Evidences that Leptin Up-regulates E-Cadherin Expression in Breast Cancer: Effects on Tumor Growth and Progression

Loredana Mauro, Stefania Catalano, Gianluca Bossi, Michele Pellegrino, Ines Barone, Sara Morales, Cinzia Giordano, Viviana Bartella, Ivan Casaburi, and Sebastiano Ando

Introduction

Leptin is an adipocyte-derived hormone (1) that, in addition to the control weight homeostasis by regulating food intake and energy expenditure (2, 3), is implicated in the modulation of many other processes such as reproduction, lactation, hematopoiesis, immune responses, cell differentiation, and proliferation (4, 5). The activities of leptin are mediated through the transmembrane leptin receptor (ObR; refs. 6, 7) by activation of the Janus-activated kinase/signal transducers and activators of transcription (STAT) and mitogen-activated protein kinase (MAPK) pathways (8, 9). Epidemiologic studies show a positive association between obesity and an increased risk of developing different cancers (10, 11). Several lines of evidence suggest that leptin and ObR are involved in the development of normal mammary gland and in mammary carcinogenesis (12–14). It has been recently reported that in primary breast tumors, leptin was detected in 86.4% of cases examined, and its expression was highly correlated with ObR (15). This indicates that leptin can influence breast cancer cells not only by endocrine and/or paracrine actions but also through autocrine pathways.

In epithelial and epithelium-derived tumors, cell-cell adhesion and tumor mass mostly depend on E-cadherin, a 120-kDa transmembrane molecule (16, 17). As it might be expected, E-cadherin seems to have a major influence on primary cancer development and evolution. Alteration in the function of E-cadherin and the cadherin-catenin complex has been implicated in cancer progression (18), invasion (19–21), and metastasis (22, 23).

In this study, we explored a new aspect of the involvement of leptin in initial steps of mammary tumorigenesis. Specifically, we asked whether leptin can affect primary tumor mass either in vivo in MCF-7 cell tumor xenograft or in vitro in MCF-7 three-dimensional cultures. Our results showed that leptin is able to promote tumor cell proliferation and homotypic tumor cell adhesion via an increase of E-cadherin expression. This combined effect may give reasonable emphasis to the important role of this cytokine in stimulating primary breast tumor cell growth and progression, particularly in obese women. [Cancer Res 2007;67(7):3412–21]

Materials and Methods

Plasmids. The plasmids containing the human E-cadherin promoter or its deletions were given by Dr. Y.S. Chang (Chang-Gung University, Republic of China; ref. 24). pHEGO plasmid containing the full length of estrogen receptor α (ERα) cDNA was provided by Dr. D. Picard (University of Geneva). pSG5 vector containing the cDNA-encoding dominant-negative STAT3, which is a variant of the transcription factor STAT3 lacking an internal domain of 50 bp located near the COOH terminus (STAT3), was given by Dr. J. Turkson (University of South Florida, College of Medicine, Tampa, FL). pCMV/myc vector containing the cDNA-encoding dominant-negative extracellular signal-regulated kinase 2 K52R (ERK2) was provided by Dr. M. Cobb (Southwestern Medical Center, Dallas, TX).

Site-directed mutagenesis. The E-cadherin promoter plasmid-bearing cyclic AMP–responsive element binding protein–mutated site (CREB mut) was created by site-directed mutagenesis using Quick Change kit (Stratagene, La Jolla, CA). We used as template the human E-cadherin
Cell and culture conditions. MCF-7, HeLa, and BT-20 cells were obtained from the American Type Culture Collection (Manassas, VA). MCF-7 and HeLa cells were maintained in DMEM/F-12 containing 5% calf serum and BT-20 cells were cultured in MEM supplemented with 10% fetal bovine serum, 1% Eagle's nonessential amino acids, and 1% sodium pyruvate (Sigma, Milan, Italy). Cells were cultured in phenol red-free DMEM (serum-free medium), containing 0.5% bovine serum albumin, 24 h before each experiment. All media were supplemented with 1% L-glutamine and 1% penicillin/streptomycin (Sigma).

