Dacarbazine Causes Transcriptional Up-Regulation of Interleukin 8 and Vascular Endothelial Growth Factor in Melanoma Cells: A Possible Escape Mechanism from Chemotherapy

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Abstract
The incidence of cutaneous malignant melanoma in the United States has increased more than any other cancer in recent years. Chemotherapy for metastatic melanoma is disappointing, there being anecdotal cases of complete remission. Dacarbazine (DTIC) is considered the gold standard for treatment, having a response rate of 15–20%, but most responses are not sustained. The mechanisms for the increased chemotherapeutic resistance of melanoma are unclear. The objective of this study was to determine the mechanisms by which melanoma cells escape the cytotoxic effect of DTIC. Here, we show that DTIC induced interleukin (IL)-8 and vascular endothelial growth factor (VEGF) protein overexpression and secretion via transcriptional up-regulation in the two melanoma cell lines SB-2 and MeWo. Luciferase activity driven by the IL-8 and VEGF promoters was up-regulated by 1.5–2- and 1.6–3.5-fold, respectively, in the SB2 and MeWo melanoma cell lines. The mitogen-activated protein kinase signal transduction pathway seemed to regulate at least partially the activation of IL-8, whereas it was not involved in VEGF promoter regulation. Electrophoretic mobility shift analysis analyses have revealed an increase in binding activity of activator protein 1 (c-Jun) and nuclear factor-κB after DTIC treatment for both melanoma cell lines. Metastatic melanoma cell lines secreting high levels of IL-8 and VEGF were more resistant to DTIC than early primary melanomas secreting low levels of the cytokines. In addition, transfection of the primary cutaneous melanoma SB-2 cells with the IL-8 gene rendered them resistant to the cytotoxic effect of the drug, whereas the addition of IL-8-neutralizing antibody to metastatic melanoma cells lowered their sensitivity to DTIC. Taken together, our data demonstrate that DTIC can cause melanoma cells to secrete IL-8 and VEGF, which might render them resistant to the cytotoxic effects of the drug. We propose that combination treatment with anti-VEGF/IL-8 agents may potentiate the therapeutic effects of DTIC.

Introduction
The incidence and mortality of melanoma continues to rise faster than that of any other cancer in the United States (1–3). Overall, melanoma accounts for 1–3% of all malignant tumors and is increasing in incidence by 6–7% each year. Although melanoma incidence is only 5% of that of nonmelanoma skin cancer, it accounts for approximately three times as many deaths (roughly 7200 versus 2300 annually).

Although early diagnosis, as thin lesions, enables cure with surgical resection in a high percentage of cases (>50%), with a 5-year survival of 80–100%, the prognosis of metastatic melanoma remains poor. When a patient presents with advanced disease, 5-year life expectancy is <10%, with a median survival of 6–8.5 months (4–6). Melanoma metastases affect skin, lymph nodes, lung, liver, brain, bone, and sometimes other organs such as the pancreas, many of which can be asymptomatic for years.

Different therapeutic approaches for metastatic melanoma have been evaluated, including chemotherapy and biological therapies, both as single treatments and in combination (4, 7–9). To date, however, none has shown a survival impact. Systemic chemotherapy is still considered the mainstay of treatment for stage IV melanoma and is used largely with palliative intent (7, 9). Numerous chemotherapeutic agents have shown some activity in the treatment of malignant melanoma with DTIC being the most widely used. DTIC is a nonclassical alkylating agent, generally considered the most active agent for treating malignant melanoma and is approved by the U. S. Food and Drug Administration for this purpose (9, 10). The drug exerts its antitumor activities by methylation of nucleic acids or direct DNA damage and results in growth arrest and cell death. However, response rates for single-agent DTIC are disappointing, ranging from 15–20%, but most responses are not sustained.

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4 The abbreviations used are: DTIC, Dacarbazine; IL, interleukin; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAP/ERK kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AP-1, activator protein 1; EMSA, electrophoretic mobility shift analysis; NF-κB, nuclear factor-κB.
10 to 25%, with complete responses seen in <5% of patients. In addition, the response duration is often brief, i.e., 5–6 months (11, 12).

