Endothelin Receptor B Inhibition Triggers Apoptosis and Enhances Angiogenesis in Melanomas

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ABSTRACT

Endothelin receptor B (ETRB or EDNRB) is overexpressed in most human melanomas and is proposed to provide a marker of melanoma progression. We have shown previously that inhibition of ETRB leads to increased human melanoma cell death in vitro and in vivo, resulting in shrinkage of tumors grown in immunocompromised mice. In the present work, we analyzed the effects of ETRB inhibition on 10 human melanoma cell lines derived from tumors at distinct stages of progression. Our observations suggest that the ETRB antagonist BQ788 induces apoptosis most effectively in metastatic melanoma cells. Microarray analysis shows that BQ788 treatment leads to a reduction in the expression of the survival factor BCL-2-A1 and the DNA repair factor poly(ADP-ribose) polymerase 3 that is more pronounced in cells derived from metastatic than primary melanoma. Decreased cell viability was observed to correlate with reduction in ETRB expression, and reduction in ETRB protein levels by small interfering RNA interfered with increased cell death. Interestingly, reduction of ETRB expression by BQ788 was accompanied by a strong induction of VEGF expression and repression of the angiogenic suppressor gravin. These changes in gene expression correlated with increased angiogenesis in tumors injected with ETRB antagonist in vivo. Taken together, our observations suggest that ETRB may provide a potential therapeutic target in high-grade melanomas and identify candidate pathways that may be implicated in the regulation of cell survival and tumor progression associated with ETRB signaling.

INTRODUCTION

Cancer progression is often associated with reactivation of developmental programs. Consistent with this notion, melanoma cells display a highly proliferative and motile phenotype that is shared with embryonic melanocyte precursors, which typically migrate over long distances within the organism (1). Studies on the mechanisms that regulate melanocyte migration have provided insight into the function of endothelins and their receptors. The endothelin family of molecules is composed of three polypeptides, ET-1, ET-2 and ET-3, of 21 amino acids each that bind to two highly homologous G-coupled protein receptors, endothelin receptor A (ETRA) and endothelin receptor B (ETRB or EDNRB), which trigger a variety of signals according to the cell type (2). ETRB promotes migration (3) and proliferation of early melanocyte precursors (4–6), and mutation in ETRB in both humans and mice results in spotting due to the inability of an elevated proportion of melanocytes to reach the skin (7, 8). On the basis of these observations, we addressed the possibility that ETRB may play a role in melanoma progression and found that the specific ETRB antagonist BQ788 (9) inhibits growth of human melanoma cells in culture and melanoma development in nude mice (10, 11). ETRB expression is enhanced in cutaneous melanoma (12). In a study that examined the expression of 6971 genes in 31 human melanoma specimens from biopsies or tumor cell cultures, an increase in ETRB was observed in all samples (13). A similar observation was made in a study of human cancer cell lines, where cutaneous melanoma samples displayed overexpression of ETRB (14), and ETRB has been proposed to be a melanoma progression marker in a histologic study of 159 human melanoma cases (15). Recently, ETRB was shown to mediate molecular events characteristic of melanoma progression (16). Taken together, these observations suggest that ETRB activation contributes to melanoma development and progression. In support of this view, one of the ETRB ligands, ET-1 is reported to be secreted by keratinocytes in response to UV irradiation (17, 18), a major triggering factor in melanoma development (19). Moreover, UV-mediated induction of keratinocyte ET-1 down-regulates E-cadherin in melanocytes and melanoma cells through ETRB activation (20). Down-regulation of E-cadherin expression is commonly observed in melanomas and is proposed to enhance their invasiveness (21).

In the present work, we used 10 graded human melanoma cell lines derived from a primary lesion and cutaneous and lymph node metastases and tested the correlation between the progression level and responsiveness to BQ788. We show that melanoma cells derived from more advanced lesions display higher sensitivity to ETRB inhibition and provide new insight into the mechanisms that may underlie melanoma cell death as a result of ETRB blockade.

