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-2A1 and the DNA repair factor poly(ADP-ribose) polymerase 3 that is more pronounced in cells derived from metastatic than primary melanomas. Decreased cell viability was observed to correlate with reduction in ETRB expression, and reduction in ETRB protein levels by small interfering RNA led to an increase in 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 skin 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 Me191-1/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, Holdrege, 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-ETB (assay design, Inc., Ann Arbor, MI) or anti-GAPDH monoclonal antibody (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 (Interchim, Montluçon, France) 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, Mountain View, CA). Labeled cDNA was obtained by reverse transcription of 6 μg of amplified RNA and incorporation of 5-amino-propargyl-2'-deoxy-

cytidine 5'-triphosphate coupled to Cy3 fluorescent dye and 5-amino-propargyl-

2'-deoxyctydine 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 Scann arrayer 4000 scanner (Perkin-

Elmer, Foster City, CA). Image analysis was performed with the ScanArray pro-
geam (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 L deoxythymidylate triphosphate mix (Promega) and incubating at 65°C for 5 minutes. Four microliters of 5X 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 384-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: EDNDRB Hs00207474, BCL-2A1 Hs00187845, PARP-3 Hs00154515, VEGF Hs00176526, HIV-1e Hs00153153, and Gravin Hs00374907) and 4 μL of nuclease-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 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 complex of nucleosomes. Briefly, cells floating in the supernatants and those attached to the dish were incubated in lysis buffer for 30 minutes in room temperature. After spinning for 10 minutes at 200 × g, the DNA content of each sample was determined. Twenty microliters containing equal amounts of DNA were added to each well (three wells per condition) coated with antihistidine antibody and incubated with additional 80 μL of anti-DNA peroxidase immunogen under gentle shaking conditions for 2 hours at room temperature. After washing and revelation with peroxidase substrate, the samples’ absorbances were measured at 405 nm against substrate containing wells as blank.

Caspase Inhibitors and Caspase-6 Activity. Caspase 3 inhibitor I (inh-
cits caspases 3, 6, 7, 8, and 10), cell-permeable (Calbiochem, San Diego, CA) and Caspase 6 inhibitor II, Cell-Permeable (Calbiochem) were dissolved in DMSO: Cells were cultured in 96 wells and the next day divided into four groups: control with solvents only DMSO + HCO60 [the solvent of BQ788 according to (9, 11)], caspase inhibitor + HCO60, BQ788 + DMSO, and BQ788 + caspase inhibitor. Cell viability was measured with MTS solution in parallel experiments at five different time points. Caspase 6 activity was measured using Mch2/Caspase-6 Colorimetric Protease Assay kit according to the manufacturer’s instructions. Briefly, 3 X 105 SK-MEL-28 cells were plated onto 10-cm culture dishes. On the following day, BQ788 was added to experimental plates and its solvent to controls. Three days later, cells were lysed, and the lysates assayed for protein concentration. Caspase 6 activity was measured in four samples containing equal amounts of protein from each condition by spectrophotometric detection of a chromophore after its release from the labeled caspase 6 substrate VEID.

RNA Interference. Two different methods were tested for RNA interfer-
ge, giving comparable results. The first approach used predesigned double-
strand RNA oligonucleotides targeting ETRB (Ambion, Austin, TX; sequence: 5'-GGAGACUUCUAUAC5CTT-3'). Cells were plated in 24-well plates with 500 μL of medium supplemented with 10% fetal bovine serum, without antibiotics, 24 hours before transfection. For each well, cells at 40 to 50% confluence were transfected with 60 pmol of siRNA duplex, with Oli-
gofectamine Reagent (Invitrogen, Carlsbad, CA), according to the manufact-
er’s recommendations. Control cells were transfected under the same conditions with double-strand RNA oligonucleotides targeting FlI (Qiagen, Valencia, CA, sequence: 5'-UUUGACUUCCAGCGCAUUG-3'). The second approach used pSuper (23). Sixty-four mer oligonucleotides were synthesized containing a target 19 mer sequence specific to ETRB (see bold characters in the sequence). Comparing this sequence to the GenBank human Expressed Sequence Tag database gave similarities only to ETRB even when the compar-
ison was reduced to only 15 mer of the 19. Additional flanking sequences were added for the creation of a stem loop structure and BGII and HindIII cloning sites (23): forward oligonucleotide, 5'-GATCCCCGTCGATCCGCTAACGGACCTCCTCAAGAGGACCGTTTCGCATGCAC-AAA-3'; and reverse oligonucleotide, 5'-AGCCTTTTCCAAAAGTGCTGACATGTTGCAAGGGGTTTCCTCCTTCGTTAAGGACCCCTGG-TGCGAACGTCGGCCTCTCCTGTIGAAGGAGGCGCTGCATTGGGGAAA-3'. The oligonucleotides were annealed, phosphorylated, and ligated into BGII and HindIII sites of the pSuper plasmid (23) containing a H1 RNA promoter [kind gift of Professor René Bernards (Netherlands Cancer Institute, Amster-
dam, the Netherlands)]. For each cell line 20 to 40 × 103 cells were cultured in each well of 96-well plate containing medium without antibiotics. On the following day, the cells were transfected with 0.4 μg of specific or control RNA interference plasmid + 0.8 μL Lipofectamine 2000 (Invitrogen) in OptiMEM I with Glutamax I (Life Technologies, Inc.). The medium was replaced with a complete medium 6 hours later. Viability was measured 48 and 96 hours after transfection by adding MTS solution (Promega) and measuring the absorbance at 492 nm.

