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Direct inhibitory effect of curcumin on Src and focal adhesion kinase activity

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Abstract

Curcumin (diferuloylmethane) is a well-known agent with anti-inflammatory, antioxidant, and anticarcinogenic properties. In this study, we observed that curcumin inhibited the kinase activity of v-Src, which led to a decrease in tyrosyl substrate phosphorylation of Shc, cortactin, and FAK. Our *in vitro* kinase experiment revealed that the inhibitory effect of curcumin on Src could be direct. Consistent with the abrogation of Src activity was the reduction of Src-Tyr-416 phosphorylation, Src-mediated Shc-Tyr-317 phosphorylation, decreased ERK activation, and cell proliferation in v-Src transformed cells. Remarkably, curcumin not only exerted its negative effect on FAK via the disappearance of Src-mediated FAK phosphorylation, but also directly inhibited its enzymatic activity. Concurrent to reduced cortactin tyrosyl phosphorylation and FAK kinase activity was the abolishment of v-Src-mediated cell mobility. To our knowledge, this is the first report indicating that curcumin can retard cellular growth and migration via downregulation of Src and FAK kinase activity.

Keywords: Curcumin; Src; Shc; ERK; FAK; Migration

1. Introduction

Curcumin (diferuloylmethane), a popular dietary spice in the East, is a well-known agent with anti-inflammatory, antioxidant, and anticarcinogenic properties. The major constituent of tumeric powder extracted from the rhizomes of *Curcuma longa* Lin. gives the unique flavor and yellow color to curry [1]. As a β-diketone moiety containing polyphenolic compound, curcumin possesses two ferulic acid molecules linked via a methylene bridge at the C atoms of the carboxyl groups (Fig. 1A) [2]. Animal experiments have shown that in addition to its anti-inflammatory and antioxidant properties, curcumin also displays chemopreventive activity in various tissues [2]. Although it is still unclear which part of the agent is crucial for biological activity, the speculated candidates include the hydroxyl groups of the benzene rings, the double bonds in the alkene part of the molecule, and/or the central β-diketone moiety. To date, the inhibitory effects of curcumin on various signaling proteins including cyclooxygenase, ornithine decarboxylase, nitric oxide synthase, transcription factors, matrix metalloproteinases, and protein kinases have been reported [2]. Conceivably, through inhibition of these molecules, curcumin can effectively suppress or revert tumor formation and retard metastasis.

The protein TK is a large and diverse multigene family that historically defines the prototypical class of oncogenes involved in most animal malignancies [3]. Phosphorylation mediated by TK is an important post-translational modification that conveys signals concerning a variety of physiologic activities such as proliferation, differentiation, adhesion, transformation, and mobility. C-Src, encoded by the cellular homologue of *v-src*, is a ubiquitously expressed cytoplasmic TK whose overexpression and enhancement of enzymatic activity have been strongly implicated in human tumors [4]. Tyr-416 and -527 are the two well-documented Src phosphorylation sites that are located

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Abbreviations: TK, tyrosine kinase; CSK, C-terminal Src kinase; FAK, focal adhesion kinase; EGFR, EGF receptor; pTyr, phosphotyrosine; ERK, extracellular signal-regulated kinase; mAb, monoclonal antibody; ECL, enhanced chemiluminescence.

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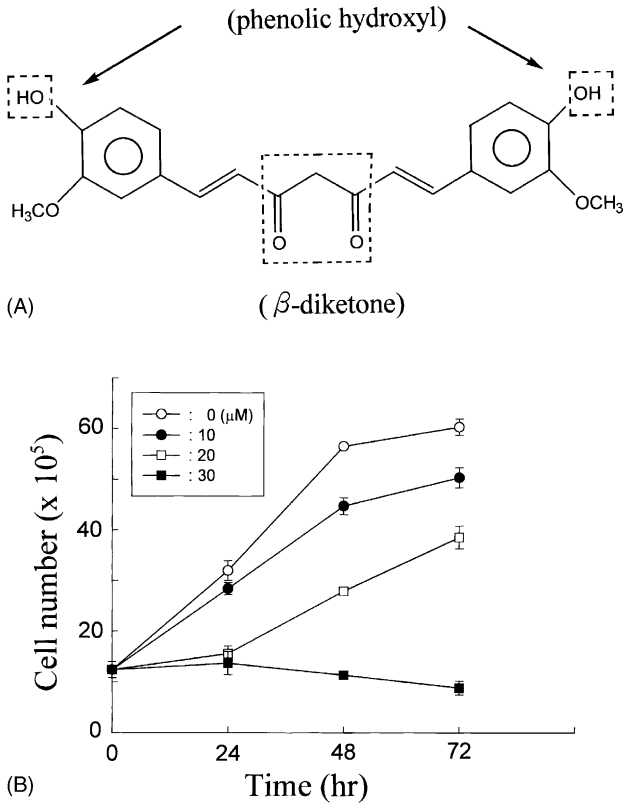

