^8 pfu (Fig. 3A). No additional benefit was derived from increasing viral doses above 1×10^9 pfu. In fact higher, Ad.FasL doses trended toward larger tumors, but this was not found to be statistically significant.

We performed dose control experiments to determine the relative effectiveness of individual treatments and combination gene therapy. Ad.FasL alone resulted in tumors that were 27 ± 11% smaller than controls (Fig. 3B). Ad.mIL12 therapy resulted in tumors that were 54 ± 7% smaller than control tumors. Combination gene therapy resulted in tumors that were 72 ± 6% smaller than control tumors.

In a survival study a single injection of Ad.FasL had no effect on survival (Fig. 4A). Surprisingly a co-injection of Ad.FasL + Ad.mIL-12 was inferior to Ad.mIL-12 (Fig. 4A; Ad.mIL-12, 22.8 ± 0.8 days vs combo Tx, 19.3 ± 0.6 days). A second injection of Ad.FasL on day 9 to target remaining Fas-positive cells likewise worsened survival over that experienced with Ad.mIL-12 alone (Fig. 4A). We reasoned that perhaps combination therapy was inferior to Ad-.mIL-12 alone due to the killing of Ad.mIL-12-transduced cells, thereby reducing both IL-12 and IFN-γ levels, and/or to the killing of activated NK/T cells important in the longer term growth suppression [3]. To test this hypothesis we injected Ad.mIL-12 into the contralateral lobe of the prostate, which did not harbor a tumor, and Ad.FasL into the tumor, as before. In this assay, as would be predicted, injection of the contralateral lobe with Ad-.mIL-12 reduced the survival-enhancing abilities of intratumoral cytokine therapy (Fig. 4B). However, the addition of repetitive intratumoral injection of Ad.FasL resulted in significantly enhanced tumor growth suppression over intratumoral treatment with Ad.mIL-12 (Fig. 4B). This study would indicate the need to either inject Ad.mIL-12 at a site remote from Ad.FasL injection or stagger the

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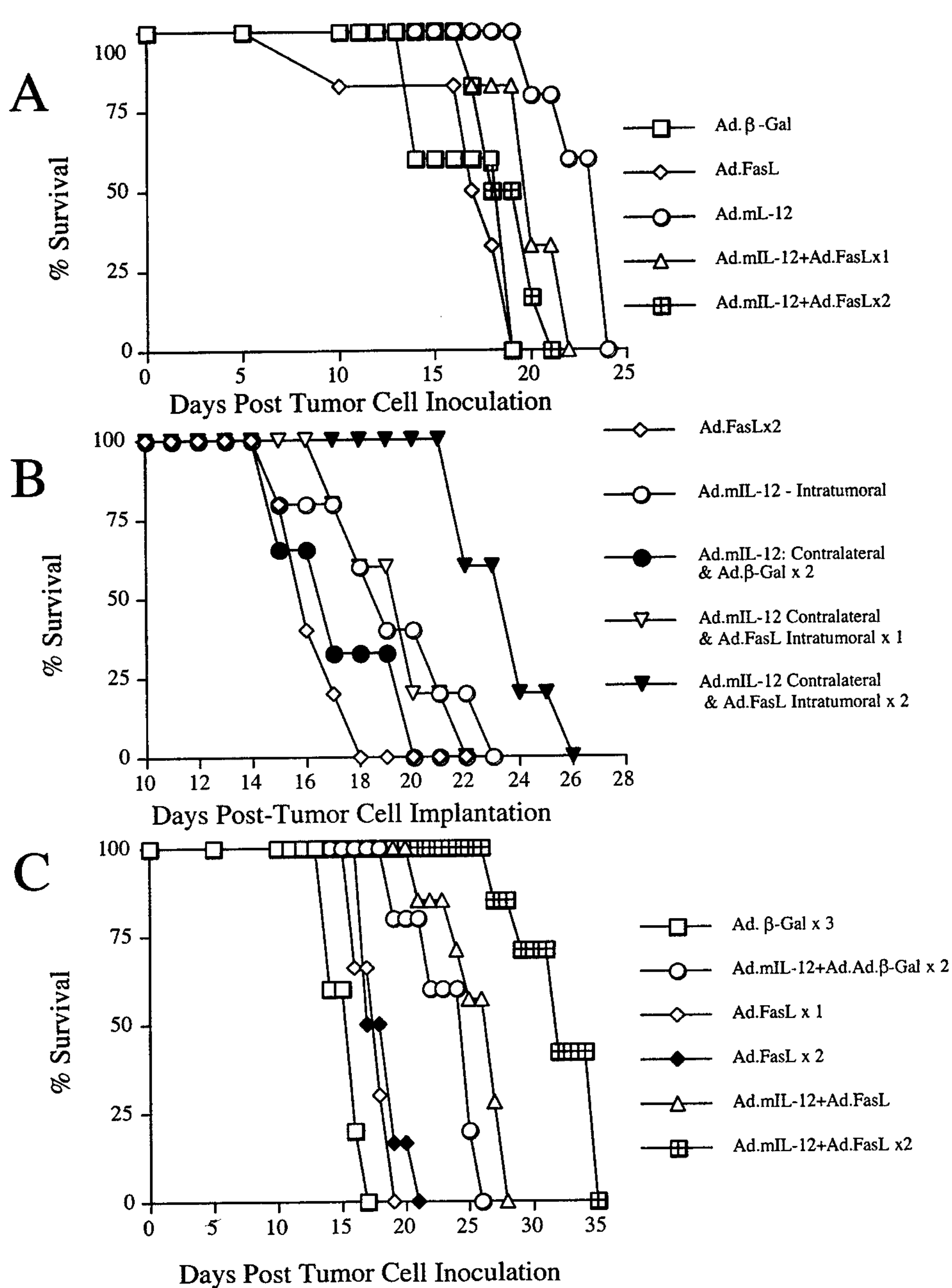