A major obstacle to a successful treatment of metastatic melanoma is its notorious resistance to chemotherapy (13–15). The field of chemoresistance is widely explored in cancer research and many mechanisms have been described by which a tumor can evade cell killing in a variety of malignancies (16–21). In malignant melanoma, the mechanisms of chemoresistance are not established. Some argue that the basis for drug resistance in melanoma is dysregulation of apoptosis at different levels of the apoptotic pathways (22, 23). Others believe that, for example, impaired drug transport, detoxification, enhanced DNA repair and multidrug resistance or multidrug resistance-like proteins play a role (24–26).

New treatments are urgently needed for the therapy of metastatic melanoma and much effort is being devoted to the development of genetic and immune therapies, but the widespread availability of these remains a distant prospect. In the meantime, chemotherapy will remain the treatment of choice, and strategies to overcome resistance offer a more immediate possibility for improving the lot of these patients. Exploring the mechanisms of overcoming tumor resistance to DTIC are therefore of great interest.

Recent studies have shown that the aggressive nature of human melanomas is related to several abnormalities in growth factors, cytokines, and their receptors. For example, metastatic melanoma cells constitutively secrete the cytokine IL-8, whereas nonmetastatic cells produce low to negligible levels of IL-8 (27–29). In fact, IL-8, originally discovered as a chemotactic factor for leukocytes, may play an important role in the progression of human melanomas (28). Several studies have demonstrated that the expression levels of IL-8 correlate with disease progression in human melanomas in vivo (29–36). Serum levels of IL-8 are also elevated in patients with malignant melanoma (34, 35). Moreover, we have previously shown that transfection of nonmetastatic, IL-8-negative melanoma cells with the IL-8 gene, rendered them highly tumorigenic and increased their metastatic potential in nude mice (36).

In addition to IL-8, aggressive melanoma cells secrete VEGF, which promotes angiogenesis and metastasis of human melanoma cells (37). Moreover, both of these angiogenic factors act on vascular endothelial cells and serve as survival factors. Thus, IL-8 and VEGF may act by autocrine and paracrine fashions to promote growth and metastasis of melanoma. Cytotoxic therapy, including radiotherapy, and other stress conditions such as hypoxia are known to induce IL-8 and VEGF by tumor cells (38–41). We hypothesized that resistance to DTIC is a result of increases in IL-8 and VEGF production in response to the drug. Here, we analyze the effect of DTIC on the production of IL-8 and VEGF in human melanoma cells. We found that treatment of melanoma cells with DTIC resulted in up-regulation of the proangiogenic cytokines IL-8 and VEGF. We propose that overproduction of these molecules is a potential mechanism for melanoma cells to evade cell death and become resistant to chemotherapy. These data have a significant clinical relevance, justifying the combination of conventional chemotherapy with anti-IL-8 and/or anti-VEGF modalities for the treatment of malignant melanoma.

**Materials and Methods**

**Cell Lines.** The human melanoma MeWo cell line was established in culture from a lymph node metastasis of a melanoma patient and was kindly provided to us by Dr. Soldano Ferrone (New York Medical College, New York, NY). MeWo cells are tumorigenic and metastatic in nude mice (36). The SB2 cell line was isolated from a primary cutaneous lesion and was a gift of Dr. Beppino Giovannella (Research Laboratory, St. Joseph’s Hospital Cancer Center, Houston, TX). In nude mice, SB2 cells are poorly tumorigenic and nonmetastatic (30). SB-2 cells transfected with a full-length IL-8 cDNA (SB-2–IL-8) were established and characterized previously (36). DTIC-resistant SB-2 and MeWo cell lines designated as SB-2-DTIC and MeWo-DTIC were established by culturing SB-2 and MeWo cells with 500 µg/ml DTIC until <10–20% confluency was achieved, after which, the medium was changed, and the cells were left to expand. The melanoma cell lines were maintained in culture as adherent monolayers in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and penicillin-streptomycin (Flow Laboratories, Rockville, MD), buffered with 10 mM HEPES (Life Technologies, Inc.), and incubated in 5% CO₂, 95% air at 37°C.

**Reagents.** DTIC was obtained from Bay Corp. (West Haven, CT) and was activated by exposure to light for 1 h before use. Antibodies to ERK and phosphorylated ERK were purchased from Cell Signaling Technology (Beverly, CA). The MEK inhibitor U0126 was purchased from Cell Signaling Technology. Neutralizing antibody to IL-8 (ABX–IL8) was obtained from Abgenix (Fremont, CA) and used as previously described (42), and anti-VEGF antibody was purchased from R&D Systems (Minneapolis, MN) and added to the cultures with the concentration of 10 µg/ml.

