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The potentiation of curcumin on insulin-like growth factor-1 action in MCF-7 human breast carcinoma cells

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Abstract

Curcumin has anticarcinogenic and chemopreventive properties in a variety of experimental cancer models. Our in vitro studies have shown that curcumin inhibits cell growth and induces apoptosis in MCF-7, a human breast carcinoma cell line. The insulin-like growth factor-1 (IGF-1) system, including IGFs (IGF-1 and IGF-2), IGF-1R (IGF-1 receptor) and IGFBPs (IGF binding proteins), has been implicated to play a critical role in the development of breast cancer. The aim of the present study was to investigate whether the growth inhibitory effects of curcumin were related to changes of the IGF-1 system in MCF-7 cells. IGF-1 at 50 μg/l in serum-free medium produced maximum proliferation and minimized apoptosis. However, curcumin exhibited a potent ability to blunt IGF-1-stimulated MCF-7 cell growth and reverse the IGF-1-induced apoptosis resistance. To determine whether curcumin intervenes in IGF-1 or IGFBP-3 secretion, MCF-7 cells were incubated in serum-free medium in the presence of various concentrations of curcumin for indicated time periods. Curcumin decreased the secretion of IGF-1 with a concomitant increase of IGFBP-3 in a dose-dependent manner. Receptor tyrosine kinase assays revealed that IGF-1-stimulated IGF-1R tyrosine kinase activation was also abrogated by curcumin in a dose-dependent manner. Real-time fluorescence quantitative reverse transcriptase-polymerase chain reaction (RFQ-RT-PCR) further revealed that curcumin suppressed IGF-1R gene expression at transcriptional level. In conclusion, the inhibition of cell growth and induction of apoptosis by curcumin in MCF-7 cells might be mediated, at least partially, by its ability to down-regulate the IGF-1 axis.
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Keywords: Curcumin; Insulin-like growth factor-1 (IGF-1); The IGF-1 axis; Human breast carcinoma

Introduction

The insulin-like growth factor-1 (IGF-1) system (also called IGF-1 axis), including IGFs (IGF-1 and IGF-2), IGF-1R (IGF-1 receptor) and IGFBPs (IGF binding proteins), has been shown to play an important role in regulating normal cell growth. Abnormal expression of the IGF-1 system has been implicated in the etiology of cancer. IGF-1 has mitogenic and survival effects on human cancer cells (Arteaga and Osborne, 1989). The cellular effects of IGF-1 are mediated by the type 1 IGF receptor (IGF-1R), which is a membrane associated receptor belonging to subclass I of the receptor tyrosine kinase (RTK) superfamily (Macaulay, 1992). Binding of IGF-1 to IGF-1R activates the RTK, and the latter in turn activates a cascade of downstream signals, which are postulated to stimulate cell proliferation and enhance resistance to apoptosis (Werner and LeRoith, 2000). Many tumor cell types secrete one or more IGF binding proteins (IGFBPs), which modulate IGF-1 effects in metabolic regulation, cell growth, and tumorigenesis (Grimberg and Cohen, 2000). Epidemiological data have shown a link between serum concentrations of IGF-1 and IGFBP-3 with increased risks of breast cancer (Hankinson et al., 1998). Compared with normal mucosa, breast tumor tissues and tumor cell lines have higher IGF-1R expression (Happerfield et al., 1997). Further, reduction

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in IGF-1 signaling appears to prevent tumor progression in a variety of tumors, including breast cancer (Mitsiades et al., 2004). As a result, the IGF-1 network is one of the targets for chemopreventive and pharmacologic interventions in human breast cancers.

Curcumin (diferuloylmethane, $C_{21}H_{20}O_6$), an active polyphenolic ingredient isolated from turmeric (the rhizome of the plant *Curcuma longa* Linn.), is traditionally used as a coloring agent and spice in food. However, curcumin has also been shown to have several pharmacological effects, including anti-inflammatory, anti-oxidant and free-radical-scavenging properties. In addition, curcumin has been reported to have important antimutagenic and anticarcinogenic activities (Kuttan et al., 1985), which stem from its ability to suppress proliferation and induce apoptosis in various tumors (Choudhuri et al., 2002). Despite the extensive analysis of anti-tumor activities of curcumin, its ability to modulate growth factor-induced human cancer cell growth remains to be fully elucidated. Recent in vitro studies have shown that treatment of cancer cells with curcumin can inhibit the intrinsic epithelial growth factor receptor (EGFR) tyrosine kinase activity and the ligand-induced activation of EGFR (Dorai et al., 2000). These findings are consistent with previous studies that curcumin could down-regulate epithelial growth factor (EGF) induced cell proliferation rate in a human lung cancer cell line (Korutla and Kumar, 1994). Moreover, platelet-derived growth factor receptor (PDGFR), also belonging to one of subclasses of the RTK superfamily, and its downstream signaling transduction have been shown to be inhibited by curcumin as well (Park et al., 2005; Yang et al., 2006). Thus, other associated growth factors such as IGF-1, and IGF-1 signaling pathway may be another critical molecular target of curcumin with respect to growth inhibition and apoptosis induction. However, direct evidence of this hypothesis has yet to be reported.

In this study, we examined the effects of curcumin on cell growth and apoptosis in MCF-7, a cell line derived from human breast carcinoma. We further investigated the involved underlying mechanism with special emphasis on its role in inhibiting IGF-1 axis activity. A possible association of curcumin's anticancer effects with suppression of IGF-1 actions was established.

Materials and methods

Materials

Curcumin, Hoechst 33342, propidium iodide (PI), and MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) were obtained from Sigma Chemical (St. Louis, MO, USA). Annexin V antibody was purchased from BD Pharmingen (Minneapolis, MN, USA). Fetal bovine serum (FBS), RPMI1640, trypsin–EDTA and penicillin–streptomycin were obtained from Gibco-BRL (Gaithersburg, MD, USA). Cell culture plastics were purchased from Corning (Corning, NY, USA). Human IGF-1 was purchased from Peprotech (London, UK). Specific radioimmunoassay kits for quantitative determination of IGF-1 and IGFBP-3 were purchased from ALPCO Diagnostics (Windham, NH, USA). Non-radioactive tyrosine kinase activity assay kit was purchased from Chemicon (Temecula, CA). Trizol reagent was from Life Technologies (Gaithersburg, MD, USA). Avian myeloblastosis virus reverse transcriptase was from Roche Diagnostic Corp (Switzerland). ABI Prism 7700 Sequence Detection System and Taqman Universal PCR Master Mix were from PE Applied Biosystems (Foster City, CA, USA). The Primer Express program (PE Applied Biosystems, CA, USA) was used to design the primers and probes.

