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Pegylated IFN-α sensitizes melanoma cells to chemotherapy and causes premature senescence in endothelial cells by IRF-1-mediated signaling

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Pegylated interferon-α2b (pIFN-α) is an integral part of the drug regimen currently employed against melanoma. Interferon regulatory factor-1 (IRF-1) has an important role in the transcriptional regulation of the IFN response, cell cycle and apoptosis. We have studied pIFN-α-induced responses when combined with the chemotherapy agent, vinblastine (VBL), in tumor and endothelial cell lines and the connection to IRF-1 signaling. Levels of IRF-1/IRF-2 protein expression were found to be decreased in tumor *versus* normal tissues. pIFN-α induced IRF-1 signaling in human melanoma (M14) and endothelial (EA.hy926) cells and enhanced cell death when combined with VBL. Upon combined IFN-α and VBL treatment, p21 expression, poly (ADP-ribose) polymerase cleavage and activated Bak levels were increased in M14 cells. An increase in p21 and cyclin D1 expression occurred in EA.hy926 cells after 6 h of treatment with pIFN-α, which dissipated by 24 h. This biphasic response, characteristic of cellular senescence, was more pronounced upon combined treatment. Exposure of the EA.hy926 cells to pIFN-α was associated with an enlarged, multinucleated, β-galactosidase-positive senescent phenotype. The overall therapeutic mechanism of IFN-α combined with chemotherapy may be due to both direct tumor cell death via IRF-1 signaling and by premature senescence of endothelial cells and subsequent effects on angiogenesis in the tumor microenvironment.
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The abysmal median survival for patients with metastatic melanoma ranges from 6 to 9 months, causing it to be one of the most aggressive human cancers. The p53 protein acts as a checkpoint in the cell cycle, either preventing or initiating programmed cell death. A p53 mutation remains the most common genetic change identified in human neoplasia[1] with melanoma exhibiting one of the highest rates of this oncogenic event.[2]

Interferon regulatory factor-1 and -2 (IRF-1 and -2) are two structurally related members of the IRF family of transcription factors. Although IRF-1 expression has been associated with tumor suppressor activity, IRF-2 expression has an oncogenic effect.[3–5] IRF-1 acts as a tumor suppressor very similar in function to p53 and regulates the expression of most of the p53 target genes.[6] It is critical to cell cycle and induction of apoptosis in response to stress signals.[7,8] The expression of several genes involved in cell-cycle regulation and apoptosis such as $p21^{WAF/cip}$,[8] $p27^{Kip1}$,[9] lysyloxidase,[10] caspase-1,[11] cyclin D1 and CDK4,[12] survivin[13] and Noxa[14] are transcriptionally regulated by IRF-1. By modulating the expression of VEGF[15] and MMP-9,[16] IRF-1 has also been shown to inhibit angiogenesis. Thus, the induction of apoptosis or modification of angiogenic controls by normal IRF-1-driven processes provide alternative pathways influential to tumor growth control. However, similar to mutant p53, this growth control may be evaded by mutant tumor cells and/or the tumor microenvironment to allow tumor progression.

The combination of cytotoxic chemotherapy with active biological agents such as IL-2 and interferon-α (IFN-α), termed biochemotherapy, has generated a great deal of interest over the last several years.[17,18] Adjuvant IFN-α treatment to prevent the recurrence of melanoma has been encouraging, yet is likely far from optimized. An additional important mechanism of the IFN-α-mediated antitumor activity appears to rely on the interference with tumor-mediated angiogenesis.[19,20] There are a number of clinical studies that focus on the use of IFN-α as an antiangiogenic agent. However, the true effects of IFN-α in combination with various standard chemotherapy regimens on the endothelium and/or the viability of tumor cells are not clearly understood. Interestingly, IFN-α (a standard of care in melanoma) is one of the agents that induces IRF-1, activating its tumor suppressor function.

In this study, using human melanoma and endothelial cell lines, we have observed that treatment with a clinically used form of pegylated IFN-α treatment in combination with the chemotherapeutic agent, vinblastine (VBL), induces cell death via IRF-1-mediated signaling in melanoma cells and concurrently induces premature senescence in endothelial cells. The induction of senescence may be a novel explanation for

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Abbreviations: pIFN-α, pegylated interferon-α2b; IRF-1, interferon regulatory factor-1; IRF-2, interferon regulatory factor-2; PARP, poly (ADP-ribose) polymerase
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the antiangiogenic effects that have been indicated with the clinical use of IFN-α.

Results

Decrease in the IRF-1/IRF-2 ratio in tumor tissues. The expression of IRF-1 and -2 can be altered by a host of factors/agents and thus they may serve as possible targets for the treatment of cancer. They have been most well studied in their role as transcription factors active in type I (IFN-α/β) and type II (IFN-γ) signal transduction pathways. There are several reports indicating that a decrease in the ratio of IRF-1/IRF-2 expression is prevalent in most tumor tissue types. In an analysis to study the protein expression profiles of IRF-1 and -2, we were able to show that although most normal and tumor tissues expressed IRF-1 and -2 the ratio of IRF-1 to IRF-2 dropped dramatically in the corresponding tumor tissue samples (Figure 1). This information builds a case for the relevance of IRF-1 and 2 in cancer biology and treatment.

Enhanced sensitivity of M14 melanoma cells to combined treatment with IFN-α and VBL. To determine the cytotoxicity of VBL and pIFN-α, the cells were incubated with varying concentrations of the drugs at 37°C for 72 h. Tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) cell proliferation assay was used to estimate the concentration required to inhibit cell growth by 50% (IC_{50}) for each drug. The IC_{50} values of VBL and pIFN-α were approximately 1 nM and 0.5 μg/ml, respectively, for M14 cells (Figure 2a and b). Cells were treated with constant concentration of pIFN-α (0.5 μg/ml) and the indicated concentration of VBL (0–10^4 pM) for 72 h. A greater than additive cell growth inhibition by MTT assay (70%) was observed when the cells were treated with a combination of 1 nM (10^3 pM) VBL and 0.5 μg/ml of pegylated IFN-α (Figure 2c).

