Epigenetic changes in tumor Fas levels determine immune escape and response to therapy

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Summary

Epigenetic regulation of gene expression significantly influences cell growth and differentiation. Here we show that epigenetic silencing of Fas determines tumor growth in vivo and apoptotic sensitivity in vitro. In established tumors with epigenetically repressed Fas, restoration of Fas activity either by transfection of *fas* or treatment with Trichostatin A (TSA), an inhibitor of histone deacetylase, suppresses tumor growth and restores chemosensitivity. The TSA-dependent chemosensitivity and tumor growth control require both tumor Fas and the host NK (natural killer) cell functions. This work demonstrates the importance of epigenetic modification of Fas in tumor progression and immune evasion, and emphasizes the essential interplay between Fas and innate immunity in the control of chemoresistant tumors.

Introduction

Tumor development is driven by genetic and epigenetic events leading to the alteration of oncogenes and tumor suppressor genes. The majority of research in cancer biology has focused on genetic mutations that contribute to human malignancy. However, epigenetic phenomena such as gene silencing (resulting from histone deacetylation and methylation) have also been shown to contribute to tumorigenesis (Fruhwald et al., 2001; Malik et al., 2000; Wong et al., 2000). Histone deacetylases (HDACs) together with histone acetyltransferases (HATs) regulate the acetylation state of nucleosomal histones, thereby altering chromatin structure. Transcriptional silencing has been found to increase as the level of acetylated histones increases (Matzke et al., 2001; Struhl, 1998). During malignant progression, transcriptional repression could promote tumorigenesis by silencing cellular apoptotic effector cascades. In support of this hypothesis, HDAC inhibitors have recently been shown to induce differentiation and/or apoptosis of a variety of transformed cell lines (Coffey et al., 2000; Marks et al., 2000).

A key apoptotic effector, Fas (also known as APO-1 or CD95), is a member of the TNF receptor superfamily (Nagata, 1997) and a downstream transcriptional target of p53 (Munsch et al., 2000). Loss of Fas function results in hyperplasia, such as lymphoproliferation, which may predispose individuals to malignancy (Adachi et al., 1993, 1995, 1996; Nagata, 1994; Watanabe-Fukunaga et al., 1992). Indeed, families of patients carrying a heterozygous mutation in the Fas gene have an increased incidence of Hodgkin's lymphoma (Fisher et al., 1995). Fas regulation has been further shown to be key in modulating immune homeostasis. Fas has been found to play an important role in the elimination of malignant cells by cytotoxic T cells and NK cells (Ashkenazi and Dixit, 1998). Recent work on innate immunity suggests that NK cell function is critical in determining the initial tumor growth (Soloski, 2001). In SCID mice, NK cells have been shown to provide antitumor immunity and their depletion has been linked to metastatic spread (Zheng et al., 1996).

We have previously shown that over time in culture, p53 wild-type cells repress Fas expression through an acetylation-related mechanism that is reversed by the HDAC inhibitor Trichostatin A (TSA) (Maecker et al., 2000). Such epigenetic silencing of Fas in vitro could parallel similar events that occur in vivo during malignant progression of p53 wild-type tumors acquiring diminished apoptotic programs. We hypothesized that, if Fas repression contributes to the apoptotic resistance of these late-passage cells, restoration of Fas should restore their apoptotic sensitivity. Further, if Fas repression contributes to the growth of these tumors in vivo, restoration of Fas to these cells should inhibit their growth in vivo. To determine if epigenetic silencing of Fas affected tumor growth in vivo, we examined whether genetic or epigenetic reversal of Fas repression impaired tumor growth. Since previous studies linked Fas signaling to the antitu-

SIGNIFICANCE

The key to developing active cancer immunotherapy is to determine why the immune system initially fails to detect transformed cells and subsequently becomes tolerant to tumor growth and metastasis. Recent work implicates a role of the host's innate immunity in the initial detection of transformed cells. Our observation of epigenetic silencing of Fas suggests a novel mechanism by which p53 wild-type tumors reduce their apoptotic sensitivity and escape immune clearance. Restoration of Fas expression with histone deacetylase inhibitors inhibits tumor growth and restores chemosensitivity in vivo in an NK cell-dependent manner. This study indicates that the innate immune system is important not only in growth control, but also in the treatment of chemoresistant solid tumors.

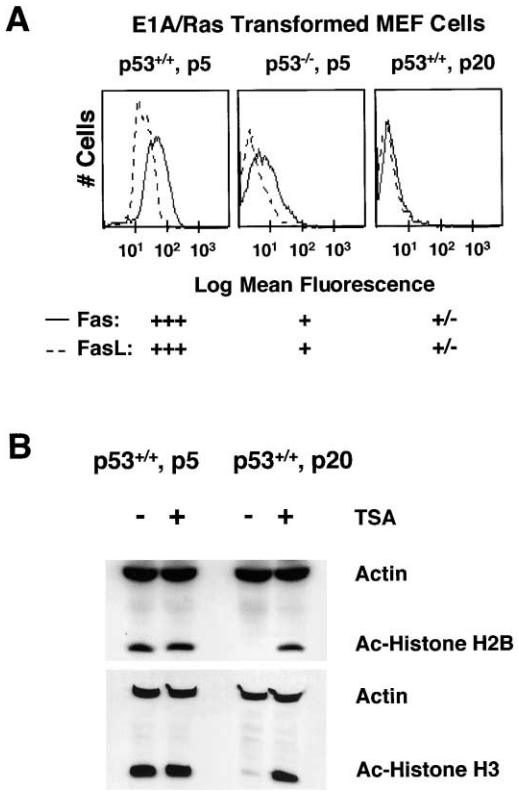

Figure 1. Epigenetic downregulation of Fas in late-passage cells

A: Surface Fas (solid lines) and FasL (dashed lines) expression of early-passage p53 wild-type and null MEFs as well as late-passage p53 wild-type MEFs. **B:** Nuclear histone H2B and H3 acetylation of p53 wild-type early- and late-passage MEFs treated for 18 hr in the presence and absence of TSA. Late-passage MEFs demonstrate deacetylated histones characteristic of transcriptional repression, which is reversed upon TSA treatment. Early-passage MEF histones, unlike the late-passage cells, are not deacetylated. Actin levels are shown to illustrate equal protein loading.

mor activity of the innate immune system (Screpanti et al., 2001), we investigated the role of NK cells in Fas-mediated control of tumor growth. In experiments with transplanted tumors derived from cells with epigenetically silenced Fas, we investigated whether restoration of Fas expression with HDAC inhibitors restored chemosensitivity and inhibited tumor growth. Taken together, our data illustrate a novel approach to restore chemosensitivity of solid tumors and reengage host immune surveillance.

