Endothelin-1 and Endothelin-3 Promote Invasive Behavior via Hypoxia-Inducible Factor-1α in Human Melanoma Cells

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Abstract
Endothelin (ET) B receptor (ET₄R), which is overexpressed in human cutaneous melanomas, promotes tumorigenesis upon activation by ET-1 or ET-3, thus representing a potential novel therapeutic target. Hypoxia-inducible factor-1α (HIF-1α) is the transcriptional factor that conveys signaling elicited by hypoxia and growth factor receptors. Here, we investigated the interplay between ET axis and hypoxia in primary and metastatic melanoma cell lines. We report that under normoxic conditions, ET₄R activation by ET-1/ET-3 enhances vascular endothelial growth factor (VEGF) up-regulation, cyclooxygenase (COX)-1/COX-2 protein expression and COX-2 promoter activity, prostaglandin E₂ (PGE₂) production, and do so to a greater extent under hypoxia. Moreover, COX-1/COX-2 inhibitors block ET-induced PGE₂ and VEGF secretion, matrix metalloproteinase (MMP) activation, and cell invasion, indicating that both enzymes function as downstream mediators of ET-induced invasive properties. The ET₄R selective antagonist BQ788 or transfection with ET₄R small interfering RNA (siRNA) block the ET-mediated effects. ETs also increase HIF-1α expression under both normoxic and hypoxic conditions and its silencing by siRNA desensitizes COX-2 transcriptional activity, PGE₂ and VEGF production, and MMP activation in response to ET-3, implicating, for the first time, HIF-1α/COX as downstream targets of ET₄R signaling leading to invasiveness. In melanoma xenografts, specific ET₄R antagonist suppresses tumor growth, neovascularization, and invasion-related factors. Collectively, these results identify a new mechanism whereby ET-1/ET-3/ET₄R axis can promote and interact with the HIF-1α-dependent machinery to amplify the COX-mediated invasive behavior of melanoma. New therapeutic strategies using specific ET₄R antagonist could provide an improved approach to the treatment of melanoma by inhibiting tumor growth and progression.

Introduction
Recent studies have shown that endothelins (ETs) and endothelin (ET) B receptor (ET₄R) pathways play a relevant role in melanocyte transformation and melanoma progression (1–5). The ET family is composed of three isopeptides, ET-1, ET-2, and ET-3, which bind to two distinct subtypes of G protein-coupled receptors [i.e., ET A receptor (ET₄R) and ET₄R]. The ET₄R is highly specific for ET-1 and ET-2, whereas it binds ET-3 with low affinity. On the contrary, ET₄R is a nonselective receptor, which binds ET-1, ET-2, and ET-3 with similar affinity (6), and is the main subtype expressed by normal and transformed melanocytes (4). Gene expression profiling of human melanoma biopsies and cell lines indicated ET₄R as one of the genes overexpressed and associated with aggressive phenotype (7), and analysis of ET₄R expression in a representative panel of melanocyte lesions has identified this receptor as a tumor progression marker (8). ET-1, which is secreted by keratinocytes in response to UV, stimulates proliferation, chemotaxis, and pigment production in melanocytes through ET₄R (9–11). Moreover, ET-1 promotes melanocyte survival and inhibits the UV-induced apoptosis by activating the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (12). Down-regulation of E-cadherin expression by UV-induced ET-1 (13) results into an enhancement of melanoma invasive capability (14). Associated with loss of E-cadherin, activation of ET₄R increases expression of N-cadherin, matrix metalloproteinase (MMP)-2, MMP-9, and α₅β₃ and α₂β₁ integrins and inhibits intercellular communication by inducing phosphorylation of gap junctional protein connexin 43, allowing tumor cells to escape growth control and to invade (2). Downstream to ET₄R pathway, activation of focal adhesion kinase and extracellular signal-regulated kinase 1/2 signaling pathways occurs leading to enhanced cell proliferation, adhesion, migration, and MMP-dependent invasion. Hence, ET₄R has emerged recently as a potential therapeutic target for melanoma (2, 15).