MATERIALS AND METHODS

Cell Culture. Human melanoma cell lines Me191-I/GG, Me300, and T921A derived from primary tumors, Me 190/DA and T640A derived from cutaneous metastases, and Me 275/Ep, Me 256, T387A, and T523A derived from lymph node and visceral metastases were established at the Ludwig Institute (Epalinges, Switzerland). The SK-MEL-28 cell line was obtained from the American Tissue Culture Collection (Manassas, VA). All cell lines were cultured in RPMI 1640 with Glutamax I (Life Technologies, Inc., Gaithersburg, MD) containing 10% fetal bovine serum (Life Technologies, Inc.) and 100 μg/mL antibiotics (Pen-Strep mix, Life Technologies, Inc.) in a humidified incubator with 5% CO2 at 37°C. Matrigel Matrix (Becton Dickinson, Holderege, NE) was used according to the manufacturer’s instructions in 8.0-μm pore size cell culture inserts in 24-multiwell plates (Becton Dickinson). Twenty thousand cells were cultured on top of Matrigel Matrix for 2 weeks. After removal of the inserts, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium (MTS) solution was added to the well to quantify the cells that migrated through the insert and were attached to the bottom of the well by measuring the absorbance at 492 nm (Promega, Madison, WI). BQ788 and BQ123 (Calbiochem, La Jolla, CA) were used as described previously (11).

Immunoblotting. Samples were subjected to SDS-PAGE according to the method of Laemmli (22). Proteins were blotted onto polyvinylidene difluoride (Millipore, Marlborough, MA) membranes and the filters were blocked with 5% nonfat dry milk. Immunostaining was performed with anti-human ETBR antibody (4 μg/mL; Assay Designs, Inc., Ann Arbor, MI) or anti-human tubulin (1 μg/mL; Oncogene Research Products, San Diego, CA) followed by incubation with a peroxidase conjugated goat antirabbit secondary antibody (1: 5000; Sigma, St. Louis, MO). Bands of Mr 51,000 and Mr 60,000 for ETRB and tubulin respectively were detected using a chemiluminescent substrate kit (Hybond ECL, Amersham, Arlington Heights, IL) according to the manufacturer’s recommendations.

cDNA Microarray Analysis. Cells were cultured in the presence of BQ788 or its solvent (HCO60) for 2 days and then lysed and subjected to RNA
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extraction with SV Total RNA isolation kit (Promega). Two rounds of RNA amplification were conducted with RiboAmp RNA Amplification kit (Arcturus, Moutainview, CA). Labeled cDNA was obtained by reverse transcription of 6 µg of amplified RNA and incorporation of 5-aminopropargyl-2'-deoxyuridine 5'-triphosphate coupled to Cy3 fluorescent dye and 5-aminopropargyl-2'-deoxyuridine 5'-triphosphate coupled to Cy5 fluorescent dye (Amer sham Biosciences, Amersham, United Kingdom). Human 10k arrays containing PCR products spotted onto glass slides were obtained from the Lausanne DNA Array Facility. Hybridization of labeled cDNA to microarrays was performed for 16 hours at 64°C in a humidified chamber (Corning Costar, Cambridge, MA). Scanning was done in a Scanarray 4000 scanner (Perkin-Elmer, Foster City, CA). Image analysis was performed with the ScanAlyze program (version 2.5). Data analysis was done with the R package: Statistics for Microarray analysis containing com.braju.sma package.4

