Curcumin and epithelial-mesenchymal transition in breast cancer cells transformed by low doses of radiation and estrogen

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Abstract. Breast cancer is a major cause of global mortality in women. Curcumin exerts anti-proliferative, anti-migratory and apoptotic effects. The aim of this study was to evaluate gene expression involved in epithelial-mesenchymal transition (EMT). An in vitro model was developed with the MCF-10F immortalized breast epithelial cell line exposed to low radiation doses of high LET (linear energy transfer) α-particles (150 keV/µm) and cultured in the presence of 17β-estradiol (estrogen). The following cell lines were used: i) MCF-10F, normal; ii) Alpha5, pre-tumorigenic, and iii) Tumor2 derived from Alpha5 injected into the nude mice. Our previous results have shown that Alpha5 and Tumor2 increased cell proliferation, anchorage independency, invasive capabilities and tumor formation in nude mice in comparison to control. Results indicated that curcumin decreased expression of EMT-related genes in Tumor2 cell line when compared to its counterpart as E-cadherin, N-cadherin, ZEB2, Twist1, Slug, vimentin, STAT-3, fibronectin; and genes p53 and caveolin-1, as well as apoptotic genes caspase-3, caspase-8, and others such as cyclin D1 and NFkB. All these changes induced a decrease in migratory and invasive capabilities of such a cell line. Thus, it seems that curcumin may impinge upon apoptosis and metastatic properties of the malignant cells exerting antitumor activity in breast cancer cells transformed by low doses of α-particles and estrogen in vitro.

Introduction

Breast cancer is the most frequent malignancy diagnosed in women in the western world. Oxidative stress is one of the important pathogenic factors of cancer development (1). Both in vitro and in vivo studies have shown that curcumin and its analogs target critical genes associated with angiogenesis, apoptosis, cell cycle, and metastasis (1). Among the antioxidants, curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; (diferuloylmethane) is a dietary natural yellow pigment derived from the rhizome of the turmeric herb known as Curcuma longa (Zingiberaceae) originating from India and South Asia. It is a polyphenol derived from several curcumin species, commonly known as turmeric which has been shown to inhibit carcinogen activation, modulate cell survival and apoptosis, with anti-invasive and anti-metastatic effects on breast, lung, colon and prostate cancer (1).

Epidemiological and experimental data demonstrated the efficacy of curcumin in chemoprevention and reversing chemoresistance of tumors of certain cancers. It possesses anti-proliferative and anti-carcinogenic potential (2,3). Curcumin interferes with multiple genes that promote carcinogenesis. It is a pleiotropic molecule with anti-proliferative, antioxidant and chemopreventive properties. Alteration of gene expressions involved in key signaling pathways render this model an important tool for monitoring effects of natural dietary compounds in breast carcinogenesis (2).

One important concept of epithelial-mesenchymal transition (EMT), which has been recognized for several decades as a fundamental process of embryogenesis, is currently considered a pivotal event in the initial step of the metastatic cascade that allows cells to acquire migratory, invasive and stem-like properties (4).

During EMT of cancer cells in situ, epithelial cell layers lose polarity together with cell-to-cell contacts and then undergo a dramatic remodeling of the cytoskeleton. Changes in gene expression that promote cell-to-cell contact, such as E-cadherin and γ-catenin, may be lost and the cells may acquire mesenchymal characteristics such as changes at N-cadherin, vimentin, fibronectin levels resulting in an enhanced ability for cell migration and invasion (5). Cadherins are glycoproteins responsible for homotypic and calcium-dependent cell-cell adhesion (6). E-cadherin is a membrane glycoprotein that plays an essential role in maintaining the integrity of cell-to-cell adhesion, which is significantly associated with tumor invasiveness and metastatic dissemination (7). Dysfunction or loss of E-cadherin is associated with an increased tendency for tumor metastasis (8). In addition, degradation of extracellular matrix and basement membranes by the tumor cells is a critical step and occurs at several stages.
of the metastatic cascade (9,10). N-cadherin (cadherin-2 or CDH-2) is a mesenchymal cadherin overexpressed in many cancers and associated with cancer cell migration and FGF receptor signaling in breast cancer metastasis (11). β-catenin is a 92 kDa protein that plays a role in both cell adhesion and intracellular signaling (12) as the classical cadherins which play fundamental roles in the development of multicellular organisms (13).