Fig. 1. Growth inhibition of IV5 cells by curcumin. (A) Chemical structure of curcumin. The phenolic hydroxyl groups and the β-diketone moiety of curcumin are indicated. (B) IV5 cells (5×10^5) were plated at the beginning. After 18 hr, some dishes were used as the control (time 0) and the remaining dishes were incubated with different concentrations of curcumin as indicated for 24, 48 and 72 hr. Total number of control and curcumin-treated cells were counted and plotted. The results were shown in means ± SD for three independent experiments performed in triplicate.

within the kinase domain and the C-terminal regulatory region, respectively. While self-mediated phosphorylation of the former enhances the catalytic activity of Src, phosphorylation of the latter that mediated by CSK (a C-terminal Src kinase) downregulates Src activity. The lack of Tyr-527 in v-Src renders v-Src to be constitutively active [5]. A number of signaling proteins such as FAK [6], cortactin [7,8], and Shc [9] have been identified as the substrates for Src family kinases. Previous studies have demonstrated that Shc Tyr-317 is an Src-mediated residue whose phosphorylation, which is associated with the SH2 domain of Grb2, propagates signaling to the proline-rich target (i.e. SOS) and leads to the activation of Ras and triggers the ERK kinase cascade [10,11].

As a substrate for Src and a unique cytoplasmic TK localized at focal contacts, FAK is an important player in integrin signal transduction [12]. Upon integrin engagement, FAK becomes activated and autophosphorylated at Tyr-397, which confers the binding site for Src family kinases [13,14]. Through the formation of a complex with FAK, Src mediates the phosphorylation of FAK at Tyr-407, -576/577, -863 and -925, and either enhances the enzymatic activity of FAK [15–17] or provides the binding site for the Grb2 SH2 domain and triggers Ras signaling [18]. Recently, accumulated evidence has indicated that the formation and activation of the Src–FAK bipartite kinase complex is important in cell spreading, migration, and survival [12].

The inhibitory effect of curcumin on EGFR and $p185^{neu}$ has been well studied [19,20]. However, unlike these two receptor TKs, the influence of curcumin on the activity of Src was poorly documented. In an attempt to address this question, we investigated the biological responses to curcumin and the molecular mechanisms involved in these responses in cells transformed with v-Src. Interestingly, following curcumin treatment, the tyrosyl phosphorylation of Shc, cortactin, and FAK progressively disappeared with a concomitant decrease of Src kinase activity. Notably, we observed that curcumin could directly inhibit the activity of both Src and FAK, which has not been revealed before. And through downregulation of the activity of these two kinases, curcumin effectively inhibited the proliferation and migration of v-Src transformed cells.

2. Materials and methods

2.1. Cell lines and drug treatment

The clonal C3H10T1/2 murine fibroblast cell line expressing v-Src (IV5) was a generous gift provided by Dr. Sarah J. Parsons and its derivation and maintenance were previously described [21]. For drug treatment, cells were incubated with or without curcumin. Their lysates were then harvested and analyzed.

2.2. Antibodies

Src-specific mouse mAb GD11 and anti-cortactin mAb (4F11) were provided by Dr. Sarah J. Parsons in University of Virginia. Polyclonal FAK antibody is directed against the C-terminal region of FAK and was described previously [17]. Antibodies specific for Tyr-397 phosphorylated (Pi-Tyr-397) FAK, Tyr-416 phosphorylated (Pi-Tyr-416) Src, Tyr-317 phosphorylated (Pi-Tyr-317) Shc and Shc were purchased from Upstate Biotechnology. Anti-phosphorylated ERK (E10) mAb was purchased from New England Biolabs, Inc. The rabbit polyclonal antibodies recognized unphosphorylated ERK and HRP-conjugated anti-phosphotyrosine antibody (PY20) were obtained from Santa Crutz.

2.3. Immunoprecipitation and immunoblot analysis

Lysis of the cells was carried out with modified RIPA buffers as described before [22] and protein concentration was determined by protein assay kit (Bio-Rad). Methods

for immunoprecipitation and immunoblotting analysis have been described [22]. The cell lysates or the immunoprecipitates of interested proteins were resolved on 8% SDS–polyacrylamide gels, transferred to nitrocellulose membranes and probed with respective antibodies followed by horseradish peroxidase (HRP)-conjugated protein A or HRP-conjugated rabbit anti-mouse IgG and detected by Enhanced Chemiluminescence method (Amersham).