Melanoma is an aggressive tumor that can metastasize early in the course of the disease and, most importantly, is resistant to most current therapeutic regimens. Thus, the identification of the genetic and environmental factors driving the natural history of this malignancy is essential for the development of new therapies (16). Among microenvironmental components, hypoxia represents a key tumor-promoting factor (17, 18), which has been associated with tumor progression (19–22). In melanoma hypoxic setting, the PI3K-Akt pathway can transform melanocytes with aggressive phenotype (17, 18), which has been associated with loss of E-cadherin, activation of ET₄R increases expression of N-cadherin, matrix metalloproteinase (MMP)-2, MMP-9, and α₅β₃ and α₂β₁ integrins and inhibits intercellular communication by inducing phosphorylation of gap junctional protein connexin 43, allowing tumor cells to escape growth control and to invade (2). Downstream to ET₄R pathway, activation of focal adhesion kinase and extracellular signal-regulated kinase 1/2 signaling pathways occurs leading to enhanced cell proliferation, adhesion, migration, and MMP-dependent invasion. Hence, ET₄R has emerged recently as a potential therapeutic target for melanoma (2, 15).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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©2007 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-06-2606
HIF-1 is a heterodimeric transcription factor, composed by HIF-1α and the constitutively expressed HIF-1β. In normoxia, HIF-1α is hydroxylated at key proline residues facilitating von Hippel-Lindau protein binding, which in turn allows ubiquitination and subsequent proteosome-targeted degradation. Under hypoxic conditions, proline hydroxylation is inhibited, thereby stabilizing HIF-1α, which can then translocate into the nucleus and bind to costitutively expressed HIF-1β, forming the active HIF-1 complex (19). The HIF-1 complex recruits the transactivator p300/CBP, resulting in enhanced transcriptional activity. HIF-1 binds a conserved DNA consensus on promoters of its target genes known as the hypoxia-responsive element (HRE). HIF-1α activates the transcription of genes that are involved in crucial aspects of cancer biology, including angiogenesis, cell survival, glucose metabolism (18), and tumor invasion (21). HIF-1α controls the expression of several genes, including VEGF, erythropoietin, and ET-1, in response to hypoxia in different tumor cells (18, 26). Although hypoxia is the major inducer of HIF-1α, other stimuli, such as growth factors, including insulin, insulin-like growth factor (IGF)-I, transforming growth factor-α (TGF-α), platelet-derived growth factor, and epidermal growth factor, and cytokines, such as interleukin-1; oncogenic activation; or loss of tumor suppressor function, hormones, nitric oxide, and reactive oxygen species, are able to regulate HIF-1α expression (18, 19). Several growth factors, such as IGF-II and TGF-2, are also HIF-1 target genes. Binding of these factors to their cognate receptors stimulates the expression of HIF-1α, which in turn activates the transcription of gene that encodes IGF-II and TGF-α through an autocrine mechanism (18).

Cyclooxygenase (COX)-2 is overexpressed in various types of cancers, including melanoma (27), and compelling evidence supports a role for COX-2 and COX-2–derived prostaglandin E2 (PGE2) in angiogenesis (28, 29) and melanoma progression (30–32). However, the mechanisms that regulate transcriptional activation of COX-2, angiogenesis, and invasiveness in low-oxygen conditions have not been determined.

We hypothesized that COX-2 may be up-regulated by hypoxia and ETs as well as through HIF-1α. Because of the EtBr relevance in melanoma progression, we also explored the role of EtBr in ET-induced aggressive phenotype. Here, we report that ET-1 and ET-3 through EtBr induce COX-1/COX-2, PGE2, and VEGF in melanoma cells grown under normal oxygen conditions and that this mechanism may be responsible for invasive behavior of primary and more so of metastatic melanoma. These effects are amplified under hypoxia. At molecular levels, through EtBr activation, ETs mimic cellular hypoxia inducing HIF-1α, which is involved in mediating ET-induced COX-2 promoter activity, COX-1/COX-2, and PGE2 expression, and MMP activity. These findings indicate that targeting HIF-1α and related signaling through EtBr blockade could effectively impair cutaneous melanoma progression.