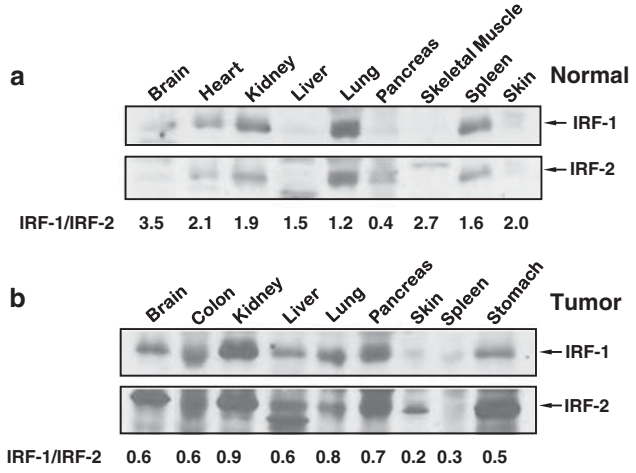

Figure 1 Western analysis of IRF-1 and -2 expression in normal human and tumor tissues. Expression profile of IRF-1 and -2 in normal human tissues (**a**) or in human tumor samples (**b**) were analyzed by western immunoblotting (ProSci) and the IRF-1/IRF-2 ratio was quantified by densitometry using the Image J software

The effect of VBL and pegylated IFN-α on cell survival was also studied by clonogenic assay. Combination of VBL (1 nM) and pIFN-α (0.5 μg/ml) was 1.75-fold more toxic to the M14 cells than treatment with VBL (1 nM) alone. Treatment with half the concentration of VBL (0.5 nM) and pIFN-α (0.25 μg/ml) was as toxic as treatment with 1 nM of VBL alone (Figure 2d). These studies suggest that combination of pIFN-α and VBL may enhance the induction of cell death compared with either treatment alone.

Induction of IRF-1 is specific to IFN-α exposure and enhances cell death via IRF-1-mediated signaling in M14 melanoma cells. IRF-1 is a critical transcriptional regulator in the IFN signaling pathway.[5] Therefore, we investigated the expression of IRF-1 in response to pIFN-α and VBL in M14 melanoma cells. A dose of 30 nM for VBL was selected for treatment to ensure complete cell death. Cells were treated at the indicated time points with VBL (30 nM) or pIFN-α (0.5 μg/ml) alone and in combination at the same (30 nM VBL + 0.5 μg/ml pIFN-α) or at half the concentration of each agent (15 nM VBL + 0.25 μg/ml pIFN-α). IRF-1 was induced by 3–6 h and then decreased by 24 h in response to pIFN-α treatment. Interestingly, treatment with 0.25 or 0.5 μg/ml pIFN-α induced the same level of IRF-1. VBL treatment alone did not cause any noticeable induction of IRF-1. In addition, IRF-2 did not show any significant change in M14 cells after treatment with either VBL or pIFN-α (Figure 3a).

Subsequently, we looked at the induction of IRF-1 downstream targets and cell death in M14 melanoma cells, as assessed by poly (ADP-ribose) polymerase (PARP) cleavage, in response to pIFN-α and VBL individually or combined against M14 melanoma cells. Transcriptional induction of p21 is dependent on both p53 and IRF-1.[8] M14 cells are p53 defective and possibly evade cell death by downregulation of p21. We have observed induction of p21 in M14 melanoma cells by 6 h and subsequent downregulation by 24 h in response to pIFN-α but not VBL (Figure 3b). Except for a delay in the induction of p21 by 3 h, the pattern of p21 protein expression in response to pIFN-α was similar to the pattern of IRF-1 induction suggestive of its transcriptional regulation by IRF-1.

Bak is a proapoptotic member of the Bcl-2 family of proteins and induces cell death by undergoing activation and homo-oligomerization.[22,23] We observed upregulation of total Bak in M14 melanoma cells upon treatment with pIFN-α but not VBL. Further, immunoblotting for PARP revealed that PARP cleavage, an indicator of cell death, occurs only upon treatment with VBL. However, combined treatment of VBL and pIFN-α at half the normal concentration of each agent (VBL (15 nM) and pIFN-α (0.25 μg/ml)) caused the same level of PARP cleavage as treatment with 30 nM VBL alone or the combined treatment (VBL (30 nM) and pIFN-α (0.5 μg/ml)). Treatment with pIFN-α alone did not cause PARP cleavage even by 48 h (Figure 3b).

To determine whether combined treatment with pIFN-α and VBL results in elevated activation of Bak, the conformationally active form was immunoprecipitated under native conditions from M14 cells treated for 36 h with VBL (30 nM) or pIFN-α (0.5 μg/ml) alone and in combination (30 nM VBL + 0.5 pIFN-α μg/ml). For immunoprecipitation, we used rabbit anti-Bak (NT) antibody (Millipore, Temecula, CA, USA), which recognizes