FIG. 4. Effect of Ad.FasL and Ad.mIL-12 on survival. (A) Established orthotopic tumors were randomized for injection with Ad.β-Gal, Ad.FasL, Ad.mIL-12, or Ad.FasL + Ad.mIL-12 on day 6. One group of Ad.mIL-12 + Ad.FasL mice received a second Ad.FasL injection on day 9. Mice were followed until death or sacrifice due to stress (n = 6–7 per group). Ad.mIL-12 versus combination therapy, P = 0.0134, Mantel–Cox. (B) Mice with established orthotopic tumors were randomized on day 6 for intratumoral injection with Ad.FasL, Ad.mIL-12, or Ad.FasL + Ad.mIL-12 and/or contralateral injection into the normal dorsolateral prostate with Ad.mIL-12 or Ad.β-Gal. Some mice injected with Ad-.mIL-12 in the contralateral lobe were reinjected on day 9 with either Ad.FasL or Ad.β-Gal. Mice were then followed as above (n = 6–7 per group). Ad.mIL-12 intratumoral versus Ad.mIL-12 contralateral + intratumoral FasL, P = 0.027, Mantel–Cox. (C) Established orthotopic tumors were randomized for injection with either Ad.β-Gal or Ad.mIL-12 on day 6. Each group was then further randomized to receive an injection with either Ad.β-Gal or Ad.FasL on day 8. In addition some mice were reinjected with either Ad.β-Gal or Ad.FasL on day 11. Mice were then followed as above (n = 6–7 per group). Ad.mIL-12 + Ad.FasL—two doses versus Ad.mIL-12, P = 0.0004; Ad.mIL-12 + Ad.FasL—two doses versus Ad.mIL-12 + Ad.FasL—one dose, P = 0.0015; Ad.mIL-12 plus two doses of Ad.FasL, P = 0.0003, Mantel–Cox.

injections of Ad.mIL12 and Ad.FasL. While the former would ensure serum levels of IFN-γ to influence Fas expression, it also would not take advantage of the multifaceted effects of IL-12 itself within the tumor.

We addressed this issue by injecting Ad.mIL-12 on day 6 as per protocol and injecting Ad.FasL on day 8 or days 8 and 11. Day 8 was chosen for the injection of Ad.FasL as it coincided with the peak serum level of IL-12 following vector injection [3]. We noted the superiority of combination therapy, especially with the second injection of Ad.FasL (Fig. 4C). Overall, the combination of Ad.mIL-12 plus two doses of Ad.FasL doubled mean survival over controls, 16 ± 0.89 days to 33 ± 1.57 days.

