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Curcumin Inhibits Invasive Phenotype of H-*ras* Transformed Human Breast Epithelial Cells and Suppresses Matrix Metalloproteinase-2 Activity

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A growing number of recent studies have focused on anticarcinogenic, antimutagenic or chemopreventive activities of phytochemicals, particularly those included in human diet. Curcumin, a dietary pigment in turmeric, has been shown to have a wide range of biological activities including anti-inflammatory, anticarcinogenic and antimetastatic effects. In the present study, we attempted to study the possible anti-invasive activity of curcumin in H-*ras* transformed MCF10A human breast epithelial cells which have previously been shown to be highly invasive. Here, we show that curcumin inhibits H-*ras*-induced invasive phenotype in MCF10A cells. A significant downregulation of matrix metalloproteinase (MMP)-2 by curcumin is observed while the activity of MMP-9 is not changed, suggesting that MMP-2 is more likely involved in the inhibitory effect of curcumin than MMP-9. We also show that curcumin treatment inhibits cell growth dose-dependently and induces internucleosomal DNA fragmentation in H-*ras* MCF10A cells and MCF7 human breast carcinoma cells. These results demonstrate that curcumin induces apoptosis in transformed human breast epithelial cells and breast carcinoma cells, suggesting a potential use of curcumin as a chemopreventive agent for breast cancer.

Key Words: Curcumin, Invasion, Matrix metalloproteinase, H-*ras*

INTRODUCTION

A growing number of recent studies have focused on anticarcinogenic, antimutagenic or chemopreventive activities of phytochemicals, particularly those included in human diet.[1,2] Curcumin (diferuloylmethane), a naturally occurring dietary pigment from the root of the plant *Curcuma longa Linn*, has been used as a coloring agent and/or spice in foods as well as in cosmetics and drugs.[3] Curcumin is a phenolic compound which has been shown to have a wide range of biological and pharmacological activities including anticancer,[4~8] anti-inflammatory[9] and antimetastatic[10] activities.

An essential part of the metastatic process includes degradation of the extracellular matrix and basement membrane. Uncontrolled degradation of the extracellular matrix and basement membrane is thought to be associated with tumor cell invasion.[11] A role for members of the matrix metalloproteinase (MMP) family on tumor invasion and

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metastasis formation has been suggested, especially, MMP-2 (72 kDa type IV collagenase, gelatinase A) and MMP-9 (92 kDa type IV collagenase, gelatinase B) which degrade type IV collagen, the major structural collagen of the basement membrane.[12~14] We have previously shown that H-*ras*, but not N-*ras*, induces an invasive phenotype in human breast epithelial cells (MCF10A) and the H-*ras*-induced invasive phenotype is associated more closely with the expression of MMP-2,[15] rather than the induction of MMP-9 expression shown previously in rat embryonic fibroblasts.[16]

Recently, curcumin has been shown to inhibit SK-Hep-1 hepatocellular carcinoma cell invasion and suppress MMP-9 secretion.[17] In the present study, we attempted to examine the effect of curcumin on the invasive phenotype of MCF10A cells transformed with v-H-*ras* (H-*ras* MCF10A) and the possible association of MMP-2 and/or MMP-9. Furthermore, we studied if curcumin induced apoptosis in H-*ras* MCF10A cells and MCF7 human breast carcinoma cells. Here, we demonstrate that curcumin inhibits invasive phenotype in parallel with a specific downregulation of MMP-2 in H-*ras* MCF10A cells and induces apoptosis in transformed human breast epithelial cells and breast carcinoma cells, suggesting a potential use of curcumin as a chemopreventive agent for breast cancer.

MATERIALS AND METHODS

1) Cell lines and culture condition

The development and characterization of a spontaneously immortalized breast epithelial cell line, MCF10A, has been described elsewhere.[18] Establishment of H-*ras* MCF10A cells was previously described.[15] Briefly, retroviral vector containing a mutant (pBW1423) containing mutation of Gly to Asp in amino acid codon 12 was introduced in MCF10A cells. More than 100 colonies were pooled together and named H-*ras* MCF10A. H-*ras* MCF10A cells were cultured in DMEM/F12 supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 2 ng/ml EGF, 0.1 μg/ml cholera enterotoxin, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin and 0.5 μg/ml fungizone. MCF7 human breast carcinoma cell line[19] was cultured in DMEM/F12 supplemented with 10% FBS and 100 units/ml penicillin-streptomycin. The cells were cultured in a humidified 5% CO_2 incubator at 37°C.