In vivo studies. The experiments in vivo were done in 45-day-old female nude mice (nu/nu Swiss; Charles River, Milan, Italy). At day 0, the animals were fully anesthetized by i.m. injection of 1.0 mg/kg Zoletil (Virbac) and 0.12% Xylor (Xylazine) to allow the s.c. implantation of estradiol (E2) pellets (1.7 mg per pellet, 60-day release; Innovative Research of America, Sarasota, FL) into the intrascapular region of mice. The day after, exponentially growing MCF-7 cells (5.0 × 10^6 per mouse) were inoculated i.p. with either solutions: recombinant human leptin (230 μg/kg) diluted in saline + 0.3% bovine serum albumin (BSA) or saline + 0.3% BSA only (control). The treatment was done for 5 days a week until the 13th week. Tumor development was followed twice a week by caliper measurements along two orthogonal axes length (L) and width (W). The volume (V) of tumors was estimated by the following formula: \( V = L × W^2 / 2 \). At the time of killing (13 weeks), tumors were dissected out from the neighboring connective tissue, frozen in nitrogen, and stored at −80°C. All the procedures involving animals and their care have been conducted in conformity with the institutional guidelines at the Laboratory of Molecular Oncogenesis, Regina Elena Cancer Institute in Rome.

Three-dimensional spheroid cell culture and growth. The cells were plated in single-cell suspension in 2% agar–coated plates and untreated or treated with 1,000 ng/mL leptin and/or E2 (100 nmol/L) for 48 h and then plated on six-well plates coated with 1.5 μg/mL recombinant human E-cadherin/Fc chimeric. Before the experiment, the wells were blocked with 1% BSA for 3 h at 37°C and then washed with PBS.

After washing out nonadherent cells, adherent cells were incubated 3 h in medium containing 500 μg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution. The reaction product was measured at 570 nm.

Total RNA extraction and reverse transcription-PCR assay. Total RNA was extracted using TRIzol reagent (Invitrogen, San Diego, CA). Reverse transcription was done using RETROscript kit (Ambion, Austin, TX). The cDNAs were amplified by PCR using the following primers: 5′-TCTAAAGTGAGGAGACCAC-3′ and 5′-GGGTAGTGGACAGAAGT-3′ (cyclin D1), 5′-TGGATACGAGGCAAAATTG-3′ and 5′-TATTGTGG-3′ (E-cadherin), and 5′-CCTAACACTCCCCCTGTC-3′ and 5′-CAAATCCGATATTCTC-3′ (36B4). The PCR was done for 30 cycles for cyclin D1 (94°C for 1 min, 60°C for 1 min, and 72°C for 2 min) and E-cadherin (94°C for 1 min, 55°C for 1 min, and 72°C for 2 min) and 15 cycles (94°C for 1 min, 59°C for 1 min, and 72°C for 2 min) to amplify 36B4, in the presence of 1 μL of first-strand cDNA, 1 μmol/L each of the primers mentioned above, deoxynucleotide triphosphate (0.5 mmol/L), Taq DNA polymerase (2 units per tube; Promega, Madison, WI) in a final volume of 25 μL.

Western blot analysis. Equal amounts of total protein were resolved on an 8% to 10% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and probed with the appropriated antibody. The antigen-antibody complex was detected by incubation of the membrane at room temperature with a peroxidase-coupled goat anti-mouse or anti-rabbit IgG and revealed using the enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom).

