Endothelin-1 Decreases Gap Junctional Intercellular Communication by Inducing Phosphorylation of Connexin 43 in Human Ovarian Carcinoma Cells*†

Received for publication, May 7, 2003, and in revised form, August 1, 2003
Published, JBC Papers in Press, August 7, 2003, DOI 10.1074/jbc.M304785200

Francesca Spinella, Laura Rosanò, Valeriana Di Castro, Maria Rita Nicotra, Pier Giorgio Natali, and Anna Bagnato

From the Laboratories of Molecular Pathology and Ultrastructure and Immunology, Regina Elena Cancer Institute, Rome 00158, Italy and the Institute of Molecular Biology and Pathology, National Research Council, Rome 00137, Italy

Endothelin-1 (ET-1) is overexpressed in ovarian carcinoma and acts as an autocrine factor selectively through the ET$_A$ receptor (ET$_A$R) to promote tumor cell proliferation, survival, neovascularization, and invasiveness. Loss of gap junctional intercellular communication (GJIC) is critical for tumor progression by allowing the cells to escape growth control. Exposure of HEY and OVCA 433 ovarian carcinoma cell lines to ET-1 led to a 50–75% inhibition in intercellular communication and to a decrease in the connexin 43 (Cx43)-based gap junction plaques. To investigate the phosphorylation state of Cx43, ovarian carcinoma lysates were immunoprecipitated and transient tyrosine phosphorylation of Cx43 was detected in ET-1-treated cells. BQ 123, a selective ET$_A$R antagonist, blocked the ET-1-induced Cx43 phosphorylation and cellular uncoupling. Gap junction closure was prevented by tyrophostin 25 and by the selective c-Src inhibitor, PP2. Furthermore, the increased Cx43 tyrosine phosphorylation was correlated with ET-1-induced increase of c-Src activity, and PP2 suppressed the ET-1-induced Cx43 tyrosine phosphorylation, indicating that inhibition of Cx43-based GJIC is mainly mediated by the Src tyrosine kinase pathway. In vivo, the inhibition of human ovarian tumor growth in nude mice induced by the potent ET$_A$R antagonist, ABT-627, was associated with a reduction of Cx43 protein. These findings indicate that the signaling mechanisms involved in GJIC disruption on ovarian carcinoma cells depend on ET$_A$R activation, which leads to the Cx43 tyrosine phosphorylation mediated by c-Src, suggesting that ET$_A$R blockade may contribute to the control of ovarian carcinoma growth and progression by preventing the loss of GJIC.

The endothelin (ET) family is composed of three isopeptides, ET-1, -2, and -3, that are potent mitogens for several human tumors, including carcinoma of the prostate (1), ovary (2, 3), colon (4), cervix (5), breast (6), and endometrium (7) as well as melanoma (8) and Kaposi's sarcoma (9). ETs and their receptors have been implicated in tumor progression through autocrine and paracrine pathways. ET-1 is produced primarily by vascular cells and in elevated amounts by different tumor cells and acts through two distinct subtypes of G protein-coupled receptors (GPCR), namely ET$_A$R (ET$_A$R) and ET$_B$R, that have different affinities for ETs (10). The ET-1/ET$_A$R autocrine pathway has a key role in the development and the progression of prostatic, ovarian, and cervical cancers (11).

We have previously demonstrated that ET-1 and ET$_A$R are overexpressed in primary and metastatic ovarian carcinomas compared with normal ovarian tissues (3). In ovarian tumor cells, ET-1 acts an autocrine factor selectively through ET$_A$R (2). Ligand binding to this receptor results in activation of a pertussis toxin-insensitive G protein that stimulates phospholipase C activity and increases intracellular Ca$^{2+}$ levels, activation of protein kinase C, mitogen-activated protein kinase, and p125 focal adhesion kinase phosphorylation (11). In this tumor, engagement of ET$_A$R promotes tumor cell proliferation (3, 12), apoptosis protection (13), invasiveness (14), and neovascularization (15–17). ET$_A$R blockade by a selective receptor antagonist, ABT-627, inhibits tumor growth, angiogenesis, and metastasis-related effectors of ovarian carcinoma in vivo (18).