Results

Modulation of Fas expression

To explore the contribution of Fas to tumor growth, we utilized genetically defined transformed MEF cells that were wild-type or null in their p53 status. While early-passage p53 wild-type MEFs express high basal levels of Fas and FasL, p53-deficient cells express low levels of Fas and FasL. Over time in culture, however, p53 wild-type cells repress Fas and FasL surface expression as a means of evading fratricide-induced cell death, becoming increasingly apoptotically resistant (Figure 1A). We determined previously (Maecker et al., 2000) that such Fas repression was due to epigenetic changes that could be reversed with Trichostatin A (TSA), an HDAC inhibitor. To confirm that TSA was in fact modulating the acetylation of core histones, we measured the levels of acetylated histone H2B and H3 from nuclear extracts of early- and late-passage p53 wild-type MEFs exposed to either vehicle or TSA (Figure 1B). While late-passage p53 wild-type cells treated with vehicle exhibit very low levels of acetylated histones, cells treated with TSA have levels of acetylated histones equivalent with those of early-passage cells. Hence, the transcriptional silencing of Fas observed in late-passage p53 wild-type cells coincided with the TSA-reversible deacetylation of core histones.

To test the contribution of Fas inactivation to apoptotic resistance in the late-passage p53 wild-type cells, we altered Fas levels either genetically (with the use of Fas-related retroviral vectors) or pharmacologically (with TSA treatment) in the genetically defined MEF cells (Figure 2A). MEF cells were infected with retroviral constructs expressing either a wild-type mouse Fas construct or a dominant-interfering Fas vector (DN-Fas). As negative controls, cells were infected with either empty vector or another member of the Fas superfamily, p75, the low-affinity nerve growth factor receptor. Since only the late-passage cells demonstrated an increase in Fas expression following TSA treatment (Maecker et al., 2000), these cells were the only ones to receive this treatment in subsequent experiments.

Following either retroviral infection or TSA treatment, surface levels of Fas were analyzed by flow cytometry (Figure 2A). With the exception of the early-passage p53 wild-type cells, retroviral transfection of Fas (as well as TSA treatment of the late-passage p53 wild-type cells) resulted in an elevation of surface Fas levels. According to our earlier findings that the early-passage p53 wild-type cells possess both elevated Fas and FasL levels (Maecker et al., 2000), it is likely that our inability to further increase Fas levels in these cells is due to apoptotic induction. This hypothesis is supported by the finding that early-passage p53 wild-type MEFs transfected with the Fas construct demonstrated increased basal apoptosis over the empty vector-transfected MEFs (Figure 3A). Also, all cells transfected with the Fas construct never exceeded the basal surface Fas levels found in the early-passage p53 wild-type cells (Figure 2A).

Since the MEF cells express FasL, we tested whether the above experimental manipulations altered surface FasL levels. Increasing a cell's surface level of Fas, either genetically or pharmacologically, resulted in a parallel elevation of FasL (Figure 2B). Likewise, decreasing a cell's surface level of Fas (with DN-Fas) resulted in a parallel downmodulation of FasL. This effect was likely an indirect one since neither p53- nor Fas-responsive elements have been reported in the FasL promoter. However, since MEF cells express both Fas and FasL, modulation of Fas in effect dictates the activity of the Fas pathway in these cells. It is possible, therefore, that a downstream target of the Fas pathway regulates FasL levels. One possible candidate is the transcription factor NF-κB. NF-κB has been shown to be activated downstream of Fas and is also capable of regulating FasL transcription (Manos and Jones, 2001).

Transfection of DN-Fas resulted in a decrease in surface Fas, regardless of the cell type transfected. This decrease in surface Fas was the result of inhibited trafficking of Fas to the cell surface (Figure 2C). Although all cell types transfected with DN-Fas exhibited decreased Fas surface expression, their total cellular Fas (as measured by intracellular staining) actually increased when compared to cells transfected with empty vector alone.

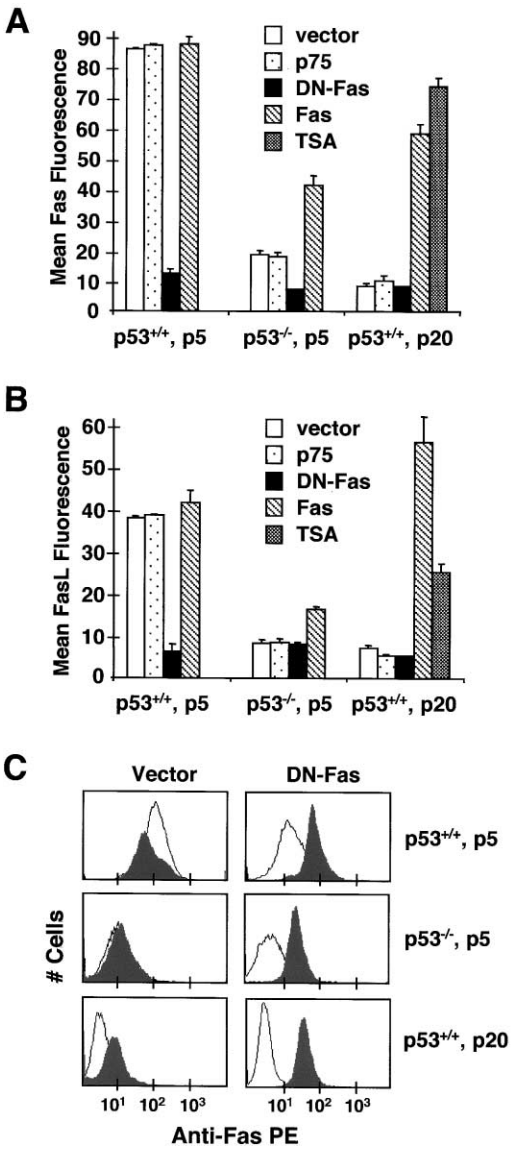

Figure 2. Genetic and epigenetic modification of Fas

A: Surface Fas levels of cells retrovirally transfected with empty vector, p75, DN-Fas, or Fas or treated with TSA. Transfection of Fas increased surface Fas levels, whereas transfection of DN-Fas resulted in a decrease in surface Fas. Empty vector and p75 failed to affect surface Fas expression. TSA treatment of $p53^{+/+}$, p20 cells resulted in an increase in their surface Fas expression. **B:** Surface FasL of genetically modified and epigenetically modified MEFs. Transfection of Fas and TSA increased surface FasL. Transfection of DN-Fas reduced surface FasL. Transfection of empty vector and p75 had no effect. **C:** Intracellular Fas staining of cells transfected with empty vector or DN-Fas. Surface Fas expression is shown by a solid black line and intracellular Fas staining by the gray fill. Transfection of DN-Fas decreased the surface Fas expression even though intracellular levels of Fas increased. DN-Fas thus inhibited Fas protein trafficking to the cell surface.

