Endothelin B Receptor Blockade Inhibits Dynamics of Cell Interactions and Communications in Melanoma Cell Progression

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ABSTRACT

Phenotypic and genotypic analyses of cutaneous melanoma have identified the endothelin B receptor (ET\(_B\)R) as tumor progression marker, thus representing a potential therapeutic target. Here, we demonstrate that activation of ET\(_B\)R by endothelin-1 (ET-1) and ET-3 leads to loss of expression of the cell adhesion molecule E-cadherin and associated catenin proteins and gain of N-cadherin expression. Exposure of melanoma cells to ET-1 leads to a 60% inhibition in intercellular communication by inducing phosphorylation of gap junctional protein connexin 43. Additionally, activation of the ET\(_B\)R pathway increases \(\alpha\)\(_{\beta}3\) and \(\alpha\)\(_{\beta}6\) integrin expression and matrix metalloproteinase (MMP)-2 and MMP-9, membrane type-1-MMP activation, and tissue inhibitor MMP-2 secretion. The ET\(_B\)R pathway results into the downstream activation of focal adhesion kinase and extracellular signal-regulated kinase 1/2 signaling pathways, which lead to enhanced cell proliferation, adhesion, migration, and MMP-dependent invasion. The small molecule A192621, an orally bioavailable nonpeptide ET\(_B\)R antagonist, significantly inhibits melanoma growth in nude mice. These findings demonstrate that ET-1 and ET-3 through ET\(_B\)R activation trigger signaling pathways involved in events associated with disruption of normal host-tumor interactions and progression of cutaneous melanoma. Pharmacological interruption of ET\(_B\)R signaling may represent a novel therapeutic strategy in the treatment of this malignancy.

INTRODUCTION

Mouse genetics and molecular profiling of human tissues have identified pathways relevant to melanocyte migration and differentiation as well as to melanoma progression. The study of melanocyte development in knock-out mice has provided insight into the functions of endothelins (ETs) and their receptors in this cell lineage (1–4). The ET family of molecules comprises three isopeptides, ET-1, ET-2, and ET-3, of 21 amino acids that bind to two highly homologous G-protein-coupled receptors, ETA receptor (ET\(_A\)R) and ET\(_B\) receptor (ET\(_B\)R), which mediate a variety of physiological activity in different cell types (5). In the adult, ET-1 secreted by keratinocytes stimulates proliferation, chemotaxis, and pigment production in melanocytes (5, 6) and plays a central role in UV-B light-induced pigmentation (7, 8). Increasing evidence points to ETs as relevant mediators in tumor progression in a variety of malignancies (9). Melanoma cells express both ET\(_A\)R and ET\(_B\)R (10). Gene expression analysis (11) and immunohistochemistry of human cutaneous melanoma (12) have recently identified ET\(_B\)R as critical in the progression of this malignancy. Through the same receptor, ET-1 acts as antiprotective factor for melanoma cells and melanocytes (13). Thus, ET\(_B\)R blockade by the ET\(_B\)R peptide antagonist BQ788 resulted in growth inhibition and death of melanoma cells in vivo and in vitro (14). Although these studies define a relevant role of the ET-1/ET\(_B\)R pathway in the biology of melanocytic tumors, the molecular events underlying this activity have not been investigated. Early melanoma growth is the result of disrupted intercellular homeostatic regulation (15). Once this balance is lost and malignant transformation has occurred, microenvironmental factors such as cell adherence to extracellular matrix, host-tumor interactions, degradation of matrix components, migration, and invasion became essential for the tumor progression to the metastatic phenotype (15). Changes in cadherin, connexin (Cx), matrix metalloproteinase (MMP), and integrin expression have emerged as key factors in melanoma progression (16–17). Therefore we analyze the role played by ETs and ET\(_B\)R in melanoma cell proliferation, cell-cell adhesion and communication, migration, tumor proteinase activation, and invasion. Because activation of focal adhesion kinase (FAK) and of extracellular signal-regulated kinase (Erk) are essential molecular signaling in melanoma progression (18–20), we analyzed the effect of ETs on these ET\(_B\)R-mediated signal transduction pathways. In view of the availability of orally active nonpeptide ET\(_B\)R antagonists (9, 21), which are more suitable in clinical setting, we use these compounds to assess their antitumor activity in vivo.