Cells and cell culture

Human breast carcinoma cells, MCF-7, were cultured in RPMI1640 medium supplemented with 0.22% sodium bicarbonate, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated at 37 °C in 5% CO_2. Curcumin was dissolved in dimethylsulfoxide (DMSO) at a concentration of 5 mM and was diluted to the required concentration with RPMI1640 medium immediately before use. Cells grown in medium containing an equivalent final volume of DMSO (final concentration <0.01%, V:V) served as control.

Determination of cell growth

To begin with, the effects of curcumin and exogenous IGF-1 on MCF-7 cell growth were determined. In brief, cells were seeded in 96-well plates ($5–8\times10^3$ per well) using RPMI1640 medium with 10% FBS for attachment. Cells were serum starved in serum-free medium (SFM) overnight. After serum starvation, cells were treated with various concentrations of curcumin for 24–48 h. To determine the abrogating effects of curcumin on IGF-1 action, cells were incubated in SFM in the absence or presence of appropriate concentrations of IGF-1 with or without curcumin for indicated time periods. At the end of treatment, the medium was aspirated from the wells and 100 μl fresh serum-free medium containing MTT (0.5 mg/ml) reagent was added to each well. Cells were incubated for 4 h at 37 °C and lysed by addition of 100 μl DMSO, and the optical density (OD) at 595 and 655 nm was then measured on a microplate reader. The OD values of DMSO read at 595 and 655 nm served as blank control. In parallel, cells were seeded in 24-well plates (5×10^4 per well) and direct cell counting employing trypan blue exclusion analysis was used to confirm significant effects. Each treatment was done in triplicate and repeated three times.

Cell cycle analysis

Cells were plated in 6-well plates ($1.5–2\times10^5$ per well) using complete medium overnight for attachment. After serum starvation, cells were treated with curcumin in the absence or presence of indicated concentrations of IGF-1 as described above. At the end of treatment, adherent cells were released by trypsinization, combined with nonadherent cells, and recovered by centrifugation at 500 g for 5 min at 4 °C and washed with PBS. Samples for DNA content analysis were fixed with 70% ethanol over 24 h. Cells were then washed with PBS and stained

with propidium iodide (PI) (50 μg/ml) and analyzed by flow cytometry.

Annexin V and PI analysis

MCF-7 cells were treated with different concentrations of curcumin in the presence or absence of IGF-1 as described above. After 6 h, cells were harvested and the percentage showing apoptosis was measured by flow cytometry after staining with fluorescein isothiocyanate (FITC)-conjugated Annexin V (5:100, V:V) and PI (5 μg/ml) as described by the manufacturer. We scored viable cells as those negative for Annexin V and PI. Percentage of apoptosis was calculated from the increase of the number of Annexin V-positive cells between the treated and control samples.

Morphologic observations

Samples for morphologic examination were stained with Hoechst 33342 (10 μg/ml) and PI (2.5 μg/ml), and examined by fluorescence microscopy for apoptotic morphologic features, such as chromatin condensation and nuclear fragmentation. Hoechst 33342 binds tightly to cellular DNA that has been damaged by exogenous stimuli, and PI stains the cellular DNA of those cells with a compromised cell membrane. This allows viable cells (Hoechst 33342+/ PI+) to be distinguished from apoptotic cells (Hoechst 33342++/ PI+) and necrotic cells (Hoechst 33342+/ PI++).

Radioimmunoassay for IGF-1 and IGFBP-3 peptides in the culture medium supernatant

Concentrations of IGF-1 and IGFBP-3 in the supernatant of culture medium were measured by radioimmunoassay (RIA) using specific IGFBP-blocked IGF-1 RIA kit and IGFBP-3 RIA kit, respectively. Briefly, MCF-7 cells were exposed to different concentrations of curcumin for 3–48 h in serum-free medium. After treatment, cell conditioned culture medium was removed and centrifuged at 500 g for 10 min at 4 °C, and stored at −70 °C for IGF-1 and IGFBP-3 secretion assay. The RIA experiments were performed according to the instructions provided by the manufacturer. The detection limit of the assay of IGF-1 was 0.02 ng/ml and that of IGFBP-3 was 0.06 ng/ml. All conditions were tested in triplicate, and the means presented represent three independent experiments.

IGF-1-induced IGF-1R receptor tyrosine kinase activity assays

The tyrosine kinase activity of IGF-1R induced by its ligand, IGF-1, was analyzed using a non-radioactive tyrosine kinase activity assay kit. After serum starvation, cells were pretreated with different concentrations of curcumin for 6 h before they were stimulated with IGF-1. After stimulation with IGF-1 (50 μg/l) for 30 min, cells were lysed with detergent lysis buffer and cell lysates were immunoprecipitated with the protein tyrosine kinase (PTK) antibody. The assay mixture was prepared and the kinase reaction was performed according to the protocol provided by the manufacturer. Absorbance of each microwell was read on a standard microplate reader using 450 nm as the primary wave length. Relative optical density (OD) values obtained were compared with the phosphopeptide standards to obtain relative activities. Each treatment was done in triplicate and the means presented represent three independent experiments.

Real-time fluorescence quantitative reverse transcriptase-polymerase chain reaction (RFQ-RT-PCR) assays

Total RNAs were isolated using Trizol reagent as described by the manufacturer. One microgram of total RNA was reverse transcribed by avian myeloblastosis virus reverse transcriptase under standard conditions. Duplicate samples of 1 μl of each cDNA were amplified by PCR in the ABI Prism 7700 Sequence Detection System. The Primer Express program was used to design the primers and probes. The sequences of primers and probes used in real-time PCR are as follows: IGF-1R-primer-F: 5′-CTCAGTTAATCGTGAAGTGGAACC-3′; IGF-1R-primer-R: 5′-GCAGTAATTGTGCCGGTAAAGG-3′. GAPDH-primer-F: 5′-GGACCTGACCTGCCGTCTAG-3′; GAPDH-primer-R: 5′-TAGCCCAGGATGCCCTTGAG-3′. IGF-1R-Probe: 5′-CCGTCCTGAGGCTGCCGCTGC-3′; GAPDH-Probe: 5′-CCTCCGACGCCTGCTTCACCACCT-3′. The amplification reaction mixture (25 μl) contained cDNAs, primers, probes, and Taqman Universal PCR Master Mix. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was coamplified as an internal control to normalize for variable amounts of cDNA in each sample. PCR reactions were performed as follows: 95 °C for 10 s; 45 cycles of 95 °C for 5 s and 60 °C for 20 s. Message RNA fold changes in target genes relative to the endogenous GAPDH control were calculated as suggested by Schmittgen and Zakrajsek (2000).