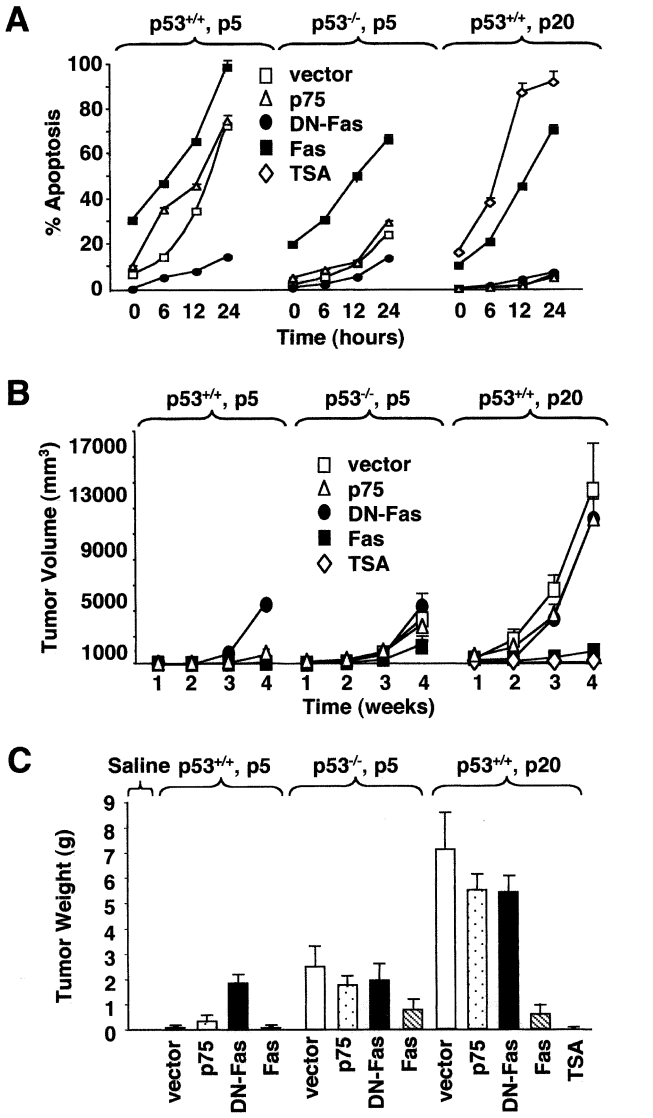

Figure 3. Fas restoration confers apoptotic sensitivity and determines tumor growth in vivo

A: Percent apoptosis in MEF cells following 6 Gy ionizing irradiation. MEF cells either transfected with Fas or treated with TSA demonstrated an increase in their apoptotic sensitivity in response to irradiation. Cells transfected with DN-Fas were protected against radiation-induced apoptosis. Transfection of empty vector or p75 didn't alter the ability of MEF cells to undergo radiation-induced cell death. Restoration of surface Fas either genetically or pharmacologically restored the ability of late-passage cells to undergo radiation-mediated apoptosis. **B:** Time course of tumor growth of SCID mice injected with MEF cells transfected with the designated constructs or treated with TSA. Elevating tumor Fas greatly inhibited growth in vivo. Likewise, inhibiting tumor Fas (DN-Fas) accelerated tumor growth. On day 27 a mouse injected with $p53^{+/+}$, p20 DN-Fas tumor cells died; on day 28 a mouse injected with $p53^{+/+}$, p20 empty vector died. The causes of these deaths were not determined. **C:** Final tumor weight of mice on day 28 postinjection.

Fas restoration confers apoptotic sensitivity and determines tumor growth in vivo

Having confirmed that we were able to modify surface levels of Fas, we next investigated apoptotic sensitivity of these cells. MEFs were exposed to 6 Gy ionizing radiation, and apoptosis was measured at 0, 6, 12, and 24 hr following treatment (Figure 3A). Increasing surface Fas enhanced both basal and radiation-induced apoptosis. Further, decreasing surface Fas (with DN-Fas) protected cells from radiation-induced cell death. This effect was specific to Fas in that p75 failed to affect apoptotic sensitivity regardless of cell type. Further, the alteration in apoptotic sensitivity was greatest in cells possessing wild-type p53, arguing for the importance of p53 in maximal activation of the Fas death machinery.

Having shown that we were able to alter Fas levels as well as apoptotic sensitivity in vitro, we tested whether this would correlate with tumor growth in vivo. One-half million p53 wild-type early-passage, late-passage, or null MEFs transfected with empty vector, Fas, DN-Fas, or p75 or treated with TSA were injected into C57BL/6J SCID mice, and tumor growth was monitored over 1 month. Tumor growth was analyzed by determining tumor volume over time (Figure 3B) and final tumor weight (Figure 3C). Consistently, tumor cells expressing the lowest levels of Fas grew the largest tumors; conversely, tumor cells expressing the highest Fas levels grew the smallest tumors or failed to implant altogether. Late-passage p53 wild-type cells with repressed Fas demonstrated the fastest growth rate (Figure 3B) as well as the largest tumor weight (Figure 3C). p53 null cells, expressing low levels of Fas, grew at a slower rate and achieved a smaller final tumor weight (approximately half that of the late-passage cells). Early-passage p53 wild-type cells, with high levels of Fas, either failed to take altogether or achieved tumor weights 10-fold smaller than the late-passage cells. Further, late-passage p53 wild-type MEFs with genetic or epigenetic Fas restoration were inhibited in their growth in vivo or, in the case of the TSA-treated cells, failed to implant. Of note, inhibition of Fas signaling (by transfection of DN-Fas) completely ablated the growth advantage of p53 null cells over early-passage p53 wild-type cells. As with our earlier in vitro data, transfection of p75 failed to affect the course of tumor growth of any of the MEF cells, demonstrating that differences in tumor growth were Fas dependent.

NK cells provide Fas-dependent tumor immunity

To understand the immune basis of Fas-mediated tumor growth in vivo, the above experiments were repeated in NK-deficient SCID beige mice. One-half million p53 wild-type early-passage, late-passage, or null MEFs were injected into SCID beige mice, and tumor growth was monitored over 1 month (Figures 4A and 4B). In the absence of NK cells, Fas levels failed to determine tumor growth, as evidenced by the lack of difference between the growth of the early- and late-passage p53 wild-type MEFs (Figures 4A and 4B).

To understand why the p53 null MEF tumors grew slower than the p53 wild-type tumors, in vitro proliferation assays were conducted (Figure 4C). It has been suggested that in immunocompetent hosts, tumor growth is a balance between proliferative and apoptotic indices (Evan and Vousden, 2001). In SCID beige mice that lack NK apoptotic effectors, we hypothesized that proliferation rates would determine tumor growth. Indeed, the early-passage p53 null MEFs were found to proliferate more slowly in vitro than the late-passage p53 wild-type MEFs, although these p53 null MEFs proliferated faster than the early-passage p53 wild-type cells (Figure 4C). However, when Fas was inactivated in the early-passage p53 wild-type MEFs (with DN-Fas to prevent fratricide-induced death resulting from cell-cell contact in vitro), their proliferative potential was identical to the late-passage cells. Thus, in SCID beige mice, Fas failed to influence proliferative potentials. These data suggest that in the absence of innate immunity, proliferative indices rather than apoptotic regulators dictate tumor growth rates.