Real-Time–PCR. RNA was prepared from the different cell lines in different culture conditions with SV total RNA isolation kit (Promega) according to the manufacturer’s protocol. For each sample, RNA concentration was determined with the total RNA nanoprobe of an Agilent bioanalyzer. A stock of 5K-MEL-28 RNA aliquots was prepared containing 10, 20, 50, 100, 200, 500, and 1000 ng/12 µL for use as a standard curve and kept in −80°C. For the experimental unknown samples, a stock of 100 ng/12 µL RNA aliquots from each cell line and condition was prepared and stored at −80°C. For each real-time experiment, one series of standard curve and unknown experimental series of aliquots were put on ice for use. cDNA was prepared by adding to each tube 0.5 µL of random hexamers (Promega) and 1 µL of 10 mM oligonucleoside triphosphate mix (Promega) and incubating at 65°C for 5 minutes. Four microliters of 5× Moloney murine leukemia virus buffer (Promega), 1 µL of the RNase inhibitor RNasin (Promega), and 1 µL of Moloney murine leukemia virus reverse transcriptase RNAse H (Promega) were then added, and the solution was incubated at 42°C for 50 minutes followed by 15 minutes at 70°C. For real-time reactions, 15 µL of each cDNA sample were transferred to 3 wells in a 386-well plate. To each well we added 10 µL of TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA), 1 µL of primers + probe mix (Assays-on-Demand from Applied Biosystems: EDRNB Hs00207474, BCL-2A1 Hs00187845, PARP-3 Hs01545151, VEGF Hs00173626, HIF-1α Hs00153153, and Gravin Hs00374507) and 4 µL of nuclelease-free water (Promega). Wells containing water instead of cDNA served as negative controls. The samples were subjected to 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. A standard curve of Ct versus RNA quantity was established, and accordingly, the Ct in the unknown samples was correlated to quantity of transcript.

Apoptosis. Cells were plated in 24 wells plate in 500 µL of medium and cultured for 5 days in the presence of BQ788 or solvent. For measuring apoptosis, we used a cell death detection ELISA PLUS kit (Roche, Basel, Switzerland) designed to reveal histone–180-bp DNA fragment complexes of each tube 0.5 L of the RNase inhibitor RNasin (Promega), and 1 L of random hexamers (Promega) and incubating at 65°C for 5

RESULTS

Endothelin Receptor B Antagonism Is Most Effective against Metastatic Melanoma. To address the relationship between the level of progression of human melanoma and responsiveness to BQ788 treatment, we initially used four cell lines of which three had been grown in culture for no more than 5 to 10 passages. The cell lines included Me 191-i/G (Primary), a low-passage cell line derived from a primary cutaneous melanoma lesion, Me 190/DA (Cut-met), a cell line derived from a s.c. metastasis proximal to the primary lesion, and Me 275/EP (LN-met), a cell line derived from a lymph node metastasis of a patient who had s.c. metastasis 2 years earlier. The fourth was the lymph node metastasis derived cell line SK-MEL28, which has been maintained in culture for an undetermined number of passages (American Tissue Culture Collection), and which we had previously shown to be highly sensitive to BQ788 (11). To determine whether the low-passage cell lines display invasiveness in vitro that reflects their stage of progression, we assessed their behavior in a Matrigel invasion assay. Consistent with the lesions from which they originated, most of the primary melanoma cells failed to penetrate the gel and remained on its surface (Fig. 1A), the s.c. metastasis-derived cells displayed an intermediate degree of invasiveness (Fig 1A) and the lymph node-derived cells were highly invasive (Fig 1A). The SK-MEL28 cells, however, did not invade Matrigel (data not shown), possibly as a consequence of prolonged maintenance in culture. Western blot analysis showed that expression of ETRB was more elevated in melanoma cells from metastatic than from primary lesions (Fig. 1B). Accordingly, the sensitivity of the cells to BQ788 was propor-
tional to the stage of progression of the tumor from which the cells were derived (Figs. 1C and 2C). Thus, although incubation with BQ788 for 7 days had little effect on primary melanoma cell viability (12% cell death), it reduced the viability of the Cut-met cells by 45% and that of the LN-met line by 96% (Fig. 1C). Taken together with the report that ETRB expression increases in human melanoma as they advance to metastatic disease (15), these observations suggest that ETRB inhibition might be most effective in metastatic melanoma.

Endothelin Receptor B Inhibition Reduces BCL-2A1 and Poly-(ADP-ribose) Polymerase-3 (PARP-3) Expression and Induces Apoptosis and Caspase-6 Activation. To explore the possible mechanisms that underlie the loss of viability of metastatic but not primary...
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Table 1 Differentially regulated genes in SK-MEL-28 and LN-met cell lines after 2 days of treatment with BQ788 as revealed by microarray analysis and confirmed by real-time reverse transcription-PCR.