**Cell Proliferation Assay.** Melanoma cells (2 × 10⁵) were plated in 96-well plates and then treated with 0–1000 µg/ml DTIC for 48 h. A MTT assay was performed at 48 h to determine the relative cell numbers based on the conversion of MTT to formazan in viable cells. MTT (40 g/ml) was added to each well and incubated for 2 h. The medium was then removed, and 100 µl of DMSO were added to lyse the cells and solubilize the formazan. A standard microplate reader was used to determine the absorbance at 570 nm.

**Measurement of Angiogenic Factors.** Tumor cells (2 × 10⁵) were plated in 35-mm dishes. When the cultures reached 70–80% confluence, fresh medium was applied with the appropriate treatment (DTIC, U0126), collected after an additional 24 h incubation, and then clarified of cells and cell debris by centrifugation. The cells were harvested with trypsin-EDTA and counted. The conditioned media samples were stored at −20°C for later analysis or used immediately for measurement of VEGF-A and IL-8, using quantitative immunometric sandwich ELISAs, following the procedure recommended by the manufacturer (R&D Systems).
Western Blot Analysis. Melanoma cell lines were seeded at $1 \times 10^5$ in 100-mm tissue culture plates in 10 ml of CMEM. After overnight incubation, the plates were washed twice in PBS, and the cells were scraped off in 400 $\mu$l of Triton lysis buffer, 1 $\mu$l of DTT, and 4 $\mu$l of protease inhibitor mixture. After a 30-min incubation on ice, the cells were centrifuged at 15,000 rpm for 15 min. The protein concentration was determined using Bradford reagent (Bio-Rad Laboratories, Hercules, CA), and BSA standards and 40 $\mu$g of protein were loaded onto a 10% SDS-PAGE gel and electrophoretically transferred to a 0.45-$\mu$m nitrocellulose membrane (Millipore, Bedford, MA). The membrane was blocked with 5% milk in Tween Tris-buffered saline for 1 h. The membrane was cut in half and incubated in 1 ml of either control IgG (1:500 dilution) or anti-ERK overnight. Membranes were probed with a secondary antibody, peroxidase-conjugated AffiniPure rabbit antihuman IgG (H+L) for 1 h and then washed with Tween Tris-buffered saline. Probed proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotechnology, Arlington Heights, IL) following the manufacturer’s protocol.

Transient Transfections and Luciferase Assay. IL-8 promoter activity was measured by transient transfection with the pGL2-IL-8 construct, where the region from +44 to −1481 of the IL-8 promoter was cloned into the pGL2-basic vector to drive expression of the firefly luciferase gene. The pGL3VEGF 3.317 construct was used to measure VEGF promoter activity. In the pGL3VEGF 3.317 construct, full-length VEGF promoter (−2362 to +955 relative to the transcription initiation site) was cloned into pGL3 basic vector.

AP-1 and NF-κB transactivation activity was measured using luciferase constructs where three AP-1 or three NF-κB consensus elements were cloned in front of the luciferase reporter gene. c-fos, c-jun, p50, p52, or p65 polyclonal antibodies against c-fos, c-jun, p50, p52, or p65 were incubated with labeled AP-1 or NF-κB consensus sequences in binding buffer for 30 min followed by incubation with concentrated polyclonal antibodies against c-fos, c-jun, p50, p52, or p65. The membrane was transferred to a 0.45-$\mu$m filter and the cytoplasmic fraction was separated from the insoluble material by centrifugation for 2 min at 15,000 rpm. The soluble nuclear protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories).

EMSA. DNA-binding activity was assayed by EMSA. AP-1 or NF-κB consensus oligonucleotides were end-labeled with [γ-32P]-ATP (Promega Corp.) and incubated with 5 μg of nuclear extract. The binding reaction was carried out in 1× binding buffer [4% glycerol, 1 mM MgCl$_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl (pH 7.5), and 1 μg/ml poly(dI-dC)] for 20 min at room temperature. For competition, the nuclear extract was incubated for 30 min with unlabeled oligonucleotide and then incubated with labeled AP-1 or NF-κB consensus sequences for an additional 30 min. After supershift assay, the nuclear extracts were incubated with the appropriate radiolabeled consensus sequence in binding buffer for 30 min followed by incubation with concentrated polyclonal antibodies against c-fos, c-jun, p50, p52, or p65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for an additional 30 min. The nucleoprotein complexes were resolved in 4% polyacrylamide gel for 4 h in 0.5× Tris–borate EDTA buffer at 4°C.

