Abstract. Curcumin (diferuloyl methane) is an antioxidant that exerts antiproliferative and apoptotic effects and has anti-invasive and anti-metastatic properties. Evidence strongly implicates that epithelial-mesenchymal transition (EMT) is involved in malignant progression affecting genes such as Slug, AXL and Twist1. These genes are abnormally expressed in many tumors and favor metastasis. The purpose of this study was to determine the potential effect of curcumin on EMT, migration and invasion. Triple-positive and triple-negative breast cancer cell lines for estrogen receptor (ER), progesterone receptor (PgR) and HER/neu were used: i) MCF-10F, a normal immortalized breast epithelial cell line (negative), ii) Tumor2, a malignant and tumorigenic cell line (positive) derived from Alpha5 cell line injected into the immunologically depressed mice and transformed by 60/60 cGy doses of high LET (linear energy transfer) α particles (150 keV/µm) of radiation and estrogen, and iii) a commercially available MDA-MB -231 (negative). The effect of curcumin (30 µM for 48 h) was evaluated on expression of EMT-related genes by RT-qPCR. Results showed that curcumin decreased E-cadherin, N-cadherin, β-catenin, Slug, AXL, Twist1, Vimentin and Fibronectin protein expression, independently of the positivity of the markers in the cell lines. Curcumin also decreased migration and invasive capabilities in comparison to their own controls. It can be concluded that curcumin influenced biochemical changes associated with EMT-related genes that seems to promote such transition and are at the core of several signaling pathways that mediate the transition. Thus, it can be suggested that curcumin is able to prevent or delay cancer progression through the interruption of this process.

Introduction

Breast cancer is the dominant cause of death in women worldwide and it is the most common cancer in big city areas (1). Breast cancer in its advanced-stage has been related to the degree of metastasis (2). The process of metastasis seems to be regulated by a variety of gene products and EMT has been recognized as a fundamental process of embryogenesis, it is an important event in the metastatic cascade where the cells acquire migratory, and invasive capabilities (3).

Curcumin, an effective component of the spice turmeric (Curcuma longa) and a dietary chemopreventive agent (4,5), has been shown to resist initiation of carcinogenesis, modulation of cell survival, induction of apoptosis, inhibition of angiogenesis and induce anti-invasive and anti-metastatic effects (6).

Cadherins are cell adhesion molecules fundamental in the development of multicellular organisms (7). Among them, E-cadherin is essential for epithelial tissue integrity (8) and N-cadherin is expressed at gastrulation stage by downregulation of E-cadherin and undergoing EMT considered in cells of mesenchymal origin (9). Both establish cell-cell adhesion with their extracellular domains and are connected with catenins at their intracellular domains (10). Both also interact with receptors for growth factors involved in the modulation of signaling pathways, E-cadherin in relation with receptors of epidermal growth factors (11), and N-cadherin with fibroblast growth factor receptors (FGFR) (12). The protein B-catenin plays a role in signaling and cell adhesion (13,14).

SLUG, a member of the SNAI family (15-17) is involved in development of EMT (16), it is an inhibitor of apoptosis (18), and is part of breast and kidney development (15,16). AXL is activated through several mechanisms, as binding of its ligand and dimerization with HER2/neu (19-21). Since AXL is over-expressed in human cancers, it has significant correlation with tumor stage in breast cancer, especially in metastases (22,24). Twist1 is another factor that induces EMT and degradation of extracellular matrix (25-27) by promoting loosening of cell-cell junctions of epithelial cells and becoming invasive (28).

Vimentin is the most important part of the cytoskeletal of the cell along with microtubules and microfilaments (29). Fibronectin exerts multiple effects in vitro and in vivo as a component of the extracellular matrix stimulating proliferation, migration and differentiation (30-33). It activates various processes associated with cell invasion and proliferation.

Curcumin inhibits invasive capabilities through epithelial mesenchymal transition in breast cancer cell lines

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cell surface receptors most notably integrins (34) as well as
development of fibrillar structures (35) and activation of
various growth factors (36).

An inducer of EMT in cancer metastasis is the ZEB1,
a transcription factor that also induces EMT-suppressing
microRNA-200s (miR-200s) (37). ZEB2 belongs to the ZEB
protein family (38) and it is involved in differentiation (39,40).
Enhancer of zeste homolog 2 (EZH2) silences gene transcrip-
tion by trimethylation of histone H3 (41). It is upregulated
in multiple malignancies (42,43), and mediated by silencing
tumor suppressor genes (44). It is implicated in transcriptional
activation whose mechanism is not known (45-48).