**Figure 1.** Effect of leptin on growth of MCF-7 cell tumor xenografts. A, xenografts were established with MCF-7 cells in female mice implanted with E2 pellet. One group was treated with 230 μg/kg leptin (5, n = 5) and a second group with vehicle (5, n = 5). *, P < 0.05, treated versus control group. Representative Western blot on protein extracts from xenografts excised from control mice and mice treated with leptin showing MAPK (B) and STAT3 (C) activation. The immunoblots were stripped and reprobed with total MAPK and STAT3, which serve as the loading control. pmAPK, phosphorylated MAPK; pSTAT3, phosphorylated STAT. Columns, mean of three separate experiments in which the band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%; bars, SE. *, P < 0.05.
Transfection assay. MCF-7 cells were transfected using the FuGENE 6 reagent (Promega) with the mixture containing 0.5 µg of human E-cadherin promoter constructs. HeLa cells were transfected with E-cadherin promoter (0.5 µg per well) in the presence or absence of HEGO (0.2 µg per well) or cotransfected with STAT3 or ERK2 dominant negative (0.5 µg per well). Twenty-four hours after transfection, the cells were treated with 1,000 ng/mL leptin and/or 100 nmol/L E2 for 48 h. Empty vectors were used to ensure that DNA concentrations were constant in each transfection. TK Renilla luciferase plasmid (5 ng per well) was used. Firefly and Renilla luciferase activities were measured by Dual-Luciferase kit. The firefly luciferase data for each sample were normalized based on the transfection efficiency measured by Renilla luciferase activity.

Electrophoretic mobility shift assay. Nuclear extracts were prepared from MCF-7 as previously described (25). The probe was generated by annealing single-stranded oligonucleotides, labeled with [γ³²P]ATP and T4 polynucleotide kinase, and then purified using Sephadex G50 spin columns. The DNA sequences used as probe or as cold competitors are as follows: CRE, 5'-TGAGGTCAGGAGTTCCAGACC-3'; Sp1, 5'-ATCAGC-GGGGGGGGGGGGGGTTCCAGACC-3'. In vitro transcribed and translated CREB protein was synthesized using the T7 polymerase in the rabbit reticulocyte lysate system (Promega). The protein-binding reactions were carried out in 20 ml of buffer [20 mmol/L HEPES (pH 8), 1 mmol/L EDTA, 50 mmol/L KCl, 10 mmol/L DTT, 10% glycerol, 1 mg/mL BSA, 50 µg/mL poly(dI/dC) with 50,000 cpm] of labeled probe, 20 µg of MCF-7 nuclear protein or an appropriate amount of CREB protein or Sp1 human recombinant protein (Promega), and 5 µg of poly(dI/dC). The mixtures were incubated at room temperature for 20 min in the presence or absence of unlabeled competitor oligonucleotides. The specificity of the binding was tested by adding to the mixture reaction–specific antibodies (anti-CREB and anti-Sp1). Mithramycin A (100 µmol/L; ICN Biomedicals, Inc., Costa Mesa, CA) was incubated with the labeled probe for 30 min at 4°C before the addition of nuclear extracts. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25× Tris borate-EDTA for 3 h at 150 V.

Chromatin immunoprecipitation assay. We followed chromatin immunoprecipitation (ChIP) methodology described by Morelli et al. (26). MCF-7 cells were untreated or treated with 1,000 ng/mL leptin and/or 100 nmol/L E2 for 1 h. The cells were then cross-linked with 1% formaldehyde and sonicated. Supernatants were immunocleared with sonicated salmon sperm DNA/protein A agarose (Upstate Biotechnology, Inc., Lake Placid, NY) and immunoprecipitated with anti-CREB or anti-Sp1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Pellets were washed as reported (26), eluted

Table 1. Effect of leptin on cell aggregation in MCF-7 breast cancer cells

<table>
<thead>
<tr>
<th>Spheroids</th>
<th>Control</th>
<th>Leptin</th>
<th>E2</th>
<th>Leptin + E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>25–50 µm</td>
<td>0.0 ± 0.0</td>
<td>0.6 ± 2.6</td>
<td>26 ± 1.8</td>
<td>0.6 ± 2.6</td>
</tr>
<tr>
<td>50–100 µm</td>
<td>0.0 ± 0.0</td>
<td>0.6 ± 2.6</td>
<td>26 ± 1.8</td>
<td>0.6 ± 2.6</td>
</tr>
<tr>
<td>&gt;100 µm</td>
<td>0.0 ± 0.0</td>
<td>0.6 ± 2.6</td>
<td>26 ± 1.8</td>
<td>0.6 ± 2.6</td>
</tr>
</tbody>
</table>