Results

DTIC Induces Up-Regulation of VEGF and IL-8 Protein in Melanoma Cells. Two human melanoma cell lines were chosen for this study, SB2 and MeWo. SB2 cells originated from a primary cutaneous melanoma and are poorly tumorigenic and nonmetastatic in nude mice. In addition, they express negligible levels of IL-8 and VEGF when compared with other melanoma cell lines originating from metastatic lesions. MeWo cells derived from a lymph node melanoma metastasis are tumorigenic and form lung metastases after i.v. injection but do not spontaneously metastasize after s.c. injection into nude mice. This cell line expresses moderate levels of VEGF and IL-8 in vitro. The rationale for choosing these melanoma cell lines with different malignant potential was to explore whether the response to the chemotherapeutic agent is cell specific, and if it is associated with tumor progression.

To determine the doses of DTIC that would induce a cyto-
static effect without killing the cells, an MTT assay was performed after treatment with doses of the drug ranging from 0 to 1000 μg/ml for 48 h (Fig. 1). Both the SB-2 and MeWo cell lines were moderately resistant to the drug in vitro, and cell death was observed at a concentration of 125 μg/ml. MeWo cells exhibited a higher resistance to the drug with an IC_{50} of ~430 μg/ml in comparison to 280 μg/ml for the SB2 cells. No viable cells were found at a concentration of 750 μg/ml for the SB2 and at 1000 μg/ml for the MeWo cells. Accordingly, we chose to use a maximum dose of 500 μg/ml in our studies.

Cytotoxic therapy and other stress conditions such as hypoxia are known to induce IL-8 and VEGF by tumor cells. To determine whether DTIC induces VEGF or IL-8 in the melanoma cells, and if so, whether the induction is dose dependent, media were conditioned by cells exposed to various drug concentrations and collected for measurement of the cytokine levels by ELISA. VEGF levels in the conditioned media exhibited a dose-dependent increase after being exposed to the drug for 48 h (Fig. 2, A and B). Maximum levels of VEGF protein, 1056.9 pg/10^6 cells and 1524.1 pg/10^6 (4.6- and 7.9-fold compared with control untreated cells) were observed after exposure to 250 μg/ml DTIC for the SB2 and MeWo, respectively (Fig. 2, A and B). After 5 days of treatment, the VEGF levels were elevated up to 4.8- and ~9.5-fold in the SB2 and MeWo cells treated with 250 μg/ml DTIC, respectively, as compared with control medium from untreated cells (Fig. 2, A and B). Treatment with 500 μg/ml DTIC resulted in a decrease of VEGF secretion in media from both cell lines because of the cytotoxic effect of the drug. Although SB2 and MeWo cells expressed different basal levels of VEGF, they both increased VEGF secretion after DTIC treatment.

Similar results were obtained for IL-8 expression in response to DTIC. Increases of 4.0- and 4.8-fold and 12.0- and 16.7-fold in IL-8 secretion were observed after exposure to 500 μg/ml DTIC for 48 h and 5 days in the SB2 and MeWo, respectively (Fig. 2, C and D). Unlike VEGF, secretion of IL-8 continued to be elevated at the high dose of 500 μg/ml DTIC. The early elevation in the cytokine protein levels secondary to DTIC exposure cannot be explained by clonal selection as not enough time elapsed for tumor cell death and expansion of high secreting cells. The increase thus, most probably, represents induction of VEGF and IL-8 in response to a stress-related protective mechanism.