MATERIALS AND METHODS

Cells. The human cutaneous melanoma cell line 1007 established from a primary lesion was a generous gift of Dr. G. Parmiani (National Cancer Institute, Milan, Italy). The melanoma cell lines SK-Mel28 (American Type Culture Collection, Rockville, MD) M10 and Mel120 were derived from a metastatic melanoma (22). Cells were grown in RPMI 1640 containing 10% FCS. All culture reagents were from Invitrogen (Paisley, Scotland, United Kingdom). Melanoma cells were starved for 24 h in serum-free medium and then incubated for indicated times with ET-1 and ET-3 (Peninsula Laboratories, Belmont, CA). When the effects of the antagonists [BQ123 and BQ788 (Peninsula Laboratories) and A192621 (Abbott Laboratories, Abbott Park, IL)] were studied, they were added 15 min before agonist. Pretreatment of cells with PD98059 (10 \(\mu\)M, Calbiochem-Novabiochem Corporation, San Diego, CA) or Iomastat (10 \(\mu\)M, Chemicon International, Temecula, CA) was performed for 20 min before the addition of ET-1 or ET-3.

Reverse Transcription-PCR. Total RNA was prepared using the TRIzol reagent (Invitrogen) as recommended. The primer sets used were 5'-TGGCTCTCCTGCTCCCTCTAGATGATTAAAGAG-3' and 5'-GGTCCATAACAGCTTCTTGAGGCGTT-3' for ET-1, 5'-4 CACTGTTGTTGATGTTGATAC-3' and 5'-GGAGATCATAAGCAGAATA-3' for ET\(_A\)R, 5'-TCAAACGGTGTGTTGATGTC-3' and 5'-ACTGAATAGCCACAACTTTC-3' for ET\(_B\)R, 5'-AACTGAGGTGCTGAGGCTCGT-3' and 5'-TTTCCAGGCTGAGGAATGCT-3' for E-cadherin, and 5'-TTCCAGGCTGAGGAATGCT-3' and 5'-GCTTTTCCACTGTCATT-3' for Snail. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control and the primer sets used were 5'-TGAAG-3' and 5'-GTCCGAGCTCAACGG-3' and 5'-GTAGGCATTGAGCTTCTC-3' for E-cadherin. The cDNA was amplified for 35 cycles of a denaturation step at 94°C for 1 min; a primer annealing step at 64°C (E-cadherin), 55°C (GAPDH), and 54°C (Snail), and 54°C (ET-1, ET\(_A\)R, ET\(_B\)R, and GAPDH) for 30 s; and an extension step at 72°C for 1 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide.

Northern Blotting. Total RNA from 1007 cells was separated on 2% agarose gel containing ethidium bromide. 2 mg RNA was applied per lane and transferred to a Hybond-N+ nylon membrane. The membranes were hybridized in the QuickHyb hybrid-
ization solution (Strategene, La Jolla, CA). The cDNA probe used for analysis of the E-cadherin, Snail, and GAPDH mRNA was prepared by reverse transcription-PCR products. Probes were labeled with [α-32P]dCTP using a random hexamer oligolabeling kit (Amersham Biosciences, Little Chalfont Buckinghamshire, United Kingdom). Densitometric scanning was performed with aMustek MES-6000CX apparatus, and the data were analyzed with Phoretix 1D software and normalized to those of GAPDH.

**Western Blotting.** Conditioned medium and protein extracts were separated by SDS-PAGE and revealed by Western blot using antibodies (Abs) to MMP-2 and MMP-9 (NeoMarkers, Fremont, CA), tissue inhibitor metalloproteinase (TIMP)-2, membrane type 1 (MT1)-MMP (Chemicon), phospho-ERK1/2 (Cell Signaling Technology, Inc., Beverly, MA), ERK1/2 (Cell Signaling Technology), αv, α2, β1, β3 (Chemicon), E-cadherin, N-cadherin, β-catenin, p120-catenin, Cx43 (BD Transduction Laboratories, Heidelberg, Germany), and β-actin (Oncogene Research Products, Boston, MA). Blots were developed with an enhanced chemiluminescence detection system kit (Amersham Biosciences).