NOTE: MCF-7 cells were cultured as three-dimensional spheroids in serum-free medium. The extent of aggregation was scored by measuring the spheroid diameters with an ocular micrometer. The values represent a sum of spheroids in 10 optical fields under ×10 magnification. The results are mean ± SE from at least three experiments. Representative three-dimensional cultures are shown in Fig. 2A.
with elution buffer (1% SDS and 0.1 mol/L NaHCO₃), and digested with proteinase K (26). DNA was obtained by phenol/chloroform extractions and precipitated with ethanol; 5 µL of each sample were used for PCR with CREB primers (5'-TGTAATCCAACACTTCAGGAGG-3' and 5'-TTGAGACG-GAGTCTCGCTCT-3') and Sp1 primers (5'-TAGCAACTCCAGGCTAGAGG-3' and 5'-AICTGACTTCCGCAAGCTACA-3'). The PCR conditions were 94°C for 1 min, 56°C for 2 min, and 72°C for 2 min for 30 cycles.

**Statistical analysis.** Data were analyzed by ANOVA using the STATPAC computer program. Statistical comparisons for in vitro studies were made by Wilcoxon-Mann-Whitney test.

**Figure 3.** Leptin up-regulates E-cadherin expression in MCF-7 spheroids and xenografts. Reverse transcription-PCR of E-cadherin mRNA was done in MCF-7 three-dimensional cultures stimulated for 48 h with 1,000 ng/mL leptin and/or 100 nmol/L E₂ (A) as well as in xenografts (B). 36B4 mRNA levels were determined as a control. C−, RNA sample without the addition of reverse transcriptase (negative control). Protein extracts obtained from MCF-7 spheroids (A) and xenografts (B) were immunoblotted with a specific antibody against human E-cadherin. Representative results. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Columns, mean of three separate experiments in which the band intensities were evaluated in terms of optical density arbitrary units and expressed as the percentage of the control assumed as 100%; bars, SE. MCF-7 cells were transiently transfected with a luciferase reporter plasmid containing the human E-cadherin promoter full-length p-1008/+49 (C) or mutated in the CREB site (CREB mut; D). Schematic representation of human E-cadherin promoter constructs. The +1 position represents the transcriptional initiation site. The cells were left untreated (control) or treated in the presence of 1,000 ng/mL leptin and/or 100 nmol/L E₂. Columns, mean of three separate experiments; bars, SE. In each experiment, the activities of the transfected plasmid were assayed in triplicate transfections. *, P < 0.05; **, P < 0.01 compared with control.
Leptin enhances cell-cell adhesion and cell proliferation. We did three-dimensional MCF-7 cultures to evaluate in vitro the effects of leptin on cell aggregation. It has been reported that multicellular spheroid culture can more closely mimic some in vivo biological features of tumors and improve the relevance of in vitro studies (27–30).

Our results evidenced that leptin and/or E2 treatment for 48 h enhances cell-cell adhesion of MCF-7 cells compared with untreated cells (Fig. 2A). The combined exposure to both hormones switches cell aggregation towards the formation of spheroids exhibiting prevalently a diameter larger than 100 μm (Table 1).

E-cadherin is a major type of adhesion molecule, which forms Ca2+-dependent homophilic ligations to facilitate cell-cell contact in epithelial cells (16, 17). Thus, to study whether E-cadherin was responsible for leptin-enhanced cell-cell adhesion, we supplemented the cell culture medium with function-blocking E-cadherin antibody or EGTA, a calcium-chelating agent. As shown in Fig. 2A, in the presence of the antibody, MCF-7 cells formed small aggregates showing limited intercellular contact, whereas EGTA treatment prevented cell-cell adhesion, and cells remained rounded and singed suspended.