The Link between Drug Resistance and Secretion of IL-8/VEGF. To further establish the correlation between the drug sensitivity and the levels of IL-8 and VEGF, we analyzed DTIC sensitivity in a panel of melanoma cell lines exhibiting different expression levels of IL-8 and VEGF (Table 1). In addition, we compared the drug sensitivity in the SB-2 cells before and after stable transfection with the IL-8 gene (Table 1). The results depicted in Table 1 show that the cell lines WM2664 and A375SM, both expressing relatively high levels of IL-8 and VEGF, exhibited a higher resistance to DTIC. Moreover, transfection of SB-2 cells with the IL-8 gene rendered them much more resistant to DTIC, which reached almost the level of the highly metastatic A375SM cells. The link between drug sensitivity and IL-8/VEGF levels was additionally investigated by using DTIC-resistant SB-2 and MeWo cells established in culture. The SB-2-DTIC and MeWo-DTIC cells were found to express higher constitutive levels of IL-8 and VEGF long after the withdrawal of the drug. These cells also exhibited a higher resistance to DTIC (Table 1). To provide a direct evidence for the involvement of IL-8 and/or VEGF in the resistance to the drug, we have treated A375SM, WM2664, and SB-2-IL-8 cells with 500 μg/ml DTIC combined with neutralizing antibodies to IL-8 or VEGF (Table 2). Neutralization of IL-8 in the culture of these cells resulted in their increased sensitivity to the drug. The addition of anti-VEGF did not alter their sensitivity to DTIC (Table 2), suggesting that IL-8 plays a major role in melanoma resistance to the drug.

The Increase in Cytokine Levels in Response to DTIC Is Transcriptionally Regulated. We next determined whether the increase in VEGF and IL-8 secretion induced by DTIC was because of transcriptional up-regulation of the corresponding genes. To that end, luciferase activities driven by the VEGF and IL-8 promoters were analyzed after treatment with DTIC. Treatment of SB2 and MeWo cells with 125 or 500 μg/ml DTIC resulted in 2.3- and 2-fold increases and 1.6- and 1.7-fold increase in VEGF promoter activity, respectively, (Fig. 3A). Similarly, luciferase activity driven by the IL-8 promoter was increased by 1.4- and 1.6-fold in SB2 cells and 1.6- and 1.55-fold in the MeWo cells, respectively, all treated with the same doses of DTIC (Fig. 3B). The increase in IL-8 and VEGF promoter activities is compatible with the increased levels of the cytokines secreted by the treated cells. These changes in promoter activities, albeit modest, were sufficient to alter the sensitivity of the cells to the drug.

DTIC Activates the MAPK in Melanoma Cells. MAPK activity has been previously shown to be up-regulated in various cancer cells in response to different chemotherapeutic agents. Moreover, some of the transcription factors regulating the promoter activity of IL-8 and/or VEGF such as AP-1 and NF-κB are known to be stimulated by MAPK. We thus sought to explore the effect of DTIC on this signaling pathway. Immunoblotting for the phosphorylated ERK 1/2 protein in the two melanoma cell lines after treatment with
Phosphorylated ERK was observed 15 min after DTIC treatment in SB2 cells and after 1 h in MeWo cells. Levels of phosphorylated ERK were sustained throughout the 24-h treatment period in both cell lines. After 24 h of treatment, an increase of 12.6- and 5.8-fold in ERK activation was observed for SB-2 and MeWo cells, respectively (Fig. 4).

Phospho-ERK Hyperactivity Is Necessary for IL-8 but not for VEGF Up-Regulation.

We next determined whether an ERK-dependent pathway mediates DTIC-induced IL-8 and VEGF expression. To that end, melanoma cells were pretreated with UO126 (a specific MEK inhibitor) and treated with DTIC for 48 h. IL-8 protein levels were significantly inhibited by UO126 in both cell lines (Fig. 5A). DTIC only partially reversed this inhibition in the SB2 cell line and did not change the protein level in the MeWo cells, suggesting that the DTIC induction of IL-8 is at least partially regulated by activation of ERK. Different results were obtained for VEGF expression. Although ERK inhibition by UO126 resulted in decreased VEGF protein secretion in both cell lines, DTIC stimulation of the UO126-pretreated cells resulted in overexpression of VEGF that overcame the inhibition by ERK blockade, suggesting that the DTIC effect on VEGF is independent of ERK phosphorylation.