Materials and Methods

Cells and cell culture conditions. The human cutaneous melanoma cell line 1007 was derived from primary melanoma (33). The melanoma cell lines SK-Mel 28 (American Type Culture Collection, Rockville, MD), M10, and Mel120 were derived from metastatic lesions (34). Cells were grown in RPMI 1640 containing 10% FCS. All culture reagents were from Invitrogen (Paisley, Scotland, United Kingdom). To expose cells to hypoxia, a 5°C. Gelatin zymography. The melanoma cell supernatants were electro-phoresed for analysis in 9% SDS-PAGE gels containing 1 mg/mL gelatin as described previously (36). Briefly, the gels were washed for 30 min at 22°C in 2.5% Triton X-100 and then incubated in 50 mmol/L Tris-HCl (pH 7.6), 1 mmol/L ZnCl2, and 5 mmol/L NaCl, for 18 h at 37°C. After incubation, the gels were stained with 0.2% Coomasie blue.
Chemoinvasion assay. Chemoinvasion was assessed using a 48-well modified Boyden’s chamber (NeuroProbe, Pleasanton, CA) and 8-μm pore polyvinyl pyrrolidone–free polycarbonate Nucleopore filters (Costar, New York, NY) as described previously (36). The filters were coated with an even layer of 0.5 mg/mL Matrigel (Becton Dickinson, Bedford, MA). The lower compartment of chamber was filled with chemoattractant (100 nmol/L ET-3) and/or inhibitors (27 μL/well). Serum-starved 1007 cells (0.5 × 10^6/mL) were harvested and placed in the upper compartment (55 μL/well). Where specified, cells were preincubated for 30 min at 37°C with the indicated concentrations of COX-1 or COX-2 inhibitors. After 6 h of incubation at 37°C, the filters were removed and stained with DiffQuick (Merz-Dade, Dudingen, Switzerland), and the migrated cells in 10 high-power fields were counted. Each experimental point was analyzed in triplicate.

M10 melanoma xenografts. Female athymic (nu/nu) mice, 4 to 6 weeks of age (Charles River Laboratories, Milan, Italy), were handled according to the institutional guidelines under the control of the Italian Ministry of Health. Mice were injected s.c. on one flank with 1.5 × 10^6 viable M10 cells expressing ET₆R. The mice were randomized in groups (n = 10) to receive treatment i.p. for 21 days with A-192621 (10 mg/kg/d; Abbott Laboratories, Abbott Park, IL; ref. 2), and controls were injected with 200 μL drug vehicle (0.25 N NaHCO₃). The treatments were started 7 days after the xenografts, when the tumor was palpable. Each experiment was repeated thrice. Tumor size was measured with calipers and calculated using the formula π / 6 × larger diameter × (smaller diameter)^2.

Immunohistochemical analysis. Indirect immunoperoxidase staining was carried out on acetone-fixed 4-μm frozen tissue sections. The avidin biotin assays were done using the Vectastain Elite kit (for nonmurine

![Figure 1. ET-1 and ET-3 promote VEGF expression through ET₆R in melanoma cell lines. A, serum-starved 1007 and M10 cells were stimulated with increasing concentrations of ET-1 or ET-3 for 6 h, and total RNA was extracted and analyzed by RT-PCR for VEGF mRNA expression. Primers for GAPDH mRNA were used as loading control. B, serum-starved 1007 and M10 cells were cultured for the indicated times in the presence of 100 nmol/L ET-3, and conditioned media were analyzed by ELISA for VEGF production. Columns, VEGF production; bars, SD. *, P < 0.005 compared with the control. C, total RNA from serum-starved 1007, SK-Mel 28, and Mel 120 cells cultured for 6 h in the presence of 100 nmol/L ET-1 or ET-3 alone or in combination with 1 μmol/L BQ788 or from cells transfected with ET₆R siRNA was extracted and analyzed by RT-PCR for VEGF transcripts. Primers for GAPDH mRNA were used as loading control. D, conditioned media from serum-starved 1007 cells cultured for 24 h in the presence of 100 nmol/L ET-1 or ET-3 alone or in combination with 1 μmol/L BQ788 or from cells transfected with ET₆R siRNA were collected and analyzed by ELISA for VEGF secretion. Columns, VEGF production; bars, SD. *, P < 0.001 compared with the control; **, P < 0.004 compared with ET-1 or ET-3.]
primary antibodies) and the Vector MOM immunodetection kit (for murine primary antibodies) obtained from Vector Laboratories (Burlingame, CA). Mayer's hematoxylin was used as nuclear counterstain. Sections incubated with isotype-matched immunoglobulins or normal immunoglobulins served as negative control. The primary antibodies used were as follows: mouse anti–COX-2 (1:200; Cayman Chemical), anti-VEGF (1:200), monoclonal rat antimouse CD31 (platelet/endothelial cell adhesion molecule 1; 1:20; generously donated by Dr. A. Mantovani, Mario Negri Institute, Milan, Italy), anti-Ki67 MoAb (clone MIB1; 1:20; Ylem, Rome, Italy), and a monoclonal anti–MMP-2 (1:20; Oncogene Research Products, Cambridge, MA). The evaluation of microvessel density (MVD) was done by two independent observers on a ×200 magnification counted at least in five fields as reported previously (37). Ki67 score was expressed as tumor cells with nuclear staining counted at least in five separate ×40 microscopic fields.

