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 \( \alpha_\beta_1 \) and \( \alpha_\beta_2 \) 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 A-192621, 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, ET_{A} 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 immunophenotyping 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 antiapoptotic factor (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 Iломastat (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 per a SUPERSCRIPT One-Step RT-PCR System (Invitrogen). The primer sets used were 5'-TGCCTCCTGCTGCCCTCAGTGAATAAGGAG-3' and 5'-GGTCACATAACGCCTCTGGGCTT-3' for ET-1, 5'-4' CACTGGTGGATGTGAATC-3' and 5'-GGAGATCAAGGCATATAGA-3' for ET_{B}R, 5'-TCAACCGGTTGGCTGCTGC-3' and 5'-ACT- GATAGCCCAACCATTCT-3' for ET_{A}R, 5'-AAATATGATGTCAGGGTGTG-3' and 5'- AAAATCAGTGCTCCTGTCGTTGCT-3' for E-cadherin, and 5'-TCCAGACGTGCTACGACGAG-3' and 5'-GGTCTTTCCACTGCTCCTACAT-3' for Snail. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control and the primer sets used were 5'-TGAAGCTCCAGTGCTGCAAGAACAAGAAG-3' and 5'-GATGCCCAGCTGACGAGGAG-3' for GAPDH. 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 (Snail), and 54°C (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 a 2% denaturing formaldehyde agarose gel (15 \( \mu \)g RNA/lane) and transferred to a nylon membrane. The membranes were hybridized in the QuickHyb hybrid-
ET_{B}R BLOCKADE IN MELANOMA CELL PROGRESSION

ET_{A}R and ET_{B}R blockade study.

**RESULTS**

**ET-1 and ET-3 Alter Cell Adhesion Molecule Expression through ET_{B}R.** 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 ET_{A}R and ET_{B}R 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-catenin and p120-catenin paralleling 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 ET_{B}R antagonist, reversed these effects, indicating that ET_{B}R activation is required for these responses (Fig. 1B). The transcription factor Snail, a master regulator of epithelial to mesenchymal 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 involves transcriptional mechanisms (Fig. 1C). Tyrosine phosphorylation of catenin proteins may result in a reduced interaction with both

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

**REFERENCES**

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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 ET₄R Binding.** Loss of GJIC has been shown to occur during melanoma progression. We studied the effects of ETs on GJIC using SL/DY 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 ET₄R was responsible for ET-induced disruption of GJIC, the ET₄R antagonist was used in SL/DY 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 casually 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 ET₄R activation results into phosphorylation of Cx43 and disruption of GJIC.

**ET-1 and ET-3 Induce Secretion and Activation of MMPs through ET₄R.** 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
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 the presence of bq788 (100 nm). cell lysates were immunoblotted with anti-cx43 and anti-β-actin for internal control.

et-1 and et-3 increase αβ1 and αβ3 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, β1, 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, β3, and β1 expression were up-regulated (data not shown). thus, in 1007 melanoma cells, ET-1 and ET-3 were able to significantly increase the heterodimeric αvβ3 and αvβ1 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 αv, β3, α3, and β1 expression that was blocked by BQ788. these results suggested that ETs through ETB receptor activated αvβ3 and αvβ1 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.
ET-1 and ET-3 Induce Melanoma Cell Proliferation, Adhesion, Migration, and Invasion through ET$_B$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 [$^{3}H$]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-$\alpha_{V}\beta_3$ and anti-$\alpha_{V}\beta_1$ Abs to interfere with adhesion and migration of 1007 melanoma cells. Abs to the $\alpha_{V}\beta_1$ and $\alpha_{V}\beta_3$ 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 $\mu$M), and in the presence of MEK inhibitor, such as PD98059 (10 $\mu$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 ET$_B$R as a relevant receptor in these events associated with melanoma progression.

A-192621, a Selective ET$_B$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 ET$_B$R antagonist (37), on the in vitro and 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
ENDOTHELIN-RECEPTOR BLOCKADE IN MELANOMA CELL PROGRESSION

To interact with wider stromal counterparts in breast carcinoma (38). ET-1-induced down-regulation of E-cadherin, which is responsible for contact-mediated regulatory control by keratinocytes (16), has been described previously. Here, we demonstrate that ET-1 and ET-3 through ETB\(R\) induce down-regulation of E-cadherin and associated catenin proteins, such as\(\beta\)-catenin and p120-catenin, with a parallel up-regulation of N-cadherin. This latter change allows homotypic adhesive contact as well as heterotypic (i.e., fibroblasts, endothelial cells) melanoma cell-cell interactions (15).

Our results now document one pathway regulating transcription of E-cadherin. Consistent with the role of the transcriptional repressor\(Snail\) in melanoma (27), we demonstrate that ETs induce a significant up-regulation of\(Snail\) mRNA that concurs with the E-cadherin mRNA down-regulation. In addition, ETs can suppress the adherent growth in vivo and activation of\(MT1-MMP\) and\(MMP-2\), leading to polarized ECM degradation and a collective movement of migration and invasion (15).

Of interest, ETs appear to impair the gap junction communication by inducing a transient and time-dependent reduction of GJIC (32). Melanoma cell invasiveness relies on a promigratory subset of cells at the tumor leading edge, which is characterized by a molecular program capable of silencing neighboring cells via cell-to-cell signaling and concomitant polarization of\(\beta1\)-integrin expression (39). Melanoma cells at the leading margin engage and cluster\(\beta1\)-integrins in anterior protrusions and show an increased expression and activity of ETB\(R\) as clinically relevant target especially in consideration of the development of small molecules capable of antagonizing ETs. Blockade of ETB\(R\) by the peptide antagonist, BQ788, in fact has been demonstrated to inhibit melanoma cell growth in vivo and in vitro (14). In view of the potential use in clinical settings, we showed that the specific nonpeptidic orally active ETB\(R\) antagonist A-192621 displays antitumor activity against established melanoma expressing ETB\(R\). In conclusion, we have identified multiple molecular pathways elicited by ET-1 and ET-3 that regulate melanoma local and metastatic growth. Because all of the molecular effectors involved in melanoma progression including cell-cell adhesion and cell-cell communication molecules, tumor proteases, and integrins are triggered by the ETB\(R\) activity, blockade of this receptor by small molecules results in inhibition of melanoma growth in vitroand in vivo, thus offering the possibility of exploring targeted therapy in this malignancy. In view of known resistance of melanoma to current therapies, this knowledge may be of clinical relevance to assess the extent to which ETB\(R\) blockade can be exploited in integrated treatments.

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REFERENCES

ETB R BLOCKADE IN MELANOMA CELL PROGRESSION


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