Recent evidence shows that ET-1 has also been implicated in the regulation of gap junctions in astrocytes, Rat-1 and cardiac cells, although the mechanism that regulates this effect has not been fully established (19–21). Moreover, the role of ET-1 in the regulation of GJIC in tumor cells has not been studied.

GJs are composed of transmembrane channel proteins, termed connexins (Cxs), that directly connect the cytoplasm of two adjacent cells, allowing the cells to review and shape the functional state of their neighbors by exchanging signaling molecules, a process called gap junctional intercellular communication (GJIC) (22).

During the transition to malignant lesions and to metastatic cancer, stepwise changes in intercellular communications provide tumor cells with the ability to overcome microenvironmental controls from the host, to invade surrounding tissues, and to metastasize (23). Various tumor-promoting agents (24, 25) and different growth factors (26) decrease GJIC, either by suppressing Cx expression or by inducing post-translational modifications such as phosphorylation, a process that is closely related...
to cellular processes such as trafficking, assembly/disassembly, gating of gap junction channels, and altered susceptibility to degradation (27). Several studies have shown that the turnover of connexins is exceptionally rapid and that degradation of Cx43 involves both the lysosome and the proteasomal pathways (28). Phosphorylation, in most cases, is a prerequisite for ubiquitination that marks the protein for proteasomal destruction. The COOH-terminal tail of Cx43 contains several serine and tyrosine phosphorylation sites, suggesting that this region of the molecule contains a complex array of potential regulatory sites (29).

Human ovarian surface epithelial cells exhibit extensive GJIC and expression of different types of Cx (e.g., Cx26, Cx32, and Cx43) (29–31). Defects in intercellular communication, including reduced or inappropriate expression of Cx43, the main gap junction protein in normal human ovarian surface epithelium, has emerged as key factors in ovarian carcinoma progression (29–33). The aim of this study was to examine whether the activation of the ET-

**EXPERIMENTAL PROCEDURES**

**Cells**—Human ovarian carcinoma cell lines HEY and OVCA 433, in which the ET-1/Et-AR autocrine pathway is biologically active, we report here for the first time in tumor cells that 1) ET-1 significantly decreases intercellular communication and Cx43 expression at the cell surface; 2) these activities require ET-1 receptor activation; and 3) Cx43 phosphorylation is mainly mediated by the Src tyrosine kinase pathway. In addition, ABT-677 (atrasantan), a nonpeptide orally bioavailable ET-1R antagonist with in vivo and in vitro antitumor activity (18, 34–37), prevents in vivo Cx43-based GJIC loss of function. Thus, our findings identify the signaling pathways linking ET-1R with cellular uncoupling, which may contribute to ET-1-mediated ovarian tumor progression.

**Scrape Loading/Dye Transfer**—Levels of GJIC in control and treated cultures were determined using the scrape-loading/dye transfer (SL/DY) technique, applying a mixture of fluorescent dye, Lucifer Yellow (LY) (Sigma) and rhodamine-dextran (RhD) (Molecular Probes, Inc., Eugene, OR) (38). HEY and OVCA 433 cell cultures, as described above, were washed thoroughly with PBS in which Ca^2+ was omitted to prevent uncoupling of the cells due to high Ca^2+. The mixture containing 0.5% LY and 0.5% RhD in Ca^2+–free PBS was added to the cells, and scrape loading was performed, applying two or three cuts on cell monolayers with a razor blade. The dye mixture was rinsed away 1 min after the scrape. Cells were washed three times with PBS and fixed with 4% paraformaldehyde, and cells stained with LY and/or with RhD were detected by fluorescence emission with an inverted fluorescent microscope equipped with a camera. Functional permeability was assessed after the scrape in control and treated cultures, applying two or three cuts on cell monolayers with a razor blade. The dye mixture was rinsed away 1 min after the scrape. Cells that received the LY from the scrape-loaded cells, excluding the RhD-stained cells, were considered communicating. The numbers of communicating cells in the untreated and treated samples were counted. Gap junction communicating capacity was expressed as percentage of the control.