Smad interacting protein 1 (SIP1; also known as ZEB2, for zinc finger E-box-binding protein 2 and ZFHX1B) belongs to the DEFT-1 or ZEB protein family (14) and are transcriptional factors characterized by containing a homeo domain flanked by two separated zinc finger clusters (15). It is expressed in various types of human tumors, such as breast cancer, gastric cancer and ovarian cancer (15). ZEB2 is a potent repressor of E-cadherin through its direct binding to the E-cadherin promoter and a key player in tumor cell invasion and metastasis (16,17).

Twist1 induces EMT and extracellular matrix degradation in tumor progression (18-20). Slug is a member of the SNAI family of C2H2-zinc finger family of transcriptional repressors (21-23). It is involved in the EMT during development (22), acts as an inhibitor of apoptosis (24), and causes tubulogenesis during breast and kidney developments (21,22). AXL is a member of the TAM (Tyro3, Axl, Mer) family of receptor tyrosine kinases (RTK), originally identified as a transforming gene in cells of chronic myogenous leukemia patients. It is activated through several mechanisms, including binding of its ligand, growth arrest specific 6 (Gas6), and extracellular domain-mediated dimerization or crosstalk with HER2/neu (25-27). Vimentin belongs to intermediate filaments that with microfilaments and microtubules are the major cytoskeletal elements of the cell (28,29).

STAT-3, a versatile member of the family known as signal transducers and activators of transcription (STAT) mediates the axial responses of cytokines. STAT-3 is involved in normal cellular responses, as well as oncogenesis (30). Fibronectin is a component of the extracellular matrix and exerts multiple effects in vitro and in vivo including stimulation of cell proliferation, migration, differentiation and survival (31-34). It affects cell behavior through activation of various cell surface receptors most notably integrins (35). Fibronectin is required for the development of fibrillar structures (36) and for the storage and activation of various growth factors (37).

The p53 gene is known as the guardian of the genome (38). A major biological function of p53 is to respond to stress signals and activate the transcription of downstream target genes involved in important cellular mechanisms like cell cycle control, DNA repair and apoptosis. Caveolin is a specialized lipid raft on the plasma membrane found in mesenchymal cells. The caveolin family consists of three members, caveolin-1 (Cav-1), caveolin-2 and caveolin-3. Cav-1 is widely expressed in various tissues and plays an essential role in a number of human diseases including cancer (39).

Apoptosis is genetic death program (40). The balance between pro- and anti-apoptotic signals maintain biological homeostasis and its imbalance is related to malignant transformation (40). The central component of the apoptotic machinery is the family of caspases (41,42). The members of the caspase family can be divided into two groups: i) upstream initiator caspases, such as caspase-8 and ii) downstream effector caspases, such as caspase-3 (43,44).

Cyclins, cyclin-dependent kinases (cdks) have been identified as regulatory subunits (cyclins) and catalytic subunits (cdks) of cell cycle-regulated kinases involved in the control of mitosis. It is involved in regulating the G1 to S transition (45,46). Abnormalities involving cyclin D1 may deregulate control of the G1-S transition and, therefore, contribute to genomic instability and tumor development (45,46). Cyclin D1 along with its binding partners CDK 4/6 partially mediate G1 to S-phase transition of the cell cycle through phosphorylation and inactivation of retinoblastoma (Rb) protein with subsequent release of E2F transcription factors (47-49). It is believed that the oncogenic properties of cyclin D1 depend to a large extent on its ability to activate cyclin-dependent kinases 4 or 6 (Cdk4/6) (50,51).

Nuclear Factor κB (NFκB) is a complex of transcription factors that function in the development of acquired resistance to several other targeted agents (52). NFκB signaling has two major pathways, one is the canonical pathway that mainly modulates cell proliferation, inflammation or anti-apoptosis, and the other one is the non-canonical pathway that mainly controls lymphogenesis (53).

To gain insights into the effects of curcumin on breast carcinogenesis an established in vitro experimental breast cancer model (Alpha model) (54) was used. It was developed with the immortalized human breast epithelial cell line, MCF-10F (55) that was exposed to low doses of high LET (linear energy transfer) α-particles (150 keV/μm) of radiation, values comparable to α-particles emitted by radon progeny, and subsequently cultured in presence or absence of 17β-estradiol (estrogen). MCF-10F was exposed to low doses of high LET α-particles (150 keV/microm) and subsequently cultured in the presence or absence of 17beta-estradiol (E) for periods of up to 10 months post-irradiation. MCF-10F cells irradiated with either a single 60 Gy dose or 60/60 Gy doses of α-particles showed gradual phenotypic changes including altered morphology, increase in cell proliferation relative to the control, anchorage-independent growth and invasive capability before becoming tumorigenic in nude mice. In addition, these cells present all the characteristics of breast epithelium in their ultra structural features (56-58). However, only those MCF-10F cells treated with a 60 Gy dose of α-particles followed by estrogen treatment and exposed to a second dose of 60 Gy dose of α-particles followed by estrogen (60 Gy + E/60 Gy + E), named Alpha5 became tumorigenic in both SCID and nude mice (54). Tumor2 developed from Alpha5 injected in the athymic mice. The aim of this work was to evaluate the effect of curcumin on epithelial mesenchymal transition and other related genes in breast cancer cells transformed by low doses of α-particles and estrogen.