Similar results were obtained by the luciferase reporter assay (Fig. 6). UO126 treatment of SB2 and MeWo transiently transfected with the IL-8 promoter reduced basal promoter activity by 43–47%. The addition of 250 μg/ml DTIC to the UO126-treated cells resulted only in a small increase in the promoter activity in the SB2 cells and no increase at all in the MeWo cells (Fig. 6A). A similar experiment performed after transfecting the cells with the VEGF promoter demonstrated different results. Although the MEK inhibitor reduced promoter activity significantly in both cells, additional treatment of these cells with DTIC resulted in an increase in the promoter activity at the same rate as if the cells were treated with chemotherapy without the UO126 ERK inhibitor (Fig. 6B). Collectively, our data show that DTIC-induced IL-8 production is dependent on ERK activation, whereas DTIC-induced VEGF production is not.

**DTIC’s Effect on AP-1 and NF-κB Transcriptional Activity and DNA Binding.** Because AP-1 and NF-κB are involved in the regulation of IL-8 and VEGF and because the
activity of these transcription factors could be affected by ERK activity, we next determined the effect of DTIC with and without ERK inhibitor on the luciferase activities driven by the AP-1- and NF-κB-responsive elements. Treatment with DTIC resulted in 1.5–2-fold increase in transcriptional activity of both AP-1 and NF-κB. However, AP-1 and NF-κB activities were inhibited by UO126, and the inhibition could not be reversed by the addition of DTIC (Fig. 7, A and B). We conclude that DTIC-induced transcriptional activity of both AP-1 and NF-κB is dependent on ERK activation.

In the last set of experiments, we analyzed the effect of DTIC with and without UO126 on the DNA binding activities of AP-1 and NF-κB. To that end, nuclear extracts from MeWo cells before and after treatment with DTIC were analyzed for their binding activity to AP-1 and NF-κB binding motifs on an EMSA gel. The results depicted in Fig. 8A demonstrate that DTIC caused an increase of NF-κB binding to its consensus binding motif (Fig. 8A, Lane 2). This binding activity was reduced by UO126 treatment (Fig. 8A, Lane 3) and could not be restored by additional treatment with DTIC (Fig. 8A, Lane 4). Both p50 and p65 were bound to DNA as evidenced by the supershift analysis (Fig. 8A, Lanes 7–10). DTIC treatment slightly increased AP-1 DNA-binding capability (Fig. 8B, Lane 2), which was somewhat reduced by UO126 (Fig. 8B, Lane 3), in an irreversible manner (Fig. 8B, Lane 4). This binding consisted mainly of c-Jun but not with c-fos as evident from the supershift analysis (Fig. 8B, Lanes 7–10). Fig. 8 shows the results obtained with nuclear extracts from MeWo cells; the same results were obtained for SB-2 cells (data not shown).

Taken together, the results imply that AP-1 and NF-κB are required for DTIC-induced IL-8 transcriptional up-regulation, whereas other transcription factors activated by DTIC are involved in the regulation of VEGF expression.

### Table 2
Neutralization of IL-8 but not VEGF sensitizes melanoma cells in vitro to DTIC

| Cell line | % cell viability after treatment with DTIC (500 μg/ml) | % cell viability in cells treated with DTIC (500 μg/ml) plus IgG Anti-IL-8 Anti-VEGF IgG Anti-IL-8 Anti-VEGF |
|-----------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| WM2664    | 55*                             | 96                              | 95                              | 97                              |
| A375SM    | 81                              | 97                              | 96                              | 96                              |
| SB-2-IL8  | 52                              | 97                              | n.d.                            | 97                              |

*Percentage of viable cells as determined by the MTT assay.

n.d., not determined.

Fig. 3. Effect of DTIC on VEGF and IL-8 promoter. Luciferase activity driven by the VEGF and IL-8 promoters was assayed after treatment with 125 and 500 μg/ml DTIC. Firefly and Renilla luciferase activities were measured by the dual-luciferase reporter assay. Fold increase was calculated relative to control cells, which were given the value of 1. DTIC caused an up-regulation of VEGF and IL-8 promoters in both cell lines.