Statistical analysis

Statistical analysis was performed using SPSS 11.5 for Windows. Data were expressed as means ± standard deviation (SD) and analyzed by one-way ANOVA with the Student–Neuman–Keuls post-hoc test. Values of $p<0.05$ were considered statistically significant.

Results

Curcumin inhibited MCF-7 cell growth

We examined the effects of curcumin on the viability of MCF-7 cell line using MTT assays. The results showed that curcumin decreased the MCF-7 cells in vitro viability in a dose- and time-dependent manner. Compared with control, cell viability inhibition caused by curcumin at 6.25 μM and above was found to be statistically significant within 24 h. However, curcumin at 3.13 μM could not cause a growth inhibition until cells were treated for 48 h. Cells exposed to the same concentrations of curcumin under each tested condition were further inhibited as the incubation time periods were extended

from 24 h to 48 h (data not shown). In parallel, significant effects were confirmed by direct cell counting employing trypan blue exclusion analysis (data not shown). These results indicated that curcumin was actually inhibiting MCF-7 cell growth.

IGF-1 stimulated MCF-7 cell growth

MCF-7 cells were growth stimulated by exogenous IGF-1 as depicted in Fig. 1. At 48 h, cells grown in SFM achieved only 27.6±4.18% (p<0.05) of cell viability found in complete medium (containing 10% FBS). When IGF-1 (2 μg/l to 50 μg/l) was added, cell viability was enhanced in a dose-dependent manner. After stimulation with IGF-1 at 20, 40 and 50 μg/l, the cell viability was 63.3±6.10%, 81.2±3.57% and 83.2±4.07% of that observed in complete medium (p < 0.05), respectively (Fig. 1A). We further observed that the increasing rate of cell growth was slowed down when high concentrations of IGF-1 (40 μg/l and 50 μg/l) were added, suggesting that IGF-1Rs on the MCF-7 cell surface might have been saturated by IGF-1 at 40 μg/l to 50 μg/l. Together with the data confirmed by direct cell counting (Fig. 1B), our results indicated that IGF-1 was actually one of the important growth factors for MCF-7 cell growth. In addition, based on these results, we selected IGF-1 at 50 μg/l in serum-free conditions for the subsequent experiments.

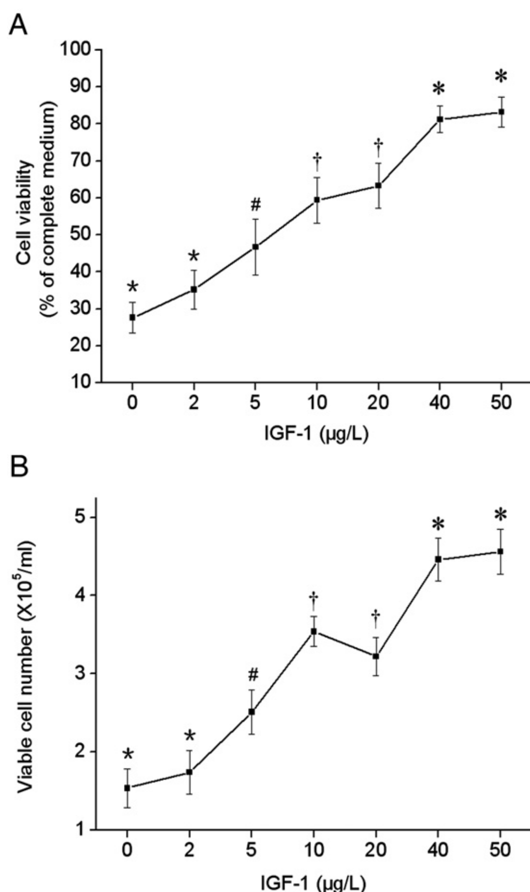

Fig. 1. The effects of exogenous insulin-like growth factor-1 (IGF-1) on MCF-7 cell growth in vitro. Cells were plated and cultured as described in Materials and methods. After serum starvation, the monolayers were incubated in serum-free medium (SFM) in the absence or presence of various concentrations of IGF-1. Cells in complete medium (10% fetal bovine serum, FBS) were standardized as control. (A): Exogenous IGF-1-stimulated MCF-7 cell viability in vitro, assessed by the MTT assay. (B): Exogenous IGF-1-stimulated MCF-7 cell growth in vitro, assessed by direct cell counting. Each graph represents data from triplicate separate experiments. Values were expressed as means±SD. Significance was determined using an ANOVA coupled to the Student–Neuman–Keuls post-hoc test in which p<0.05 was required for a statistically significant difference. The signs (*, #, †, *) denote significant difference from control and other treatment groups at a level of p<0.05.

Curcumin abrogated IGF-1-stimulated MCF-7 cell growth

We further investigated whether curcumin could abrogate the growth-stimulatory effects of IGF-1. MCF-7 cell viability was estimated by using MTT assay after simultaneously incubated with curcumin (10 μM, 20 μM and 40 μM) and IGF-1 (50 μg/l) in SFM for 24–48 h. As shown in Fig. 2A, curcumin treatment at 10 μM for 24 h and 48 h inhibited IGF-1-stimulated viability by 26.0±3.40% and 20.5±5.51% (p<0.05), whereas 20 μM curcumin abrogated IGF-1-stimulated viability by 45.7±7.77% and 45.9±6.22% (p<0.05), respectively. Furthermore, curcumin at 40 μM suppressed the IGF-1-induced growth-stimulatory effects by 63.2±3.74% and 73.7±1.27% at 24 h and 48 h, a decrease as low as that of cells grown in SFM alone at 24 h and 48 h (p>0.05). These abrogating effects were also confirmed by direct cell counting (Fig. 2B). Our results thus documented that curcumin could inhibit the exogenous IGF-1-induced growth-stimulatory effects, suggesting that curcumin might counteract the IGF-1 action.