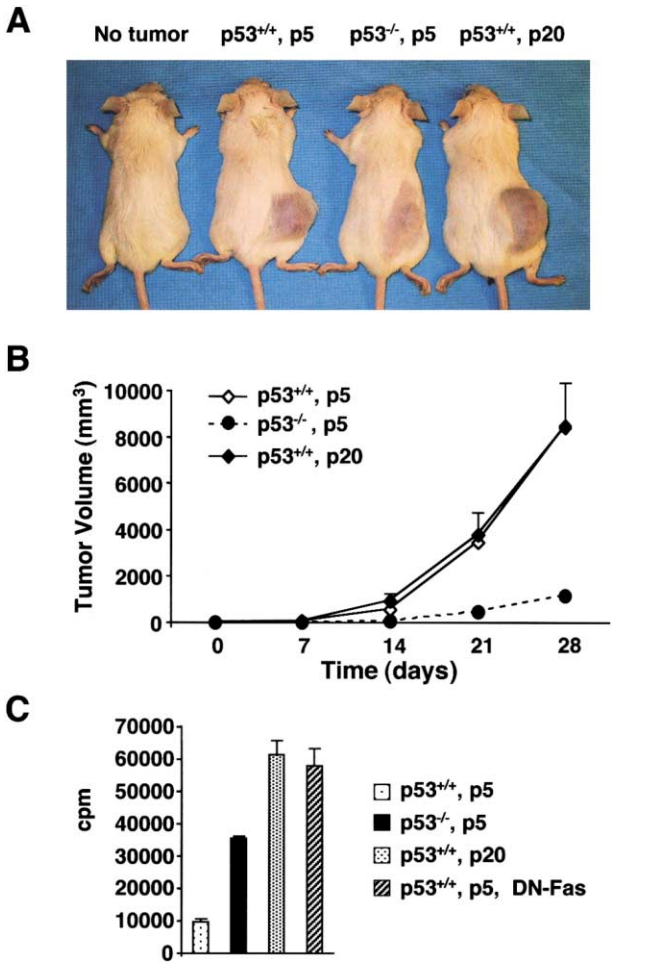

Figure 4. NK cells regulate Fas-mediated tumor clearance

A: The median of five NK-deficient SCID beige mice is shown for each experimental group. **B:** Time course of tumor growth in SCID beige mice. Tumor Fas levels no longer determined tumor growth. On day 22, one mouse injected with $p53^{+/+}$, p5 tumor cells died; on day 28 one mouse injected with $p53^{+/+}$, p5 tumor cells died. The causes of these deaths were not determined. **C:** In vitro basal proliferation of MEF cells. Suppression of surface Fas levels with DN-Fas abolished the difference in growth rate between $p53^{+/+}$, p5 and $p53^{+/+}$, p20 cells.

Epigenetic modification of Fas restores chemotherapeutic responsiveness of established tumors in vivo

We next investigated if Fas modulation in established, apoptotically resistant tumors would sensitize them to chemotherapeutic attack and immune clearance. To address this issue, we first measured apoptosis in the late-passage p53 wild-type MEFs (with repressed Fas) induced by various chemotherapeutic agents in the presence and absence of TSA. In the absence of TSA, the late-passage cells were resistant to chemotherapy-induced killing, whereas in the presence of TSA they were effectively eliminated (Figure 5A). This sensitization was directly attributable to reversal of Fas repression (and not a secondary effect of TSA), since late-passage cells transfected with the DN-Fas construct were protected against chemotherapeutic attack even in the presence of TSA.

Having confirmed in vitro that we were able to sensitize late-

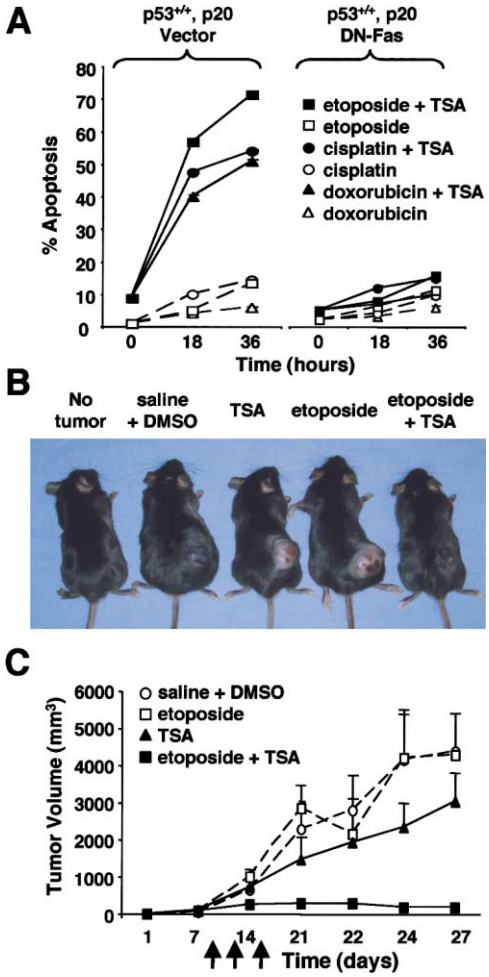

Figure 5. The in vitro and in vivo roles of Fas in tumor chemosensitivity

A: In vitro apoptosis of late-passage MEFs in response to chemotherapy was analyzed in the presence and absence of TSA. Late-passage MEFs resistant to chemotherapeutic drugs are sensitized in the presence of TSA. This effect is directly attributable to the reexpression of Fas in that late-passage cells transfected with the DN-Fas construct are protected from killing even in the presence of TSA. **B:** The median mouse of five of each experimental group receiving the designated treatment on day 10, 13, and 16 post-tumor injection. **C:** Time course of tumor growth. Arrows represent when treatment was administered. On day 28, one mouse receiving TSA treatment died, of undetermined cause.

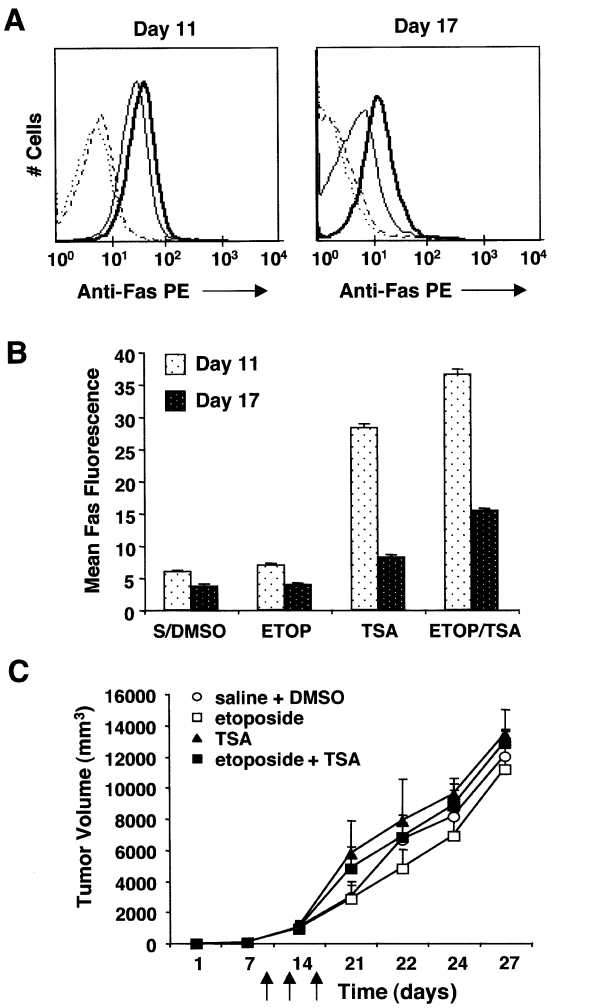

Figure 6. Epigenetic restoration of Fas in established tumors and chemosensitivity