Statistical analysis. Statistical analysis was done using the Student’s t test. Repeated measures ANOVA followed by Scheffe post hoc testing for multiple comparisons was used to evaluate the statistical significance of observed differences (SSPS, Chicago, IL). All statistical tests were two sided. P < 0.05 was considered statistically significant.

Results

ET-1 and ET-3 increase VEGF expression through ET₃R in human melanoma cells. The effect of ET-1 and ET-3 on VEGF expression was investigated in primary (1007) and metastatic (M10) melanoma cell lines. RT-PCR analysis for VEGF revealed that in both cell lineages, ET-1 and ET-3 increased the VEGF transcript levels in a concentration-dependent manner (Fig. 1A). As shown by ELISA, VEGF release was induced by ET-3 in a time-dependent fashion, reaching the maximum stimulation (4-fold above control levels) after 48 h in both cell lines (Fig. 1B). To investigate the functional relevance of ET₃R blockade on VEGF production, we used pharmacologic and molecular approaches inactivating ET₃R by specific siRNA (Supplementary Fig. S1), blocked ET-1/ET-3–induced VEGF mRNA expression and secretion (Fig. 1C and D), clearly showing that in melanoma cells, ET-1 and ET-3 induce VEGF through the binding with ET₃R and that blockade of ET₃R significantly inhibits VEGF production.
ET-1 and ET-3 increase COX-1 and COX-2 expression, COX-2 promoter activity, and PGE\(_2\) production through ET\(_A\)R. We next investigated whether ET-1 and ET-3 could affect COX-1 and COX-2 expression and PGE\(_2\) production in the 1007 and M10 melanoma cells. As shown by RT-PCR and Western blot analysis, ET-1 and ET-3 significantly induced COX-1 and COX-2 mRNA and protein up-regulation (Fig. 2A and B). ET-1 and ET-3 stimulation resulted also in a significant increase of PGE\(_2\) production (>2-fold above the control; Fig. 2C). In the presence of BQ788, or in silenced ET\(_A\)R-cells, these effects were significantly inhibited (Fig. 2A–C). To determine whether ETs may regulate COX-2 promoter activity, cells were transiently transfected with the human COX-2 promoter (phPES2, _−1432/+59_ ) reporter and pCMV-β-galactosidase plasmids. Treatment with ET-1 or ET-3 for 24 h induced 3-fold increase in luciferase activity compared with untreated cells, which was fully prevented by BQ788 (Fig. 2D), showing that ET\(_A\)R-mediated pathways regulate COX-1 and COX-2 expression, COX-2 promoter, and PGE\(_2\) release in melanoma cells.

COX-1 and COX-2 mediate ET-induced PGE\(_2\), VEGF production, MMP activation, and melanoma cell invasion. To assess whether COX-mediated pathways may regulate ET-induced PGE\(_2\) and VEGF production, as well as invasiveness, we used selective or nonselective COX inhibitors in 1007 melanoma cells. The COX-2 inhibitor, NS-398, or silenced COX-2 by specific siRNA (Supplementary Fig. S2), as well as the COX-1 inhibitor, SC-560, and the non-COX isotype selective inhibitor, indomethacin, significantly blocked both VEGF and PGE\(_2\) production after 24 h of ET-3 treatment (Fig. 3A and B). These data indicate that both enzymes, although by a different extent, participate to ET-mediated PGE\(_2\) and VEGF production in these COX-1/COX-2–positive melanoma cells. Impairment of COX pathways by selective inhibitors has been shown to reduce melanoma cell invasiveness (27). Because we showed previously that ETs promote MMP activity and invasion in melanoma cells (2), we analyzed the contribution of ET-3–induced COX enzymes to invasive activity. By using gelatin zymography and chemoinvasion assays, we showed that treatment with COX inhibitors reduced ET-3–induced MMP-9 and MMP-2 activation as well as cell invasion (Fig. 3C and D). Similar results were obtained in 1007 melanoma cells stimulated with ET-1 (data not shown). These results show that ET-1/ET-3 signaling elicits an ET\(_A\)R-dependent activation of PGE\(_2\) pathway promoting cell invasiveness, MMP activity, and VEGF production through the activation of both COX enzymes.