**Gelatin Zymography.** The 1007 and M10 cell supernatants were electro- phoresed for analysis in 9% SDS-PAGE gels containing 1 mg/ml gelatin. The gels were washed for 30 min at 22°C in 2.5% Triton X-100 and then incubated in 50 mM Tris (pH 7.6), 1 mM ZnCl2, and 5 mM CaCl2 for 18 h at 37°C. After incubation, the gels were stained with 0.2% Coomassie Blue. Enzyme-digested regions were identified as white bands on a blue background. Molecular sizes were determined from the mobility, using gelatinzymography standards (Chemicon).

**Immunoprecipitation and SDS-PAGE.** Serum-starved 1007 melanoma cells were treated with ET-1 and ET-3 for different times. Lysates were immunoprecipitated with anti-FAK (Upstate Biotechnology) or anti-β-catenin or anti-p120-catenin. The blots were incubated for 1 h with antiphosphoty-rosine mAb (PY20; BD Transduction Laboratories) or with anti-FAK (Upstate Biotechnology) and developed with an enhanced chemiluminescence system.

**Flow Cytometry Analysis.** Serum-starved 1007 melanoma cells were treated with ET-1 and ET-3 (10 nM) for 24 h and incubated with primary Ab to αv, α, α2, α5, αv, β1, β3, β2, or αvβ5 or αvβ3 (Chemicon) for 40 min at 4°C. FITC-conjugated secondary Ab (Chemicon) was applied to the cell for 30 min at 4°C. Labeled cells were scanned on a FACScan cytometer (Becton Dickson).

**ELISA.** MMP-2 and TIMP-2 levels in conditioned medium were measured using a Biotrak Human MMP-2 ELISA kit (Amersham Biosciences) and a human TIMP-2Immunooassay Kit (Chemicon), respectively. TIMP-2 expression was measured by an ELISA capable of recognizing TIMP-2 complexes with active MMP-2. The range of detection of the assays was of 1.5–320 ng/ml for TIMP-2. The experiments were performed in quadruplicate three times. Tumor size was measured with calipers and was calculated using the formula πd2 × larger diameter × (smaller diameter)2.

**Statistical Analysis.** Statistical evaluations of data were made by the two-sided Student’s test and by two-way ANOVA as appropriate.

## RESULTS

**ET-1 and ET-3 Alter Cell Adhesion Molecule Expression through ETaR.** ET-mediated activities were assessed on the primary melanoma 1007 cells and in the metastatic cell lines M10, SK Mel 28, and Mel 120, which express ETaR and ETbR mRNA but no ET-1 mRNA (Fig. 1A). The tumor suppressor E-cadherin, which is often down-regulated in most melanoma (25), has recently been shown to be modulated by ET-1 (26). In 1007 and M10 cells, ETs induced E-cadherin down-regulation upon 24 h of treatment (Fig. 1B). Because the catenins are cytoplasmic proteins that bind E-cadherin and are critical for cellular adhesion, we investigated the effects of ETs on β-catenin and p120-catenin. In both melanoma cell lines, ET-1 and ET-3 stimulation for 24 h decreased the protein levels of β- and p120-catenin parallelizing those of E-cadherin down-regulation (Fig. 1B). The shift in cadherin profile from E- to N-isofrom during melanoma progression has been described both in vitro and in vivo, as a mechanism that not only frees melanoma cells from control by keratinocytes but also provides new adhesion properties enhancing the malignant phenotype (16). In 1007 and M10 melanoma cells, we observed that ET-1- and ET-3-induced progressive loss of E-cadherin was concomitant with gain of N-cadherin expression (Fig. 1B), which may enable melanoma cells to interact with N-cadherin-positive neighboring melanoma cells, fibroblasts, and endothelial cells. BQ788, a specific ETaR antagonist, reversed these effects, indicating that ETaR activation is required for these responses (Fig. 1B). The transcription factor Snail, a master regulator of epithelial to me-enchymal transition, has recently been found to repress E-cadherin expression. Screening of a panel of melanoma cells from different progression stages revealed that the expression of Snail negatively correlates with expression of E-cadherin (15, 27). By Northern blot analysis, we demonstrated that ET-treatment of 1007 cells induced a significant increase in the expression of Snail mRNA that closely correlated with down-regulation of E-cadherin at mRNA and protein levels, suggesting that down-regulation of E-cadherin protein is involved transcriptional mechanisms (Fig. 1C). Tyrosine phosphory-lation of catenin proteins may result in a reduced interaction with both...
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**Fig. 1.** A, ETaR and ETbR mRNAs detected by reverse transcription-PCR in melanoma cell lines. PCR products of 367 bp for ETaR, 529 bp for ETbR, and 533 bp for GAPDH are shown as visualized by ethidium bromide. All of the melanoma cell lines tested, 1007, M10, SKMel 28, and Mel120, expressed mRNA for ETaR and ETbR but no ET-1 mRNA. B, down-regulation of E-cadherin and catenin proteins and up-regulation of N-cadherin by ET-1 and ET-3 in melanoma cells. Serum-starved 1007 and M10 cells were stimulated with 10 nM ET-1 or ET-3 for 24 h in the absence or presence of 100 nM BQ788. Lysates were immunoblotted with Abs to E-cadherin, N-cadherin, β-catenin, or p120-catenin and for internal control Ab to β-actin. C, Snail mRNA levels expression inversely correlate with E-cadherin after ET-treatment. Snail and E-cadherin mRNA were analyzed by Northern blotting in 1007 cells treated with ET-1 or ET-3 (10 nM) for 6 h. GAPDH mRNA expression levels were used as a loading control. The relative density of mRNA content from C was statistically analyzed and represents the average value of three independent Northern blots ± SD. *, P < 0.001 compared with control. D, ET-1 and ET-3 induce the tyrosine phosphorylation of catenin proteins. Serum-starved 1007 cells were incubated for 5 min with 10 nM ET-1 or ET-3. Cell lysates were immunoprecipitated with anti-β-catenin and anti-p120-catenin and then immunoblotted with antiphosphotyrosine. The membranes were reprobed with the specific anti-β- and p120-catenin to ensure equal amounts of proteins.