Cell cycle progression and apoptosis induction by curcumin in MCF-7 cells

Following treatment with various concentrations of curcumin for 24 h, the cell cycle distribution of the combined adherent and detached populations was determined by flow cytometry using PI staining. Compared to control, the number of MCF-7 cells remaining as an adherent monolayer was greatly reduced after treatment with curcumin (10–40 μM) for 24 h. Further, DNA content histogram showed a profound G2/M arrest and a significant increasing of sub-G1 peak, and this increasing of sub-G1 peak was accompanied by a progressive loss of the normal S phase (data not shown). As an indicator of apoptosis, the sub-G1 peak represents a late stage of apoptosis, in which the nucleus has fragmented into small packets containing less than the 2N complement of DNA. However, Annexin V and PI analysis was a tool employed for evaluation of earlier stage of apoptosis. Compared to control, cells treated with curcumin at 10–40 μM showed significantly enhanced Annexin V positivity (data not shown).

Curcumin reversed IGF-1-induced apoptosis resistance in MCF-7 cells

Examination of the sub-G1 peak by flow cytometric analysis provided the first clue that curcumin could counteract the prosurvival effects exerted by IGF-1. As predicted, MCF-7 cells

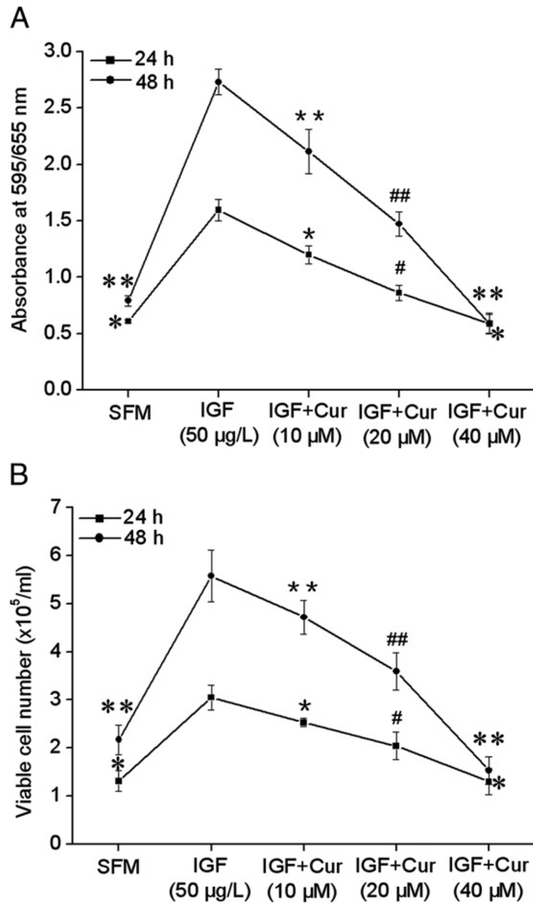

Fig. 2. Curcumin abrogated insulin-like growth factor-1 (IGF-1)-stimulated cell growth in MCF-7 cells. Cells were plated and cultured as described in Materials and methods. After serum starvation, the monolayers were incubated for 24–48 h in the presence of IGF-1 (50 μg/l, IGF) and increasing concentrations of curcumin (0, 10, 20, 40 μM, Cur). The IGF-1-treated samples served as control. (A): Curcumin inhibited IGF-1-stimulated cell viability in vitro as assessed by the MTT assay. (B): Curcumin inhibited IGF-1-stimulated cell growth as assessed by direct cell counting. Each graph represents data from triplicate separate experiments. Values were expressed as means±SD. Significance was determined using an ANOVA coupled to the Student–Neuman–Keuls post-hoc test in which $p<0.05$ was required for a statistically significant difference. The signs (*, #, * and **, ##, **) denote significant difference from control and other treatment groups at 24 h and 48 h at a level of $p<0.05$.

grown in serum-free conditions (compared to IGF-1-treated cells) were arrested in G0/G1 and exhibited a sub-G1 peak of 4.7±0.51%, whereas IGF-1 addition initially increased the proportion of cells in S phase ($p<0.05$). However, simultaneous treatment with curcumin and IGF-1 produced a sub-G1 peak of 12.9±1.80% ($p<0.05$). Annexin V analysis was also employed to evaluate whether curcumin could abrogate IGF-1-induced apoptosis resistance. The results demonstrated that cells grown in SFM showed increased Annexin V positivity, which was significantly prevented by IGF-1 (50 μg/l), whereas inclusion of curcumin potently increased Annexin V staining (data not shown). In parallel, PI and Hoechst 33342 staining and fluorescence microscopy were also used to confirm the presence of nuclear morphological features associated with apoptosis, such as chromatin condensation and nuclear fragmentation (data not shown). Taken together, these results suggested that curcumin could antagonize the prosurvival effects induced by IGF-1 in breast carcinoma cells.

Curcumin intervened in IGF-1 and IGFBP-3 secretion in MCF-7 cells

Since curcumin could inhibit IGF-1-induced cell growth and apoptosis resistance, we hypothesized that curcumin could have ability to modulate the IGF-1 axis. We thus investigated whether curcumin could cause any changes of IGF-1 and