2) Materials

Curcumin was purchased from Sigma Co. (St. Louis, MO) and resuspended in DMSO to make 100 mM stock solution which was diluted with PBS before treatment. Caspase inhibitors, Ac-YVAD- CHO[20] and Ac-DEVD-CHO[21] were purchased from Takara Co. (Shiga, Japan).

3) *In vitro* invasion assay

In vitro invasion assay was performed as described previously[22,23] with a slight modification using a 24-well transwell unit with polycarbonate filters, 6.5 mm in diameter and a pore size of 8.0 μm (Corning Costar, Cambridge, MA). The lower side of the filter was coated with 10 μl of 0.5 mg/ml type I collagen, and the upper side was coated with 10 μl of 0.5 mg/ml reconstituted basement membrane substance (Matrigel: Collaborative Research, Lexington, KY). The coated filters were air-dried for 1 hr prior to the addition of the cells. The lower compartment contained 600 μl of DMEM/ F12 containing 0.1 mg/ml BSA. Fifty thousand cells pretreated with various concentrations of curcumin for 48 hr were resuspended in 100 μl of DMEM/ F12 containing the corresponding concentrations of curcumin and placed in the upper part of a transwell plate. Cells were incubated for 17 hr in a humidified atmosphere of 5% CO_2 at 37°C to allow invasion. Cells were fixed with methanol and stained with hematoxylin for 10 min followed by eosin. Cells on the upper surface of the filter were mechanically removed by wiping with a cotton swab, and the invasive phenotypes were determined by counting the cells that migrated to the lower side

of the filter with microscopy at ×400. Thirteen fields were counted for each assay. Each sample was assayed in triplicate.

4) Gelatin zymography

After the cells were treated with 100 μM curcumin for 48 hr, the conditioned media were collected, centrifuged at 3,000 rpm for 10 min to eliminate cell debris and the total protein content was determined using BCA protein assay reagents (Pierce, IL). Gelatin zymogram assay was performed as described previously.[24)] Briefly, samples were electrophoresed in a 10% SDS-PAGE gel co-polymerized with 0.1% gelatin. After electrophoresis, gels were washed twice (2×30 min) in 2.5% triton X-100 to remove SDS and incubated for 18 hr at 37°C in 40 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.5, which permits enzymatic activity. After staining with 0.1% Coomassie brilliant blue followed by destaining with 10% acetic acid, areas of lysis were observed as white bands against a blue backgound.

5) Cytotoxicity

Cells (1×10^4) cultured in a 96-well plate were treated with curcumin for 20 hr. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (5 mg/ml) was added to the media and the cells were further incubated for 4 hr. After 100 μl of supernatant was replaced with 100 μl of DMSO, absorbance of each well was read at 540 nm with a micro-ELISA reader (Molecular Devices, Sunnyvale, CA). Percent of cell survival was defined as the relative absorbance of treated versus untreated cells.

6) DNA fragmentation assay

Cells in a 100-mm dish were treated with 50 μM curcumin for 48 hr, trypsinized and collected with ice-cold PBS. After centrifugation (2,000 g) for 10 min at 4°C, cells were transferred to an eppendorf tube and recentrifuged at 15,000 rpm for 15 min at 4°C. Cell pellets were resuspended in 0.5~1 ml of isolation buffer (10 mM EDTA, 50 mM Tris- HCl, pH 8.0, 0.5% SDS, 0.5 mg/ml proteinase K) and incubated overnight at 50°C. The lysate was centrifuged at 15,000 rpm for 15 min at 4°C to separate the soluble fragmented DNA from the intact chromatin pellet. DNA was extracted with phenol/chloroform/isoamylalcohol (25 : 24 : 1) and precipitated with ethanol. Purified DNA was treated with 1 μg/ml RNase A for 1 hr at 37°C prior to electrophoresis on a 1.8% agarose gel containing ethidium bromide.

RESULTS

1) Curcumin inhibits H-*ras*-induced invasive phenotype of MCF10A cells

We examined the effect of curcumin on invasive phenotype of H-*ras* MCF10A cells which have been shown to be highly invasive. After pretreatment with curcumin for 48 hr, viable cells (5×10^4) were resuspended in DMEM/F12 media containing curcumin and subsequently incubated in a transwell chamber coated with Matrigel for 17 hr to allow cell invasion. As shown in Fig. 1, curcumin treatment significantly reduced the number of