ET-1– and ET-3–induced VEGF expression is mediated by HIF-1α. To analyze more in-depth the mechanisms by which ET-1 and ET-3 may influence the cellular hypoxic response, we...
investigated the effects of its major mediator HIF-1α. Although in normoxic conditions, HIF-1α protein levels were barely detectable in 1007 and M10 melanoma cells, following exposure to ET-1 or ET-3 rapidly increased its expression (Fig. 4A). Under hypoxic conditions, the level of HIF-1α induction by ET-1 and ET-3 was increased to an even greater extent than that induced by hypoxia alone (Fig. 4B). Both in normoxic and hypoxic melanoma cells, ET-3–induced accumulation of HIF-1α protein paralleled the ET-3–induced VEGF production (Fig. 4C). At the end of 24 h of stimulation with ET-3, the VEGF production in 1007 and M10 melanoma cells reached a level (~3.5-fold increase) higher than that observed under hypoxic treatment alone (~3-fold increase; Fig. 4C). To address the functional role of ETAR blockade on HIF-1α protein accumulation, we examined the effect of ETAR antagonist, BQ788, or ETAR siRNA under normal oxygen and low-oxygen environment. ETAR blockade inhibited the ET-1– and ET-3–induced HIF-1α protein expression in both conditions (Fig. 4A and B), indicating that ET-1 and ET-3 mimic and cooperate with hypoxia to induce HIF-1α and to enhance VEGF expression through ETAR. To evaluate whether HIF-1α was required in hypoxia and ET-mediated VEGF up-regulation, HIF-1α protein levels were silenced by the use of a specific siRNA (Supplementary Fig. S3). This treatment decreased the capacity to up-regulate HIF-1α under hypoxia and abolished hypoxia and ET-3–mediated VEGF induction under both normoxic and hypoxic stimuli (Fig. 4D), showing that ET- and hypoxia-inducible expression of VEGF is mediated by HIF-1α.

HIF-1α mediates ET-driven COX and PGE₂ pathway and MMP activity. The mechanism leading to COX-2 overexpression and induction by hypoxia in melanoma is unknown. To assess whether COX-1 and COX-2 expression could be modulated by hypoxia, we measured the expression of both enzymes in different oxygen conditions. As shown in Fig. 5A, exposure to hypoxia increased the intracellular levels of COX-1/COX-2 and this effect was amplified in the presence of ET-3. To address the role of HIF-1α in hypoxia- and ET-3–mediated COX-1/COX-2 expression, we transfected 1007 cells with HIF-1α siRNA. The reduced HIF-1α protein levels resulted in inhibition of ET-3– and hypoxia-induced COX-1 and COX-2 expression (Fig. 5A; Supplementary Fig. S3), showing for the first time that both hypoxia and ET-3 increase COX-2 expression, through HIF-1α under normoxic and hypoxic condition. To achieve a greater understanding of the regulatory mechanism underlying the HIF-1α–mediated COX-2 up-regulation, we transfected 1007 cells with HIF-1α siRNA and ET-3 promoter under normal oxygen and low-oxygen environment. Exposure to hypoxia for 24 h resulted in an ~3-fold increase in COX-2 promoter activity compared with normoxia (Fig. 5B) comparable with that induced by ET-3 in normoxic conditions. Under hypoxic environment, 1007 cells treated with ET-3 displayed a further 4-fold increase of COX-2 promoter activity compared with the hypoxic cultured cells, suggesting that COX-2 can be transcriptional enhanced by exposure to hypoxia and that ET-3 and hypoxia may share a common transcriptional mechanism to potentiate COX-2 up-regulation. Cotransfection with HIF-1α siRNA and COX-2 promoter
significantly inhibited ET-3- and hypoxia-induced COX-2 transcriptional activity (Fig. 5B), indicating that HIF-1α is a major mediator of ET-3–driven COX expression and reporter gene activation in both oxygen-deprived and normoxic conditions. In hypoxia, ET-3 treatment potentiated the enhanced effect of hypoxia on PGE2 production. In the presence of HIF-1α siRNA, hypoxia- and ET-3–induced PGE2 levels were inhibited (Fig. 5C), indicating that in response to both stimuli, a HIF-1α–dependent increase in PGE2 production occurs. We finally investigated the pathways whereby ET-3 and hypoxia could affect the invasive behavior of melanoma cells. ET-3 and hypoxia induced MMP activity, and in hypoxic conditions, the presence of ET-3 promoted MMP activity to a greater extent than hypoxia alone that was inhibited by HIF-1α siRNA (Fig. 5D).