STAT3, member of the family known as signal transducers,
is involved in oncogenesis (49). Cyclins have been identified as
regulatory subunits and catalytic subunits of cell cycle-regu-
lated kinases. The cyclin/cdk complexes are implicated in the
control of mitosis. G1 to S transition is regulated by Cyclin D
since abnormalities involving cyclin D1 deregulate control of
the G1-S transition contributing to tumor development (50,51).

Notch proteins are a family of transmembranes with five
ligands. Notch signaling is activated in human breast cancer
with the accumulation of Notch1 intracellular domain in tissue
(52). It has been shown that Notch1 activates Akt and survivin
(53,54), and has also been involved in chemoresistance.
Increased Notch ligands have been shown to be correlated
with poor overall survival in breast cancer patients (55).

Thus, the purpose of this study was to evaluate the effect
of curcumin on EMT when a comparison was done between a
triple-positive and a triple-negative breast cancer cell lines for
ER, PgR and Erb-B2 in relation to this process. Curcumin effect
was evaluated with triple-negative cell line the immortalized
breast epithelial cell line MCF-10F, Tumor2, a triple-positive
cell line derived from Alpha5 injected into the nude mice, and
MDA-MB231, a triple-negative for the same markers.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Product length (bp)a</th>
<th>Primer sequenceb</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td>93</td>
<td>F: AGTGGGCAAGATGGTGTGA R: TAGTGGAGTCCCAGGCCTTA</td>
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<tr>
<td>N-cadherin</td>
<td>67</td>
<td>F: TCG ATG TGT TGC ACC ACG G R: GAC GTG TCC CCA AGA C</td>
</tr>
<tr>
<td>β-catenin</td>
<td>94</td>
<td>F: GCCAGGTGCTGAGGTGCTA R: TCTGTCAGGTGAAGTCCTAAG</td>
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<tr>
<td>Slug</td>
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<td>F: GACCCTGGTTGCTTCAAGGA R: TTGTCCAGTGAGGCAGAAAG</td>
</tr>
<tr>
<td>AXL</td>
<td>121</td>
<td>F: GTTTGGAAGCTGTGATGGAAGGC R: CGCTTACCTAGGAAATCCT</td>
</tr>
<tr>
<td>Twist1</td>
<td>118</td>
<td>F: TCCCGTGTCCTAGCA R: AGTATCCAGCTCCAGAGTCCTAGAC</td>
</tr>
<tr>
<td>Vimentin</td>
<td>117</td>
<td>F: TGTCACAAATCGATGGATGTTTTC R: TGGTACCATTTCCTGCTCCTG</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>105</td>
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<td>141</td>
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<td>ZEB2</td>
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</tr>
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<td>84</td>
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<td>163</td>
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<tr>
<td>Cyclin D1</td>
<td>60</td>
<td>F: GTGGCCTCTAAGATGAGGA R: GGTGTAGATGCAAGGCCTT</td>
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<tr>
<td>Notch1</td>
<td>140</td>
<td>F: GAGGCGTGGCAAGACTATGC R: CTGTACTCCTACGGTGA</td>
</tr>
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</table>

aLength of cDNA product amplified by gene-specific RT-qPCR analysis. bPCR primer sequences used to generate a product of the indicated
size, listed in 5'→3' orientation. F, forward; R, reverse.
Materials and methods

Cell cultures and treatment. MCF-10F, Tumor2 and MDA-MB-231 human breast cancer cell lines were maintained in Dulbecco’s (DMEM; Gibco, USA) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 0.1 mM non-essential amino acids, 0.2 mM glutamine, 1 mM pyruvate, and 10% heat-inactivated fetal bovine serum and incubated in a 5% CO2 humidified atmosphere at 37˚C.

RNA extraction and cDNA synthesis. Total RNA was isolated by using TRIzol reagent (Invitrogen Corp., Carlsband, CA, USA) according to the manufacturer’s instructions. Total RNA (2 µg) was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsband, CA, USA) and RNase inhibitor (Applied Biosystems) were used in these studies.

RT-qPCR. The cDNA (2 µl) was used in 20 µl qPCR reaction containing SYBR Green PCR Master Mix (Agilent, La Jolla, CA, USA) and 5 µM of each primer for the target genes such as E-cadherin, N-cadherin, β-catenin, Slug, AXL, Twist1, Vimentin, Fibronectin, ZEB1, ZEB2, EZH2, STAT3, Cyclin D1, and Notch1. Table I shows the primers for the gene selected to develop cDNA probes. The reaction was performed in a CFX 96 Real-Time PCR (Bio-Rad Laboratories, Hercules, CA, USA) at 95˚C for 10 min and 40 cycles of a 2-step program of 95˚C for 10 sec and 61˚C for 45 sec when fluorescence-reading occurs. After amplification, PCR product was monitored through dissociation curve analysis (measurement of fluorescence during an increasing heating of 2˚C/min from 61 to 95˚C). At this step, undesirable DNA contamination (if present) could be detected since primers were designed to encompass an intron. Reactions were performed in triplicate and the threshold of the cycle was obtained using Bio-Rad CFX Manager 2.1 software and the average gene expression was normalized with a reference housekeeping gene β-actin. Relative expression was a normalized to the average in cells.