**Immunostaining of Cx43**—HEY cells were grown to 80% confluence in 8 wells of tissue culture (LabcTek). After the addition of 100 nM ET-1 for 30 min, the cells were rapidly washed with ice-cold PBS and fixed on acetone. The cells were incubated overnight at 4 °C with mouse monoclonal anti-Cx43 (Chemicon International, Temecula, CA) diluted 1:10 in PBS and then for 1 h with a fluorescein-labeled goat anti-mouse IgG (Fab′) fraction (Cappel; ICN Biomedicals GmbH) diluted 1:200 in PBS at room temperature. Cells stained directly with the secondary antibody or with the isotype-matched murine immunoglobulins were used as a negative control. Indirect immunofluorescence staining was carried out on acetone-fixed 4-μm frozen tissue sections employing rabbit anti-Cx43 antiserum and fluorescein-labeled goat anti-rabbit IgG (Fab′) fraction (Sigma).

**Immunoprecipitation and Immunoblotting**—Cells were washed with ice-cold PBS and lysed in lysis buffer (50 mM Tris·HCl (pH 7.4), 250 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 0.15% Triton X-100, 1 mM orthovannadate, 0.06 units of aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin). For immunoprecipitation of Cx43, cells were lysed in lysis buffer. Cell lysates, obtained after centrifugation at 14,000 × g for 10 min to remove insoluble material, were incubated overnight at 4 °C with protein A-Sepharose (Amersham Biosciences). Immunoprecipitation was performed by incubation overnight at 4 °C of the precleared lysates with a polyclonal anti-pan-Cx43 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), which detects nonphosphorylated and phosphorylated Cx43 species, insolubilized on protein A-Sepharose. After washing six times with lysis buffer, the immunoprecipitated material was solubilized in a lysis buffer under reducing conditions and analyzed by electrophoresis on 12.5% SDS-PAGE gels and transferred to nitrocellulose for immunoblotting. Nonspecific binding of Abs was prevented by incubating the blotted membrane in 3% bovine serum albumin TBS-T (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween) for 1 h at room temperature. The blots were then incubated for 1 h with anti-phosphotyrosine or with anti-pan-c-Src, a nonphosphospecific antibody or with anti-c-Src (Tyr(P)104) antibody (BIOSOURCE) or anti-Cx43 monoclonal Ab (Transduction Laboratories). Membranes were then washed with TBS-T three times for 15 min each at room temperature and incubated for 1 h with peroxidase-conjugated secondary antibody, washed again, and subjected to the ECL (Amersham Biosciences).

For detection of Cx43 or c-Src or activated form of c-Src, an equal amount of cell lysate proteins from ovarian carcinoma cells or homogenized HEY tumor specimens was subjected to 12% SDS-PAGE and transferred to nitrocellulose. The filters were blocked with 3% bovine serum albumin in TBS-T and incubated with either Cx43 (Transduction Laboratories), anti-pan-c-Src (BIOSOURCE), or an activated form of c-Src (BIOSOURCE) antibodies or 1 h. After washing with TBS-T, blots were incubated with appropriate peroxidase-conjugated secondary antibody, washed again, and subjected to the ECL (Amersham Biosciences) procedure. After being stripped, the membranes were reprobed with β-actin monoclonal antibody (Oncogene, CN Biosciences, Inc., Darnaststedt, Germany) to ensure equal loading. The relative intensity of signals was quantified using the Scion image analysis program. The quantification data was statistically analyzed using Student’s t test and represent the average value of three independent Western blots.

**HEY Xenografts in Nude Mice**—Female athymic (nu/nu) mice, 4–6 weeks old, were purchased from Charles River Laboratories (Milan, Italy). The treatment protocol followed the guidelines of animal experimentation adopted by the Regina Elena Cancer Institute under the control of the Ministry of Public Health. Mice were given injections subcutaneously into one flank with 1.5 × 10^5 viable HEY cells, as determined by trypan blue staining, resuspended in 200 μl of PBS. After 7 days, when established tumors of 0.2–0.3 cm^3 in diameter were detectable, mice were randomized in groups (n = 10) to receive different treatments. One group was treated intraperitoneally for 21 days with ABT-677 (atrasantan, provided by Abbott, at daily doses of 2 mg/kg/day) dissolved in 0.25% NaHCO_3·Control mice were injected in the same way with 200 μl of drug vehicle. On day 21, after the final injection, tumors were removed from control and treated mice and snap frozen in liquid nitrogen for immunohistochemical and Western blot analysis.