<table>
<thead>
<tr>
<th>Gene function</th>
<th>Down-regulated</th>
<th>Up-regulated</th>
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<tbody>
<tr>
<td>Cell death</td>
<td>BCL2-A1</td>
<td>PARP-3</td>
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<tr>
<td>Angiogenesis</td>
<td>VEGF</td>
<td></td>
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<tr>
<td>Development</td>
<td>Gravin</td>
<td>EDNRB</td>
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NOTE. Differentially regulated genes in human melanoma cells upon EDNRB antagonism.

melanoma cells in response to ETRB antagonism, we addressed changes in gene expression profiles of BQ788-treated and untreated cells by cDNA microarray analysis. The LN-met and SK-MEL28 cells were treated with BQ788 and RNA was extracted 2 days after treatment, a time point that precedes significant changes in cell viability by 3 to 5 days. For each cell line, transcripts from BQ788-treated cells were compared with those derived from control (solvent-treated) cells.

Comparing the differentially regulated genes in the two different cell lines resulted in the identification of only a few genes that appeared to be significantly affected by BQ788 at this early time point (Table 1). Two genes that were found to be down-regulated upon treatment with BQ788 included the survival factor BCL-2A1 (24–32) and ADP ribosyltransferase 3 (PARP-3; ref. 33). PARP enzymes are activated in response to DNA damage and are implicated in the repair of DNA strand breaks. PARP cleavage, leading to its inactivation and thereby preventing DNA repair and improving endonuclease access to chromatin, is an early event in apoptosis (34, 35).

If the observed reduction in BCL-2A1 and PARP-3 expression is implicated in ETRB blockade-dependent cell death, one would expect the more resistant cell lines to display less reduction in BCL-2A1 and PARP-3 levels. To test this possibility and to validate our microarray results, we used quantitative real-time PCR. We found that the degree of reduction in BCL-2A1 RNA correlates with the reduction in viability of the four different cell lines treated with BQ788 (Fig. 2A).

The primary melanoma cells displayed low expression of BCL-2A1, which did not change with BQ788 treatment. The Cut-met line displayed slightly higher levels of BCL-2A1, which was reduced 1.8-fold by BQ788. The more sensitive cell lines LN-met and SK-MEL-28 displayed a 2.9- and 5.7-fold decrease in BCL-2A1 levels, respectively, as a result of BQ788 treatment. A similar response pattern was observed for PARP-3 (Fig. 2B).

Because BCL-2A1 and PARP are known regulators of apoptosis (36, 37), we used an ELISA that detects histone-associated DNA fragments to measure apoptotic cell death in the different cell lines treated with BQ788. The levels of apoptosis were observed to correlate with the levels of reduction in BCL-2A1 and PARP-3 RNA (Fig. 2C). The primary melanoma cells, which did not show a decrease in BCL-2A1 or PARP-3 levels, did not undergo apoptosis in response to BQ788 treatment. In contrast, the other cell lines displayed a gradual increase in apoptosis, ranging from a small increase in the Cut-met line through an intermediate increase in the LN-met line to the highest induction in SK-MEL-28 cells.

BCL-2-A1 inhibits the activation of caspase 9 but not of caspases 3 or 8 in endothelial cells (38). To verify the association between caspase activity and ETRB blockade-dependent cell death, we tested the effect of the pan caspase 3 inhibitor I (which blocks caspases 3, 6, 7, 8, and 10) at various time points ranging from 6 hours to 7 days in culture on apoptosis triggered by BQ788 in melanoma cells. At the early time points, when the BQ788 effect was still modest, the pan-inhibitor rescued cell viability (Fig. 2D). However, after 5 to 7 days, the pan inhibitor was no longer effective (data not shown).

Although addressing the possible activation of effector caspasas, we observed that at all time points tested, caspase 6 inhibitor was more effective at rescuing cells than the pan-caspase inhibitor (Fig. 2E). After 3 days of treatment with BQ788, caspase 6 activation could be detected (Fig. 2F). Taken together, these results suggest that ETRB antagonism induces melanoma apoptosis through reduction in BCL-2A1 expression and, at least in part, caspase 6 activation.