2.4. In vitro kinase assay

The v-Src or FAK immunoprecipitates from IV5 cells were preincubated with or without various amounts of curcumin for 10 min in ice basket. Then the kinase reaction of v-Src or FAK immunocomplexes was performed in a 20-μL kinase buffer, containing 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 7.2), 5 μM ATP, 10 μCi [γ-^{32}P]-ATP (6000 Ci/mmol, NEN), and either 10 mM $MgCl_2$ (for v-Src) or 10 mM $MnCl_2$ (for FAK), for 15 min at room temperature. Enolase (1 μg) and the bacterial GST fusion protein (0.2 μg), GST-397Y [17], were added as the exogenous substrates for Src and FAK, respectively. Incubations were terminated by addition of sample buffer followed by 5-min boiling. Then the labeled products were resolved by SDS–PAGE, visualized by autoradiography, and quantitated by densitometry.

2.5. Migration

Chemotactic migration of IV5 cells were assayed by modify Boyden chamber [23]. Briefly, IV5 cells were trypsinized to obtain single-cell suspension, and suspended in serum-free DMEM. IV5 cells were added to the upper wells (48-multiwell Boyden microchambers) at 2×10^4 cells per well in the presence or absence of curcumin while fibronectin (10 μg/mL) was present in the lower chamber. The migrated cells will traverse a polycarbonate filter (8 μm) from the upper chamber to the lower chamber. After 6 hr at 37° in 5% CO_2, non-migratory cells on the upper membrane surface were removed with a cotton swab and the cells that traversed and spread on the lower membrane surface were fixed with methanol and stained with Giemsa stain (Modified solution) (Sigma). By utilizing a microscope with a 40× objective, the number of migratory cells per membrane was enumerated. Four random fields in each filter were examined. Each experiment was performed in triplicate and migration was expressed as the mean ± SD of total cells counted per field.

2.6. Statistical analysis

Values given represent the mean ± SD of experiments done in triplicate. Statistical significance was tested by Student's *t*-test for either paired or unpaired data as appropriate.

3. Results

3.1. Curcumin inhibits the proliferation and v-Src kinase activity in IV5 cells

Since curcumin (Fig. 1A) is a well-established food constituent with chemopreventive properties and its effect on Src is poorly described, thus, we utilized cells transformed with v-Src (IV5) to evaluate the possibility that curcumin might inhibit the proliferation of IV5 cells. As shown in Fig. 1B, compared to control cells, curcumin significantly inhibited cellular growth of IV5 cells in a dose-dependent manner (the IC_{50} for 24 hr growth inhibition is approximately 15 μM). Because tyrosyl phosphorylation plays a critical role in mitogenesis, we investigated the influence of various concentrations of curcumin on the profile of tyrosyl-phosphorylated proteins in IV5 cells after 24 hr treatment. Whole cell extracts prepared from curcumin-treated and untreated IV5 cells were resolved in SDS–PAGE and analyzed by pTyr Western immunoblotting. As demonstrated in Fig. 2, ~20 μM curcumin could cause the decrease in the pTyr content of a number of cellular proteins suggesting that curcumin might inhibit any or multiple tyrosine kinases. Due to no significant change of the pTyr profile in curcumin-treated normal control cells (data not shown), thus, this prompted us to speculate that curcumin might negatively influence v-Src activity. Indeed, reduced Src-Tyr-416 phosphorylation in response to curcumin was observed while no alteration of the amount of v-Src was detected (Fig. 3A). When the concentration of curcumin reached ~20 μM, its inhibition of Src phosphorylation was ~30% after normalization ($P < 0.05$). To further confirm the reduction of v-Src enzymatic activity following curcumin addition, the level of tyrosyl-phosphorylated cortactin in curcumin-treated and untreated IV5 cells was compared. While similar amounts of cortactin were detected in all the samples analyzed, reduced tyrosyl phosphorylation of cortactin following curcumin treatment was dose-dependent (Fig. 3B). Further confirmatory evidence for the curcumin-mediated reduction of Src kinase activity was provided by a time-dependent experiment in which Shc Pi-Tyr-317 was significantly diminished in curcumin-treated IV5 cells, though the amount of Shc was not altered (Fig. 4). Despite 100 μM curcumin was utilized in the time course study, ~20 μM curcumin was statistically significant to reduce Shc Pi-Tyr-317 (data not shown). And interestingly, we observed that the curcumin-mediated inhibition of Src Pi-Tyr-416 preceding the inhibition of Shc Pi-Tyr-317 (Fig. 4).