Cell migration and invasion assays. The modified Boyden’s chambers to analyze migration and invasiveness were used as described (56) (Corning, NY, USA). Cells (3x10^5) in 100 µl of medium were used for migration and invasion assays; 8-µm membrane pores were pre-coated with 60 µl Matrigel matrix gel (BD Biosciences, Grand Island, NY, USA) at least one hour before seeding the cells for upper chambers; 600 µl of medium with 10% FBS was placed in the lower chambers as chemo-attractant. Cells treated with 30 µM curcumin were cultured for 48 h in a humidified incubator. Then, the upper chambers were wiped using cotton swabs. The membranes were fixed with 100% methanol at room temperature for 15 min, visualized and quantified using DAPI. Ten fields of each chamber were photographed (x40 magnification). This experiment was independently repeated two times.

Statistical analysis. Results of gene expression of control and treated group were compared with ANOVA followed by Dunnet’s test. The average ± standard error of the mean was used to express numerical data. A p-value <0.05 was considered statistically significant.
Results

Effect of curcumin on growth of breast cancer cells in vitro. We analyzed the effect of curcumin on cell proliferation of MCF-10F, Tumor2 and MDA-MB-231 cell lines after 48 h. Graded concentrations of curcumin (0-70 µM/l) were used to determine cell viability by MTT assay. The growth curves showed that cell proliferation was inhibited in a dose-dependent manner by curcumin with inhibition at doses ≥30 µM/l.

Curcumin inhibits the expression of markers of EMT in breast cancer cells. To confirm the effects of curcumin on EMT, we...
sequentially analyzed gene expression of EMT markers by RT-qPCR analysis. Results indicated that curcumin decreased gene expression of twelve genes, E-Cad, N-Cad, Slug, AXL, Twist1, Vimentin, Fibronectin, ZEB2, EZH2, STAT3, Cyclin D1 and Notch1 in Tumor2 triple-positive for ER, PgR and ErbB2 protein expression (Figs. 1-4). However, curcumin only decreased gene expression of four genes, β-catenin and Slug (Fig. 1), AXL and Vimentin (Fig. 2) in MDA-MB-231 triple-negative cells for the same markers used in clinic. Curcumin increased gene expression of E-Cad and N-Cad (Fig. 1), ZEB1 (Fig. 3), Cyclin D1 (Fig. 4) in MDA-MB-231 triple-negative. MCF-10F decreased gene expression of four genes, E-Cad, Slug, AXL and Twist1 (Fig. 2), and it increased gene expression of eight genes, N-Cad, β-catenin, Vimentin, Fibronectin (Fig. 2), ZEB2, EZH2 and STAT3 (Fig. 3) and Notch1 (Fig. 4).

Effect of curcumin on migration and invasion of breast cancer cells. EMT is associated with metastasis. The motile phenotypes of cells treated with curcumin were evaluated. The number of migratory (Fig. 5) and invasive (Fig. 6) capabilities of cells was significantly reduced in cells after treatment with curcumin. Thus, our study suggested that curcumin could delay cancer progression through its ability to disrupt EMT. It can be concluded that curcumin influenced biochemical changes associated with EMT.

Discussion

Curcumin has been shown to inhibit carcinogen activation and angiogenesis, modulate cell survival and apoptosis, with anti-invasive and anti-metastatic effects on breast, lung, colon and prostate cancer (57). Curcumin reduced cell proliferation of MCF-10F, Tumor2 and MDA-MB-231 cell lines after 48 h when cell viability was measured by MTT assay. Cell proliferation was inhibited in a dose-dependent manner with evident inhibition at dose ≥30 µmol/l). Curcumin has demonstrated antioxidant and anti proliferative properties in breast cancer and seems to induce a G2/M phase arrest (58-60).