Endothelin Receptor B Expression Levels Are Important for Metastatic Melanoma Cell Viability. Our microarray data suggest that ETRB transcription was reduced in melanoma cells treated with BQ788 (Table 1). Additional examination of the four different melanoma cell lines with real-time–PCR confirmed this observation and showed that sensitivity of the melanoma cell lines to BQ788 treatment correlated with the reduction in ETRB mRNA expression (Fig. 3A).

Although the primary melanoma cells did not show any change in ETRB expression levels as a result of BQ788 treatment, the Cut-met line displayed a 1.49-fold reduction, the LN-met line a 1.85-fold decrease, and SK-MEL-28 cells an 11-fold decrease. Western blot analysis showed that ETRB protein expression was reduced upon treatment of SK-MEL-28 cells with BQ788 (Fig. 3B), providing support to the notion that decreased melanoma viability correlates with reduction in ETRB expression.

To address the functional relevance of this correlation, we used small interfering (si)RNA to lower the endogenous ETRB levels and found that a reduction in ETRB expression results in reduced melanoma cell viability (Fig. 4). LN-met cells were transiently transfected with either pSuper (23) containing 21-bp fragments that had been shown by BLAST analysis to be ETRB-specific or ETRB-targeting siRNA oligonucleotides and tested for ETRB expression by real-time PCR and Western blot analysis 48 and 96 hours later. Both approaches resulted in a strong decrease in ETRB transcript levels as early as 48 hours after transfection (Fig. 4A and data not shown), accompanied, at 96 hours, by a significant decrease in ETRB protein levels (Fig. 4B) and reduced cell viability (Fig. 4C). Transfection of Cut-met and SK-MEL-28 cells with ETRB-targeting pSuper RNA interference
resulted in a similar reduction in viability 96 hours after transfection (Fig. 4, C and D). These observations are consistent with the notion that ETRB expression may play an important role in the survival of metastatic melanoma cells.

**Endothelin Receptor B Antagonism Leads to Enhanced Angiogenesis.** Surprisingly, microarray analysis of BQ788-treated cells revealed an up-regulation of vascular endothelial growth factor (VEGF) expression. Real-time PCR analysis confirmed that VEGF expression levels increased 18-fold in BQ788-treated LN-met cells and 65-fold in SK-MEL-28 cells, compared with only 2-fold in primary melanoma, and no change in the Cut-met line (Fig. 5A). The observed changes in VEGF expression may be explained, at least in part, by changes in the expression levels of its known regulator hypoxia-inducible factor-1α (HIF-1α; ref. 39). HIF-1α RNA expression levels showed small but significant changes upon treatment of the cells with BQ788 (Fig. 5B), which correlated with the corresponding changes in VEGF expression. These results suggest that down-regulation of ETRB leads to up-regulation of HIF-1α and VEGF, which is supported by the finding that in ETRB-deficient rats, HIF-1α and VEGF expression levels are higher than normal (40). Because VEGF and HIF-1α are regulated by ET-1 via the activation of the ETRA (40–42), we assessed the effect of ETRA blockade on the melanoma cell lines used in this study. The ETRA inhibitor BQ123 in combination with BQ788 did not alter the changes in VEGF RNA levels induced by BQ788 alone, suggesting that VEGF expression induced by ETRB inhibition is independent of ETRA activity (data not shown).

Interestingly, expression of the A kinase anchoring protein gravin, which has been observed to inhibit VEGF expression in astrocytes (43), was down-regulated in our cell lines upon treatment with BQ788 (Fig. 5C). Down-regulation of gravin, at least in the most sensitive cell lines, LN-met and SK-MEL-28, correlated with induction of VEGF. We therefore analyzed tissue sections of tumors derived from a human melanoma cell line grown in nude mice and injected with BQ788 for signs of changes in angiogenesis (11). Despite inhibition of growth, BQ788-treated tumors showed an increase in CD31-positive vascular structures, as revealed by immunohistochemistry, consistent with stimulation of angiogenesis (Fig. 5, D and E). These results suggest a direct correlation between the induction of VEGF transcription in vitro and increased angiogenesis in vivo resulting from ETRB inhibition in metastatic melanoma cells.