E-cadherin and actin-cytoskeleton (28). In this context, we observed that in serum-starved 1007 cells, both isopeptide ET-1 and ET-3 (10 nM) induced an increase in the tyrosine phosphorylation of β-catenin and p120-catenin that contributes to loss of functional cell adhesions (Fig. 1D).

**Intercellular Communication Is Impaired by ET-1 and ET-3 through ETaR Binding.** Loss of GJIC has been shown to occur during melanoma progression. We studied the effects of ETs on GJIC using SL/DT methods in 1007 and M10 cells. For the evaluation of GJIC function, only cells stained with LY, excluding the artificially wounded cells and GJIC were inhibited by 60–70% with respect to the control. GJIC of melanoma cells returned to basal level within 1 h (Fig. 2A). To determine whether ETaR was responsible for ET-induced disruption of GJIC, the ETaR antagonist was used in SL/DT experiments. ET-1- and ET-3-induced dye transfer inhibition was completely prevented by BQ788 (Fig. 2B). Gap junction formation requires Cx molecules aligning in hemi-channels. Both melanocytes and keratinocytes express Cx43 in coculture (25). The phosphorylation of Cx43 is believed to be causally linked with disruption of GJIC (29). Recent evidence shows that ET-1 decreases GJIC in ovarian carcinoma cells by inducing Cx43 phosphorylation (30). Therefore to evaluate the effect of ETs on Cx43 expression in melanoma cells, 1007 and M10 cells were exposed to ET-1 and ET-3 (10 nM). Non-phosphorylated Cx43 was detected in untreated 1007 and M10 cells. ETs significantly induced increase in electrophoretic mobility shift of Cx43 (Fig. 2C). Exposure of melanoma cells to 10 nM ET-1 and ET-3 resulted in a rapid induction of two phosphorylated species that we refer to collectively as Cx43-P (Fig. 2C). BQ788 blocked this effect, indicating that ETaR activation results into phosphorylation of Cx43 and disruption of GJIC.

**ET-1 and ET-3 Induce Secretion and Activation of MMPs through ETaR.** A critical step during tumor invasion is the degradation of the extracellular matrix (ECM) by MMPs. To identify the role of ETs on the activation status of MMP-2 and MMP-9, conditioned media of 1007 and M10 cells were analyzed by gelatin zymography. When 10 nM ET-1 and ET-3 was added, zymography showed that both melanoma cell lines secreted high levels of gelatinolytic proteases corresponding to the active forms of MMP-2 and MMP-9 (Fig. 3A). These results were confirmed by Western blotting, demonstrating that treatment with ET-1 and ET-3 induced an increased secretion of both latent and active forms of MMP-2 and MMP-9 in the conditioned medium of 1007 cells as compared with untreated cells (Fig. 3B). MT1-MMP is a transmembrane MMP known to bind and activate MMP-2 at the cell surface (31). ET-1 and ET-3 enhanced expression of both the latent MT1-MMP and to an even greater extent, the activated form of MT1-MMP. Recent studies have demonstrated that activation of pro-MMP-2 by MT1-MMP depends upon the presence of critical amounts of TIMP-2, which is required for the formation of the ternary complex that leads to the activation of MMP-2 (32). Both ET-1 and ET-3 induced a significant increase of TIMP-2 expression as demonstrated by Western blotting (Fig. 3B). By ELISA, we confirmed that both isopeptides were capable of eliciting MMP-2 and TIMP-2 secretion in the conditioned medium of 1007 cells. Addition of BQ788 completely blocked the ET-induced conversion of