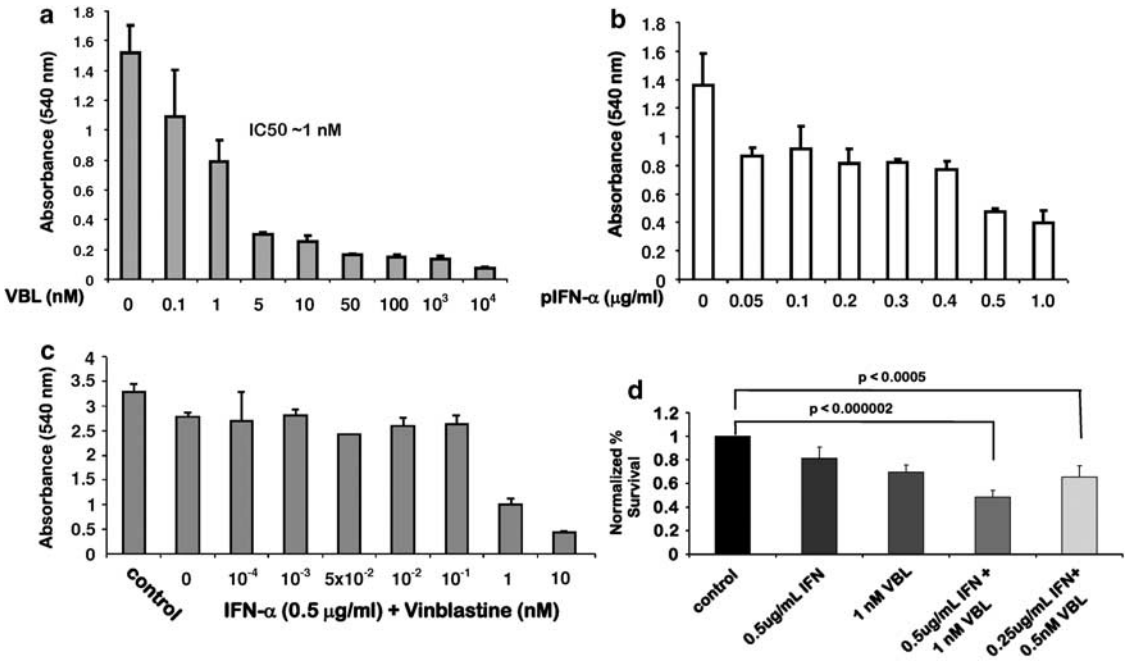

Figure 2 Enhancement of M14 cell sensitivity to VBL cytotoxicity by pegylated IFN-α. A total of 3000 cells per well in a 96-well plate were treated with indicated concentrations of VBL (**a**) or pegylated IFN-α2b (**b**) for 72 h and subjected to MTT cell viability assay. (**c**) Cells were treated with the pegylated IFN-α2b (0.5 mg/ml) in combination with the indicated concentrations of VBL (0–10 nM) for 72 h. Cell proliferation was estimated by MTT assay. (**d**) Cells (200 cells per well) were treated with the indicated concentration of drugs for 24 h. The cells were rinsed, and fresh medium was added, and after 2 weeks, colonies were stained with crystal violet, counted and the surviving fraction was calculated. Results in each of the above experiments are the means ± S.D. ($n = 6$). The *P*-values are shown

the conformationally active form of Bak. Cells treated with pegylated IFN-α had an increased level of inactive Bak (Supplementary Figure 1). Treatment with VBL caused inactive Bak to undergo a conformational change and increased levels of activated protein were detected. Combined treatment with pIFN-α and VBL induced a further accumulation of active Bak leading to an increase in the total amount of cell death (Figure 3c).

Using the technique of BH3 profiling developed by Letai and colleagues,[24,25] we observed that a buildup of inactive Bak in the mitochondria as a consequence of pIFN-α treatment made the M14 melanoma cells more sensitive to VBL-induced cell death (i.e., pIFN-α conditions or primes the cells to death signaling induced by other agents). The essence of this technique is to use isolated mitochondria, which contain inactive Bak, and induce cytochrome *c* (cyt *c*) release through the addition of tBid or Bid (BH3 peptide), which promotes Bak activation. Mitochondria isolated from M14 melanoma cells treated with 0.5 μg/ml pIFN-α, and subsequently incubated with an activator (BH3 peptide) of Bak, induced cyt *c* release in a dose-dependent manner (Figure 3d). In contrast, only low background levels of cyt *c* are released from the mitochondria of untreated cells under similar conditions (Figure 3d).

Evaluation of the response of EA.hy926 endothelial cells to VBL and/or pIFN-α. Using the MTT assay, EA.hy926 human endothelial cells were found to be relatively more sensitive to VBL than the M14 melanoma cells with an IC_{50} between 500 pM and 1 nM (Figure 4a). The IC_{50} for pIFN-α in these cells was ~0.5 μg/ml (Figure 4b). To test the combined effect of the drugs, cells were treated with a constant concentration of pIFN-α (0.5 μg/ml) and indicated concentration of VBL (0–500 pM) or indicated concentrations of VBL alone for 72 h. Cell growth inhibition measured by MTT assay was 40% greater with the combined treatment of 500 pM VBL and 0.5 μg/ml of pIFN-α in comparison with treatment with 500 pM VBL alone (Figure 4c). In subsequent clonogenic studies, cells were treated with the indicated concentration of pIFN-α and VBL alone and in combination, and the medium was replaced with fresh medium without the drugs after 24 h. In contrast to the results from the MTT assay, the clonogenicity of EA.hy926 cells was found to be markedly reduced after pIFN-α treatment alone. Combination of VBL (500 pM) and pIFN-α (0.5 μg/ml) had a similar effect to that of pIFN-α alone (Figure 4d).

Induction of IRF-1 in response to pIFN-α and synergistic response of IRF-1 downstream targets to VBL and pIFN-α in EA.hy926 endothelial cells. Induction of IRF-1 in EA.hy926 cells that have wild-type p53 was found to occur by 6 h in the presence of pIFN-α but not VBL (Figure 5a, top panel). Cyclin D1, like p21, is a downstream target of p53[26] and IRF-1.[12] We observed the induction of p21 and cyclin D1 by both VBL and IFN-α. Upregulation of p21 at 6 h after treatment was much greater when EA.hy926 endothelial cells were treated with VBL and pIFN-α. Half the normal concentration of the drugs in the combined treatment was sufficient to cause maximum induction of p21 (Figure 5a, upper middle panel). Upregulation of cyclin D1 at 6 h was followed by its downregulation by 24 h. However, the