Fig. 4. Effect of DTIC on ERK activation. SB-2 and MeWo melanoma cells were treated with 250 μg/ml DTIC for 15 min to 24 h, and ERK phosphorylation was measured by Western blot analysis. Phosphorylated ERK was observed 15 min after DTIC treatment in SB-2 cells and after 1 h in MeWo cells. The expression of unphosphorylated ERK serves as an indication of equal loading. Fold of ERK activation is indicated at the bottom of the gels for each time point.
Discussion

In this study, we demonstrated that DTIC treatment transcriptionally induced VEGF and IL-8 protein expression in melanoma cell lines. This finding has significant therapeutic implications, especially as DTIC is still considered the drug of choice for metastatic melanoma. Our study provides a potential explanation for the high resistance of melanomas to chemotherapy. We have observed IL-8 and VEGF up-regulation in both cell lines tested, suggesting that this is a general phenomenon in melanoma cells. This notion is additionally supported by recent studies demonstrating elevated levels of IL-8 in the sera of a cohort of patients with metastatic melanoma after chemotherapy that included DTIC (34, 35).

Tumor and host cells that infiltrate and surround a tumor mass express a variety of growth factors and cytokines, among them IL-8 and VEGF, which play significant roles in regulating tumor growth, angiogenesis, and metastasis. However, although the role of VEGF in the process of tumor-induced angiogenesis is well established, several recent studies suggest the intriguing possibility that VEGF has a direct effect on tumor cells themselves (43). VEGF tyrosine kinase receptors (flt-1 and KDR) and the neuropilin receptor have been found to be coexpressed with VEGF on several human tumor cells such as leukemic cells, ovarian carcinoma Kaposi’s sarcoma, prostate carcinoma, breast cancer, pancreatic cancer, and melanoma, suggesting that VEGF may directly influence tumor cell growth by an autocrine mechanism (44–50). The biological relevance of VEGF receptor expression on melanoma cells is now being studied with controversial results. One study found that VEGF increased the proliferation of KDR-positive melanoma cells in vitro (51); although another study, however, did not support these results (52). In our studies, neutralizing antibody to VEGF did not alter the sensitivity of melanoma cells to DTIC in vitro. In vivo, however, VEGF induction by the drug can affect angiogenesis and hence tumor growth and metastasis.

The second cytokine found to be up-regulated by DTIC in our study is IL-8. This CXC chemokine, originally identified as a neutrophil chemotactic factor, has since been shown to contribute to many functions promoting tumor growth and metastasis. This has been primarily explained by its action as an autocrine growth factor for cancer cells and its induction of haptotactic migration (28). Only later was it shown that IL-8 also exhibits potent angiogenic activities both in vitro and in vivo (53). Several studies have demonstrated that the expression levels of IL-8 correlate with disease progression in human melanomas (35, 37). Moreover, we have previously shown that transfection of the nonmetastatic and IL-8-
negative, SB2 melanoma cell line with the IL-8 gene rendered it highly tumorigenic and increased its metastatic potential in nude mice (36). In this study, we show that neutralizing antibody to IL-8 rendered melanoma cells much more sensitive to DTIC, thus providing direct evidence for the involvement of IL-8 to the resistance of melanoma cells to the drug.

VEGF and IL-8 are known to be inducible in tumor cells in response to various stimuli derived from the tumor microenvironment, of which, hypoxia is the best characterized (38, 39). Hypoxia leads to a rapid increase in VEGF and IL-8 expression in numerous cells both by increasing the transcription of the gene and by prolonging mRNA half-life. We have also previously shown that UVB irradiation induces IL-8 mRNA and protein secretion in SB2 cells (30) and enhances their tumorigenicity and metastatic potential in nude mice. However, the specific signaling pathways that contribute to cytokine up-regulation have not been fully elucidated and seem to involve several signal transduction pathways, including the phosphatidylinositol 3'-kinases and MAPK (54–57).

The human VEGF promoter contains binding sites for AP-2, SP-1, or SP-1-related factors in addition to binding sites for AP-1, signal transducers and activators of transcription 3, and hypoxia-inducible factor 1 (56). Although no direct binding site for NF-kB on the human VEGF promoter has been identified, a recent study by Huang et al. (58) determined that blockade of NF-kB activity in human ovarian cells can suppress expression of VEGF in vitro and in vivo, suggesting a role for NF-kB in the transcription of VEGF. As for the IL-8 gene, it is regulated at both the transcriptional and posttranscriptional levels. Transcriptional activation is primarily mediated by a steroid responsive element, a HFN-1 element, two IRF-1 elements, an AP-1 sequence, an AP-3 site, a C/EBP sequence, and an NF-kB-NF-IL-6 overlapping sequence (59, 60). Examination of the regulatory regions of both promoters reveals that they share common promoter sites for transcription factors, which explains their similar response to various stimuli. In our studies, we demonstrated that the elevation in the protein level was at least partially transcriptionally controlled by the elevation of the activity of the transcription factors AP-1 and NF-kB. However, other transcription factors may also play a role in this response. Taken together, the results seem to show that DTIC-induced up-regulation of IL-8 and VEGF resembles their up-regulation in other stress-related conditions.