**Statistical Analysis**—All statistical analyses were assessed using a two-tailed Student’s t test and were performed by the Instat software system (GraphPad Software Inc., San Diego, CA).
**ET-1 Induces Phosphorylation of Cx43—**To determine whether ET-1 can regulate Cx43 expression in ovarian carcinoma cells, HEY and OVCA 433 cells were exposed for different times to ET-1 (100 nM). ET-1 significantly induced dose-dependent increase in the electrophoretic mobility shift of Cx43 (Fig. 2A). As analyzed by SDS-PAGE, Cx43 is normally reported as being represented by a faster migrating, nonphosphorylated species (Cx43-P0) and two more slower migrating, phosphorylated species (Cx43-P1 and Cx43-P2) (39) that we refer to collectively as Cx43-P. ET-1 treatment resulted in a time-dependent Cx43 phosphorylation reaching the maximum level after 30 min and returning to basal level after 1 h of exposure to ET-1 (Fig. 2A). Treatment with alkaline phosphatase results in the total disappearance of the slower migrating bands, indicating that these bands correspond to the phosphorylated forms of Cx43 (Fig. 2B). The differences in Cx43 banding patterns between the two cell lines may reflect differential steady-state levels probably related to kinetic variability in Cx43 turnover. Nonphosphorylated Cx43 (Cx43-P0) was detected in untreated HEY cells, whereas the phosphorylated Cx43 species (Cx43-P) were much less apparent (Fig. 2, A and B). Exposure of HEY cells to from 0.1 nM up to 100 nM ET-1 for 30 min resulted in a dose-dependent increase of Cx43 phosphorylation that reached the highest intensity at 100 nM of ET-1 (Fig. 2C). Densitometric analysis of Fig. 2C shows that ET-1 increased the Cx43-P at the expense of Cx43-P0 (Fig. 2D, left), and the total cellular Cx43 content only slightly decreased following treatment with ET-1 (Fig. 2D, right).

We further examine the other connexin subtypes that participate in gap junction formation in HEY and OVCA 433 cells. Western blotting showed that Cx32 and Cx26 expression was barely detectable in these cells, and ET-1 did not modify their levels of expression (data not shown).
Endothelin-1 Inhibits GJIC in Human Cancer Cells

ET-1-induced Cx43 Phosphorylation and GJIC Inhibition Are Mediated through ET₁ₐR—To investigate which receptor subtype mediates ET-1-induced disruption of GJIC and Cx43 phosphorylation, a selective ETₐR receptor antagonist, BQ123, and a selective ETₐR antagonist, BQ788, were used in the presence or absence of 100 nM ET-1. BQ123 was able to completely block ET-1-induced Cx43 phosphorylation, whereas BQ788, were used in the presence or absence of 100 nM ET-1. BQ123 was able to completely block ET-1-induced Cx43 phosphorylation, whereas BQ788 did not (Fig. 3A). To determine whether ET₁ₐR was responsible for the inhibition of GJIC, BQ 123 was used in SL/DT experiments. ET-1-induced dye transfer inhibition was prevented by the ET₁ₐR antagonist (Fig. 3B). Taken together, these findings indicate that ET-1 acts through ET₁ₐR to induce the phosphorylation of Cx43 and to disrupt the GJIC.

ET-1 Alters the Localization of Cx43 Expression in Ovarian Carcinoma Cells—To examine the possibility that ET-1-induced Cx43 phosphorylation modified the distribution of membrane gap junction plaques, we investigated HEY cells with Cx43-specific antibody by indirect immunofluorescence. A fine punctate Cx43-specific staining characteristic of gap junctional plaques was observed often outlining cell boundaries of the untreated cells. After 30 min of ET-1 treatment, the fine punctate staining pattern was significantly reduced, now appearing granular and randomly distributed (Fig. 4, A and B). This change was concomitant with the phosphorylation of Cx43 and the complete inhibition of dye transfer to adjacent cells.