**Discussion**

The steady increase of melanoma incidence in the last decades, the early metastasization of the tumor, and the resistance of advanced melanoma to current treatment regimens underscore the
importance of acquiring a better understanding of the pathogenesis of this disease (16). Research in this area has identified ET axis as one of the key regulators of melanoma progression (2–4), suggesting that the inhibition of ET<sub>R</sub> signaling pathway may improve treatment of this malignancy. In this study, we investigated as to whether ETs activities may be influenced by the key micro-environmental factor, hypoxia, in modulating the invasive behavior of melanoma cells. Here, we show that ET<sub>R</sub> activation triggers a HIF-1α–mediated up-regulation of VEGF levels in primary and metastatic human melanoma cells. Moreover, HIF-1α seems to act in concert to ETs also in inducing COX-1/COX-2 expression, COX-2 transcriptional activity, PGE<sub>2</sub> production, MMP activity, and cell invasion, indicating a central role for ETs to potentiate hypoxia-induced melanoma progression through HIF-1α. Finally, blockade of ET<sub>R</sub> inhibits tumor growth, neovascularization, and invasive molecular determinants.

Neoangiogenesis and invasion in melanoma is strictly dependent on the interplay of a variety of stimuli, including local hypoxia (38). In addition to the classic hypoxia-mediated induction of HIF-1α, different growth factors, including ET-1, which is capable of inducing an angiogenic phenotype on endothelial cells and tumor neovascularization (37, 39), have been shown to enhance HIF-1α stabilization with resulting accumulation and activation (18–22, 40). Recent studies have provided evidence that the epidermal microenvironment of melanocytes is hypoxic and that a low oxygen level is required for melanocyte transformation initiated by Akt through a HIF-1α–dependent mechanism (25). In the present study, we show that ET-1 and ET-3 are inducers of HIF-1α expression equipotent to hypoxia and behave so even to a greater extent under hypoxic conditions, indicating that ETs and hypoxia exert additive effects on HIF-1α–dependent machinery to promote melanoma angiogenic determinants and cell invasion, which occur at early stages of melanomagenesis.

The COX-1 and COX-2 enzymes are involved in tumor progression by inducing proliferation, survival, angiogenesis, invasion, and metastasis in several solid tumors (27–32, 41–44). To elucidate the regulatory mechanisms that underlie COX-1/COX-2 regulation, we identify COX enzymes as downstream signals of ETs/ET<sub>R</sub> pathway, providing evidence that ET-1 and ET-3 induced COX-2 promoter activity and COX-1/COX-2 expression with resulting PGE<sub>2</sub> production. Furthermore, the decrease of ET-3–induced VEGF production, MMP activation, and cell invasion by COX inhibitors shows that COX-mediated pathway by ET<sub>R</sub> stimulates angiogenesis-related factor expression and migratory activities, thus identifying a novel mechanism responsible for ETs/ET<sub>R</sub> tumor-promoting properties. Differently from results of