Assay of apoptosis in vivo. We sacrificed random mice from the staggered injection scheme (Fig. 4C) representing each treatment group on day 11 or day 14 for analysis of apoptotic activity. Three days following vector injection Ad.FasL alone increased apoptotic activity by ~1.5×, while combination therapy doubled activity (Fig. 5).

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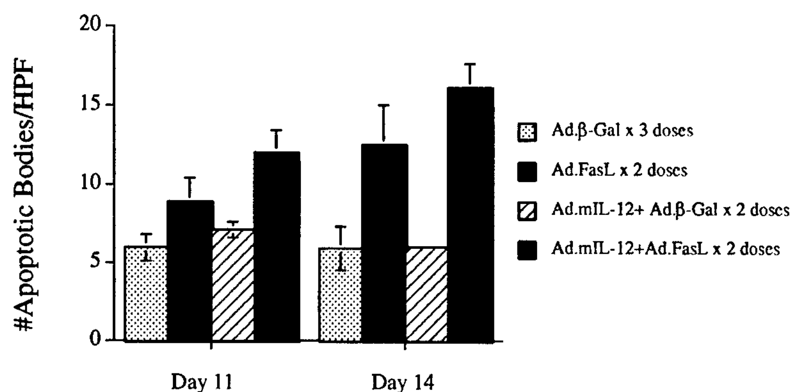

FIG. 5. Effect of staggered vector dosing on apoptosis. Animals from each treatment arm were sacrificed on either day 11 or day 14 and the primary tumors prepared for paraffin embedding, sectioning, and TUNEL assay. Specimens harvested on day 11 were injected with Ad.FasL or Ad.β-Gal only once, while those from day 14 were injected twice. Tumor sections were scanned across both long and short axes and the number of apoptotic bodies per high-power field (HPF) per tissue section was recorded. For each treatment condition at the two time points each bar represents the average number of apoptotic bodies per HPF ± SD ($n = 4$ tumors per time point and treatment condition). Three days after Ad.FasL injection Ad.β-Gal versus Ad.FasL, $P = 0.036$; control versus combination therapy, $P = 0.0016$; Ad.FasL versus combination therapy, $P = 0.028$ (*t* test). Three days after the second injection of Ad.FasL, Ad.FasL ×2 versus Ad.mIL-12 + Ad.FasL ×2, $P = 0.03$ (*t* test).

Analysis of tissues 3 days following the second injection of Ad.β-Gal or Ad.FasL noted a near tripling (2.72×) of apoptosis by a second injection in combination-treated tumors compared to a doubling for tumor injected twice with Ad.FasL only (Fig. 5).

Discussion

A major practical issue of *in situ* cancer gene therapy that must be addressed to ensure its success is the lack of high transduction efficiency following injection of a tumor. The approach of combining Ad.mIL-12 and Ad.FasL exploits the ability of a single injection of IL-12, which would transfect 25% of cells at best, to then influence a potential target on virtually all cells within the same tumor. Vector-mediated expression of FasL would thus be capable of killing many surrounding Fas^+ cells. Such a process would represent a bystander effect as an important mechanism to achieve tumor control following *in situ* gene therapy. Results from the *in vitro* studies indicate that Ad.FasL alone results in the maximum killing of approximately 40% of the cells at the highest vector dose. This activity closely correlates with the constitutive level of Fas expression by RM-1 of 30% [3]. The addition of IFN-γ increased the level of maximum cell kill, shifted the attainment of maximum cell kill to a twofold lower vector dose, and maintained significant cytotoxicity even at very low vector doses. At m.o.i. 6.25 Ad.FasL alone caused cell loss of only 5% but in combination with IFN-γ resulted in nearly 60% cell loss (Fig. 1), indicating the presence of synergistic killing as hypothesized.