In addition, the role of E-cadherin was confirmed using an adhesion assay in which cells were allowed to adhere to E-cadherin/Fc protein–coated dishes. This assay showed a greater binding of cells pretreated with leptin and/or E2 for 48 h with respect to untreated cells (Fig. 2B). The adhesion was blocked using either a function-blocking E-cadherin antibody or EGTA (data not shown). Thus, the increased aggregation observed in the presence of leptin and/or E2 was dependent on E-cadherin.

In three-dimensional cultures, we also observed a significant increase of cell growth upon leptin and/or E2 treatment. The leptin-induced cell proliferation was completely abrogated when E-cadherin function was blocked (Fig. 2C).

Furthermore, in MCF-7 spheroids and in xenografts, we observed an increase of cyclin D1, a regulator of cell cycle progression, in terms of mRNA and protein content in the presence of leptin and/or E2 (Supplementary Fig. S1).

Leptin up-regulates E-cadherin expression. To investigate if an enhanced expression of E-cadherin occurred in the above-mentioned conditions, we did reverse transcription-PCR and Western blotting analysis. Our results showed that either leptin or E2 and, in higher extent, the exposure to both hormones increased expression of E-cadherin in terms of mRNA and protein content (Fig. 3A). The latter results were also evident in MCF-7 xenografts (Fig. 3B).

To evaluate whether both hormones were able to activate E-cadherin promoter, we transiently transfected MCF-7 cells with human E-cadherin promoter plasmid (p-1008/+49). A significant increase in promoter activity was observed in the transfected cells exposed to leptin and/or E2 for 48 h (Fig. 3C).

In contrast, we observed that leptin was unable to activate the constructs containing different deleted segments of human E-cadherin promoter (p-164/+49 and p-83/+49) with respect to the full length, whereas E2 induced activation in the presence of p-164/+49 construct (Supplementary Fig. S2).

Leptin enhances CREB-DNA and Sp1-DNA binding activity to E-cadherin promoter. The role of leptin and E2 on the transcriptional activity of the E-cadherin gene was explored analyzing the nucleotide sequence of the E-cadherin gene promoter. We evidenced, upstream to the initiation transcription site, one CRE (−925/−918) and two Sp1 (−144/−132 and −51/−39) as putative effectors of leptin and estrogens. For instance, in MCF-7 cells transiently transfected with E-cadherin promoter plasmid-bearing CREB-mutated site (CREB mut), we observed that the stimulatory effect of leptin was abrogated, whereas the activation of E2 still persisted, although in a lower extent with respect to the intact promoter (Fig. 3D).

To characterize the role of these motifs in modulating E-cadherin promoter activity, we did electrophoretic mobility shift assay (EMSA). Nuclear extracts from MCF-7 cells, using as probe a CRE-responsive element, showed two protein-DNA complexes (Fig. 4A, lane 1), which were abolished by the addition of a nonradioabeled competitor (Fig. 4A, lane 2). Leptin treatment induced a strong increase in CREB-DNA binding activity (Fig. 4A, lane 3), which was immunodepleted in the presence of CREB antibody (Fig. 4A, lane 4). Using transcribed and translated in vitro CREB protein, we obtained two bands migrating at the same level as that of MCF-7 nuclear extracts (Fig. 4A, lane 9). In the presence of the MAPK inhibitor PD98059, the complex induced by leptin treatment was reduced (Fig. 4B, lanes 5 and 9). These findings addressed a specific involvement of leptin signaling in the up-regulation of E-cadherin expression.