Materials and methods

Breast cancer cell lines. The spontaneously immortalized breast epithelial cell line, MCF-10F cells was grown in DMEM/F-12 (1:1) medium supplemented with antibiotics 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B (all from Life Technologies, Grand Island, NY, USA) and 10 μg/ml and 5% equine serum (Biofluids, Rockville,
Table I. Primers for genes selected to develop cDNA probes.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Product length (bp)a</th>
<th>Primer sequenceb</th>
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| E-cadherin | 93 | F: AGTGGGCACAGATGGTGTGA  
R: TAGTGGGAGTCGCCAGGCTA |
| N-cadherin | 67 | F: TCGATTGTGGTACCGGACG  
R: GAGGTTGTCGCACTTGAGC |
| β-catenin | 94 | F: GCAGACGTGGTCGAAGTGTCA  
R: TCTGGTCAGTGTAGGTCCTAC |
| ZEB2 | 128 | F: CAAGAGGGCAGACAAGGC  
R: GGTGGGCAATACCGTCATCC |
| Twist1 | 118 | F: TCCGCGTCCACTAGCA  
R: AGTATCATCTCAGCTCCAGAC |
| Slug | 72 | F: GACCGCTGTCTTCAAGAGA  
R: TGTGCAGTAGGCAAGAAA |
| AXL | 121 | F: GTTTGGAAGCTGGGATGTAAGGA  
R: CGGTTCAATCTCGAAGAAC |
| Vimentin | 117 | F: GTGTAACCAATCTGTTGATGTTC  
R: TTGGTACCTTCCTGCGCTC |
| STAT-3 | 163 | F: GGTTGGACATGATGCACACTAT  
R: TGTTGCAGTGAGGGCAAGAA |
| Fibronectin | 105 | F: GAGGAGACGTGGTGTTTAAAC  
R: AGCCTCAAAGGTCTCACCCA |
| mp53 | 93 | F: CCTGACGATTTTTCGCA  
R: TAGATGGGTGTTACAGTCAGG |
| Caveolin-1 | 79 | F: AACGAATGCAGTTGCTCAAGAT TTG  
R: TCCAAAAGCCGTCAAAACCTTG |
| Caspase-3 | 192 | F: CAGGAACGTGGTGACATGGTTAGG  
R: GCTTGTCCGCACTTTCTG |
| Caspase-8 | 128 | F: CATCCAGTCTTCTGAGAAGGA  
R: GCTTGTGATGCAAGCTTCT |
| Cyclin D1 | 60 | F: GTGGCCCTTAAGATGGAAGGA  
R: GGTGTAGATGCAAGCTTCT |
| NFkB | 114 | F: ATCTGGCGAGTGACCGAAGACT  
R: CCAGGCTGGTCGCGGAAA |

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' to 3' orientation; F, forward; R, reverse.

RNA extraction and cDNA synthesis. RNA from cells were obtained using TRizol reagent (Invitrogen Corp., Carlsbad, CA, USA) following to the manufacturer's protocol. RNA (2 µg) were reverse-transcribed to cDNA with kit high capacity cDNA Reverse Transcription (Applied Biosystems, Carlsbad, CA, USA).

RT-qPCR. Synthesized cDNA (2 µl) was mixed in 20 µl qPCR reaction containing SYBR Green PCR Master Mix (Agilent, La Jolla, CA, USA) and 5 µM of primers for the target genes such as E-cadherin, N-cadherin, β-catenin, ZEB2, Twist, Slug, Axl, vimentin, STAT-3, fibronectin, p53, caveolin-1, caspase-3, caspase-8, cyclin D1 and NFkB. Table I shows the primers to develop cDNA probes. CFX 96 Touch Real-time PCR Detection Systems (Bio-Rad Laboratories, Hercules, CA, USA) to perform the reaction with the following conditions: 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. Reactions were performed in triplicate. Threshold cycle (Ct) was obtained using Bio-Rad CFX Manager 2.1 software. Gene expression was normalized using β-actin. Relative expression was always normalized to the average breast cells and its counterparts.