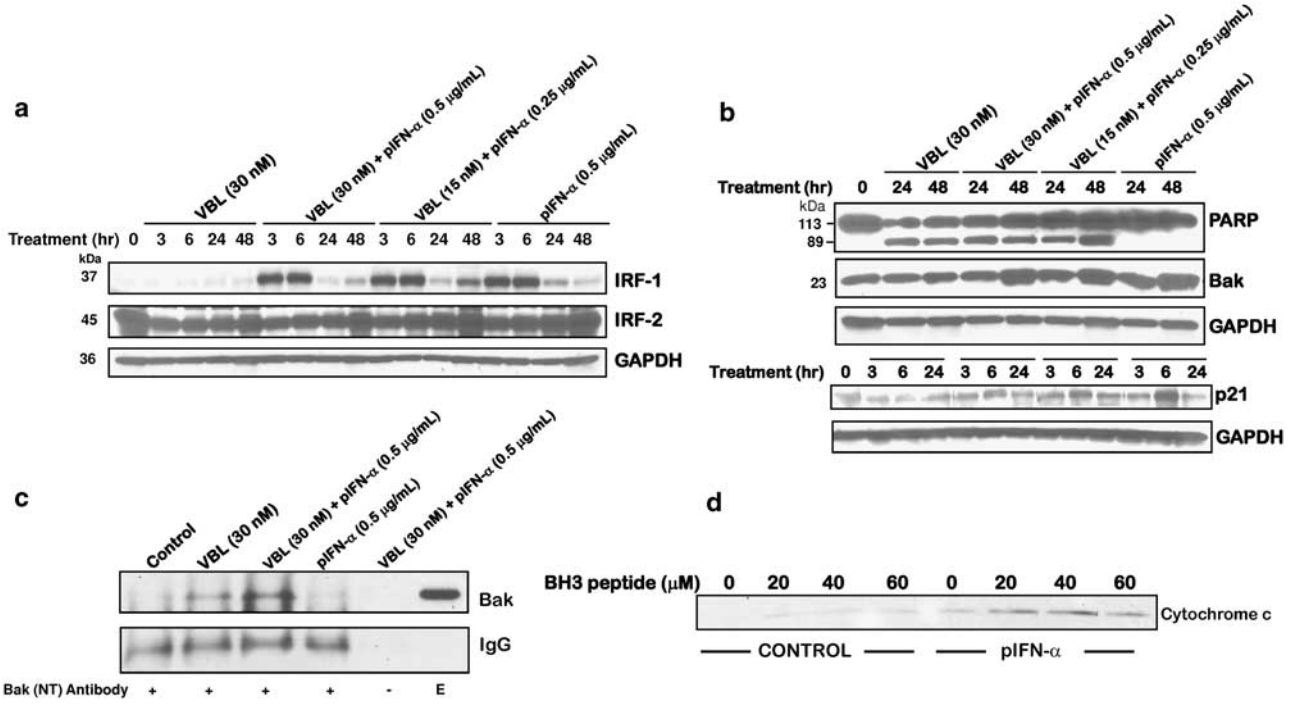

Figure 3 Molecular response of M14 melanoma cells to vinblastine (VBL) and IFN-α. (**a**) IRF-1, and not IRF-2, is induced specifically in response to pegylated IFN-α in M14 melanoma cells. M14 cells were treated for the indicated time with either VBL, pegylated IFN-α2b (pIFN-α), or a combination of both at the indicated concentrations. Cell extracts (50 mg per lane) were immunoblotted for IRF-1 or IRF-2. Immunoblotting for GAPDH served as the loading control. (**b**) Induction of IRF-1 downstream targets in response to IFN-α and combined effect of IFN-α and VBL on PARP-induced apoptosis in M14 cells. Cells were treated with the indicated drug concentrations of VBL or pegylated IFN-α (pIFN-α) and harvested at the given time points. Cell extracts were subjected to immunoblotting for PARP, Bak and p21. Immunoblotting for GAPDH served as the loading control. (**c**) Treatment of M14 cells with IFN-α and VBL causes induction and activation of Bak. M14 melanoma cells were untreated or treated with VBL (30 nM) or pIFN-α (0.5 mg/ml) alone or in combination (30 nM VBL + 0.5 pIFN-a mg/ml) for 36 h. Lysates were immunoprecipitated with anti-Bak-NT antibody for active form of Bak followed by immunoblotting for Bak. Precipitates prepared in the absence of the antibody (−) and whole-cell extract (E) were also examined as controls. (**d**) BH3 peptide-mediated cytochrome *c* (cyt *c*) release from the mitochondria of M14 melanoma cells. Mitochondria (25 μg per reaction) isolated from M14 cells without and after treatment with 0.5 mg/ml IFN-α for 48 h were incubated with the indicated concentrations of BH3 peptide for 30 min at room temperature. Supernatant was collected and cyt *c* release was observed by immunoblotting. DMSO vehicle was 2% final in all samples

combined treatment caused a greater reduction in cyclin D1 at 24 h (Figure 5a, lower middle panel). Although the level of Bak expression tended to vary in the presence of pIFN-α alone or in combination with VBL, it was completely absent at 48 h after treatment with VBL alone (Figure 5b). This suggests that IFN-α treatment increases and maintains the level of Bak expression to some extent, a function likely mediated through IRF-1 activity and also indicated by our results from western blotting described in Supplementary Figure 1 and the BH3 profiling results shown in Figure 3.

Exposure to IFN-α induces a senescent phenotype in EA.hy926 endothelial cells. We did not see substantial cell death even at 1 μM IFN-α treatment in the 72 h MTT assay (Figure 4b) but observed a significant reduction in cell survival in the clonogenic studies (Figure 4d). These perplexing results led us to inspect the doubling time of the cells after treatment with pIFN-α. Interestingly, although the control cells doubled in 0.9167 days (about 24 h), the pIFN-α-treated cells failed to increase in total number (Figure 6a). This loss of the proliferative capacity of EA.hy926 cells was subsequently observed to be at least partially caused by pIFN-α-induced senescence. We observed that upon exposure to pIFN-α, cells became enlarged, flattened, irregularly shaped and significantly more cells expressed SA β-gal +, suggesting a senescent phenotype (Figure 6b, middle and lower panel) as compared with untreated cell cultures (Figure 6b, upper panel).