ET-1-induced GJIC Reduction Is Regulated by Tyrosine Kinase Pathways—A number of kinases and signal transduction pathways are known to affect GJIC and Cx43 phosphorylation (26). To identify the kinase responsible for ET-1-induced GJIC reduction, we examined the effects of tyrosine and serine/threonine kinase and tyrosine-phosphatase inhibitors on GJIC activity. Pervanadate (1 mM), a tyrosine-phosphatase inhibitor, induced a complete inhibition of LY spreading between LY-loaded HEY cells by the same extent observed in ET-1-treated cells (Fig. 5). Conversely, ET-1-induced gap junction closure was fully prevented by tyrosine kinase inhibitor tyrphostin 25 (100 μM). These results suggest that tyrosine kinase pathways mainly regulate ET₁ₐR-mediated gap junction closure in ovarian carcinoma cells. The serine/threonine kinase inhibitor, staurosporine (100 nM) only partially reversed the effect of ET-1 on GJIC (Fig. 5), suggesting that ET-1 can also phosphorylate Cx43 on serine residues after activation of several serine/threonine kinases, such as protein kinase C.

ET-1 Induces Tyrosine Phosphorylation of Cx43—To prove that the observed ET-1-induced phosphorylation of Cx43 occurs on tyrosine residue, we performed immunoprecipitation and immunoblot experiments with anti-Cx43. HEY cells were incubated for increasing time periods with 100 nM ET-1, and cell lysates were immunoprecipitated with anti-Cx43 and immunoblotted with anti-phosphotyrosine (Fig. 6A). Immunoblot analysis indicated that ET-1 treatment resulted in a time-dependent induction of Cx43 tyrosine phosphorylation. Fig. 6B shows the amount of the ET-1-induced Cx43-P as quantified by the Scion image analysis program.

Involvement of c-Src on ET-1-induced GJIC Reduction—Several lines of evidence demonstrated that c-Src plays a crucial role in signaling via GPCR to inactivate GJIC in Rat-1 fibroblast (20). Moreover tyrosine phosphorylation of Cx43 in cardiomyocytes was mediated by c-Src (40). Therefore, we investigated whether ET-1, through ET₁ₐR, could induce loss of GJIC by the c-Src-mediated signaling in ovarian carcinoma cells. SL/DT experiments demonstrated that the selective Src kinase inhibitor, PP2 (50 nM), prevented the ET-1-induced reduction of GJIC in HEY cells (Fig. 7A). Signaling molecules involved in ET₁ₐR-stimulated tyrosine kinase pathways include Src in different cell types (41, 42). Using specific anti-phospho-Src antibody (anti-Src (Tyr(P)418)) (43), we found that exposure of HEY cells to ET-1 induced a time-dependent increase of c-Src kinase activation reaching a maximum after 30 min of stimulation, which concurred with kinetics of Cx43 phosphorylation (Fig. 7B). Moreover, pretreatment of ovarian cancer cells with PP2 (50 nM) blocked the ET-1-induced tyrosine phosphorylation of c-Src (Fig. 7C) and Cx43 tyrosine phosphorylation (Fig. 7D). Because Cx43 has been reported to interact directly with c-Src and this interaction is a necessary and sufficient condition to phosphorylate Cx43 (20), we investigated the effects of ET-1 on the Cx43 interaction with c-Src (Fig. 7E). ET-1 stimulation of HEY cells led to an activation of c-Src that communoprecipitated with Cx43 (Fig. 7F), indicating c-Src as a suitable candidate for ET-1-induced GJIC inhibition and Cx43 phosphorylation.