Cell migration and invasion assays. Migration and invasiveness were performed using modified Boyden's chambers (Corning, Inc., Corning, NY, USA) constructed with multiwell cell culture plates and cell culture inserts. The upper chambers of Transwells with 8-µm membrane pores were pre-coated with 60 µl Matrigel matrix gel (BD Biosciences) at least 1 h before seeding of the tested cells (54). A total of 3x105 in 100 µl of medium without fetal bovine serum was added into the upper chambers and 600 µl of medium with 10% FBS was placed to lower chambers as chemoattractant. Cells were cultured for 48 h following treatment. Curcumin (30 µM) was added to the cell culture. Normal culture medium was added at the bottom chamber to induce the cancer cell lines. Cells which were pretreated were seeded in the top chamber. The matrigel invasion chamber was incubated for 16 h in a humidified tissue culture incubator. Then, the upper chambers were removed from lower chambers and then wiped using cotton swabs. The invaded and migrated cells were fixed using 100% methanol at room temperature for 15 min, visualized and quantified using DAPI. Three fields of each chamber were photographed (x40 magnification). This experiment was independently repeated at least twice.

Statistical analysis. Numerical data were expressed as the average ± standard error of the mean (SEM). Comparison between treated groups and controls was carried out by ANOVA and Dunnet's test. A P<0.05 was considered to be significant.

Results

Curcumin inhibited the expression of markers of EMT such as E-cadherin, N-cadherin, β-catenin and ZEB2 in breast cancer cells (Fig. 1A-D). Results in Fig. 1A-D show a decrease in MD, USA), 0.5 µg/ml hydrocortisone (Sigma, St. Louis, MO, USA) and 0.02 µg/ml epidermal growth factor (Collaborative Research, Bedford, MA, USA). An in vitro experimental breast cancer model developed by exposure of the immortalized human breast epithelial cell line was used. MCF-10F was exposed to low doses of high LET (linear energy transfer) α-particles radiation (150 keV/µm) and subsequent growth in the presence or absence of 17β-estradiol at 10−8 M (E or Estrogen) (Sigma-Aldrich, St. Louis, MO, USA). The following cell line consisted of human breast epithelial cells in different stages of transformation: i) a control cell line, MCF-10F; ii) Alpha5 and iii) Tumor2 (54).
E-cadherin (Fig. 1A), N-cadherin (Fig. 1B), β-catenin (Fig. 1C), and ZEB2 (Fig. 1D) gene expression in MCF-10F and Tumor2 (P<0.01) in comparison with its counterparts. However, there was no difference in Alpha5 cell line. Results in Fig. 2A-D show a decrease in other EMT-related genes Twist (Fig. 2A), Slug (Fig. 2B), Axl (Fig. 2C), and vimentin (Fig. 2D) gene expression in MCF-10F and Tumor2. However, there was no difference in Alpha5 cell line.
Curcumin induced a decrease in gene expression as shown in Figs. 3 and 4A-D for STAT-3 (Fig. 3A), fibronectin (Fig. 3B), mp53 (Fig. 3C), and Cav1 (Fig. 3D), caspase-8 (Fig. 4A), caspase-3 (Fig. 4B), cyclin D1 (Fig. 4C) and NFkB (Fig. 4D) in the malignant and tumorigenic cell line Tumor2 (P<0.01) in comparison with its counterpart. However, there was no difference in Alpha5 cell line in comparison with its counterpart. Since EMT is associated with cellular progression we studied...
the effect on migration and invasion of breast cancer cells. Curcumin decreased the number of migratory and invasive cells significantly (P<0.05) compared to the untreated cells as can be observed in Fig. 5.

**Discussion**

Accumulating evidences suggest that curcumin has a diverse range of molecular targets, supporting the concept that it acts upon numerous biochemical and molecular cascades. Despite our increasing knowledge on this interesting substance there still remain many unknown effects that deserve intense investigation. The multi-targeting of curcumin comes from its structure, chemistry and influence on multiple signaling molecules as well as its ability to bind directly to carrier proteins that improves its solubility and bioavailability. It binds to DNA and RNA. Both in vitro and in vivo studies have shown that curcumin and its analogs target critical genes associated with angiogenesis, apoptosis, cell cycle, and metastasis.