A: In vivo tumor Fas levels. Tumor cells harvested from a parallel group of treated mice 12 hr following the first treatment (day 11) and 24 hr following the third treatment (day 17). Flow cytometry data displayed from the median tumor (of five treated per group). Dotted line, saline/DMSO treated; dashed line, etoposide treated; solid line, TSA-treated; bold solid line, etoposide plus TSA treated. Only one mouse of five receiving the combination of etoposide plus TSA still had tumor cells to recover on day 17 for analysis of Fas/MHC I expression. **B:** Histogram summary of in vivo Fas levels of tumors isolated from above treated mice. Tumor cells isolated from mice receiving the combination of etoposide plus TSA demonstrate the highest levels of Fas. **C:** Growth of DN-Fas-transfected tumors over time in SCID mice receiving the above treatments on day 10, 13, and 16 post-tumor injection as indicated by arrows. TSA enhancement of etoposide chemosensitivity observed in Figures 5B and 5C was directly attributable to Fas modification as demonstrated by the finding that tumor cells transfected with DN-Fas failed to regress or retard their growth.

passage MEFs to chemotherapy-induced killing, the question arose whether established, late-passage tumors in vivo would exhibit such sensitization. To test this hypothesis, one-half million late-passage p53 wild-type MEFs were injected into the hind flank of C57BL/6J SCID mice. Ten days postinjection, the mice were randomly assigned to one of four treatment groups: saline plus DMSO, etoposide, TSA, or etoposide plus TSA. Tumor-bearing mice were treated on day 10, 13, and 16. Tumors were measured over 1 month (Figures 5B and 5C). Additionally, in order to ascertain if the effectiveness of chemotherapeutic treatments correlated with in vivo modification of tumor Fas expression, a second group of mice was treated in parallel so that tumors could be analyzed at designated times following the treatments (Figures 6A and 6B).

Tumors derived from late-passage MEFs were chemotherapy resistant (Figures 5B and 5C). Tumors treated with saline plus DMSO grew at similar rates, achieving near identical volumes as tumors receiving etoposide chemotherapy. While TSA-treated mice grew smaller tumors, on average, they failed to differ statistically from mice receiving either saline plus DMSO or etoposide alone. However, mice receiving both TSA and etoposide dramatically responded to treatment by either arresting further growth or regressing altogether (Figures 5B and 5C).

Fas was indeed found to be repressed during the course of tumor progression, as evidenced by the significantly lower

levels of tumor Fas exhibited on day 17 when compared to levels on day 11 post-tumor implantation (Figures 6A and 6B). These in vivo data replicate what we previously had found in vitro (Maecker et al., 2000). Further, tumor Fas modification indeed correlated with therapeutic response (Figures 6A and 6B). Tumors harvested from mice receiving the combination TSA and chemotherapy treatment exhibit greatly elevated Fas levels over tumors isolated from mice receiving the other treatments. These results demonstrate that epigenetic factors are not only capable of regulating tumor growth, but they also influence therapeutic response.

To test whether Fas modification was in fact required for enhancement of chemotherapeutic responses by TSA, the chemotherapy experiments were repeated in C57BL/6J SCID mice with tumor cells retrovirally transfected with DN-Fas. Reversal of tumor Fas repression was found to be critical to therapeutic response in that late-passage tumor cells transfected with DN-Fas failed to arrest or regress when treated with the combination of TSA and etoposide (Figure 6C). These data demonstrate that Fas restoration is required for the restoration of tumor chemotherapeutic responsiveness.

Modification of MHC I levels fail to mediate restoration of chemotherapeutic responsiveness

One explanation for the enhanced therapeutic response observed in mice receiving the combination TSA/chemotherapy treatment is modification of tumor MHC I levels. To address this issue, we analyzed tumor MHC I levels from the treated mice in conjunction with Fas. However, unlike Fas, tumor MHC I levels failed to differ statistically, among any of the groups at any of the tumor harvest times, with the exception of 24 hr following the third treatment (Figure 7A). MHC I levels of tumors receiving the combination TSA and chemotherapy treatment were greater than levels of mice receiving the other treatments. One explanation is that selection occurred during the course of treatment, with cells expressing the lowest levels of MHC I having been eliminated earlier by NK cells during the first or second round of treatment. In support of this, most of the tumor cells isolated on day 17 from mice receiving the combination treatment were dead (as evidenced by staining with a vital dye 7-ADD; data not shown) and excluded from Fas/MHC I analysis.

In order to delineate the effects of TSA and chemotherapy upon MHC I modulation apart from confounding effects resulting from effector-mediated tumor elimination that occur in vivo, analysis of the mouse surface MHC I proteins were conducted in vitro (Figures 7B and 7C). Primary p53 wild-type MEFs as well as E1A/Ras-transformed MEFs were treated for 24 hr with saline plus DMSO, etoposide, TSA, and the combination of etoposide and TSA. Surface MHC I levels of the H-2K^b (Figure 7B) and H-2D^b (Figure 7C) proteins were analyzed by FACS. Consistent with published work (Ishido et al., 2000; Johnsen et al., 1999; Maudsley and Pound, 1991), transformation resulted in a surface downmodulation of both MHC I proteins. Also in line with reported data (Ishido et al., 2000; Johnsen et al., 1999), surface MHC I levels decreased with increasing passage in transformed cells promoting a role of MHC I downmodulation as a means of enhancing tumor immune escape. However, although transformation and increasing passage both modulated surface MHC I levels, chemotherapy and/or TSA treatment did not. These findings support the hypothesis that modulation of tumor Fas levels, and not surface MHC I, is responsible for the enhancement of chemotherapeutic responsiveness.

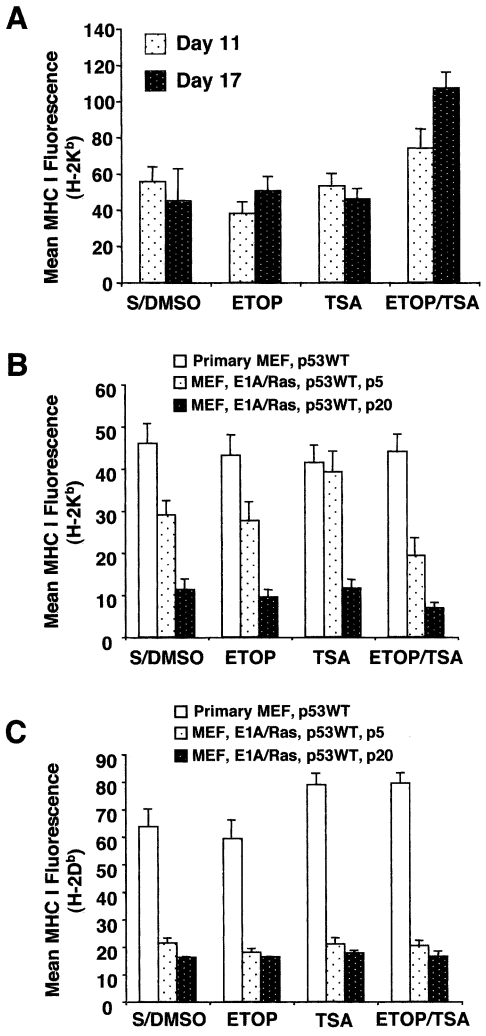

Figure 7. MHC I levels do not change in response to chemotherapy, TSA, or the combination chemotherapy plus TSA

A: Histogram summary of in vivo MHC I (H-2K^b) tumor levels from treated mice. **B:** Histogram summary of in vitro flow cytometry data of surface MHC I (H-2K^b) levels of primary and transformed p53 wild-type MEFs 24 hr following treatment with saline plus DMSO, etoposide, TSA, and etoposide plus TSA. **C:** Histogram summary of in vitro flow cytometry data of surface MHC I (H-2D^b) levels of primary and transformed MEFs. Chemotherapeutic treatment and/or TSA exposure fails to affect surface levels of MHC I proteins.