The *in vivo* experiments demonstrated the positive growth effects of manipulating Fas/FasL interactions but also demonstrated some of its limitations. The dose escalation of Ad.FasL noted a maximum effect with 1×10^9pfu and trended toward less effect with higher doses. In initial survival studies co-injection of Ad.mIL-12 and Ad.FasL significantly negated the effects of Ad.mIL-12 therapy. One would postulate two underlying reasons for this phenomenon. First, it is possible that as greater numbers of cells are infected with FasL they are killed off too rapidly to exert any effect on surrounding Fas^+ cells. Second, and more likely, FasL itself may neutralize some of the positive effects of IL-12. Experience with Ad.mIL-12 alone had demonstrated several factors mediating growth responses in addition to increasing Fas expression, including induction of neutrophil-mediated apoptosis within the first 24–48 h following injection and the induction of both NK and T cell responses after 7–8 days [3]. These events undoubtedly were dependent on the ability of treatment to induce a double peak of IL-12: the first peak occurs on the second day post-Ad.mIL-12 injection and the second peak occurs on the eighth day postinjection [3]. Approximately 2 days following each peak of IL-12, a similar peak of IFN-γ could be measured in serum of treated mice. The first cytokine peak was produced in response to the vector itself and the second by induced NK/T cell immune response [3]. Therefore, we reasoned that the presence of FasL at high concentrations within the prostate could first neutralize Ad.mIL-12-transfected/producing cells to reduce the levels of both IL-12 and IFN-γ and second, the reductions of these cytokines would lessen the induction of NK/T cell responses critical to IL-12-mediated survival.

We addressed this hypothesis in two ways. Injecting Ad.mIL-12 into the non-tumor-bearing contralateral prostate lobe but continuing the injection of Ad.FasL into the tumor maintained the interaction of IL-12–IFN-γ–Fas + FasL to result in superior growth effects over co-injection of both vectors into the cancer. In this scenario growth effects are obtained without any direct benefit from IL-12 itself, and the power of Fas/FasL interactions is cleanly illustrated in this aggressive model of prostate cancer. In the second scenario the vectors, Ad.mIL-12 and Ad.FasL, are injected in a staggered fashion, directed to avoid high levels of FasL at the time of the first peak of IL-12 secretion. In this study the clear benefits of Fas/FasL interaction combined with the effects of IL-12 are identified to increase apoptotic activity and double survival from 16 ± 0.89 to 33 ± 1.57 days. An important caveat learned from these studies points to the need for careful thought when approaching combination strategies for cancer: some de-

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signs may actually be detrimental and should be assembled knowing the variety of pathways each may influence.

Exploitation of the interaction of Fas and its ligand, FasL, to initiate an intracellular cascade resulting in apoptosis [2] has attracted interest from a variety of investigators against many cancers. However, use of soluble FasL or agonist-acting anti-Fas antibodies results in severe toxicity due to the wide variety of vital tissues, primarily the liver, that express Fas, requiring restrictive expression of FasL [17]. Furthermore, while virtually all of the human prostate cancer cell lines express Fas, most are resistant to Fas transactivation by agonist anti-Fas antibodies [4,5,8]. Studies have demonstrated that the apoptotic pathway appears intact within these resistant cells, but is actively inhibited through an unknown mechanism [4,18]. The block of the apoptotic pathway appears to occur upstream of caspase 8 [19], and in comixing of resistant and sensitive cell lines the dominant nature of Fas resistance phenotype was demonstrated [20]. Some investigators have found that use of vector-mediated FasL expression increased Fas-mediated killing over that achieved by agonist antibody [21,22], as was experienced with RM-1. Moreover, sensitization to Fas-mediated cell death has been achieved in many human prostate cancer cells by a variety of drugs, including Adriamycin, etoposide, mitoxantrone, camptothecin, and VP-16, though the pathways involved are unknown [5,18,23,24]. In the present study IFN-γ enhanced Fas-directed cell kill in a sensitive cell line (PC3) and restored sensitivity in a resistant cell lines (RM-1 and LNCaP) under conditions of both anti-Fas IgG (RM-1) and sFasL transactivation. In RM-1 the enhancement in cytotoxicity was directly related to increased expression of Fas as measured by FACS, the level of which closely correlated with the degree of cell kill. The human cell lines, however, demonstrated high levels of constitutive Fas, which were unchanged by IFN-γ exposure. The mechanism behind this process is the subject of further investigation.