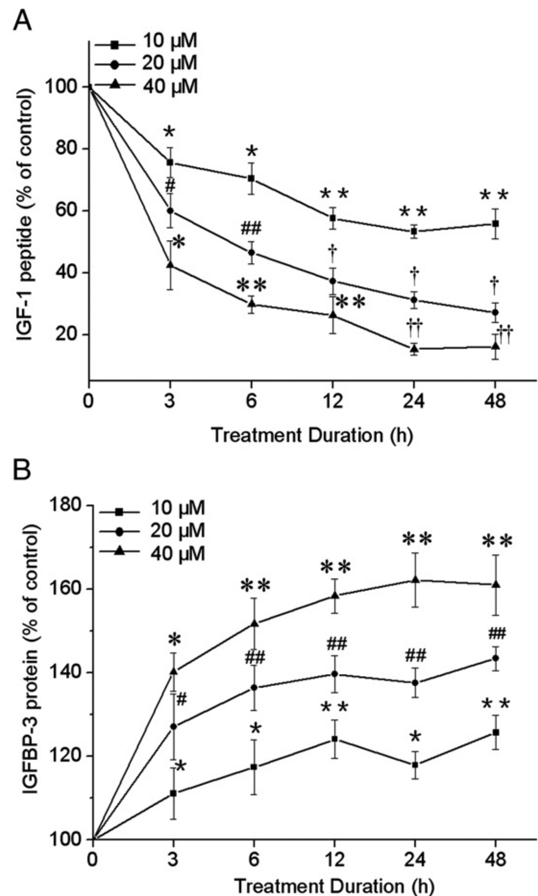

Fig. 3. The effects of curcumin on insulin-like growth factor-1 (IGF-1) and IGF binding protein-3 (IGFBP-3) secretion in MCF-7 cell culture medium. Cells were plated and cultured as described in Materials and methods. After serum starvation, the monolayers were incubated with various concentrations of curcumin in serum-free medium (SFM). At each indicated time point, the conditioned medium was collected for radioimmunoassay. (A): Curcumin inhibited IGF-1 secretion in MCF-7 cell culture medium. The absolute values (on which per cent is based) of IGF-1 peptide secretion at each time point (0, 3, 6, 12, 24, 48 h) were expressed as means±SD (ng/ml). (B): Curcumin stimulated IGFBP-3 secretion in MCF-7 cell culture medium. The absolute values (on which per cent is based) of IGFBP-3 peptide secretion at each time point (0, 3, 6, 12, 24, 48 h) were expressed as means±SD (ng/ml). Each graph represents data from triplicate separate experiments. Values were expressed as a percentage of the peptides secretion by untreated control wells. Significance was determined using an ANOVA coupled to the Student–Neuman–Keuls post-hoc test in which $p<0.05$ was required for a statistically significant difference. All the signs (*, #, *, † and **, ##, **, ††) denote both significant difference from control at each time point and significant difference from different treatment duration with curcumin at the same concentration ($p<0.05$).

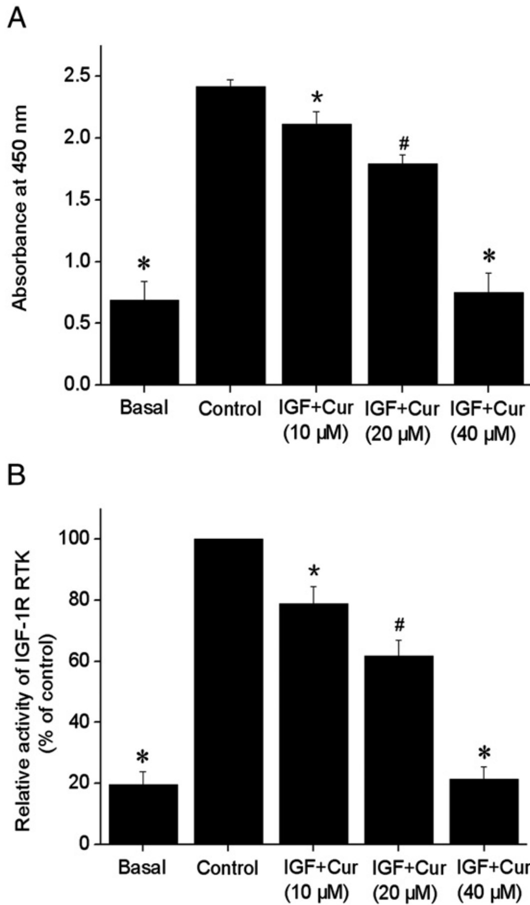

Fig. 4. Curcumin inhibited insulin-like growth factor-1 (IGF-1)-stimulated IGF-1 receptor tyrosine kinase (RTK) activity. MCF-7 cells were incubated for 30 min in the presence of IGF-1 (50 μg/l, IGF) after pretreated for 6 h with increasing concentrations of curcumin (0, 10, 20, 40 μM, Cur). Those untreated cells were expressed as basal level and the IGF-1/SFM-treated samples served as control. (A): Inhibition of IGF-1-stimulated IGF-1R RTK activity by curcumin, as expressed by absorbance at 450 nm. (B): The relative RTK activity of IGF-1R was also inhibited by curcumin, as expressed by percentage of stimulation over control. Each graph represents data from triplicate separate experiments. Values are means ± SD. Significance was determined using an ANOVA coupled to the Student–Neuman–Keuls post-hoc test in which $p<0.05$ was required for a statistically significant difference. The signs (⋆, #, *) denote significant difference from control and other treatment groups at a level of $p<0.05$.

IGFBP-3 concentrations in MCF-7 cells conditioned culture medium. Results of radioimmunoassay revealed that MCF-7 cells secreted both IGF-1 and IGFBP-3. Compared to control, treatment with curcumin (10 μM, 20 μM and 40 μM) significantly decreased the concentrations of IGF-1 in the supernatant of culture medium in a dose-dependent manner ($p<0.05$) (Fig. 3A). A prominent decrease in the level of the IGF-1 protein was observed after curcumin treatment for 3 h ($p<0.05$), and this decrease persisted for at least 48 h. However, levels of IGFBP-3 were significantly increased by curcumin ($p<0.05$) (Fig. 3B). A significant stimulatory effect on IGFBP-3 secretion was detectable when cells were treated with curcumin (10 μM, 20 μM and 40 μM) in SFM for 3 h, and this increase of IGFBP-3 secretion was further stimulated in a dose-dependent manner within at least 48 h ($p<0.05$). These data suggested that curcumin-induced decrease of IGF-1 was associated with a concomitant increase of IGFBP-3 in MCF-7 cell culture supernatant. Moreover, the elevated IGFBP-3 protein can further inhibit the activity of IGF-1 by binding free IGF-1 to down-regulate IGF-1 bioavailability.

Curcumin inhibited IGF-1-activated IGF-1R tyrosine kinase activity

To explore the possible effects of curcumin on IGF-1-stimulated IGF-1R tyrosine kinase activity in vitro, cells were pretreated with curcumin for 6 h before they were stimulated with IGF-1. As shown in Fig. 4, the MCF-7 cell line appeared to contain basal level of IGF-1R tyrosine kinase activity. The