NK cells mediate chemotherapeutic responsiveness

To determine whether NK effector function was essential for the restoration of chemotherapeutic response, NK-deficient SCID beige mice bearing late-passage tumors were treated with saline plus DMSO, etoposide, TSA, or the combination of TSA and etoposide. None of these groups differed in tumor size (Figure 8A). These data suggest that although tumors from mice receiving the combination of TSA plus etoposide treatment elevated their surface levels of Fas in vivo (Figures 6A and 6B), in the absence of FasL-expressing NK effectors, their apoptotic programs fail to be engaged. Thus, NK cell function is required for the Fas-mediated restoration of chemotherapeutic responsiveness of these tumors.

To address the possible issues of strain background upon the chemotherapeutic effect, NK tumoricidal activity was mea-

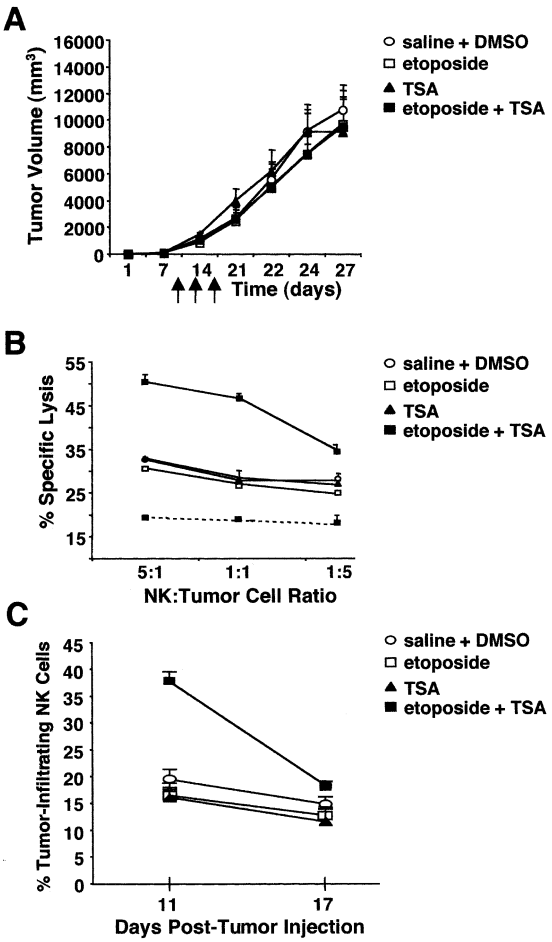

Figure 8. NK cells are required for Fas-mediated sensitization of established tumors to chemotherapy and immune clearance

A: Time course of tumor growth of late-passage MEFs in SCID beige mice (NK-deficient). Treatment was administered as on day 10, 13, and 16 post-tumor injection as indicated by arrows. NK cell function was found to be critical for TSA enhancement of etoposide chemosensitivity in that SCID beige mice receiving the combination of TSA and etoposide failed to regress or slow tumor growth. **B:** NK tumoricidal activity. TSA restoration of tumor Fas expression in etoposide-treated tumor cells was found to enhance NK-mediated cell death that was not observed in tumor cells treated with etoposide alone or in tumor cells transfected with DN-Fas receiving both TSA and etoposide. The solid lines represent tumor cell lysis of late-passage MEFs transfected with empty vector, whereas the dashed lines represent tumor cell lysis of late-passage MEFs transfected with DN-Fas. **C:** Percentage of tumor infiltrating NK cells in vivo (from Figures 6A and 6B, treated mice). Tumors isolated from mice receiving the designated treatments were co-stained for CD45 (common leukocyte antigen) and the Pan-NK cell antibody (DX5) to calculate the percentage of tumor-infiltrating NK cells. Only tumors isolated from mice receiving the combination of etoposide plus TSA treatment exhibited increased NK cell infiltration (only one mouse of five receiving the combination of etoposide plus TSA still had tumor cells on day 17 for analysis).

sured directly (Figure 8B). NK cells harvested from C57BL/6J SCID mice were utilized as effector cells in chromium release assays. Late-passage MEF cells (retrovirally transfected with either empty vector or DN-Fas) treated with saline plus DMSO, etoposide, TSA, or etoposide plus TSA were used as targets. NK-mediated killing was substantially enhanced in tumor cells receiving the combination of etoposide plus TSA. This enhanced death was attributable to reversal of tumor Fas repression as tumor cells transfected with DN-Fas were protected from NK-mediated elimination. Thus, in tumor cells with repressed Fas, epigenetic modulation of Fas restored chemosensitivity by engaging NK-mediated clearance.

Lastly, to confirm that NK cells were indeed infiltrating into the tumors of treated mice to exert potential tumoricidal effects, tumors isolated from mice receiving the various chemotherapeutic regimens (Figures 5B, 5C, 6A, and 6B) were harvested and stained for their presence of NK cells (Figure 8C). Surprisingly, elevated NK infiltration was only found to be present in tumors isolated from mice receiving the combination of chemotherapy plus TSA. This finding, coupled with the in vitro finding that NK tumoricidal activity is only enhanced against cells receiving the combination therapy, raises the possibility that the combination of TSA and chemotherapeutic treatment provides additional costimulatory signals to the NK cells that are not provided by the other treatments alone. Our data further emphasize that the synergy between tumor Fas and infiltrating NK cells is able to enhance chemosensitivity and modulate tumor growth.

Discussion

This work, although based on a highly idealized p53 model system, provides a mechanism for the chemotherapeutic resistance observed in p53 wild-type tumors and suggests modulation of Fas expression as a therapeutic target. Our data suggest that HDAC inhibitors, such as TSA, potentially increase the efficacy of chemotherapeutic agents by restoring apoptotic effector arms silenced during tumor progression.

During malignant progression, epigenetic changes in the expression of p53's downstream target genes may indeed occur before p53 inactivation. Specifically, Fas repression may be an early event in some tumors, resulting in the malignant growth of tumor cells with wild-type p53. Interestingly, oncogenic H-Ras has recently been found to be capable of suppressing Fas mRNA through hypermethylation of the Fas gene in vitro (Peli et al., 1999). Oncogenic activation could thus potentially predispose a cell to malignant transformation by silencing Fas and hence disabling an effector arm of p53's apoptotic program (Johnstone et al., 2002).

Elegant studies have suggested that the Fas pathway can signal either proliferation or apoptosis, depending upon the developmental and activation state of lymphocytes (Siegel et al., 2000). Freshly isolated human T cells in vitro have been reported to proliferate in response to activating Fas antibodies and anti-CD3 stimulation (Alderson et al., 1993). These findings raise the possibility that tumor downregulation of Fas may not only suppress the tumor's apoptotic sensitivity, but may also hinder the expansion of immune effectors. The Fas apoptotic effector-dependent control of tumor growth is supported by our previous studies in which we showed that FasL was required for Fas-mediated killing (Maecker et al., 2000).