To observe the effect of pIFN-α on the morphology of EA.hy926 endothelial cells over time, 1×10^5 cells were treated for 1, 2 and 6 days with 0.25 and 0.5 μg/ml of pIFN-α and observed at × 200 magnification. Cells treated with IFN-α for 1 and 2 days were rinsed and grown in fresh medium until the sixth day. There were significantly more cells exhibiting a senescent phenotype in cultures treated continuously with 0.5 μg/ml of pIFN-α compared with cells treated with 0.25 μg/ml of pIFN-α (Supplementary Figure 2a). The cells were subsequently harvested and replated with fresh medium into new culture vessels for 2 weeks and again observed by phase-contrast microscopy. In order to prevent the cells from being over confluent, only one-fifth of the total cells in the untreated plate were replated. The senescent phenotype continued to persist in these freshly plated cells previously exposed to pIFN-α independent of total length of the initial exposure to pIFN-α (Supplementary Figure 2b).

Treatment with the microtubule inhibitor, VBL, causes the cells to arrest at G2/M. Cells tend to get rounded, start floating and eventually undergo cell death. In EA.hy926 cells, a similar

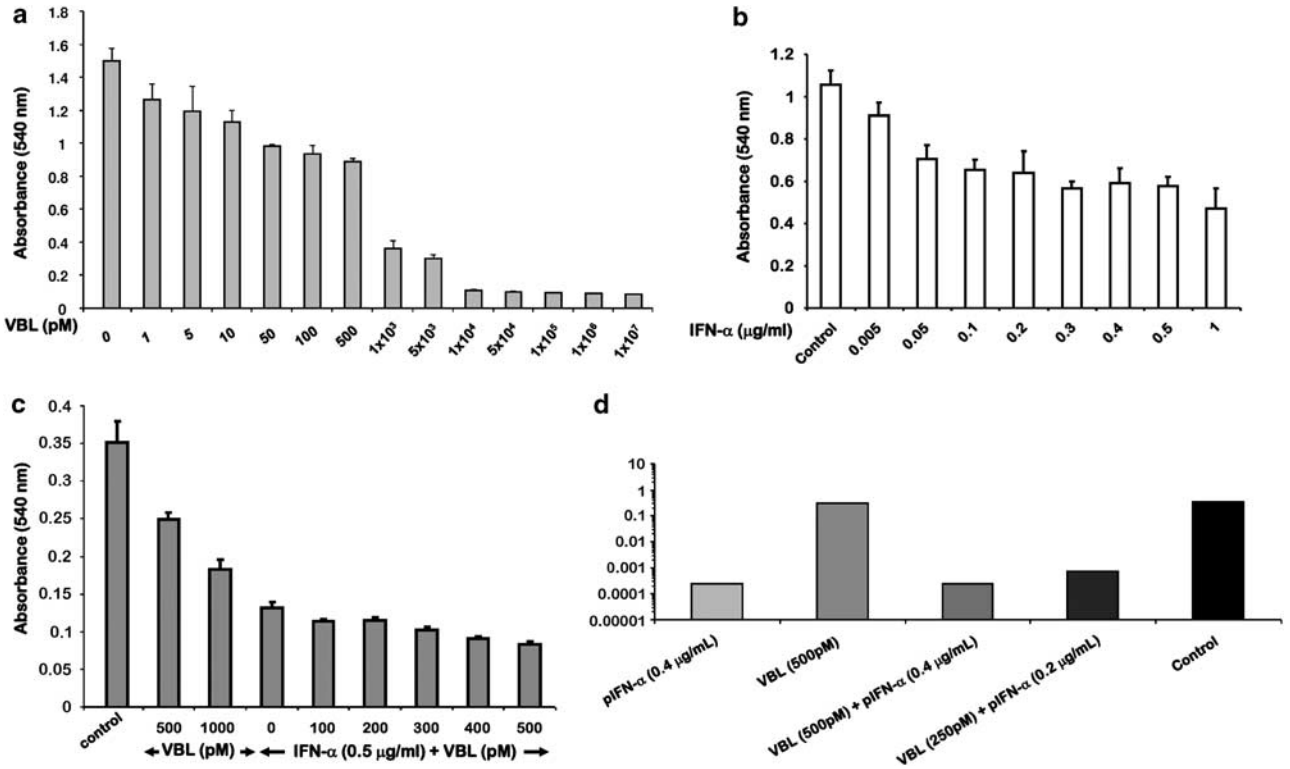

Figure 4 Sensitivity of EA.hy926 endothelial cells to VBL and pegylated IFN-α. A total of 3000 cells per well in a 96-well plate were treated with indicated concentrations of VBL (**a**) or pegylated IFN-a2b (**b**) for 72 h and subjected to MTT colorimetric assay. (**c**) Cells were treated with the pegylated IFN-a2b (0.5 mg/ml) in combination with the indicated concentrations of VBL (0–500 pM) for 72 h. Cell viability was estimated by MTT assay. The above three figures were representative of three independent experiments, the results of each are the means ± S.D. ($n = 6$). (**d**) A total of 2000 cells per well in a six-well plate were treated with the indicated concentration of drugs for 24 h. The cells were rinsed, fresh medium was added, and after 2 weeks, colonies were stained with crystal violet, counted and the surviving fraction was calculated

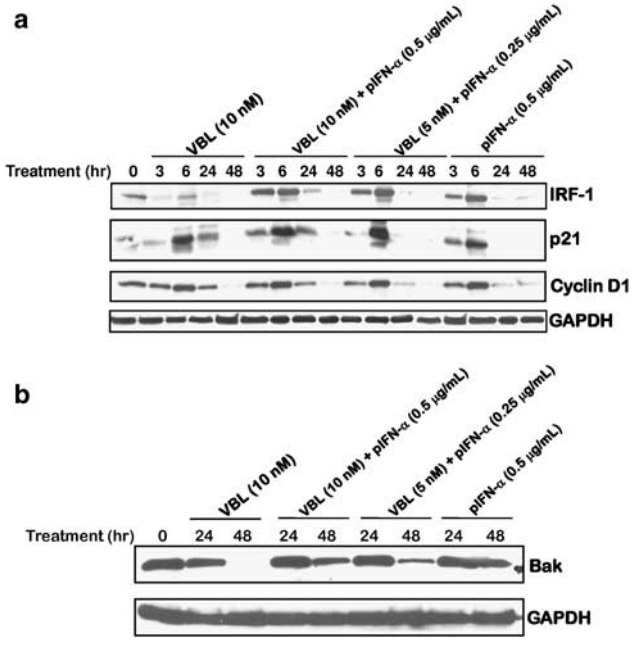

Figure 5 Induction of IRF-1 and its downstream targets in response to pIFN-α and VBL in EA.hy926 endothelial cells. Cells were treated with the indicated concentrations of VBL or pegylated IFN-α (pIFN-α) and harvested at the given time points. Cell extracts were subjected to immunoblotting for (**a**) IRF-1, p21 and cyclin D1, and (**b**) Bak. Immunoblotting for GAPDH served as the loading control