Analysis of Cx43 Protein Expression in Ovarian Carcinoma Xenografts of Animals Treated with ET₁ₐR Antagonist—To evaluate in vivo the effect of the potent ET₁ₐR antagonist, ABT-627, we examined immunohistochemically Cx43 expression in murine ovarian carcinoma xenografts. HEY ovarian carcinoma...
cells were grown as subcutaneous tumors in nude mice. At day 7, when well established HEY xenografts were palpable with a tumor size of 0.25 cm$^3$, mice were randomized into treated and vehicle-injected control groups of 10 animals each. The treated mice were injected intraperitoneally for 21 days with 2 mg/kg/day of ABT-627. A 2 mg/kg/day dose of ABT-627 was selected because it induced a 65% inhibition of the tumor growth, was well tolerated, and corresponded to that used in human clinical trials (18). Immunohistochemical evaluation of the expression of Cx43, performed on HEY tumors at day 40 after the tumor cell injection, revealed an increase in Cx43-
based gap junction plaques in HEY tumors treated with ABT-627 (Fig. 8A). Western blot analysis of the expression of Cx43 protein, performed on HEY tumor xenografts freshly excised on day 40 after tumor cell injection, revealed a marked reduction in the phosphorylated forms of Cx43 in ABT-627-treated mouse compared with control (Fig. 8B). To prove that the observed ABT-627-induced reduction of Cx43 phosphorylation occurs on a tyrosine residue, HEY xenograft lysates were immunoprecipitated with anti-Cx43 and immunoblotted with anti-phosphotyrosine (Fig. 8C). Immunoprecipitation and immunoblot analysis indicated that ABT-627 treatment resulted in a significant reduction of Cx43 tyrosine phosphorylation. These data indicate that ETAR antagonist prevents in vivo Cx43 tyrosine phosphorylation and subsequent degradation related to cellular uncoupling.

DISCUSSION

During tumor progression, epithelial cancer cells leave their local “neighborhood” to move into new microenvironments by acquiring a local invasive and metastatic phenotype. The acquisition of migratory abilities in epithelial cells is accompanied by the loss of expression of cell-cell junctional molecules. Several growth factors that bind tyrosine kinase receptors or GPCR have been shown to induce deregulation or loss of function of GJIC in several cell types by inducing Cx43 phosphorylation (20, 24–26, 40). In the present study, we demonstrated that in ovarian carcinoma cells, ET-1 via ETAR induces a transient and a dose-dependent reduction of GJIC. Western blot and immunolocalization analysis clearly showed that Cx43 becomes more phosphorylated, and fewer gap junction plaques were apparent when ovarian cancer cells were treated with ET-1, suggesting that ET-1 promotes the cellular uncoupling at

**Fig. 6.** Kinetics of Cx43 tyrosine phosphorylation in HEY cells. HEY cells were stimulated for up to 120 min with 100 nM ET-1. Cx43 was immunoprecipitated (IP) from cell lysates and immunoblotted (IB) with anti-phosphotyrosine (anti-PY; upper panel) or with anti-Cx43 (lower panel) in Western blot. Heavy chain of immunoglobulin (IgG) migrates upper tyrosine-phosphorylated Cx43 as indicated by the arrowheads (A). The relative density of Cx43-P content from A was statistically analyzed and represents the average value of three independent Western blots ± S.D.; *, p < 0.001 compared with control (B).