The MCF-10F is unique in the sense that it retains all the characteristics of normal breast epithelium in vitro including dome formation in confluent cultures, three-dimensional growth in collagen gel (55), dependence upon hormones and growth factors for growth in vitro, lack of anchorage-independence or invasive capabilities and non-tumorigenic in the nude or SCID mice (54). It was previously shown (59,60) that among all the various transformed human breast cell lines only Alpha5 cell line and Tumor2 increased cell proliferation, adhesion, presented anchorage-independency, invasive capabilities and tumor formation in nude mice. These cell lines were also positive for estrogen receptor, progesterone receptor and HER, c-Ha-ras and Rho-A gene and protein expression.

EMT is associated with enhanced cellular progression. Curcumin inhibited EMT gene expression in breast cancer cells as E-cadherin, N-cadherin, β-catenin and ZEB2 in Tumor2 in comparison with its counterparts. Curcumin also inhibited Twist1, Slug, Axl and vimentin gene expression in the same cell line. It is known that Twist1 promotes stationary epithelial cells to lose cell-cell junctions and gain migratory and invasive capacities (61); Slug acts as an inhibitor of apoptosis (24) and Axl is overexpressed in a wide variety of human cancers with significant correlation with tumor stage in breast cancer patients playing an important role in cancer progression and metastases (62-64). Curcumin decreased STAT-3 expression.
and fibronectin gene expression in Tumor2 in comparison to its counterpart. It is known that STAT-3 mediates the axial responses of cytokines involved in normal cellular responses and oncogenesis (30).

The antioxidant inhibited Cav-1 gene expression of malignant and tumorigenic cell line Tumor2. It has been reported that inhibition of the tumor promoter Cav-1 expression in Hca-F cells prevents EMT formation by increasing stabilization of Cav-1 with β-catenin (65,66). Curcumin induced tumor cell apoptosis since it decreased caspase-3, caspase-8 and cyclin D1 expression in Tumor2 in comparison to its control. Abnormalities involving cyclin D1 may alter G1-S transition and contribute to genomic instability and tumor development (45,46). The ability of curcumin to induce apoptosis in tumor cells and/or potentiate apoptosis induction by classical chemotherapeutic drugs, support its potential in anticancer therapies (67,68). This substance also inhibited the ability of p53 to activate both the extrinsic and intrinsic apoptotic pathways (71). It has been reported that p53-mediated apoptosis may be associated with the activation of caspase-3 and caspase-8 is suggested by the ability of p53 to activate both the extrinsic and intrinsic apoptotic pathways (71). It has been reported that p53 activates effector caspases by possibly inducing the release of mitochondrial cytochrome-c, including caspase-3, caspase-8 and through the apoptotic effector machinery engaged by p53 (72,73). It has been reported that p53 enhances cancer cell apoptosis and prevents cell replication by stopping the cell cycle at G1 or interphase (74).

Curcumin induced a reduction of tumor cell invasion and metastasis along with apoptosis. The motile phenotypes of cells treated with curcumin were evaluated by migration and invasion assay. After treatment with curcumin the number of migratory and invasive cells decreased significantly compared to the untreated cells. Curcumin has been reported to inhibit cell proliferation and promote accumulation of cells in the G2/M phase of the cell cycle (75).

Thus, the mechanism of apoptosis induced by curcumin seems to be through reduction of tumor cell invasion and metastasis by NFκB. The authors (76) showed that NFκB, a transcription factor in the cell was altered by curcumin. Curcumin plays an important role in the inhibition of EMT in breast cancer cells through the downregulation of NFκB-Snail activity (77). These data provide a new perspective of the anti-invasive mechanism of curcumin, indicating that the effect is partly due to its ability to intervene in the EMT process (77). The inhibition of human breast cancer cell growth by curcumin is mediated via certain signaling cascades including the modulation of the NFκB signaling pathway.

Several studies in vitro and first clinical investigations confirm the antitumor effects of curcumin, either as an isolated chemoprevention substance or in combination with chemotherapeutic agents as supportive measure reducing pharmaceutical resistance of tumor cells to certain chemotherapeutics. The ability of curcumin to induce apoptosis in tumor cells by a classical chemotherapeutic drug, or an antioxidant such as curcumin supports its potential in anticancer therapies. Despite our increasing knowledge on this interesting substance there still remain many unknown effects that deserve intense investigation. These studies reveal the inhibitory effect of curcumin with emphasis on multi-targeted biological and molecular effects in a breast cancer model. Thus, it seems that curcumin may impinge upon several processes including apoptosis and metastatic properties of the malignant cells exerting antitumor activity in breast cancer cells transformed by low doses of α-particles and estrogen in vitro.

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