EMT has a role in embryonic development and cancer progression, where epithelial cells acquire mesenchymal phenotypes. It reduces cell-to-cell adhesion, loses cell polarity, enhances migratory and invasive capabilities (61); then tumor cells migrate from their site of origin to other tissues activating specific genetic changes (62). During EMT, epithelial cancer cell layers lose polarity, cell-to-cell contact and then undergo a dramatic remodeling of the cytoskeleton. Expression of E-cadherin and γ-catenin are lost and cells acquire mesenchymal markers such as N-cadherin, vimentin and fibronectin enhancing the ability for cell migration and invasion (63). Once tumor cells migrate they re-express E-cadherin and other epithelial markers through a process that is often referred to as mesenchymal-epithelial transition (MET) (64). Therefore, agents that block or reverse these processes offer a therapeutic strategy to avoid cancer progression. Curcumin can re-establish an epithelial phenotype from mesenchymal cells by blocking EMT-related gene expression.

EMT induction is driven by interplay between tumor environment and cancer cells which mechanisms may activate different transcriptional factors such as Twist, Slug and Snail, through multiple cellular signaling pathways (65-69). Analysis of the expression of EMT-related genes indicated that curcumin decreased gene expression of E-cadherin, Slug, AXL and Twist1 in MCF-10F cell line (four genes). While the substance decreased N-cadherin, β-catenin, Slug, AXL, Twist1, Vimentin, Fibronectin, ZEB2, EZH2 and STAT3 in Tumor2 (ten genes) and in MDA-MB-231, triple-negative cell lines such as E-cadherin, N-cadherin, Twist1, AXL and Fibronectin (five genes) gene expression in comparison to its counterpart. It is important to conclude that EMT was triggered by curcumin in MDA-MB-231 cells since it not only decreased the expression of EMT genes but induced morphological changes and inhibited cell motility and invasiveness.

Curcumin did not induce significant difference in Fibronectin gene expression in MCF-10F or Tumor2. However, curcumin decreased gene expression of AXL, and Fibronectin in MDA-MB-231 cell line. During cancer progression carcinoma cells seem to enter into an EMT program, acquiring features of mesenchymal-like cells that influenced invasiveness (62). Evidence has shown that EMT is involved in malignant progression by inducing genes such as Slug, AXL.
Figure 5. Effect of curcumin on migration in MCF-10F and Tumor2 cell lines analyzed by modified Boyden's chambers. (A) Cell lines were stained with DAPI after curcumin treatment. (B) Graph that represents the relative grade of luminescence of cell lines in relation to migration from 100% of the counterpart, respectively. Bars represent the mean ± SEM. **P<0.01 versus counterpart.

Figure 6. Effect of curcumin on invasion in MCF-10F and Tumor2 cell lines analyzed by modified Boyden's chambers. (A) Cell lines were stained with DAPI after curcumin treatment. (B) Graph that represents the relative grade of luminescence of MCF-10F and Tumor2 cell lines in relation to invasion from 100% of the counterpart, respectively. Bars represent the mean ± SEM. **P<0.01 versus counterpart.
and Twist1 since such genes are expressed in numerous tumor types favoring the metastatic process.

E-cadherin plays an important role in epithelial cell adhesion and acts as a metastatic suppressor in epithelial carcinomas since loss of E-cadherin is associated with advanced diseases (70). Vimentin is found in mesenchymal cells and its expression has been observed in the progression of EMT, tumor cells that are highly proliferative and invasive (71). E-cadherin and Vimentin are markers of EMT and directly regulated by Slug (72,73) since many preventive agents effectively inhibit EMT by inhibiting Slug transcription factors. In this study curcumin inhibited Slug expression, affecting E-cadherin and vimentin to retard cancer cell invasion and providing new mechanistic bases for therapeutic use in breast cancer patients.

Cyclin D1 gene plays a critical role in breast carcinogenesis. It seems that the antiproliferative effects of curcumin are due to inhibition of Cyclin D1 expression (74). Thus, decreased expression of Cyclin D1 was observed in Tumor2 and MDA-MB-231. Others reported a decrease in the Cyclin D1 protein expression with curcumin treatment (75). Curcumin inhibited the expression of several EMT markers such as β-catenin and Slug in both Tumor2 and MDA-MB-231. Our data demonstrated the efficacy of curcumin since it reduced Notch1 expression suggesting its antimetastasis function through downregulation of EMT genes and by promoting other genes. It was also found that curcumin inhibited migration and invasion of breast cancer cells. It was reported that curcumin inhibits the migration and invasion of lung (76) and breast (77) cancer cells. These data provide a new perspective on the role of curcumin in the anti-invasive properties of breast cancer cells by its ability to interfere with the EMT process. Most importantly, this study demonstrated and clarified the potential effect of curcumin to inhibit EMT-related gene expression in a triple-positive and a triple-negative cell line.

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References