**Fig. 7.** Involvement of protein-tyrosine kinase c-Src on ET-1 effects. A, HEY cells were pretreated for 30 min with the c-Src inhibitor PP2 (50 nM) and then treated with 100 nM ET-1 for 30 min. GJICs were measured by the SL/DT method, and the data were quantified and expressed as the relative percentage of the control. The data show a representative experiment of three different experiments, and results are means of triplicate samples ± S.D. Statistical comparisons were made in reference to untreated cells. *, p < 0.001 compared with control; **, p < 0.05 compared with ET-1. B, HEY cells were stimulated with 100 nM ET-1 for the indicated time periods. The activated form of c-Src was detected by using specific anti-c-Src (Tyr(P)418), and total c-Src was detected by using anti-c-Src (lower panel). C, whole cell lysates from HEY cells treated with ET-1 (100 nM) or PP2 (50 μM) alone or in combination with ET-1 were immunoblotted (IB) with specific anti-c-Src (Tyr(P)418; upper panel) or total c-Src by using anti-c-Src (lower panel). D, the same lysates as in C were immunoprecipitated (IP) with anti-Cx43 and immunoblotted either with anti-phosphotyrosine (upper panel) or with anti-Cx43 (lower panel). E, cell lysates from ET-1-treated and untreated HEY cells were immunoprecipitated with anti-Cx43 and immunoblotted with anti-c-Src (Tyr(P)418; upper panel), anti-c-Src (middle panel), or anti-Cx43 (lower panel). The positions of c-Src and c-Src (Tyr(P)418) are indicated by the arrows. In D and E, heavy chain of immunoglobulin (IgG) is indicated by the arrowheads.
the level of connexin maturation and subsequent degradation. Cx43 tyrosine phosphorylation was mainly responsible for ET-1-induced loss of cell-cell communication in these tumor cells as ET-1, transiently disrupt GJIC in Rat-1 cells through a c-Src tyrosine kinase pathway and that GPCR use c-Src kinase to act with the adherens junction protein, β-catenin (49). It has been demonstrated that ET-1 induces Cx43 phosphorylation in vitro through mitogen-activated protein kinase signaling pathways in cardiomyocyte.

Degradation of phosphorylated Cx43 has been reported to be correlated with the rapid turnover/disassembly of gap junction plaques, with the consequent decrease in intercellular communication (27). Data presented in this study cannot exclude the possibility that ET-1-induced Cx43 phosphorylation could destabilize gap junction plaques by a mechanism involving the degradation pathway. The intrinsic mechanism whereby Cx43 gets targeted for degradation in response to ET-1-induced phosphorylation remains to be investigated in future studies.

Interestingly, we found that addition of a specific ET₄₅R antagonist, BQ 123, blocked the ET-1-induced loss of GJIC and Cx43 phosphorylation, demonstrating that ET₄₅R activation by ET-1 contributes to loss of growth control via a Cx43-mediated disruption of GJIC. In a previous study, we demonstrated that ET₄₅R blockade by the potent ET₄₅R antagonist, ABT-627, resulted in therapeutic activity against established ovarian carcinoma expressing ET₄₅R. This effect was associated with a significant decrease in microvessel density and in vascular endothelial growth factor and matrix metalloproteinase-2 expression and with an increased percentage of apoptotic cells (18). In this study, we found that ABT-627 treatment, concomitantly with a reduction of tumor growth, increases the Cx43-based gap junction plaques and decreases Cx43 tyrosine phosphorylation, indicating that the antitumoral activity of ABT-627 may also be due to the prevention of post-transcriptional modification of Cx43. In addition to its well established role as a channel-forming protein, Cx43 might function as a microtubule-anchoring protein (44). In this model, it has been proposed that Cx43 is part of a multiprotein complex. For example, the c-Src can bind directly and phosphorylate the Cx43, a mechanism that seems responsible for the disruption of Cx43 interaction with the scaffolding protein zona occludens protein 1 (ZO-1), which associates with tight junction, cytoskeleton, and signal transduction molecules in several cell types (45–47).

Recent results demonstrated that c-Src acts by affecting trafficking of Cx43 to the plasma membrane or by altering connexin-connexin assembly within the plasma membrane through regulation of the Cx43-ZO-1 interaction. Thus, the cytoplasmatic-terminal region of Cx43 is also a multimeric interaction site for cytoskeleton structures like ZO-1. Cx43 is also directly stimulated by Wnt-1 signaling (48) and can interact with the adherens junction protein, β-catenin (49). It has been demonstrated that restoration of cadherin-based cell-cell adhesion induces the assembly of Cxs into gap junctions (50). Similarly, in prostate cancer cells, α-catenin expression triggered the trafficking and assembly of Cxs into gap junctions and recruited ZO-1 to the cell surface (51). The significance of this finding is not understood. Because the dynamic behavior of cell interactions and communication is affected in migrating cells that lack cell-cell contacts, one can envision that a GPCR, such as ET₄₅R, which mediated inhibition of Cx43-based junctional communication, might alter intercellular interactions that are responsible for contact-mediated regulatory control. The molecular mechanisms by which cadherins and their associated proteins may facilitate the assembly of Cxs into gap junctions are likely to be complex. The possibility that ET-1 alters cell adhesion and that it consequently alters the assem-
E-cadherin-mediated adhesive interactions. Here, we demonstrated that ABT-627 is able to block Cx43 tyrosine phosphorylation and to increase Cx43-based intercellular communication, which are correlated with tumor growth reduction in ovarian carcinoma xenografts. New therapeutic strategies using specific ET<sub>R</sub> antagonists with suitable pharmacological and toxicity profiles for clinical use (36, 37) may provide a novel approach to the treatment of ovarian carcinoma in which ET<sub>R</sub> blockade could result in tumor growth inhibition also by preventing the disruption of cell-cell communication.