While much speculation can be made concerning the presence or absence of Fas sensitivity in the few human prostate cancer cell lines available, the status of Fas sensitivity in prostate cancer tissue in patients is entirely unclear. Most of the human cell lines have been derived from metastatic lesions, are highly passaged, and bear little resemblance to prostate cancers by other parameters. By immunohistochemistry human prostate tissue, both benign and malignant, expresses Fas but not FasL [4–7]. Human cell lines derived from benign prostate tissue undergo apoptosis following infection with Ad.FasL and the injection of normal rat prostate with Ad.FasL results in apoptosis of epithelial cells lining glandular acini [25]. Analysis of cancer tissue obtained via laser capture microdissection noted loss of heterozygosity (LOH) of the Fas gene in 31% of cases, but there was no relation between immunohistochemical analysis for Fas presence or absence and LOH [26]. Therefore, until fresh explants of prostate cancers or epithelial cells from such tissues are analyzed for Fas sensitivity this question will remain unanswered.

MATERIALS AND METHODS

Cell lines. The RM-1 cell line, an androgen-insensitive mouse prostate cancer cell line, was established from a ras^+ myc-induced primary tumor, derived in the mouse prostatic reconstitution model in C57BL/6 mice ([9,10]; obtained from Dr. Timothy Thompson, Baylor College of Medicine, Houston, TX). RM-1 cells were grown in DMEM with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). LNCaP and PC-3, human prostate cancer cells (obtained from ATCC), were grown in RPMI 1640 or DMEM, respectively, supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). TC268, a human sarcoma cell line ([11]; obtained from Dr. Nicholas Mistsiades, NIH, Bethesda, MD, USA), and 293 cells, a human embryonic kidney cell line, were grown in DMEM with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). Jurkat cells (obtained from ATCC), a human T cell leukemia cell line, were grown in RPMI 1640 supplemented with 10% FBS. A549 cells, a human lung cancer cell line (obtained from ATCC) were grown in Ham's F12 medium supplemented with 10% FBS. Cells were passaged by trypsinization and maintained with routine medium changes. All media were obtained from CellGro (Mediatech, Herndon, VA, USA).

The influence of IFN-γ on Fas sensitivity in vitro. We first addressed Fas-mediated cytotoxicity in RM-1, PC3, and LNCaP cells by exposing plated cells to PBS or IFN-γ (recombinant human or mouse; Pharmingen, San Diego, CA, USA) for 24 h. Each treatment group was then randomized to agonist anti-Fas IgG (10 μg/ml, either hamster anti-mouse or mouse anti-human; Pharmingen) or isotype control IgG (hamster or mouse, 10 μg/ml; Pharmingen). Viable cells, as per trypan blue exclusion, were counted the following day.

The ability to kill LNCaP or PC3 cells through Fas transactivation was also ascertained by exposing plated cells to sFasL. Soluble FasL was derived by concentrating medium from TC268 sarcoma cells, which are known to excrete active sFasL into conditioned medium, as previously described [11]. Twenty-five 20-cm plates of TC268 or 293 cells (sFasL negative) were prepared. When cells were 80% confluent, plates were washed two times with PBS and fresh serum-free medium was applied for a 4-h wash. Fresh serum-free medium was then applied and harvested 48 h later. The medium was then centrifuged at 2000 rpm for 5 min to remove cellular debris. The resulting supernatant was concentrated 30-fold with Centriprep-10 filters (Amicon, Beverly, MA, USA). PC3 and LNCaP cells were grown in regular medium or medium + 200 u/ml IFN-γ for 24 h, followed by labeling with chromium (^{51}Cr). A known number of targets were plated and exposed at a 1:1 volume ratio with TC268 concentrated medium (+sFasL) or 293 cell concentrated medium (no sFasL) for 18 h. To serve as a positive control, Jurkat cells were labeled with ^{51}Cr as above and exposed to concentrated medium from either TC268 or 293 cells for 18 h. The percentage of specific lysis was calculated as (experimental release − spontaneous release)/(maximal release − spontaneous release) from gamma counter measurements of supernatant harvested by the Skatron system (Skatron Instruments, Inc., VA, USA).