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Fig. 2. ETs induce GJIC inhibition in melanoma cells. A, serum-starved 1007 and M10 cells were treated with 10 nM ET-1 for up to 60 min, and GJ capacity was assayed by SL/DT method. Gap junction function was evaluated by analyzing net transfer of LY, excluding dextran-stained cells, as described in “Materials and Methods.” The data, reported as the relative percentage of the control, represent the average value of three different assays each performed in triplicate samples ± SD; *, P < 0.005 compared with control. B, serum-starved 1007 and M10 cells were incubated for 30 min with ET-1 or ET-3 (10 nM) in the absence or in presence of BQ 788 (100 nM). Photographs show a representative experiment of SL/DT assay performed on 1007 cells; bar = 100 μm. The data, reported as the relative percentage of the control, represent the average value of three different assays each performed in triplicate ± SD; *, P < 0.001 compared with ET-1 or ET-3. C, ET-1 and ET-3 induce the phosphorylation of Cx43. Serum-starved 1007 and M10 cells were incubated for 5 min with 10 nM ET-1 or ET-3 in the absence or in presence of BQ 788 (100 nM). Cell lysates were immunoblotted with anti-Cx43 and with anti-β-actin for internal control.

Fig. 3. ET-1 and ET-3 increase the secretion and activation of MMP-9, MMP-2, and MT1-MMP to their active form and TIMP-2 expression (Fig. 3C), as analyzed by Western blot and ELISA (Fig. 3, B and C). These data indicate that the activation of MMP, involved in a complex proteolytic cascade, is mediated by ET βR activation.

ET-1 and ET-3 Increase αvβ1 and αvβ3 Integrin Expression. Because tumor cell invasiveness and metastasis formation depend on cell adhesive properties to the ECM (33–35), we investigated whether ETs were capable of modulating integrin expression on 1007 melanoma cells. Among the integrin subunits evaluated by flow cytometry, the levels of α1, α2, α3, α5, and β3 integrin subunit expression in cells stimulated with ET-1 and ET-3 (10 nM) for 24 h remained unaltered, whereas the levels of αv, β1, α2, and β3 expression were up-regulated (data not shown). Thus, in 1007 melanoma cells, ET-1 and ET-3 were able to significantly increase the heterodimeric αvβ1 and α3β3 integrin expression (Fig. 4A), which play a major role in melanoma progression (33–35). These results were confirmed by Western blotting (Fig. 4B), demonstrating that both peptides significantly increased αvβ1, α3β3, and β3 expression that was blocked by BQ788. These results suggested that ETs through ET βR activated αvβ1 and α3β3 integrins that could promote rapid adherence and increased motility of melanoma cells.
ET-1 and ET-3 Induce FAK and MAPK Activation through ET B R.