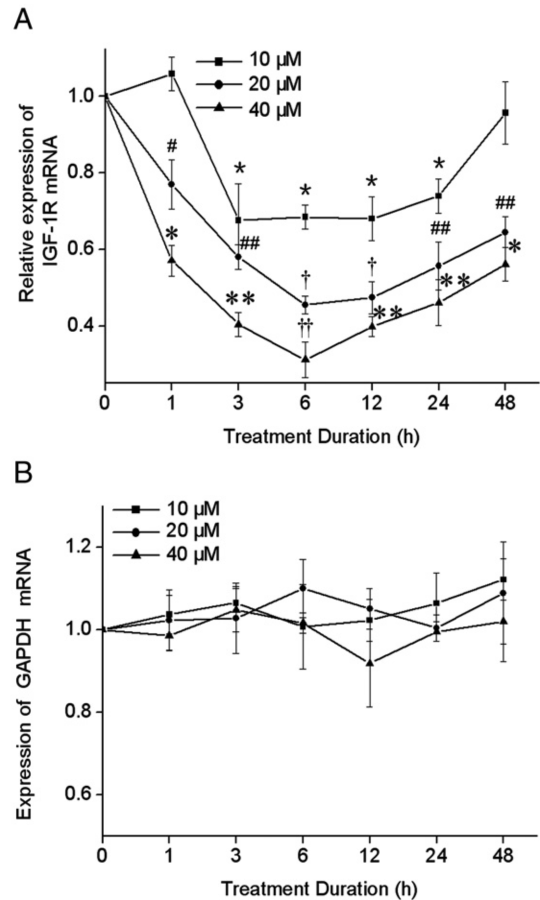

Fig. 5. The effects of curcumin on insulin-like growth factor-1 receptor (IGF-1R) mRNA and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in MCF-7 cells. MCF-7 cells were treated with various concentrations of curcumin for indicated time periods, and the mRNA levels of IGF-1R and GAPDH were assessed by real-time RT-PCR assay. After normalization with the endogenous control gene (GAPDH), the levels of IGF-1R mRNA were determined and expressed as means ± SD. The mRNA levels of IGF-1R and GAPDH in MCF-7 cells without curcumin treatment were standardized as 1, respectively. (A): Curcumin suppressed IGF-1R mRNA in MCF-7 cells. (B): Curcumin had no effects on GAPDH mRNA in MCF-7 cells. Each graph represents data from triplicate separate experiments. Significance was determined using an ANOVA coupled to the Student–Neuman–Keuls post-hoc test in which $p<0.05$ was required for a statistically significant difference. All the signs (⋆, #, *, † and ⋆⋆, ##, **, ††) denote both significant difference from control at each time point and significant difference from different treatment duration with curcumin at the same concentration ($p<0.05$).

lower basal level of the tyrosine kinase activity was significantly enhanced by IGF-1 (50 μg/l) within 30 min, and this increase of RTK activity was inhibited by pretreatment with curcumin at doses as low as 10 μM with a further inhibition at 20 μM, whereas 40 μM curcumin even appeared to decrease the kinase activity to as low as the basal level. Since the activated IGF-1R tyrosine kinase by IGF-1 binding can subsequently activate a cascade of downstream intracellular signals leading to cell proliferation and resistance to apoptosis, these inhibitory effects on the IGF-1R tyrosine kinase activity by curcumin might elucidate one of the mechanisms underlying that curcumin counteracted IGF-1-induced breast cancer cell growth and apoptosis resistance as observed above.

Curcumin down-regulated IGF-1R mRNA expression

Since IGF-1-mediated signaling transduction is ultimately determined by the IGF-1R, we investigated the potential IGF-1R gene expression regulation by curcumin. At the end of indicated treatment, total RNAs was isolated, and IGF-1R mRNA levels were determined by quantitative real-time RT-PCR analysis. As shown in Fig. 5A, compared to control, curcumin at 10 μM caused a significant inhibition of the IGF-1R gene expression at the 3-hour time point ($p<0.05$), and this suppression lasted for 24 h ($p<0.05$) and recovered at 48 h ($p>0.05$). However, treatment with 20 μM curcumin for 1 h was sufficient to inhibit the IGF-1R mRNA by 23.0±6.50% ($p<0.05$), with a further inhibition up to 54.6±2.27% and 52.6±4.20% at 6 h and 12 h ($p<0.05$), then the IGF-1R gene expression slightly increased between 24 h and 48 h ($p<0.05$). Although similar results were obtained with curcumin at 40 μM, significant differences were detectable only at 1 h and 6 h as doses of curcumin increased from 20 μM to 40 μM ($p<0.05$), indicating that curcumin at even higher concentration could not completely inhibit the IGF-1R gene expression. In addition, the inhibition of IGF-1R mRNA by curcumin was not due to an unspecific down-regulation of RNA synthesis, since curcumin treatment did not significantly modify the expression of GAPDH mRNA which served as endogenous control gene ($p>0.05$) (Fig. 5B).

Discussion

We aimed to investigate whether curcumin could modulate IGF-1-mediated mitogenic and antiapoptotic effects in human breast cancer cells and to explore the possible inhibitory effects of curcumin on the IGF-1 axis. We not only demonstrated that curcumin exhibited a potent ability to reverse the IGF-1-induced cell growth and apoptosis resistance, but also further elucidated that these inhibitory effects were in association with suppression of several critical elements involving the IGF-1 axis and even its downstream IGF-1R tyrosine kinase.

Curcumin has cancer chemopreventive properties in a variety of animal models of chemical carcinogenesis, including those resulting in tumors of the mammary gland (Singletary et al., 1996). Curcumin has also been shown to inhibit the growth of human breast cancer cell lines in vitro (Choudhuri et al., 2002). As expected, we documented that curcumin did inhibit MCF-7 cell growth in a dose-and time-dependent manner. These growth inhibitory effects were associated with a G2/M cell cycle blockage and a sub-G1 apoptotic peak induction. Our data are consistent with previous studies that reported curcumin exerted its anticancer effects via proliferation inhibition and apoptosis induction in breast cancer cells (Simon et al., 1998). Interestingly, previous studies reported that the human multidrug-resistant breast cancer cell line was more sensitive to curcumin than the mammary epithelial cell line, even though normal and malignant breast cell lines accumulated a similar amount of curcumin (Ramachandran and You, 1999). This selectivity between normal and malignant tissues indicates that curcumin is a promising chemopreventive agent.

Although there have been extensive studies to investigate the mechanism of curcumin as a chemopreventive agent, its ability to modulate growth factor-induced proliferation in human cancer cells has remained to be fully elucidated. We proposed a possible underlying molecular mechanism that curcumin-induced MCF-7 cell growth inhibition and apoptosis might be via down-regulation of the IGF-1 axis. There is compelling evidence that IGF-1 stimulates proliferation and inhibits apoptosis in a variety of normal and cancer cells. We observed that MCF-7 cells grown under serum-free conditions responded to IGF-1 by increasing cell number and proportion of cells in the S phase. We also observed that exogenous IGF-1 completely reversed the tendency of MCF-7 cells to express markers of apoptosis, such as sub-G1 DNA cell fragments and Annexin V positivity. These data were supported by previous studies showing IGF-1 stimulation of thymidine incorporation and mitogenic effects in human breast cancer cells (Nie et al., 1997). Our results also confirmed that IGF-1 was one of major regulators of breast cancer cell growth and survival (Sachdev and Yee, 2006).