**Sensitivity to Endothelin Receptor B Inhibition Characterizes Metastatic Melanoma Cells.** To provide additional evidence that melanoma cell sensitivity to ETRB inhibition increases with tumor progression, we assessed the response to BQ788 of six additional melanoma-derived cell lines that had undergone no more than 5 to 10 passages in vitro. These included cells from two primary melanomas (Me300 and T921A), one cutaneous metastasis (T640A), one lymph node metastasis (T387A), and two visceral metastases [Me 256 (in intestine) and T523A (pleural effusion)]. Treatment of these cells with BQ788 recapitulated the observations made on the initial cell lines, with the primary tumor-derived cells showing minimal sensitivity and metastatic lesion-derived cells displaying 60 to 80% sensitivity (Fig. 6A). Consistent with these results, real-time PCR analysis of ETRB, BCL-2A1, PARP-3, and GRAVIN transcripts in Me300, T640A, and Me256 cells (representing a primary lesion, and a local and distal metastasis) revealed that the strongest decrease in expression of all four genes in response to BQ788 occurred in distal metastasis-derived cells, with little or no change being detected in cells derived from primary tumors (Fig. 6B–E). Accordingly, the strongest increase in VEGF expression was observed in metastatic cells (Fig. 6F).
ETRB expression has been shown to increase with melanoma progression, being highest in metastatic lesions (15). We have shown here that blocking ETRB with BQ788 can induce melanoma cell death in culture and that melanoma sensitivity to ETRB inhibition increases with tumor progression. Repression of ETRB with siRNA decreased melanoma cell viability, supporting the notion that high-grade melanoma cells depend, at least in part, on ETRB-derived signals for their survival. Cell death induced by inhibition of ETRB was preceded by the repression of the ETRB, BCL-2A1, PARP-3, and GRAVIN genes and induction of the VEGF gene, suggesting hitherto unexplored links between ETRB engagement and intracellular signaling.

Endothelin is increasingly recognized to promote proliferation, survival, migration, and invasion of a broad range of tumors by stimulating ETRA, ETRB, or both, depending on the tumor type (44). Stimulation of ETRB in astrocytes activates Rap1/B-Raf and Ras/Raf-1 complexes, protein kinase (PK)C, and the mitogen-activated protein kinase pathway, resulting in the activation of extracellular signal-regulated kinases (45). In melanomas, which preferentially express ETRB, ET-1 stimulates proliferation and survival by mechanisms that remain to be elucidated. The observations made in the present work may provide clues toward understanding some of the mechanisms involved.

Interestingly, gravin, which serves as a scaffold to coordinate the location of PKA, PKC, and actin (48), was observed to be repressed in response to ETRB inhibition. Subcellular localization of signaling enzymes plays a central role in the control of cellular physiology. Correct intracellular targeting of kinases and phosphatases to their preferred substrates is essential to reduce indiscriminate phosphorylation and dephosphorylation that could alter the activation and function of vital cellular mechanisms and potentially compromise cell survival itself. Consistent with this notion, gravin may play a role in localizing PKA and PKC to substrates that are relevant to ETRB...
signaling. PKC is implicated in the regulation of cell survival (49), and it is conceivable that in high-grade melanoma cells, suboptimal localization of PKC might alter the effectiveness of ETRB signaling, contributing to increased susceptibility to proapoptotic stimuli. In support of this view, intracellular location of PKC-β, which provides survival signals in T cells, has recently been shown to play a critical role in the regulation of its function (50). It is also noteworthy that the mouse orthologue of gravin, SSeCKS, interacts with and regulates G-protein–coupled receptors (43, 48). This observation raises the interesting possibility that gravin may foster communication between ETRB on the one hand and PKC and PKA on the other. Thus, the observed changes in BCL-2A1, PARP-3, and GRAVIN gene expression in response to ETRB inhibition suggest several possible mechanisms that may contribute to ETRB-dependent survival of metastatic melanoma cells.