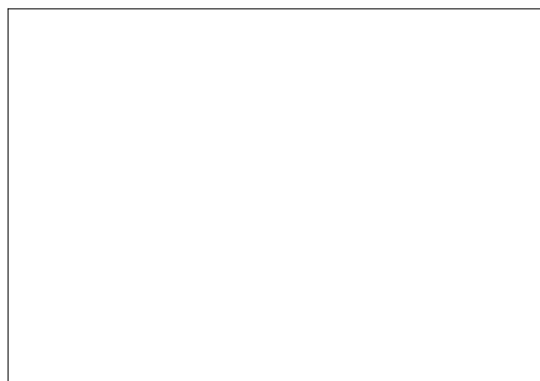

Fig. 1. Curcumin inhibits invasive phenotype of H-*ras* MCF10A cells. Fifty thousand viable cells pretreated with curcumin for 48 hr were incubated in a transwell chamber for 17 hr. The number of invaded cells per field were counted (400×) in thirteen fields and the mean values were determined. Experiments were performed triplicate. Bar is mean number of invaded cells per field, and line is SE for triplicate determinations.

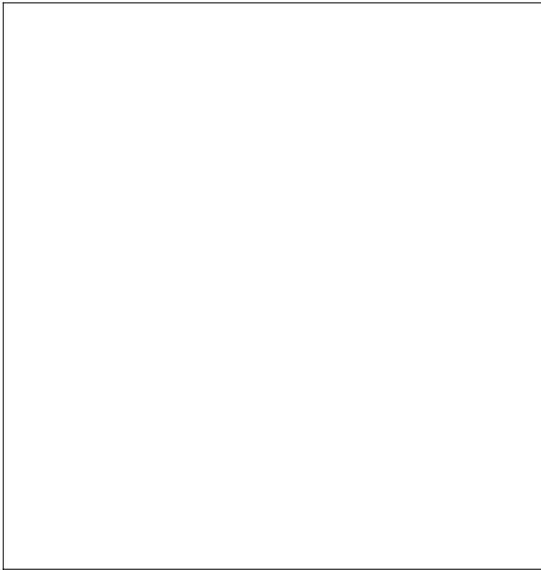

Fig. 2. Curcumin downregulates MMP-2 activity, but not MMP-9, in H-*ras* MCF10A cells. A, Cells were treated with curcumin (100 μM) for 48 hr and the conditioned media were subjected to gelatin zymogram assay. B, Relative activities of MMP-2 (72 kDa) and MMP-9 (92 kDa) were determined by densitometry of gelatin zymograph.

invaded cells through a reconstituted basement membrane in a dose-dependent manner. Treatment of H-*ras* MCF10A cells with 50 and 100 μM of curcumin inhibited cellular invasion to 29% and 3% of control, respectively. The IC_{50} value of curcumin for inhibiting invasive phenotype of H-*ras* MCF10A cells was 36 μM.

2) Curcumin downregulates MMP-2, but not MMP-9

In order to determine the possible association of MMP-2 and/or MMP-9 in the anti-invasive effect of curcumin, we examined the enzymatic activities of MMPs in H-*ras* MCF10A cells treated with 100μM curcumin for 48 hr. A significant down- regulation of MMP-2 by curcumin was observed in gelatin zymograph while the activity of MMP-9 was not changed (Fig. 2). These results are consistent with our previous finding that MMP-2 is more closely associated with the H-*ras*-induced invasive phenotype than MMP-9 in human breast epithelial cells.[15] At a concentration of 100 μM, curcumin blocked H-*ras* MCF10A cell invasion almost completely while the MMP-2 activity remained about 50% of control, suggesting that MMP-2 is not the only element reponsible for the anti-invasive effect of curcumin in these cells. The data demonstrate that curcumin inhibits invasive phenotype of H-*ras* MCF10A human breast epithelial cells in which MMP-2 is more likely involved, at least in part, rather than MMP-9.

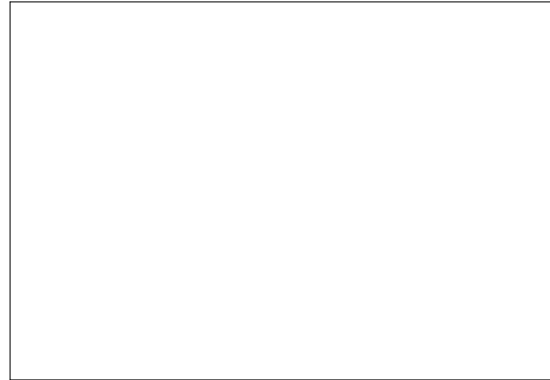

Fig. 3. Curcumin induces cell death in H-*ras* MCF10A and MCF7 cells. Cells (1×10^4) were treated with curcumin for 20 hr. MTT assay was performed and the cytotoxicity was determined by relative absorbance normalized to the control cells. The results presented are means±SE of triplicates.