morphological change was observed by 48 h (Supplementary Figure 3a) upon treatment with 30 nM VBL or with a combination of 15 nM VBL and 0.25 μg/ml pIFN-α. A quantification of adherent *versus* nonadherent cells after treatment with pIFN-α and VBL is shown in Supplementary Figure 3b and represents data from three replicates per condition. As unsynchronized cells were used for plating, we did observe a percentage (11%) of nonadherent cells upon IFN-α treatment as compared with almost 50% of nonadherent cells after VBL or combined treatment with IFN-α and VBL.

Discussion

The use of pegylated IFN-α2b in monotherapy[27] and in combination with chemotherapy has been evaluated in several different types of cancers.[28,29] Pegylated IFN-α2b with its improved pharmacokinetic profile has been successfully introduced into melanoma therapy.[30] However, the most common adverse effects of neuropsychiatric, hematological and hepatic toxicity as a consequence of IFN therapy have not been able to be negated and therefore hold the full potential of this agent in check. Development of optimal protocols for the use of pegylated IFN-a2b to maximize benefit and minimize toxicity will allow for wider use with fewer adverse effects. A detailed understanding of the mechanism of action of IFN-α alone and in combination with chemotherapy is required to achieve this goal.

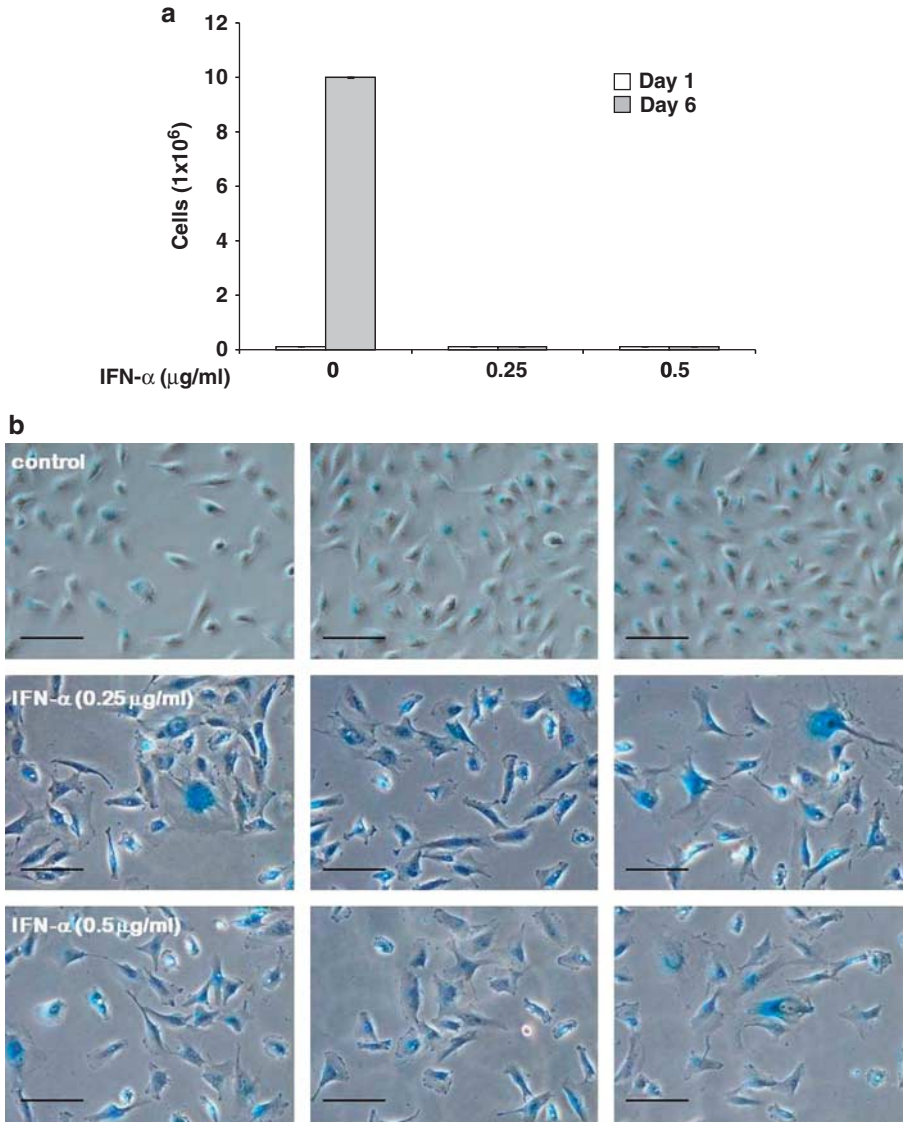

Figure 6 Induction of senescence-like growth arrest in EA.hy926 endothelial cells in response to pIFN-α. (**a**) In all, 5×10^4 EA.hy926 cells were plated onto 60 mm Petri plates and on the next day treated with 0.25 or 0.5 mg/ml of IFN-α and counted on the sixth day after treatment. (**b**) The cells treated in the same manner were stained for senescence-associated β-galactosidase at 48 h after treatment and phase-contrast images of cells were taken at × 200 magnification. Scale bar = 100 μm

Owing to the ability of IFNs to enhance immunogenicity, IFN signaling and IFN therapy have been extensively studied with regard to levels of tumor immunity attained. Other non-immune-based molecular mechanisms have been studied at the cellular level in response to IFNs, but have not readily been connected with tumor/tumor microenvironment response. Recent evidence has broadened the scope of IRF-1 tumor suppressor functions in a variety of cancers, including ovarian cancer,[31] melanoma,[4] breast cancer[32] and cervical cancer.[33] Here, we have shown that there is a substantial fall in the IRF-1/IRF-2 ratio in tissues from nine different types of human tumors when compared with the corresponding normal tissue (Figure 1). This may not just serve as a useful indicator for diagnosis and prognosis of most tumors (Figure 1) but enables their use as possible targets in cancer treatment.