**Figure 6.** Blockade of ET<sub>R</sub> by A-192621 inhibits tumor growth, neovascularization, and invasion-related marker expression in vivo. A, antitumor activity of ET<sub>R</sub> antagonist treatment on established M10 human melanoma xenografts. Mice received injection s.c. with 1.5 × 10<sup>6</sup> cells. Seven days when tumor became palpable, mice were treated i.p. for 21 d with vehicle or with A-192621 (10 mg/kg/d). Points, averages of three different experiments; bars, SD. The 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 < 0.001. B, immunoblotting for COX-2, HIF-1α, and VEGF expression in M10 tumor xenografts. Anti–β-actin was used as loading control. C, comparative immunohistochemical analysis of Ki-67, CD31, VEGF, COX-2, and MMP-2 expression in M10 tumor xenografts. Original magnification, ×250 and ×160 for CD31. D, quantitative assessment of immunohistochemical analysis for MVD and proliferation index.
Denkert et al. (27), indicating that in melanoma, COX-2 is the major source of PGE2, we showed by using selective COX-1/COX-2 inhibitors that both enzymes are involved in PGE2-mediated invasiveness of these tumor cells. Our findings show that ETs and hypoxia contribute to COX-1/COX-2 up-regulation in melanoma cells. We further provide evidence that HIF-1α plays a major role in melanoma response to hypoxia as well as to ETs, demonstrating that down-regulation of HIF-1α in ET-stimulated cells inhibits the capability to induce COX-2 expression and transcriptional activity, VEGF and PGE2 production, and tumor protease activity. Furthermore, ET-3 and hypoxia have additive effects on the induction of these events through HIF-1α. These data agree with recent results showing a close relationship between hypoxia and COX-2 gene induction. In ovarian carcinoma, the effect of PGE2 on VEGF is potentiated by hypoxia and is associated with HIF-1α expression (45), suggesting that COX-dependent prostanooids may play an important role in the regulation of hypoxia-induced VEGF expression. In hypoxic lung cancer, COX-2 is up-regulated in a HIF-1α-dependent manner, thus providing the first evidence that COX-2 is a target gene of HIF-1α (46). In addition, while this report was in preparation, Kaidi et al. (47) reported that HIF-1α directly binds a specific HRE located at −506 on the COX-2 promoter, highlighting the biological significance of COX-2 up-regulation during hypoxia in colorectal cancer cells. Recent data show that PGE2 can directly induce expression of HIF-1α protein (48, 49). These findings suggest the possibility of an autocrine stimulation, in which high PGE2 levels due to increased COX-2 overexpression stimulate expression of HIF-1α responsible of continuous COX-2 transcription resulting into promotion of invasiveness. As shown for several growth factors (18), HIF-1α therefore contributes to autocrine signaling pathways that are crucial for cancer progression. Collectively, these results present evidence that signaling pathways associated with angiogenesis and invasiveness can be activated by HIF-1α in melanoma cells exposed to ET-1/ET-3, disclosing a yet unidentified regulatory mechanism, which relays on the convergence of microenvironmental hypoxia and ETs, influencing the behavior of melanoma cells through HIF-1α-COX signaling cascade (Supplementary Fig. S4).

Gaining a better understanding of the complexities of tumor context can improve the development of more effective antitumor treatments. In this regard, ETaR seems clinically relevant because by connecting with hypoxia, it can modulate melanoma progression. This is also supported by a gene array profiling of melanoma that identified ETaR as one of the genes associated with multiple aggressive phenotypes, including the plasticity of melanoma cells to engage in vasculogenic mimicry (7). The invasive melanoma cells that are capable of generating tubular networks in vitro expressed in fact both MMPs and ETaR (50). Immunohistochemical and immunoblot analysis of melanoma xenografts provides in vivo evidence for this concept because that treatment with ETaR antagonist induces a significant tumor growth inhibition associated with a reduction of MVD, VEGF, COX-2, HIF-1α, and MMP-2 expression. Therefore, the antitumor effect of ETaR antagonist on melanoma cell growth in vivo and in vitro (2, 15) is likely to result also from its interference with the formation of microvascular channels lined by tumor cells overexpressing ETaR and MMPs. In conclusion, the present study delineates the link between hypoxia and ETaR-triggered molecular events producing the activation of other signaling molecules, such as COX-2 and its downstream targets, to expanding the cellular communication network responsible for the invasive phenotype. In view of these findings, ETaR antagonists, which have shown to induce concomitant antitumor activity and suppression of neovascularization in vivo, may represent a promising HIF-1α-targeted therapeutic approach in the treatment of melanoma.

Acknowledgments
Received 7/14/2006; revised 12/4/2006; accepted 12/6/2006.
Grant support: Associazione Italiana Ricerca sul Cancro, Ministero della Salute.

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