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-l/CG (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. West-
ern 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-

4 Internet address: http://www.r-project.org.

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Fig. 1. BQ788 is most effective against metastatic melanoma. A. Cells were cultured on Matrigel in 24-well culture plate inserts for 2 weeks, after which, the inserts were removed and cells in the lower wells that had traversed the matrix were quantified in an MTS assay. B. Cell lysates of the three lines were subjected to Western blot analysis with an anti-ETRB antibody (top panel) and an anti-tubulin antibody (bottom panel). C. Viability of melanoma cells that were untreated, vehicle treated (control), and BQ788-treated cells for 7 days as assessed in an MTS assay. The mean values were calculated and plotted as the percentage of the vehicle treated value ± SE. BQ788 values for the three cell lines were significantly different from control (P < 0.05).

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 advance to metastatic disease (15), these observations suggest that ETRB inhibition might be most effective in metastatic melanoma.
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 BCL2-A1 (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 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 PARP-3 levels. To test this possibility and to validate our microarray results, we used quantitative real-time PCR.

To address the functional relevance of this correlation, we used small interfering (si)RNA to lower the endogenous ETRB levels and observed 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. Both approaches resulted in a strong decrease in ETRB transcript levels and reduced melanoma viability.

**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>
</tr>
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<tbody>
<tr>
<td>Cell death</td>
<td>BCL2-A1</td>
<td>PARP-3</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>Gravin</td>
<td>VEGF</td>
</tr>
<tr>
<td>Development</td>
<td>EDNRB</td>
<td>HIF-1α</td>
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NOTE. Differentially regulated genes in human melanoma cells upon EDNRB antagonism.
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 (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*, *PAPR-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).
DISCUSSION

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.

There appears to be a direct correlation between the reduction of ETRB expression and that of the survival factor BCL-2A1 and the DNA repair factor PARP-3 in response to ETRB blockade. BCL-2A1 protects several cell types from apoptosis, including monocytes, macrophages, endothelial cells, neutrophils, and B-cell lymphomas (25, 27, 28, 31, 32), and mediates chemoresistance in some human cancer cell lines (24, 29, 30). PARP-3 belongs to a family of constitutive factors of the DNA damage surveillance network (46). PARP-1 promotes transcriptional activation of nuclear factor-kB (34, 47), a known inducer of BCL-2A1 (24, 27, 29–32). It therefore seems likely that at least two consequences of ETRB inhibition include impairment of DNA repair and reduction of resistance to proapoptotic signals. Down-regulation of BCL-2A1 results in caspase 9 activation but neither caspase 3 nor 8 cleavage (31, 38). Consistent with these observations, Western blot analysis did not reveal caspase 3 activation (data not shown) but a colorimetric assay detected caspase 6-like activity and caspase 6 inhibitors rescued cells from BQ788-induced apoptosis. These observations suggest that caspase 6 may be implicated in the induction of apoptosis mediated by ETRB inhibition. Whether caspase 6 activation occurs via reduction in BCL-2A1 expression or by alternative pathways remains to be determined.

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,
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which is reported to enhance HIF-1α and VEGF expression (44). However, combined inhibition of ETRA and ETRB had no effect on the increase in VEGF expression elicited by 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 astrocytes while augmenting angiopoietin 1 expression, thereby reducing 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 will be worth investigating in the clinic.

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