An unexpected observation was that the repression of genes implicated in protection against apoptosis by BQ788-mediated inhibition of ETRB was accompanied by the induction of VEGF expression. Accordingly, the growth inhibitory effect of BQ788 on human melanoma xenografts was associated with increased angiogenesis. These observations are consistent with reports suggesting that ETRB deficient rats display higher basal VEGF expression levels than wild-type counterparts (40). In addition, examination of expression data from 31 human melanomas (13) indicates that tumors with relatively low ETRB expression have lower BCL2-A1 and higher VEGF expression levels than tumors with high ETRB expression. The relationship between ETRB, BCL-2A1, and VEGF expression observed in our melanoma-derived cell lines may therefore reflect the physiologic in vivo situation.

It is plausible that increased melanoma cell production of VEGF in response to ETRB blockade may stimulate endothelial cell proliferation in paracrine fashion, leading to the observation that tumors injected with BQ788 display increased angiogenesis. However, these observations raise several questions. Given that VEGF promotes growth of a range of tumor types independently of its proangiogenic properties, how can increased VEGF expression be reconciled with reduced survival? A simple explanation may be that the conflicting signals generated by ETRB blockade, i.e., VEGF induction on the one hand and BCL-2A1 and gravin repression on the other, trigger apoptosis by default. An alternative explanation may lie in an imbalance between pro- and antiapoptotic forces, where the effect of suppression of antiapoptotic signals resulting from BCL-2A1 and gravin repression overrides the survival promoting effect of VEGF.

A related issue is the mechanism whereby ETRB blockade induces VEGF expression. A possibility that we had envisaged was that selective blocking of ETRB might result in increased ETRA signaling,
treatment could induce HIF-1α expression during BQ788 treatment alone, rendering such a mechanism unlikely. A second possibility might be that HIF-1α and VEGF expression constitute part of a stress response program in metastatic melanoma cells. The stress imposed by BQ788 treatment could induce HIF-1α expression, which would in turn augment VEGF production. Finally, a recent study has shown that SSeCKS represses VEGF expression in astrocytomas while augmenting angiopoietin-1 expression, thereby repressing angiogenesis and increasing vascular stability (43). It is plausible that gravin may control, in part, VEGF expression in high grade melanoma cells and that its down-regulation may participate in augmenting VEGF transcription. Clearly, several possible mechanisms may contribute to the observed increase in VEGF expression in response to ETRB blockade, and additional work will be required to elucidate the relationship between ETRB signaling and VEGF expression. Nevertheless, the potential implication of gravin in ETRB signaling and VEGF expression control offers an attractive lead for future investigation.

Currently, there is no effective cure for metastatic melanoma, and although various approaches are being implemented, an efficient way to eliminate or even reduce metastatic lesions has yet to be developed. Our present observations that melanoma progression depends, at least in part, on ETRB-related survival signals suggest that ETRB inhibition may provide a potentially promising new therapeutic avenue for the management of invasive and metastatic melanoma. The observation that ETRB inhibition induces VEGF could be important for the design of clinical trials. VEGF induces angiogenesis, which promotes tumor growth and invasiveness, but also increases vascular permeability, which could potentially enhance drug delivery (51). BQ788 administration results in inhibition of tumors grown in nude mice and leads to shrinkage of tumors treated systemically. It is tempting to speculate that by stimulating angiogenesis and vascular permeability, BQ788 action may help create conditions that enhance tumor cell accessibility and thereby amplify its own proapoptotic action. It is also conceivable that BQ788 may be of value in combination with other drugs whose delivery it may facilitate by promoting angiogenesis. BQ788 has already been assessed in clinical trials for hypertension in patients and healthy volunteers and was not found to be toxic (52, 53). The potential effectiveness of BQ788 and its analogues alone or in combination with conventional approaches may be worth investigating in the clinic.

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