Acknowledgments—We gratefully acknowledge Dr. Perry Nisen (Global Oncology Development) of Abbott for kindly providing the ABT-627, Marco Varmi and Giacomo Elia for excellent technical assistance, and Maria Vincenza Sarcone for secretarial assistance.

REFERENCES

1. Nelson, J. B., Hedican, S. P., George, D. J., Reddi, A. H., Plantadosi, S., Eisenberger, M. A., and Simons J. W. (1995) Nat. Med. 1, 994–999.
2. Bagnato, A., Tecce, R., Moretti, C., Di Castro, V., Spergel, D., and Catt, K. J. (1995) Clin. Cancer Res. 1, 1059–1066.
3. Bagnato, A., Salani, D., Di Castro, V., Wu-Wong, J. R., Tecce, R., Nicotra, M. R., Venuti, A., Natali, P. G., and Bagnato, A. (1999) Cancer Res. 59, 720–727.
4. Egady, G., Jullierat-Jeanneret, L., Jeannin, J. F., Korth, P., Bosman, F. T., and Pinet, F. (2000) Am. J. Pathol. 157, 1863–1874.
5. Venuti, A., Salani, D., Manni, V., Poggiali, F., and Bagnato, A. (2000) J. Pathol. 194, 2277–2283.
6. Baley, P. A., Resink, T. J., Eppenberger, U., and Hahn, A. W. (1990) J. Clin. Invest. 85, 1320–1323.
7. Econsomo, K., MacDonald, P. C., and Casey, M. L. (1992) Cancer Res. 52, 554–557.
8. Lahav, R., Heffner, G., and Patterson, P. H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11496–11500.
9. Bagnato, A., Salani, D., Di Castro, V., Albini, A., Salani, D., Varmi, M., Nicotra, M. R., and Natali, P. G. (2001) Am. J. Pathol. 158, 841–847.
10. Rubanji, G. M., and Polokoff, M. A. (1999) J. Cell. Physiol. 179, 219–223.
11. Del Bufalo, D., Di Castro, V., Birocco, A., Varmi, M., Salani, D., Rosano, L., Trisciuoglio, D., Spinella, F., and Bagnato, A. (2002) J. Membr. Biol. 219–223.
12. Bagnato, A. (2001) Mol. Pharmacol. 61, 524–532.
13. Rosano, L., Varmi, M., Salani, D., Di Castro, V., Spinella, F., Natali, P. G., and Bagnato, A. (2001) Cancer Res. 61, 8340–8346.
14. Salani, D., Di Castro, V., Nicotra, M. R., Rosano, L., Tecce, R., Venuti, A., Natali, P. G., and Bagnato, A. (2000) Am. J. Pathol. 157, 1537–1547.
15. Salani, D., Taraboletti, G., Rosano, L., Di Castro, V., Borsotti, P., Giavazzi, R., and Bagnato, A. (2000) Am. J. Pathol. 157, 1703–1711.
16. Bagnato, A., Salani, D., Di Castro, V., Colli, M., Borsotti, P., and Giavazzi, R. (2001) J. Biol. Chem. 276, 27850–27855.
17. Carducci, M. A., Nelson, J. B., Bowling, M. K., Rogers, T., Eisenberger, M. A., Simons J. W., and Finan, S. (1999) Cancer Res. 59, 1369–1373.
18. Bagnato, A. (2001) Cell Sci. 114, 1189–1209.