A series of viability and survival studies indicated that pIFN-α enhances the sensitivity of the M14 melanoma cells to cell death by the cytotoxic agent, VBL (Figure 2). At the molecular level, although IRF-1 gets specifically induced in response to pIFN-α, the level of IRF-2 remains more or less constant (Figure 3a) and it is known that IRF-1 is involved in cell-cycle regulation and induction of apoptosis**.** For instance, transcriptional induction of p21 by γ-irradiation was dependent on both IRF-1 and p53.[8] p21 is a cyclin-dependent kinase inhibitor and regulates cell-cycle progression at S phase.[34] The M14 melanoma cell line is p53 defective and thus one possible alternate route to induction of p21 is through upregulation of IRF-1 (Figure 3b).

We also observed distinct PARP cleavage, suggestive of cell death induction, upon VBL treatment after 24 h. pIFN-α treatment alone did not cause any PARP cleavage even by 48 h (Figure 3b). We investigated the possible role of Bak induction and activation in the greater than additive cell death caused by VBL combined with pIFN-α. Bak, a distal mediator of apoptosis, undergoes

conformational changes and homo-oligomerization in response to diverse apoptotic signals, leading to pore formation in the mitochondria and release of apoptosis-promoting factors.[22,23] The expression of Bak has been shown to be upregulated by IRF-1[35] (Figure 3b). Activation of Bak is a critical step toward apoptosis involving the exposure of its N-terminal epitope. Previously, we showed that VBL induces activation of Bak in KB-3 cells.[23] Importantly, although there is a significant increase in Bak expression in response to pIFN-α (Figure 5b), we show in the present report, using a conformationally dependent Bak antibody, that only VBL induces Bak activation in M14 melanoma cells (Figure 3c). It appears that the combined treatment of pIFN-α and VBL allows for an accumulation of total Bak and a subsequent increase in overall activation of Bak in M14 melanoma cells, which leads to enhanced cell death by the combined treatment.

Using an alternate strategy, we have also demonstrated that the buildup of inactive Bak in response to pIFN-α treatment sensitizes the M14 melanoma cell mitochondria to BH3 peptide-mediated cyt *c* release (Figure 3d). This relationship thus suggests that the pIFN-α treatment primes M14 melanoma cells to more rapidly respond to cell death stimuli such as that exerted by VBL and possibly other chemotherapeutics. Induction of p21 and Bak via upregulation of IRF-1 in response to pIFN-α might partially explain how the IFN signaling pathway crosstalks with the other apoptotic and cell-cycle pathways to enhance cell death in M14 melanoma cells and may have implications for other tumor cell types.

The progressive growth and metastasis of most tumors are angiogenesis dependent and therefore controlled by not only tumor cell proliferation but also proliferation and activity of cells that form the microvasculature.[19,20] We selected the human EA.hy.926 endothelial cell line to better understand the possible effects of pIFN-α exposure on tumor microvasculature mediated by endothelial cell responses (Figure 4). EA.hy926 is an immortalized endothelial cell line harboring wild-type p53. It has been shown that the transcriptional induction of the p21 gene is dependent on both p53 and IRF-1.[8] Cyclin D1, which works in concert with p21 to promote progression through G1,[36] has also been shown to be transcriptionally regulated by p53[26] and IRF-1.[12] These two tumor suppressor transcription factors therefore converge functionally to regulate cell cycle and possibly senescence through the activation of common target genes. It appears that sensitivity to cell death is enhanced in cells in which both p53 and IRF-1-mediated pathways can be activated such as in normal cells of the stromal compartment that supports the tumor microenvironment (Figure 5).

Our initial results from the treatment of EA.hy926 cells with pIFN-α were perplexing. While on one hand MTT cell viability assay did not show complete or substantial cell death even at 1 μM IFN-α treatment (Figure 4b), long-term survival studies showed a significant reduction in number of colonies when treated with 0.5 μg/ml IFN-α (Figure 4d). This increase in sensitivity to IFN-α was not due to the extent of apoptotic cell death (data not shown) but rather associated with enlarged, multinucleated, β-galactosidase-positive senescent EA.hy926 endothelial cells (Figure 6a). Although the normal cells doubled in less than 1 day, the number of cells in tissue culture plates after 5 days of IFN-α treatment remained constant. This suggested an alternate mechanism to apoptotic or clonogenic cell death may be at play. Indeed, we observed a premature senescence phenotype induced in IFN-α-treated cells that persisted for long term over subsequent passages of the cells. Induction of a senescent phenotype has previously been observed after treatment with several DNA-binding drugs.[37] The EA.hy926 endothelial cells studied here exhibited a similar phenotype and an induction followed by a subsequent decrease in the levels of p21 and cyclin D1 upon treatment with pIFN-α and VBL (Figure 5). This biphasic response of p21 and cyclin D1 is a further characteristic of cellular senescence.[38,39] Therefore, our results suggest that the normally proliferative tumor endothelium in a growing malignant tissue environment may be sensitive to pIFN-α-induced senescence, which would be expected to modify the angiogenic activity in the tumor. We have also demonstrated (Supplementary Figure 3) that VBL treatment, which causes G2/M arrest, followed by apoptosis results in nonadherent cells that do not undergo senescence.