3) Curcumin shows cytotoxicity in H-*ras* MCF10A cells and MCF7 cells

A dose response study was conducted to examine the cytotoxic effect of curcumin in two breast cell lines: H-*ras* MCF10A breast epithelial cell line and MCF7 breast carcinoma cell line. MTT assay was performed on the cells treated with various concentrations of curcumin for 20 hr. As shown in Fig. 3, curcumin exerted cytotoxicity to both cell lines in a dose-dependent manner. The IC_{50} values for growth inhibition of H-ras MCF10A cells and MCF7 cells were 35 μM and 77 μM, respectively. At a higher concentration (100 μM), the potency of curcumin was similar in the two cell lines.

4) Curcumin induces internucleosomal DNA fragmentation

To determine the mode of cell death in H-*ras* MCF10A cells and MCF7 cells induced by curcumin, we examined whether curcumin induces internucleosomal DNA fragmentation, one of the characteristics of apoptosis. As shown in Fig. 4, treatment with 50 μM curcumin for 48 hr prominently induced internucleosomal DNA fragmentation in these two cell lines, indicating that curcumin induced apoptosis in these cells. Our result is contraditory to the preview finding by Janicke *et al.*[27] that no DNA fragmentation was observed in MCF7 cell line due to the absence of caspase-3. In contrast, caspase-3 activation in MCF7 cells was shown in response to etoposide treatment,[32] suggesting that curcumin-induced apoptosis in MCF7 cells needs more investigation.

5) A caspase-3-inhibitor, Ac-DEVD-CHO, attenuates curcumin-induced apoptosis in H-*ras* MCF10A cells, but not in MCF7 cells

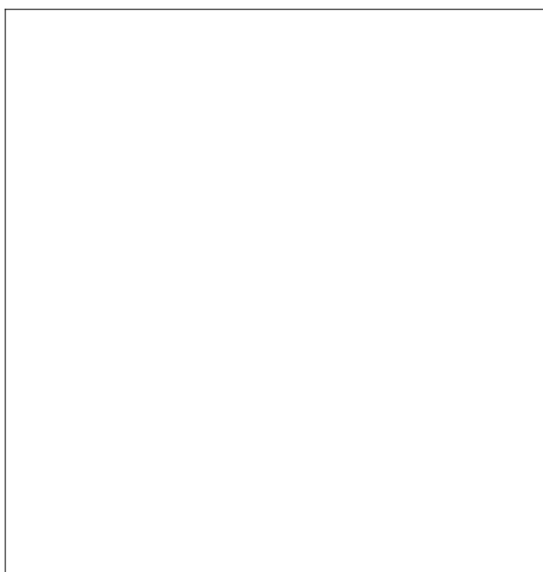

Fig. 4. Curcumin induces DNA fragmentation in H-*ras* MCF10A and MCF7 cells. Cells were treated with 50 μM curcumin for 48 hr. Cellular DNA was extracted and analyzed by 1.8% agarose gel electrophoresis.

Mounting evidence shows that activation of caspases trigger the apoptotic process in various cells.[25] Caspase-3, a member of the caspase family, has been shown to play an essential role in apoptosis induced by a variety of stimuli.[21,26] To examine whether caspase-1- or -3-like activity was critical for the curcumin-induced apoptosis, we pretreated the H-*ras* MCF10A and MCF7 cells with 200 μM caspase inhibitors for 2 hr. As shown in Fig. 5A, Ac-DEVD-CHO (a caspase-3 inhibitor) significantly attenuated