Several lines of evidence have implicated that Erk and FAK pathways play a critical role in the oncogenic behavior of malignant melanoma (18–20). Because Erk and FAK activation represents a key event in ET-1-induced mitogenic signaling pathway (36), we investigated the effect of ETs on these ET B R-mediated pathways in melanoma cells. ET-1 and ET-3 (10 nM) treatment resulted in a time-dependent induction of Erk1/2 and FAK phosphorylation, which reached a peak after 5 min and returned to baseline levels by 60 min (Fig. 5A). In presence of the constitutive activation of FAK and Erk1/2 in metastatic melanoma cell lines (18, 19), ET-1 and ET-3 were capable of increasing FAK and Erk1/2 activation in all four cell lines deriving from either primary (1007) or metastatic (M10, SKMel28, and Mel120) tumors (Fig. 5B). The phosphorylation of Erk1/2 induced by ETs was inhibited by PD98059 (10 μM), a specific MAPK kinase inhibitor (Fig. 5B). To investigate which receptor subtype mediates FAK and Erk1/2 phosphorylation, BQ123 (100 nM), a specific ET A R antagonist, and BQ788 (100 nM) were used in the presence or in absence of ET-1 and ET-3 (10 nM). BQ788, but not BQ123, was able to completely block ET-1- and ET-3-induced FAK and Erk1/2 activation (Fig. 5C), indicating that these signaling pathways are mediated through ET B R. Furthermore, by treating 1007 adherent cells with cytochalasin-D to disrupt actin filaments, a reduced level of FAK phosphorylation after ET-1 and ET-3 (10 nM) stimulation was observed (Fig. 5D), indicating that the integrity of the cytoskeleton is required for ET-1- and ET-3-induced FAK activation in melanoma cells.

Fig. 4. ET-1 and ET-3 enhance αβ1 and αβ3 integrin expression. A, fluorescence-activated cell sorter analysis of αβ1, αβ3, and β-actin integrin expression by serum-starved 1007 melanoma cells treated with 10 nM ET-1 (black) or ET-3 (gray) for 24 h. B, serum-starved 1007 cells were treated with ET-1 or ET-3 (10 nM) in the absence or presence of BQ788 (100 nM). Cell lysates were analyzed by Western blot for the expression of αβ1, αβ3, and β-actin integrin subunits and for internal control with anti-β-actin. The relative density of integrin content was statistically analyzed and represents the average value of three independent Western blots ± SD; * P < 0.001 compared with control; ** P < 0.001 compared with ET-1.

Fig. 5. A, kinetics of ET-1- and ET-3-induced FAK phosphorylation. Serum-starved 1007 cells were incubated for the indicated times with ET-1 or ET-3 (10 nM). Analysis of FAK phosphorylation was performed by immunoprecipitation with anti-FAK and subsequent immunoblotting by an antiphosphotyrosine. Analysis of the phosphorylated form of Erk1/2 was performed by immunoblotting with anti-phospho-Erk1/2. The membranes were reprobed with the specific anti-Erk1/2 and anti-FAK to ensure equal amounts of proteins. B, serum-starved primary (1007) and metastatic (M10, SKMel28, and Mel120) cell lines were incubated for 5 min with ET-1 or ET-3 (10 nM) and analyzed for FAK and Erk phosphorylation as described previously. Analysis of phosphorylated forms of Erk1/2 was performed also on melanoma cells preincubated with 10 μM PD98059 for 20 min after incubation with ET-1 (10 nM). C, ET A R antagonist blocks ET-induced FAK and Erk phosphorylation. Serum-starved 1007 cells were pretreated for 15 min with BQ123 (100 nM) or BQ788 (100 nM) in the absence or presence of 10 nM ET-1 or ET-3 for 5 min and then analyzed for FAK and Erk1/2 phosphorylation. The membranes were reprobed with the specific anti-Erk1/2 and anti-FAK to ensure equal amounts of proteins. D, ET-induced FAK phosphorylation is regulated by the cytoskeleton. Adherent 1007 cells, untreated or treated with 0.5 mg/ml cytochalasin-D (Cyto D), were stimulated with ET-1 or ET-3 (10 nM) for 5 min and then analyzed for FAK phosphorylation. The membranes were reprobed with the specific anti-FAK to ensure equal amounts of proteins.
ET-1 and ET-3 Induce Melanoma Cell Proliferation, Adhesion, Migration, and Invasion through \( \text{ET}_{\beta}\text{R} \). To examine the effects of ETs on cell proliferation, primary (1007) and metastatic (M10, SK-Mel28, and Mel120) cell lines were treated with different concentrations of ET-1 and ET-3. As shown in Fig. 6A, a significant and dose-dependent increase in \(^{3}H\text{thymidine incorporation was observed in all melanoma cell lines tested.}\)