In summary, our results suggest that the overall therapeutic mechanism of IFN-α combined with chemotherapeutic agents may be due to direct tumor cell death via IRF-1-based signaling and by inducing premature senescence in endothelial cells, which could reduce the angiogenic activity in the tumor. These results further uncover alternate mechanisms by which IFN-α functions in cancer therapy. An increased understanding of how the IFN-α pathway crosstalks with other pathways via IRF-1-based signaling to control apoptosis in the tumor microenvironment appears to be a valid approach to better identify drug regimens suited to block angiogenesis and cell proliferation in melanoma and other solid tumors. Efforts are underway to study these interconnections in cocultures and in *in vitro* angiogenic coculture constructs of tumor and endothelial cells.

Materials and Methods

Cell lines and culture. M14 is a human malignant melanoma cell strain, originally denoted as UCLA-SO-M14, later called M14, which expresses most melanoma-associated markers.[40,41] EA.hy926 is an immortalized human endothelial-like cell line derived from the fusion of primary human endothelial vein endothelial cells with the lung carcinoma cell line A549.[42] The cell lines were maintained in monolayer culture at 37°C and 5% CO_2. The M14 human melanoma cell line was maintained in RPMI (Cellgro, Manassas, VA, USA), supplemented with 10% fetal bovine serum (Atlas, Fort Collins, CO, USA), 50 units/ml penicillin and 50 μg/ml streptomycin (Hyclone, Logan, UT, USA). The EA.hy926 endothelial cell line was cultured in DMEM/F-12 (Cellgro) with 10% bovine calf serum (Hyclone), 2 mM L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin and 1 : 50 HAT (Cellgro).

Chemicals and reagents. Pegylated IFN-α2b (PEGINTRON) referred as pIFN-α was purchased from Schering-Plough (Kenilworth, NJ, USA). VBL and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). IRF-1, IRF-2 and cyclin D1 (DCS-6) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bak (NT) antibody for immunoprecipitation was from Millipore and Bak (Ab-1) antibody for immunoblotting was from Oncogene (Boston, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase antibody was from Cell Signaling Technology (Danvers, MA, USA). Cytochrome *c* and p21 antibodies were from BD Pharmingen (San Jose, CA, USA). Crystal violet (Fisher Scientific, Fairlawn, NJ, USA) and protein A/G PLUS-agarose beads were from Santa Cruz Biotechnology.

MTT cell viability assay. Cells (2000 per well) were seeded in 96-well plates and on the following day VBL and/or pIFN-α was added. After 72 h, MTT (50 mg per well) was added for 4 h followed by dimethylsulfoxide solubilization of the cells, and an absorbance reading was done at 540 nm.

Clonogenic assay. Cells were seeded at 200 cells per well in six-well tissue culture plates and on the next day treated with the indicated concentration of drugs for 24 h, and then colony formation was assessed. Cells were allowed to grow for 2 weeks after rinsing and replenishing the wells with fresh media without the drugs at 24 h. Colonies were stained with crystal violet and counted to calculate the surviving fraction.

Expression of IRF-1 and -2 in normal human and tumor tissues. Customized western blots (ProSci Inc., Poway, CA, USA) of 15 normal human and tumor tissues each were probed for IRF-1 (1 : 1000). The blots were stripped and reprobed for IRF-2 (1 : 1000). The level of IRF-1 and -2 protein expression was estimated by analyzing the relative band intensities using the Image J software (NIH, Bethesda, MD, USA).

Preparation of cell extracts and immunoblotting. Whole-cell extracts were prepared by suspending cells in 0.25 ml of lysis buffer (25 mM HEPES, pH 7.5, 0.5% sodium deoxycholate, 5 mM EDTA, 5 mM dithiothreitol, 20 mM glycerophosphate, 1 mM Na_3VO_4, 50 mM NaF, 1% Triton X-100, 20 μg/ml aprotinin, 50 μg/ml leupeptin, 10 μM pepstatin, 1 μM okadaic acid and 1 mM phenylmethylsulfonyl fluoride). Cells were incubated at 4°C on a nutator for 1 h, insoluble material was removed by centrifugation (15 min at 12 000 $\times$ *g*) and protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

BH3 peptide-mediated mitochondrial cyt *c* release. Mitochondria (25 μg per reaction) isolated from M14 cells without and after treatment with 0.5 μg/ml pIFN-α for 48 h were incubated with the indicated concentrations of BH3 peptide for 30 min at room temperature. Supernatant was collected and cyt *c* release was observed by immunoblotting. DMSO was used as the vehicle (2% final concentration in all samples).[24]

Immunoprecipitation. To evaluate Bak activation status, the cells were lysed in 0.5 ml of 10 mM HEPES (pH 7.4), 150 mM NaCl, 1% CHAPS, plus protease inhibitors (as above) and incubated at 4°C for 45 min on nutator. The cell extract was centrifuged at 12 000 $\times$ *g* for 15 min, and 500 μg of cell lysate was precleared using protein A/G-PLUS agarose beads for 30 min followed by incubation with 4 μg of Bak (NT) polyclonal antibody (Millipore) in a total of 500 μl of lysis buffer for 1 h. The lysates were then incubated with 25 μl of protein A/G-agarose beads (Santa Cruz Biotechnology) overnight at 4°C. The beads were pelleted by centrifugation and washed three times with 0.2 ml of lysis buffer. The beads were resuspended in 50 μl of 2 $\times$ SDS loading buffer and electrophoresed on 12.5% SDS-PAGE precast acrylamide gels (Bio-rad). Following electrophoresis, immunoblotting was performed using mouse anti-Bak (Ab-1) monoclonal antibody (Oncogene) at 1 : 1000 dilution.[23]

Senescence associated-β-Gal assay. The SA-β-Gal assay was performed as previously described in the manufacturer's instructions (Senescence β-galactosidase staining kit, Cell Signaling Technology). Briefly, cells were washed in phosphate-buffered saline and fixed in 2% formaldehyde–0.2% glutaraldehyde. Then the cells were washed and incubated at 37°C overnight with fresh senescence-associated β-Gal stain solution (1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal per ml), 40 mM citric acid–sodium phosphate (pH 6.0), 150 mM NaCl, 2 mM $MgCl_2$, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide). For all visualization and photography, a Nikon Eclipse Te2000-U microscope (Kanagawa, Japan) was used.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)