MAPK-signaling cascades are commonly involved in eukaryotic cell cycle regulation (61–63). They are activated by many different stimuli (e.g., mitogens, differentiation factors, and stress signals) and participate in a diverse array of cellular programs, including cell proliferation and growth, cell differentiation, cell movement, cellular senescence, and cell death (61). Up-regulation of MAPK activity is known to occur in tumor cells after treatment with various chemotherapeutic agents (62). For example, induction of ERK phosphorylation by cisplatinum was found in various cancer cells, including...
melanoma, and the inhibition of ERK activity in some of these cells resulted in sensitization of the tumor cells to the apoptotic effect of the drug (63). We have examined the role of the MAPK pathway in response to DTIC and showed an increased activity of ERK that peaked at 3 h after the initiation of chemotherapy. This elevation may have been responsible for the up-regulation of IL-8 as reflected by the sustained inhibition of IL-8 protein production and promoter activity after MEK blockade. In contrast, DTIC reversed the inhibition of VEGF protein production and promoter activity in response to UO126, suggesting that elevation of MEK activity is not responsible for VEGF up-regulation. Our results are in agreement with previous work showing that in different cellular contexts, VEGF transcriptional control is more sensitive to other signal transduction pathways such as the phosphatidylinositol 3'-kinase than to ERK signaling (64). In one study, however, epidermal growth factor-induced VEGF production by head and neck cancer cells was inhibited by UO126 (65), suggesting that ERK-induced VEGF production is cell-type specific.

In addition to DTIC, other cytotoxic modalities were previously found to induce the expression of cytokines by diverse tumor types, including melanoma. For example, radiation was shown to induce VEGF expression by melanoma cells, rendering the tumor-associated endothelial cells more resistant to radiation’s effects (40, 41). In these studies, endothelial cell survival and proliferation after in vitro irradiation was enhanced by supplementation of VEGF, whereas anti-VEGF antibody enhanced the cytotoxic effects of irradiation. In another study, radioimmunotherapy induced VEGF expression in colon cancer-bearing nude mice (66).

What is the clinical relevance of this cytokine up-regulation? We propose that it renders the melanoma cells more resistant to chemotherapy through several growth supportive mechanisms. First, as angiogenesis inducers, these cytokines can enhance tumor growth. VEGF’s and IL-8’s ability to act as survival factors can protect tumor-associated endothelial cells against cytotoxicity. In addition, as permeability factors (especially VEGF), they can induce an increase in the interstitial fluid pressure and thus might impaire the delivery of drug to the tumor. Moreover, the majority of human melanoma cell lines, including the two used in this study, SB2 and MeWo, have been shown to express one or more of the IL-8 receptors (CXCR1 and CXCR2). They also were found to express one or more of the VEGF receptors (flt-1, KDR, and neuropilin), suggesting a role for an autocrine loop in these cells. In this case, up-regulation of IL-8 and VEGF in response to DTIC can initiate autocrine survival signals.

Adjuvant therapy, although still not a common practice in patients with localized cutaneous melanoma, may diminish recurrence and metastasis, which are frequent in this disease (67). A number of studies have shown that antiangiogenic therapy increases the efficacy of conventional chemotherapy and radiotherapy (40, 41, 68). Our data suggest that chemotherapy may stimulate expression of survival factors that may promote chemoresistance. One suggestion stemming from our results is to use combined anti-VEGF/anti-IL-8 therapies with DTIC to treat malignant melanoma. We have shown recently that fully humanized anti-IL-8 antibody suppresses the tumorigenic and metastatic potential of metastatic melanoma cells in nude mice (42). We observed that treatment with anti-IL-8 increased apoptosis, decreased microvessel density, and reduced expression of the metalloprotease MMP-2 (42). We are currently exploring whether combination treatment of DTIC plus anti-IL-8 inhibits melanoma growth and metastasis in nude mice.

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References


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