Using a DNA probe containing an Sp1 site, we observed in MCF-7 nuclear extracts, a specific protein-DNA complex that was slightly enhanced by leptin, increased upon E2 exposure and furthermore by the combined treatments (Fig. 4C, lanes 1, 3, 5, and 7). In the presence of Sp1 human recombinant protein, we observed a single complex that causes the same shift with respect to the band revealed in MCF-7 nuclear extracts (Fig. 4C, lane 10). The addition of a double-stranded CREB-specific (A and B) or Sp1-specific (C and D) consensus sequence probe labeled with [32P]ATP and subjected to electrophoresis in a 6% polyacrylamide gel (lane 1). A, we used as positive control a transcribed and translated in vitro CREB protein (lane 9). Competition experiments were done by adding as competitor a 100-fold molar excess of unlabeled probe (lanes 2 and 10). MCF-7 nuclear extracts treated with 1,000 ng/mL leptin (Lep) and/or 100 nmol/L E2 for 48 h incubated with probe (lanes 3, 5, and 7), respectively. The specificity of the binding was tested by adding to the reaction mixture a CREB antibody (lanes 4, 6, and 8). MCF-7 cells were serum starved overnight with 10 μmol/L PD 98059 (lanes 2, 3, 5, 7, and 9). Lanes 11 (A) and 12 (B) contain probe alone. C, Sp1 human recombinant protein was used as positive control (lane 10). Competition experiments were done by adding as competitor a 100-fold molar excess of unlabeled probe (lanes 2 and 11). MCF-7 nuclear extracts treated with 1,000 ng/mL leptin and/or 100 nmol/L E2 for 48 h incubated with probe (lanes 3, 5, and 7), respectively. The specificity of the binding was tested by adding to the reaction mixture a Sp1 antibody (lanes 4, 6, and 8). The formation of DNA-Sp1 complexes was blocked by the addition of 100 μmol/L mithramycin A (lane 9). D, the pure antiestrogenICI 182,780 (1 μmol/L) was added in leptin-treated (lane 5) and/or E2-treated (lanes 7 and 9) MCF-7 nuclear extracts. Lane 12 contain probe alone.
of mithramycin A (100 μmol/L), which binds to GC boxes and prevents sequential Sp1 binding, to nuclear extracts treated with leptin and E2 blocked the formation of DNA-Sp1 complexes (Fig. 4C, lane 9). The original band DNA-protein complex was supershifted by Sp1 antibody (Fig. 4C, lanes 4, 6, and 8). In all hormonal treatments done, the pure antiestrogen ICI 182,780 reduced the Sp1-DNA binding complex (Fig. 4D, lanes 5, 7, and 9), evidencing that leptin induced an activation of ERα, as we previously showed (25).

Effects of leptin on CREB and Sp1 recruitment to the E-cadherin promoter. To corroborate EMSA results, we did ChIP assay. We found that the stimulation of MCF-7 cells with leptin increased the recruitment of CREB to E-cadherin gene promoter (Fig. 5A). Furthermore, we observed that leptin or E2 stimulated the recruitment of Sp1 to the E-cadherin promoter, and the combined treatment induced an additive effect (Fig. 5B). The latter event suggests that leptin and E2 may converge in activating ERα to recruit Sp1 on E-cadherin promoter.

Involvement of ERα in the leptin-induced up-regulation of E-cadherin expression. Stemming from the data provided by EMSA and ChIP assays, we evaluated the involvement of ERα in the enhanced E-cadherin expression induced by leptin. Our results showed that in three-dimensional cultures, in the presence of the pure antiestrogen ICI 182,780, the up-regulatory effect of leptin on E-cadherin protein expression still persisted, whereas the stimulatory effect of E2 was abrogated (Fig. 6A).

In addition, the specific role of leptin signaling in up-regulating E-cadherin expression was also confirmed by functional studies in ERα-negative HeLa cells. We evidenced that leptin was able to activate E-cadherin promoter (Fig. 6B), which was abrogated in the presence of ERK2 and STAT3 dominant negative (Fig. 6C), sustaining furthermore the involvement of leptin signaling. It is worth to note how the ectopic expression of ERα in HeLa cells was able to potentiate the effect of leptin (Fig. 6B). To test the activity of the transfected ERα, we did Western blotting analysis for phosphorylated ERα, whereas for dominant-negative ERK2 and STAT3 genes, we evaluated the expression of c-fos, as target of both pathways (31–33). Moreover, in BT-20 cells lacking of ERα, leptin-enhanced E-cadherin protein content was reduced in the presence of either ERK2 or STAT3 dominant negative. In the same cells, cotransfected with ERα and ERK2 or STAT3 dominant negative, E2 alone or in combination with leptin was unable to maintain the up-regulatory effect on E-cadherin expression (Supplementary Fig. S3).