We further demonstrated that curcumin blunted IGF-1-stimulated MCF-7 cell growth and apoptosis inhibition. When MCF-7 cells were simultaneously treated with curcumin and IGF-1, IGF-1 failed to increase the cell number, and curcumin treatment at 40 μM for 48 h even completely inhibited IGF-1-induced cell growth. Furthermore, inclusion of curcumin in the incubation medium could also abrogate the IGF-1-induced apoptosis resistance. Overall, our results documented that curcumin could alter the apoptotic threshold of MCF-7 cells and reverse the mitogenic and survival benefits of IGF-1 in breast cancer cells. A hypothesis is that down-regulation of the IGF-1 axis by curcumin might be a possible mechanism via which curcumin exerted its anticancer effects.

As a polypeptide growth factor, IGF-1 has important effects on cell proliferation, differentiation, and apoptosis. Previous studies have indicated curcumin exerted its anticancer effects not only via the repression of p53 mRNA and p53 proteins (Jee et al., 1998; Choudhuri et al., 2002), but also by down-regulation of the transcription factors AP-1 and nuclear factor kappaB (NF-κB) (Han et al., 2002). Since the IGF-1 gene contains AP-1 like element (Morishima et al., 1994), inhibition of AP-1 activity by curcumin might be involved in the reduction of IGF-1 secretion. Alternatively, curcumin inhibited cycloxygenase-2 (COX-2) expression and reduced the synthesis of prostaglandin E (2)

(PGE_2), one of the major products of COX-2 (Lev-Aris et al., 2006). Prostaglandin E (2) can subsequently stimulate IGF-1 transcription (Bichell et al., 1993). In summary, it is more likely that curcumin inhibits the cell's ability to respond to IGF-1 via changing the secretion of this growth factor in MCF-7 cells. Additional experiments are necessary to determine whether curcumin could affect the production of IGF-1 in the cells. Furthermore, the biological activities of IGF-1 are also modulated by a family of high-affinity IGFBPs, which bind to IGF-1 and thereby inhibit its bioactivity. Results from our study demonstrated that IGFBP-3, the predominant IGFBP, was secreted into the cell culture medium by curcumin. The elevated IGFBP-3 proteins could further inhibit IGF-1 action by controlling its availability for receptor binding, thereby exhibiting inhibitory effects on proliferation and apoptosis. Moreover, IGFBP-3 is subject to limited, and potentially regulated, proteolysis by various proteases, such as metalloproteases-9 (MMP-9) (Manes et al., 1999), which can be inhibited by curcumin through the suppression of ERK1/2 phosphorylation and NF-κB trans-activation in human breast epithelial cells (Lee et al., 2005). Therefore, suppression of metalloproteases (such as MMP-9) expression by curcumin might be associated with the increased IGFBP-3 secretion. In addition, IGFBP-3 can also inhibit cell proliferation and induce apoptosis in an IGF-1-independent way (Rajah et al., 1997), so induction of IGFBP-3 by curcumin could also directly contribute to the growth inhibitory effects of curcumin on breast cancer cells.

Biological actions of IGF-1 are ultimately mediated by IGF-1R, which has been identified as a central player in breast carcinogenesis. Cytoplasmic tyrosine kinase domain of IGF-1R is linked to the downstream signal pathway, and activation of the IGF-1R tyrosine kinase via IGF-1 binding is thought to be the mechanism by which IGF-1R transduces signals that activate cell proliferation (Hernández-Sánchez et al., 1995). Thus, the inhibition of IGF-1R kinase activity in curcumin-treated MCF-7 cells could probably block a pathway ordinarily associated with the cell growth state. In the present study, substantial inhibition of IGF-1-stimulated RTK activity was achieved at a dose of curcumin at 10 μM, while curcumin at 40 μM further suppressed the RTK activity to as low as the basal level. Therefore, inhibition of IGF-1R kinase activity by curcumin might be, at least partially, a consequence of direct kinase inhibition. Alternatively, repression of IGF-1R expression by curcumin might also contribute to the reduction in the receptor kinase activity.

Since studies have shown that the IGF-1R expression is determined, to a large extent, at the transcriptional level (reviewed in Werner and Roberts, 2003), we explored the possible effects of curcumin on the IGF-1R gene expression using real-time RT-PCR. Our results demonstrated that curcumin significantly reduced IGF-1R mRNA, indicating that curcumin could down-regulate the IGF-1R gene expression at transcriptional level. In addition, p53, one of transcription factors involved in the IGF-1R gene transcription (Werner et al., 1996), was also suppressed by curcumin (Choudhuri et al., 2002), suggesting curcumin might reduce IGF-1R transcription via suppression of p53. Moreover, the tumor suppressing activity of p53 on cell cycle arrest and apoptosis are also potentially mediated through suppression of the IGF-1R promoter (reviewed in Werner and Roberts, 2003). Based on these results, a model was proposed to explain, at least partially, the mechanisms by which curcumin inhibited breast cancer cell growth. The model is that curcumin inhibited IGF-1R transcription by suppressing p53 expression and resulted in reduction of MCF-7 cell growth.

In conclusion, our data supported the hypothesis that growth inhibition and apoptosis induction by curcumin in breast cancer cells were associated with down-regulation of the IGF-1 axis. These results provided a novel insight into the roles and mechanisms of curcumin in inhibition of breast cancer cell growth and induction of apoptosis, and potential therapeutic strategies for treatment of breast cancer.

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References

Arteaga, C.L., Osborne, C.K., 1989. Growth inhibition of human breast cancer cells in vitro with an antibody against the type 1 somatomedin receptor. Cancer Research 49 (22), 6237–6241.

Bichell, D.P., Rotwein, P., McCarthy, T.L., 1993. Prostaglandin E2 rapidly stimulates insulin-like growth factor-I gene expression in primary rat osteoblast cultures: evidence for transcriptional control. Endocrinology 133 (3), 1020–1028.

Choudhuri, T., Pal, S., Agwarwal, M.L., Das, T., Sa, G., 2002. Curcumin induces apoptosis in human breast cancer cells through p53-dependent Bax induction. FEBS Letters 512 (1–3), 334–340.

Dorai, T., Gehani, N., Katz, A., 2000. Therapeutic potential of curcumin in human prostate cancer. II. Curcumin inhibits tyrosine kinase activity of epidermal growth factor receptor and depletes the protein. Molecular Urology, 4 (1), 1–6.

Grimberg, A., Cohen, P., 2000. Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. Journal of Cellular Physiology 183 (1), 1–9.

Han, S.S., Keum, Y.S., Seo, H.J., Surh, Y.J., 2002. Curcumin suppresses activation of NF-kappaB and AP-1 induced by phorbol ester in cultured human promyelocytic leukemia cells. Journal of Biochemistry and Molecular Biology 35 (3), 337–342.