Although the Fas-deficient *lpr* and FasL-deficient *gld* systems have been used to evaluate the role of Fas/FasL in apoptosis, the *lpr* defect is leaky both at the mRNA and protein levels (Adachi et al., 1996; Booker et al., 1998). Notably, *lpr* cells express significant amounts of Fas in response to genotoxic stress such as γ-irradiation (Booker et al., 1998). Therefore, we reasoned that genetic and/or epigenetic manipulation of Fas in the isogenic MEF cells would provide a much "cleaner" system to address the role of tumor Fas in chemosensitivity and tumor

control by the innate immune system. An alternative experiment would be the implantation of the MEF tumors in *gld* mice lacking FasL. However, this approach would be difficult to interpret due to their different genetic backgrounds. To overcome these problems, we chose to manipulate tumor Fas both genetically with wild-type or dominant-negative Fas and epigenetically with TSA, and to address the role of NK cell function using the NK-positive SCID and NK-deficient SCID beige mice.

The role of Fas/FasL in DNA damage-induced apoptosis has been established in a variety of cell types including many tumor cells (Bennett et al., 1998; Eichhorst et al., 2001; Friesen et al., 1996; Houghton et al., 1997; Kolbus et al., 2000). Cells derived from *lpr* mice such as MEFs in vitro and thymocytes in vivo exhibit resistance to apoptosis induced by DNA damage (Bennett et al., 1998; Eichhorst et al., 2001). However, Newton and Strasser (2000) showed that normal lymphocytes from *lpr* mice were sensitive in vitro to γ-irradiation and DNA-damaging agents in a p53-dependent manner. Such reported discrepancies are likely due to differences in cell types and/or in cellular microenvironments found in vitro versus in vivo. For example, the p53-dependent bax expression may potentially account for the apoptotic sensitivity of the *lpr* lymphocytes observed by Newton and Strasser (2000).

HDAC inhibitors, such as TSA, potentially have a multitude of cellular effects. However, studies have found that only a small portion (about 2%) of genes alter their expression patterns in response to HDAC inhibitors (Marks et al., 2000). In this study, we also analyzed class I MHC (H-2D^b and H-2K^b) expression after TSA treatment both in vivo and in vitro. Our results indicate that TSA has little effect on the expression of MHC I molecules, despite the fact that MHC I molecules are further reduced in late-passage cells. In addition, genetic manipulation of Fas expression with wild-type and dominant-negative Fas lends further support for the role of the Fas apoptotic effector arm in tumor chemosensitivity and tumor control in vivo.

It has been reported that many tumor cells are sensitive in vitro to killing by NK cells (Bradley et al., 1998; Long et al., 1997; Ravetch and Lanier, 2000; Tanaka et al., 1995). Upon activation, NK cells elevate their surface expression of FasL and initiate apoptosis in their target via the Fas pathway (Montel et al., 1995). Further, NK cell depletion has been found to promote metastatic spread (Zheng et al., 1996). Most recently, Fas has been shown to play a central role in the rejection of tumors by NK cells (Screpanti et al., 2001). We report here that epigenetic regulation of Fas determines tumor growth as well as chemotherapeutic resistance of established tumors. Both of these processes are dependent upon NK cells.

The differences between in vivo and in vitro phenomena can be attributed, at least in part, to differences in the tumor microenvironment. Tumor cells are in direct cell-cell contact with each other when cultured in vitro, and are limited by contact inhibition. Thus, expression of Fas (with the concomitant expression of FasL) is sufficient in vitro for etoposide-induced apoptosis. However, tumor cells in vivo are not necessarily regulated in the same manner. Rather, a synergistic role of tumor Fas and host NK cells is needed to achieve chemosensitivity in vivo. This observation underscores the importance of the tumor microenvironment.

In summary, our data illustrate the importance of Fas and innate immunity in modulating tumor growth kinetics and chemosensitivity in vivo. Our study implies that upregulation of tumor Fas coupled with engagement of host immunity can provide a viable approach for efficient tumor control. In the future, it will be important to determine if the chemosensitivity of human spontaneous tumors is also influenced by Fas and NK cells.

Experimental procedures

Cell lines

Cell lines were generously provided as follows: mouse embryonic fibroblasts (MEFs) on a C57BL/6J background transformed with E1A/Ha-Ras, either wild-type or null for p53 (Scott Lowe, MIT, Cambridge, MA); amphotropic Phoenix retrovirus-producing cells (Gary Nolan, Stanford University, CA); amphotropic Phoenix cells lines stably transfected with the mouse ecotropic receptor (J. Norton, University of Pennsylvania, PA); and ecotropic Phoenix retrovirus-producing cells (Gary Nolan, Stanford University, CA). The E1A/ras-transformed MEF cells were originated from clonal populations. MEF cells at passage 5 or passage 20 were generated by consecutive in vitro culture without any selection (Maecker et al., 2000). All cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics (GIBCO-BRL, Grand Island, NY).

Plasmid constructs

The mouse Fas and DN-Fas constructs were a gift of Robert Wiltrout, NCI. The p75 construct was a gift of Moses Chao, New York University School of Medicine. These constructs were subcloned into the EcoRI site of the pBabe puro retroviral vector backbone (Morgenstern and Land, 1990a, 1990b) and were confirmed by DNA sequence analysis.

Retroviral infection of MEF cells

Retroviral production and infection were modified from existing protocols (Baker et al., 1992; Pear et al., 1993). Briefly, amphotropic Phoenix cells (Pear et al., 1993) were transfected with the mouse ecotropic viral receptor (Baker et al., 1992) and stable clones were generated. These amphotropic Phoenix cells expressing the mouse ecotropic receptor were then used in coculture with ecotropic viral-producing cells to generate high-titer retrovirus according to the published protocol (Pear et al., 1993). Viral supernatant was collected from these cocultures, supplemented with 8 μg/ml polybrene, and added undiluted to MEF cells plated into 6-well plates. These plates were then centrifuged at room temperature at 300 × g for 45 min. Cells were placed into a 32°C incubator for 11 hr to allow viral infection. This procedure was repeated every 12 hr with fresh virus for two additional times. Following the third infection, remaining virus was removed and replaced with fresh medium. Cells were then allowed to recover for 12 hr in a 37°C incubator prior to experimental analysis. The pool of retrovirally transfected cells was used in subsequent experiments.

Cell treatments

MEF cells transfected with various retroviral constructs (see above) were irradiated with 600 cGy at a dose rate of 281 cGy/min from a ^{137}Cs source in the presence and absence of 25 nM TSA. Apoptosis (see below) was measured 6, 12, and 24 hr following irradiation. Chemotherapeutically treated MEFs in vitro received 2 μg/ml cisplatin, etoposide, or doxorubicin dissolved in saline plus 10% DMSO, and apoptosis was measured at 18 and 36 hr following drug administration. In vitro proliferation assays were conducted by plating 10,000 MEF cells/100 μl/well in triplicate on a 96-well plate. Cells were allowed 1 hr to adhere prior to loading with one μCi/well ^{3}H-thymidine in 100 μl medium. Following 18 hr, cells were harvested and read on a β-scintillation counter.