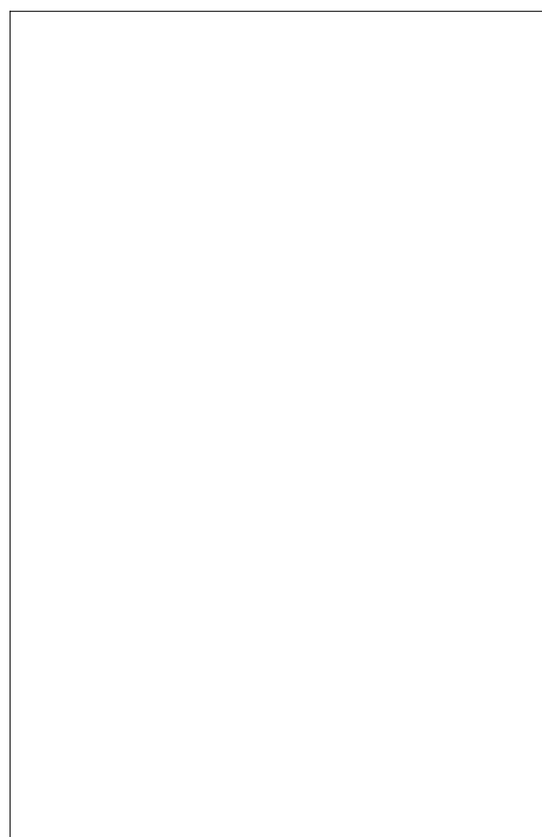

Fig. 5. Ac-DEVD-CHO attenuates the curcumin-induced growth inhibition in H-*ras* MCF10A cells, but not in MCF7 cells. A, H-*ras* MCF10A cells; B, MCF7 cells. Cells (1.5×10^5) in a 48-well plate were preincubated with 200 μM Ac-DEVD-CHO (a caspase-3 inhibitor) or Ac-YVAD-CHO (a caspase-1 inhibitor) for 2 hr followed by curcumin treatment (50 μM) for 20 hr. Cell viability was determined by trypan blue exclusion assay. The results presented are means±SE of triplicates.

curcumin-induced cell death in H-*ras* MCF10A cells while Ac-YVAD-CHO (a caspase-1-inhibitor) had little effect at the same concentration. Since Ac-DEVD-CHO was not able to fully reverse the cytotocixity of curcumin, caspase-3 does not seem to make an essential contribution to cell death. These results suggest that apoptosis induced by curcumin may involve a DEVDase (caspase- 3-like) activity in H-*ras* MCF10A cells. Data obtained from MCF7 cells, however, did not support the possible involvement of a caspase-3-like activity in curcumin-induced MCF7 cell death: Ac-DEVD- CHO did not significantly alter the cytotoxicity of curcumin (Fig. 5B). Consistently with these results, MCF7 cell line was shown to have lost caspase-3 owing to deletion within the *CASP*-3 gene.[27]

DISCUSSION

In recent years there has been a growing body of evidence that phytochemicals in our diet can reduce the risk of cancers. Chemoprevention is arguably one of the major weapons in the anticancer arsenal. Since metastasis represents the most important cause of cancer death, chemopreventive agents that may inhibit invasion have been pursued. Curcumin has been recently shown to inhibit SK- Hep-1 hepatocellular carcinoma cell invasion and suppress MMP-9 secretion.[17] In the present study, we showed that curcumin inhibited H-*ras*-induced invasive phenotype of MCF10A human breast epithelial cells with a parallel downregulation of MMP-2. Taken in conjunction with the fact that uncontrolled *ras* activation is probably the most common genetic defect in human cancer cells, our finding may be critical for chemopreventive potential of curcumin.

Numerous studies show a correlation between the levels of MMP-2 and/or MMP-9 and the invasive phenotypes of cancer cells.[28] H-*ras* mediated transformation and invasiveness were shown to be associated with enhanced expression of MMP-9 mRNA and protein in rat and human embryonic fibroblasts[16,29] and in human fibrosarcoma cells.[24,30] In contrast, the results presented in this study along with our previous finding[15] demonstrate that MMP-2 is more likely associated with H-*ras* mediated invasiveness in human epithelial cells. Since *ras*-mediated cellular responses differ between epithelial cells and fibroblasts,[33] the role of MMP-2 and/or MMP-9 on H-*ras*-signaling may be cell type-specific.

The differentiated cells of multicellular organisms apparently share the ability to carry out their own death through activation of an internally encoded suicide program, known as apoptosis.[31] Apoptosis is known to be the most potent natural defense against cancer development. Thus, efforts have been made to develop a chemoprevention strategy that triggers apoptosis in malignant/premalignant cancer cells.

In the present study, we showed that curcumin induced apoptosis in H-*ras*-transformed human breast epithelial cells and in MCF7 human breast carcinoma cells as evidenced by internucleosomal DNA fragmentation. Our results suggest that apoptosis induced by curcumin involves a DEVDase (caspase-3-like) activity in H-*ras* MCF10A cells, though caspase-3 may not make a critical contribution. Opposing results have been reported regarding caspase-3 activity in MCF7 cells: the absence of caspase-3 in MCF7 cell line owing to deletion in the gene[27] and the activation of caspase-3 responding to etoposide treatment.[32] Our result showing that caspase-3 is not involved in curcumin-induced apoptosis in these cells supports the former observation. Curcumin-induced apoptosis in MCF7 cells needs more investigation. Taken together, we demonstrated that curcumin inhibited invasive phenotype in parallel with a specific downregulation of MMP-2 in H-*ras* MCF10A cells and induced apoptosis in transformed human breast epithelial cells and breast carcinoma cells, suggesting a potential use of curcumin as a chemopreventive agent for breast cancer.

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