Discussion

Leptin stimulates cell growth, counteracts apoptosis, and induces migration and angiogenic factors in different cellular cancer models (10). For instance, hyperleptinemia is a common feature of obese women who have a higher risk of breast cancer than women with normal weight (34), but the association between circulating leptin and breast cancer is still not clear. It has been reported that in interstitial fluid of the adipose tissue, leptin concentration is higher than the circulating levels (35). Thus, we may reasonably assume that in the presence of an abundant adipose tissue surrounding epithelial breast cancer cells, the paracrine leptin effects become crucial in affecting local and primary tumor progression.

The aim of this study was to evaluate whether leptin can influence local primary breast cancer development and progression, using an in vivo model of MCF-7 xenografts implanted in female nude mice and an in vitro system represented by MCF-7 three-dimensional cultures. Our results showed in MCF-7 xenografts that leptin treatment significantly potentiated the E2-increased tumor size. In the same view, in vitro studies revealed that the combined exposure to both hormones enhanced cell-cell aggregation with respect to the separate treatments.

E-cadherin is an intercellular adhesion molecule generally implicated as tumor suppressor in several types of epithelial tumors, based on findings that the expression of this homotypic adhesion molecule is frequently lost in human epithelial cancers (18, 20, 21). However, it has well been shown in ovarian epithelial tumors that E-cadherin expression is much more elevated than normal ovaries, suggesting that E-cadherin can play a role in the development of ovarian carcinomas (36). For instance, it is worth to mention that E-cadherin may serve not only as an intercellular adhesion molecule, but it may also trigger intracellular activation of proliferation and survival signals (37).
In our study, the increased cell-cell aggregation, observed in MCF-7 three-dimensional cultures upon leptin and/or E₂ treatments, seems to be dependent on E-cadherin molecule that has an indispensable role in this process. Indeed, the addition of a function-blocking E-cadherin antibody or a calcium-chelating agent (EGTA) blocked cell-cell adhesion induced by both hormones. Besides, we showed by adhesion assay a greater binding of cells pretreated with leptin and/or E₂ on E-cadherin/Fc protein–coated dishes.

In the same experimental conditions, an increased proliferative rate was observed upon leptin or E₂ exposure, which was completely abrogated when E-cadherin function was blocked.

An important cell cycle regulator, such as cyclin D1, resulted to be up-regulated in three-dimensional cultures and in xenografts.

Besides, in both models, we showed that leptin and/or E₂ enhanced E-cadherin expression in terms of mRNA, protein content, and promoter activity.

The analysis of E-cadherin promoter sequence revealed the presence of CRE and Sp1 sites as potential target of leptin and E₂ signals. It is well documented how leptin and E₂ through nongenomic effects are able to activate the MAPK pathway that induces activation of CREB kinase, a member of the p90RSK family that corresponds to RSK2 and thereby phosphorylates CREB Ser₁³³ (38–40). This well fits with our functional studies showing that leptin was no longer able to activate the E-cadherin gene promoter mutated in the CREB site, whereas E₂ maintained an activatory effect although in a lower extent with respect to the intact promoter. The latter data suggest that the activatory effect of E₂ may persist through its binding to Sp1-DNA complex.

The important role of the Sp1-responsive element in activating E-cadherin promoter was shown by EMSA and ChIP assays. Our results evidenced that E₂, as extensively documented, acts in a nonclassic way through the interaction of ERα with Sp1 (41–45). It is worth to note that upon leptin exposure, we also observed an increase in Sp1-DNA binding activity, clearly reduced in the presence of the pure antiestrogen ICI 182,780, as well as an enhanced recruitment of Sp1 to E-cadherin promoter. These observations are supported by our previous findings reporting that leptin is able to transactivate, in a unliganded-dependent manner, ERα through MAPK signal (25).