To assess the effect of ET-1 and ET-3 on cell adhesion, 1007 cells were cultured with ET-1 and ET-3 for 24 h, and adhesion to type I collagen-coated surfaces was measured. A significant increase in cell adhesion was observed in ET-1- and ET-3-treated cells relative to control (Fig. 6B). We therefore examined the effect of ET-1 and ET-3 on the functional consequences of enhanced integrin expression and MMP activity on cellular events associated with metastatic spreading. Addition of 1 up to 100 nm ET-1 and ET-3 to melanoma cells induced a marked and dose-dependent increase in cell motility (Fig. 6C) and invasiveness (Fig. 6D). We investigated the ability of anti-\( \alpha_5\beta_1 \) and anti-\( \alpha_6\beta_3 \) Abs to interfere with adhesion and migration of 1007 melanoma cells. Abs to the \( \alpha_5\beta_1 \) and \( \alpha_6\beta_3 \) integrins strongly reduced adhesion to type I collagen, as well as cell migration induced by ETs. Moreover, ET-1-stimulated-invasion was reduced to the control level in the presence of a potent chemical broad-spectrum MMP inhibitor, such as Ilomastat (10 \( \mu \text{M} \)), and in the presence of MEK inhibitor, such as PD98059 (10 \( \mu \text{M} \)), demonstrating that ETs are able to induce MMP-dependent invasion through MAPK signaling pathways. The stimulatory effect of 10 nm ET-1 and ET-3 on 1007 cell proliferation, adhesion, migration, and invasion was completely blocked by BQ788, whereas BQ123 only partially inhibited ET-induced effects, indicating \( \text{ET}_{\beta}\text{R} \) as a relevant receptor in these events associated with melanoma progression.

A-192621, a Selective \( \text{ET}_{\beta}\text{R} \) Antagonist, Inhibits Cell Proliferation and Melanoma Growth in Nude Mice. On the basis of the previous findings, we investigated the effect of the potent A-192621, an orally active nonpeptide \( \text{ET}_{\beta}\text{R} \) antagonist (37), on the \textit{in vitro} and \textit{in vivo} growth of M10 cells, which rapidly grow in nude mice. M10 cell line was incubated with ETs and/or with A-192621. The ET-induced proliferation was significantly inhibited in the presence A-192621 (100 nm; \( P \leq 0.01 \); Fig. 7A). We translated these results into a model of nude mice xenografted with M10 melanoma cells. Treatment with A-192621 produced a 60% inhibition of M10 tumor growth with either low (10 mg/kg/day) or high (20 mg/kg/day) doses (Fig. 7B). A-192621 treatment was generally well tolerated with no signs of acute or delayed toxicity, even at the highest A-192621 dose. Comparison of time course of tumor growth curves by two-way ANOVA with group and time as variables showed that the group-by-time interaction for tumor growth was statistically significant (\( P < 0.0001 \)). Furthermore, the tumor growth inhibition obtained with A-192621 persisted for up 3 weeks after the termination of the treatment.

DISCUSSION

Progression of cutaneous melanoma requires the accumulation of a variety of phenotypical alterations that free the transformed melanocytes from the control of the surrounding normal microenvironment and enable them to acquire a stable invasive phenotype (15). Due to the resistance of melanoma to current therapies, the identification of molecular mechanisms underlying local and metastatic growth is mandatory for the development of novel treatments. Here, we have investigated whether the G-protein-coupled receptor activated by ETs may represent a relevant therapeutic target in this malignancy.

Loss of interactions between tumor cells and the surrounding normal microenvironment characterizes the progression of a number of malignancies (17). Changes in cell surface adhesion molecules that modulate these interactions are likely to be a prerequisite for invasive growth. The shift in cadherin molecules from the \( E^- \) to \( N^- \)isotype has been correlated with tumor cell motility, invasiveness, and capability
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Fig. 7. A-192621, a selective ETB R antagonist, inhibits cell proliferation and tumor growth in vivo. A. A-192621 blocks cell proliferation. Ten μM ET-1 or ET-3 was added to serum-starved M10 cells. A-192621 (100 μM) was incubated 15 min before the addition of ETs. Data are means of results from three experiments each performed in sextuplicate. *, P ≤ 0.001 compared with control. ***, P ≤ 0.01 compared with ET-1 or ET-3. B. Antitumor activity of ETB R antagonist treatment on established M10 human melanoma xenografts. Mice received injection s.c. with 1.5 × 10⁶ cells. Seven days after the tumor injection, mice were treated i.p. for 21 days with vehicle or with A-192621 (10 mg/kg/day, ■, or 20 mg/kg/day, □). Controls are indicated as △. Three different experiments were performed. Data represent the averages. Bars indicate ± SD. *, P < 0.001.

REFERENCES

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