Flow cytometry analysis

Surface levels of Fas and FasL were measured by incubating 1 × 10^6 cells on ice for 30 min with 1 μg/ml of biotinylated anti-Fas Ab, Jo2 (BD Pharmingen, San Diego, CA), anti-FasL Ab, Kay-10 (BD Pharmingen), or an irrelevant isotype control. Cells were subsequently washed and incubated for an additional 30 min with Streptavidin-PE (BD Immunocytometry Systems, San Jose, CA). Following a final wash, cells were analyzed on a FACSCalibur flow cytometer (BD Immunocytometry Systems). All experiments were conducted a minimum of three times.

Intracellular Fas staining of MEF cell lines retrovirally transfected with either empty vector or the DN-Fas construct was conducted according to the previously published method of Bennett et al. (1998).

Staining for MHC alleles was done using antibodies to H-2K^b and H-2D^b (0.5 μg each) along with 7-AAD to exclude dead cells (all reagents from BD Pharmingen). Antibody staining was done for 30 min on ice, followed by washing and addition of 7-AAD as recommended by the supplier.

Apoptosis assays

Apoptosis was determined by staining cells with the APO-DIRECT Kit (BD Pharmingen) followed by analysis on a FACSCalibur flow cytometer (BD Immunocytometry Systems). The APO-DIRECT assay detects DNA strand breaks. All experiments were repeated three times.

Analysis of Fas and FasL expression in late-passage cells

Genomic sequencing of Fas and FasL was conducted to confirm that no mutations occurred in these genes in the late-passage wild-type MEFs. Protein surface expression and mRNA expression were analyzed by flow cytometry and RNase protection assay (BD Pharmingen). DNA acetylation status was analyzed by comparing mRNA levels of Fas and FasL treated in the presence and absence of TSA as well as by analysis of acetylated Histone H3 levels of nuclear extracts of cells treated in the presence and absence of TSA. mRNA was isolated from late-passage cells treated with the HDAC inhibitor TSA (Sigma Chemical Company, St. Louis, MO), administered at 25 ng/ml for 24 hr. Acetyl-Histone H3 levels were determined from Western blots of nuclear extracts of cells either untreated or treated with TSA (50 ng/ml) for 18 hr. Five micrograms of nuclear extract were loaded per well onto an Invitrogen 10% Tris-Bis gel with MES running buffer and run according to the manufacturer's instruction. Protein was transferred onto nitrocellulose (Invitrogen, Carlsbad, CA) and blocked overnight at 4°C in Tris-buffered saline containing Tween-20 with 5% (w/v) nonfat dry milk. Following the overnight incubation, the membrane was incubated with either anti-Acetyl-Histone H2B or anti-Acetyl-Histone H3 pAb (Cell Signaling Technology, Beverly, MA) at a dilution of 1:1000. The secondary antibody employed was an anti-rabbit IgG HRP (New England Biolabs, Beverly, MA) at a dilution of 1:5000. The membrane was reprobed with an anti-actin antibody (ICN, Carlsbad, CA) at a dilution of 1:20,000 followed by secondary incubation with anti-mouse IgG HRP (BD Pharmingen) used at a dilution of 1:20,000 to ensure equal loading. The membrane was then processed with ECL reagent and film-scanned.

Mouse experiments

The ability of Fas expression to regulate tumor growth was analyzed with 0.5×10^6 MEF cells (either retrovirally transfected with the constructs described above or treated with 25 nM TSA for 24 hr). These cells were suspended in 100 μl saline and injected s.c. into the right hind flank of 8-week-old C57BL/6J SCID mice (Jackson Labs, Bar Harbor, ME). Five mice were injected per treatment group. Tumor volume was calculated by multiplying length by width by height caliper measurements. At day 28, tumors were harvested and weighed. In vivo experiments were conducted two additional times, once utilizing a different background strain of mice (Balb/c SCID) as well as differing the starting inoculation of tumor cells (1×10^6 cells/mouse; 2×10^6 cells/mouse) to confirm that results observed were not due to the background strain of mice chosen or the starting tumor inoculum. Results presented are of the last of three experiments conducted, although all data replicated the findings presented.

The immune contribution to tumor growth was assessed by measuring the growth of the MEF tumors in 8-week-old CB.17 SCID beige mice (Taconic Farms, Germantown, NY). 0.5×10^6 MEF cells (p53 wild-type early- and late-passage as well as p53 null early-passage) were injected s.c. into the right hind flank. Five mice were injected per treatment group, and tumor growth was monitored as above over 1 month.

The ability of TSA to sensitize established tumors to chemotherapy was analyzed by injecting late-passage p53 wild-type MEFs into the hind flank of C57BL/6J SCID mice. Ten days following tumor injection, the mice were randomly assigned to one of four treatment groups (each group containing five mice): saline plus DMSO, etoposide, TSA, and etoposide plus TSA. Doses of TSA and etoposide administered were 1 mg/kg body weight and 12.5 mg/kg body weight, respectively. Injections were given intratumor on days 10, 13, and 16 post-tumor injection in a final volume of 0.1 ml. Tumor growth was monitored as above over 1 month. In order to correlate potential therapeutic effects with tumor Fas and/or MHC I levels, a parallel group of mice (five per treatment group) were injected. Twelve hours after the first treatment as well as 24 hr following the third treatment, tumors were harvested from the treated mice for analysis of surface Fas and MHC I (H-2K^b) levels by FACS analysis. Infiltrating immune cells were identified by staining with CD45 FITC, and NK cells were further classified by staining with the Pan-NK antibody, DX5 PE (BD Pharmingen).

To confirm the importance of Fas to the enhancement of chemosensitivity observed with TSA, late-passage MEF cells retrovirally transfected with DN-Fas were injected into the hind flank of C57BL/6J SCID mice. Ten days following tumor injection, the mice were randomly assigned to one of four treatment groups (each group containing five mice): saline plus DMSO, etoposide, TSA, and etoposide plus TSA, as described above. Tumor growth was monitored over 1 month.

To test the importance of NK cell activity in the enhancement of chemosensitivity observed with TSA, late-passage p53 wild-type MEFs were injected into the hind flank of CB.17 SCID beige mice (lacking NK cells). Again, 10 days following tumor injection, the mice were randomly assigned to one of the four treatment groups (each group containing five mice), and treatment was conducted as described above. Tumor growth was monitored over 1 month.

^{51}Cr release assays

Splenocytes were harvested from C57BL/6J SCID mice, depleted of erythrocytes by ammonium chloride lysis, and cultured (2.5×10^6/ml) for 4 days in 1000 U/ml of human rIL-2 (PeproTech, Rocky Hill, NJ) to generate NK effectors. Twelve hours prior to the initiation of the assay, 5000 tumor cells were plated per well onto flat-bottom 96-well plates and allowed to adhere overnight. The following day, tumor cells were exposed for 6 hr to one of the four treatments: saline plus DMSO, etoposide (2 μg/ml), TSA (25 nM), or etoposide plus TSA (all conditions done in triplicate). Following treatment, tumor cells were washed and loaded with 25 μl of medium containing 1 mCi/ml Na[^{51}Cr]O_4 for 1.5 hr. After loading, cells were washed four times and NK effectors were added directly to the wells. The NK effectors were incubated with the tumor targets for 10 hr prior to harvesting. Specific lysis was calculated according to the formula: % lysis = [(experimental release − spontaneous release) / (maximum release − spontaneous release)] × 100.

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