Hankinson, S.E., Willett, W.C., Colditz, G.A., Hunter, D.J., Michaud, D.S., Deroo, B., Rosner, B., Speizer, F.E., Pollak, M., 1998. Circulating concentrations of insulin-like growth factor I and risk of breast cancer. The Lancet 351 (9113), 1393–1396.

Happerfield, L.C., Miles, D.W., Barnes, D.M., Thomsen, L.L., 1997. The localization of the insulin-like growth factor receptor 1 (IGFR-1) in benign and malignant breast tissue. The Journal of Pathology 183 (4), 412–417.

Hernández-Sánchez, C., Blakesley, V., Kalebic, T., Helman, L., LeRoith, D., 1995. The role of the tyrosine kinase domain of the insulin-like growth factor-I receptor in intracellular signaling, cellular proliferation, and tumorigenesis. Journal of Biological Chemistry 270 (49), 29176–29181.

Jee, S.H., Shen, S.C., Tseng, C.R., Chiu, H.C., Kuo, M.L., 1998. Curcumin induces a p53-dependent apoptosis in human basal cell carcinoma cells. Journal of Investigative Dermatology 111 (4), 656–661.

Korutla, L., Kumar, R., 1994. Inhibitory effect of curcumin on epidermal growth factor receptor kinase activity in A 431 cells. Biochimica et Biophysica Acta 1224 (3), 597–600.

Kuttan, R., Bhanumathy, P., Nirmala, K., George, M.C., 1985. Potential anticancer activity of turmeric (*Curcuma longa*). Cancer Letters 29 (2), 197–202.

Lee, K.W., Kim, J.H., Lee, H.J., Surh, Y.J., 2005. Curcumin inhibits phorbol ester-induced up-regulation of cyclooxygenase-2 and matrix metalloproteinase-9 by blocking ERK1/2 phosphorylation and NF-kappaB transcriptional activity in MCF10A human breast epithelial cells. Antioxidants and Redox Signaling 7 (11–12), 1612–1620.

Lev-Aris, S., Maimon, Y., Strier, L., Kazanov, D., Arber, N., 2006. Down-regulation of prostaglandin E_2 by curcumin is correlated with inhibition of cell growth and induction of apoptosis in human colon carcinoma cell lines. Journal of the Society for Integrative Oncology 4 (1), 21–26.

Macaulay, V.M., 1992. Insulin-like growth factors and cancer. British Journal of Cancer 65 (2), 311–320.

Manes, S., Llorente, M., Lacalle, R.A., Gomez-Mouton, C., Kremer, L., Mira, E., Martinez, A.C., 1999. The matrix metalloproteinase-9 regulates the insulin-like growth factor-triggered autocrine response in DU-145 carcinoma cells. Journal of Biological Chemistry 274 (11), 6935–6945.

Mitsiades, C.S., Mitsiades, N.S., McMullan, C.J., Poulaki, V., Shringarpure, R., Akiyama, M., Hideshima, T., Chauhan, D., Joseph, M., Libermann, T.A., Garcia-Echeverria, C., Pearson, M.A., Hofmann, F., Anderson, K.C., Kung, A.L., 2004. Inhibition of the insulin-like growth factor receptor 1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies and solid tumors. Cancer Cell 5 (3), 221–230.

Morishima, T., Yamasaki, Y., Kajimoto, Y., Kamada, T., 1994. Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. Journal of Biological Chemistry 269 (23), 16433–16442.

Nie, L., Oishi, Y., Doi, I., Shibata, H., Kojima, I., 1997. Inhibition of proliferation of MCF-7 breast cancer cells by a blocker of Ca(2+)-permeable channel. Cell Calcium 22 (2), 75–82.

Park, S.D., Jung, J.H., Lee, H.W., Kwon, Y.M., Chung, K.H., Kim, M.G., Kim, C.H., 2005. Zedoariae rhizoma and curcumin inhibits platelet-derived growth factor-induced proliferation of human hepatic myofibroblasts. International Immunopharmacology 5 (3), 555–569.

Rajah, R., Valentinis, B., Cohen, P., 1997. Insulin-like growth factor (IGF)-binding protein-3 induces apoptosis and mediates the effects of transforming growth factor-beta1 on programmed cell death through a p53-and IGF-independent mechanism. Journal of Biological Chemistry 272 (18), 12181–12188.

Ramachandran, C., You, W., 1999. Differential sensitivity of human mammary epithelial and breast carcinoma cell lines to curcumin. Breast Cancer Research Treatment 54 (3), 269–278.

Sachdev, D., Yee, D., 2006. Inhibition of Insulin-like growth factor signaling: a therapeutic approach for breast cancer. Journal of Mammary Gland Biology Neoplasia 11 (1), 27–39.

Schmittgen, T.D., Zakrajsek, B.A., 2000. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. Journal of Biochemical and Biophysical Methods 46 (1–2), 69–81.

Simon, A., Allais, D.P., Duroux, J.L., Basly, J.P., Durand-Fontanier, S., Delage, C., 1998. Inhibitory effect of curcuminoids on MCF-7 cell proliferation and structure-activity relationships. Cancer Letters 129 (1), 111–116.

Singletary, K., MacDonald, C., Wallig, M., Fisher, C., 1996. Inhibition of 7,12-dimethylbenz[*a*] anthracene (DMBA)-induced mammary tumorigenesis and DMBA-DNA adduct formation by curcumin. Cancer Letters 103 (2), 137–141.

Werner, H., LeRoith, D., 2000. New concepts in regulation of the insulin-like growth factors: implication for understanding normal growth and neoplasia. Cellular and Molecular Life Sciences 57 (6), 932–942.

Werner, H., Roberts Jr., C.T., 2003. The IGFI receptor gene: a molecular target for disrupted transcription factors. Genes, Chromsomes and Cancer 36 (2), 113–120.

Werner, H., Karnieli, E., Rauscher, F.J., LeRoith, D., 1996. Wild type and mutant p53 differentially regulate transcription of the insulin-like growth factor 1 receptor gene. Proceedings of the National Academy of Sciences of USA 93 (16), 8318–8323.

Yang, X., Thomas, D.P., Zhang, X., Culver, B.W., Alexander, B.M., Murdoch, W.J., Rao, M.N., Tulis, D.A., Ren, J., Sreejayan, N., 2006. Curcumin inhibits platelet-derived growth factor-stimulated vascular smooth muscle cell function and injury-induced neointima formation. Arteriosclerosis, Thrombosis, and Vascular Biology 26 (1), 85–90.