Adenoviral vectors. To construct a replication-incompetent adenovirus-expressing mouse FasL, we amplified the FasL mRNA by PCR, followed by ligation into an adenovirus backbone driven by the Rous sarcoma virus promoter. The pAd.RSV-FasL was cotransfected with pBHG10 into 293 cells by the calcium phosphate precipitation method to yield Ad.FasL. To inhibit apoptosis of Fas^+ 293 cells during the entire process, culture medium was supplemented with the general caspase inhibitor Z-VAD-FMK (20 μM; Enzyme System Products, Livermore, CA, USA). Due to the potential selection force of FasL to result in the production of wild-type adenovirus, we tested the Ad.FasL lot for replication-competent adenovirus (RCA) by standard techniques in the Vector Core of the Institute.

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Briefly, Ad.FasL (1×10^9 pfu) was added to subconfluent cultures of A549 cells at 10 pfu per cell. Cultures were observed for 2 weeks without evidence of cytopathic effect (CPE). The cultures and media were harvested and freeze/thawed three times. This material was added to fresh monolayers of A549 cells and cultures were observed for CPE for an additional 2 weeks. No CPE was observed, demonstrating <1 RCA in 1×10^9 pfu of vector.

Replication-incompetent adenoviruses expressing either mouse IL-12 (Ad.mIL-12) or β-galactosidase (Ad.β-Gal), a vector control, were constructed as previously described [12-14]. Large-scale production of all vectors was achieved following expansion in 293 cells and double cesium gradient ultracentrifugation purification. The viral titer was ascertained in 293 cells via a plaque assay and expressed as plaque-forming units per milliliter.

***In vitro* cytotoxicity of Ad.FasL.** We assayed the potential for FasL alone or in combination with IFN-γ to mediate toxicity *in vitro* by preincubating for 24 h in IFN-γ (25 or 50 u/ml; Pharmingen) or PBS. The following day each group was subdivided for a 1-h exposure to escalating doses of Ad.FasL or Ad.β-Gal. Dosing of virus was defined in terms of the m.o.i. (=number of pfu per cell). Cells were harvested 24 h later and viable cells, as determined by trypan blue (0.4%; Sigma Chemical Company, St. Louis, MO, USA) exclusion were counted.

***In vivo* gene therapy.** We induced orthotopic tumors and treated them with adenoviral vectors as previously described [15]. Briefly, tumors were induced by an injection of 7500 RM-1 cells into the dorsolateral prostate of adult C57/BL6 mice (The Jackson Laboratory). Visible tumors were then (co-)injected with vector on day 6, as per routine. All such procedures were performed under pentobarbital-induced anesthesia (50 mg/kg). Endpoints for studies were either sacrifice at 14 days post-tumor-cell inoculation, with recording of tumor wet weight, or longer term survival analysis. For survival studies animals either were found dead or were sacrificed when tumors were thought by palpation to approach 10% body weight or individual animals appeared stressed by weight loss, ruffled fur, and/or lethargy. Where indicated, alternative vector injection schemes were followed. In some studies Ad.β-Gal or Ad.FasL was injected into the primary tumor a second time on day 9. Alternatively, either Ad.β-Gal or Ad.mIL-12 was injected into the non-tumor-bearing contralateral prostate lobe, while either Ad.β-Gal or Ad.FasL was injected into the tumor on day 6 and/or 9. In other studies repetitive staggered injections of either Ad.FasL or Ad.β-Gal were performed by the following protocol: day 6, injection of tumor with Ad.β-Gal or Ad.mIL-12, and days 8 and 11, injection with Ad.β-Gal and/or Ad.FasL.

Detection of apoptosis *in vivo*. At the indicated times we sacrificed the animals and fixed primary prostate tumors in 10%, embedded them in paraffin, and sectioned them. After sectioning, tissues were stained with hematoxylin and eosin for routine histopathologic screening. Presence of apoptotic activity was achieved by TUNEL assay as per the manufacturer's instructions (Trevigne Industries, Gaithersburg, MD, USA). The number of positive bodies was counted at 400× by viewing across the long and short axis of tissue sections and expressed as number of bodies per high-power field.

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