A cross-talk between leptin and E₂ has been well documented in neoplastic mammary tissues and breast cancer cell lines (15, 25, 46, 47). For instance, E₂ up-regulates leptin expression in MCF-7 cells (15), whereas leptin is an amplifier of E₂ signaling through a double mechanism: an enhanced aromatase gene expression (46) and a direct transactivation of ERα (25). Thus, we investigated whether the up-regulatory effect induced by leptin on E-cadherin expression can be modulated by ERα. We found that E-cadherin protein seems up-regulated still by leptin in the presence of the pure antiestrogen ICI 182,780. Moreover, in HeLa cells, leptin was able to activate E-cadherin promoter, which was abrogated in the presence of ERK2 or STAT3 dominant negative, suggesting that...
leptin signaling is involved in enhancing E-cadherin expression. These latter data are supported by Western blotting analysis done in BT-20 cells lacking of ERα in which ERK2 and STAT3 dominant negative reversed leptin-enhanced E-cadherin protein content. The up-regulatory effect induced by E2 on E-cadherin expression in the presence of ectopic ERα seemed inhibited in the presence of ERK2 and STAT3 dominant negative. The latter findings may be a consequence of the enhanced expression of leptin receptor upon E2 exposure (15), which may have an impaired signaling on E-cadherin expression. An additional explanation, which could coexist with the previous one, may be that both ERK2 and STAT3 dominant negative could interfere with ERα-Sp1 interaction at level of E-cadherin gene transcription (48).

A hypothetical model of the possible mechanism through which leptin and E2 may functionally interact in modulating E-cadherin expression in breast cancer is shown in Fig. 7. Leptin through MAPK activation may phosphorylate CREB and induce its transactivation. For instance, CREB phosphorylated at Ser332 is often reported not only as an index of PKA but also as an effector of MAPK activation (49). Concomitantly, leptin in the presence of E2 may potentiate the transactivation of ERα, which in turn may interact with Sp1 and bind DNA in a nonclassic way. On the other hand, it is well known that E2α, in the presence of its natural ligand, interacts with Sp1.

Thus, we may reasonably propose that upon leptin exposure, the increased E-cadherin-mediated cellular adhesion and activation of proliferation signals may enhance the transformation of normal epithelial cells to neoplastic cells and then stimulate the growth of tumor mass. Distinct from its role as a tumor suppressor, E-cadherin may function as tumor enhancer in the development of primary breast cancer.

In conclusion, all these data address how leptin and E2 signaling may represent a target of combined pharmacologic tools to be exploited in the novel therapeutic adjuvant strategies for breast cancer treatment particularly in obese women.

Acknowledgments

Received 8/4/2006; revised 1/17/2007; accepted 1/23/2007.

Grant support: Associazione Italiana per la Ricerca sul Cancro grants 2005 and 2006 and Spanish Ministry of Education Postdoctoral Research Grant (S. Morales).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Domenico Sturino for the English revision of the article and Dr. Pasquale Cicirelli for technical assistance.

References

43. Parnell ZN, Nardulli AM. Sp1 binding sites and an estrogen response element half-site are involved in regulation of the human progesterone receptor A promoter. Mol Endocrinol 2000;14:972–85.
45. Parnell ZN, Nardulli AM. Sp1 binding sites and an estrogen response element half-site are involved in regulation of the human progesterone receptor A promoter. Mol Endocrinol 2000;14:972–85.
Evidences that Leptin Up-regulates E-Cadherin Expression in Breast Cancer: Effects on Tumor Growth and Progression

Loredana Mauro, Stefania Catalano, Gianluca Bossi, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/7/3412

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2007/04/06/67.7.3412.DC1

Cited articles
This article cites 49 articles, 23 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/7/3412.full.html#ref-list-1

Citing articles
This article has been cited by 13 HighWire-hosted articles. Access the articles at:
/content/67/7/3412.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.