3.2. Curcumin can directly inhibit Src kinase activity

The abrogation of total tyrosyl phosphorylation and the reduced level of tyrosyl-phosphorylated cortactin, Tyr-416 phosphorylated Src, and Tyr-317-phosphorylated Shc in curcumin-treated IV5 cells implied that curcumin could directly or indirectly abolish Src kinase activity. To assess

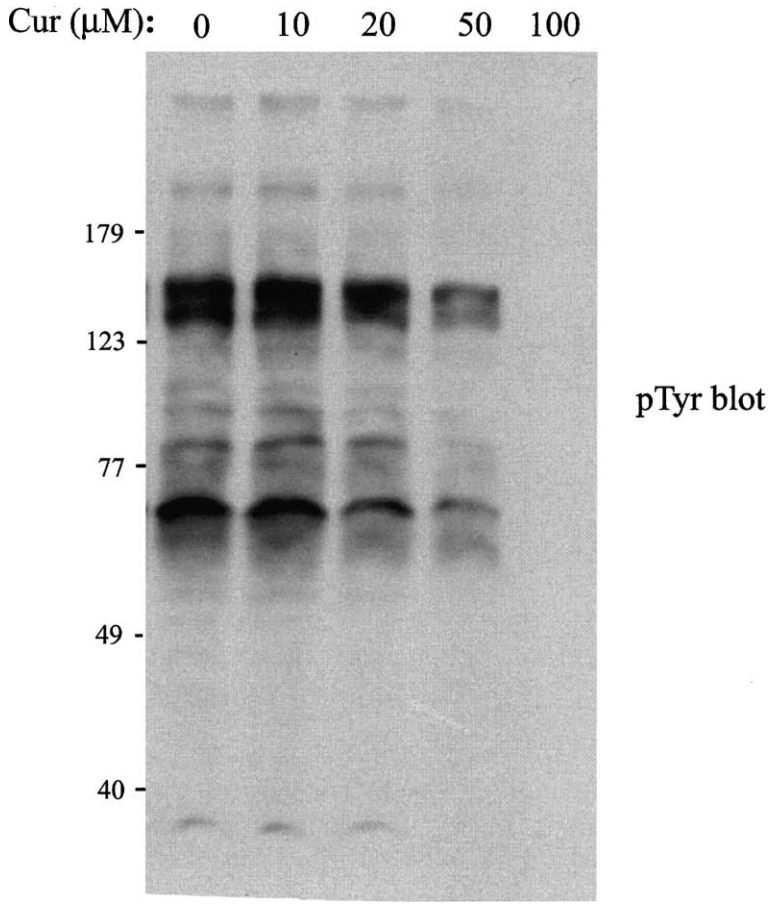

Fig. 2. Curcumin treatment led to the reduction of protein tyrosyl phosphorylation in IV5. IV5 cells were treated without or with various concentration of curcumin (10, 20, 50, 100 μM) for 24 hr as indicated. Equal amounts of lysates (100 μg) from each sample were resolved by SDS–PAGE and probed with anti-pTyr Ab. Similar results were repeated three times and the representative was demonstrated.

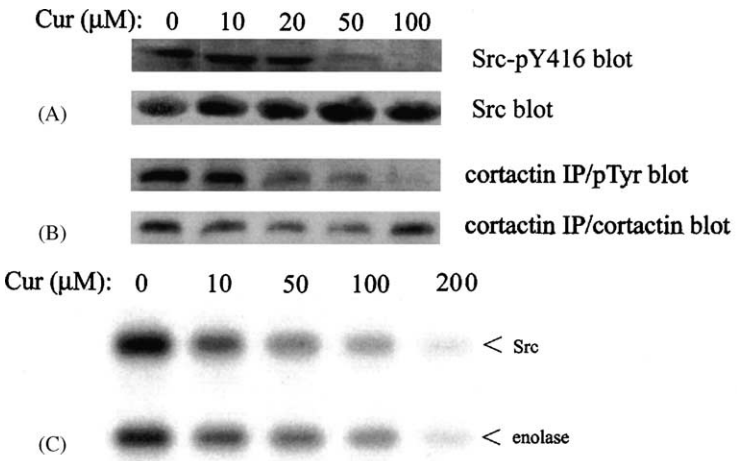

Fig. 3. Curcumin treatment reduced the level of Tyr-416 phosphorylated Src and tyrosyl phosphorylated cortactin and directly inhibited v-Src kinase activity *in vitro*. Lysates of control and 24 hr curcumin-treated IV5 cells were prepared and were (A) resolved by SDS–PAGE and immunoblotted with anti-Src Pi-Tyr-416 Ab and anti-Src Ab separately or (B) immunoprecipitated with anti-cortactin Ab and each half immunoprecipitate was probed with anti-pTyr Ab or anti-cortactin Ab. (C) The v-Src immunocomplexes derived from IV5 cells were aliquot and incubated with various concentration of curcumin at 4° for 10 min. Then, v-Src immunocomplexes were subjected to *in vitro* kinase reactions in the presence of enolase. The ^{32}P-incorporated proteins were analyzed by SDS–PAGE and detected by autoradiography. For all these experiments, similar results were repeated three times and the representative was demonstrated.

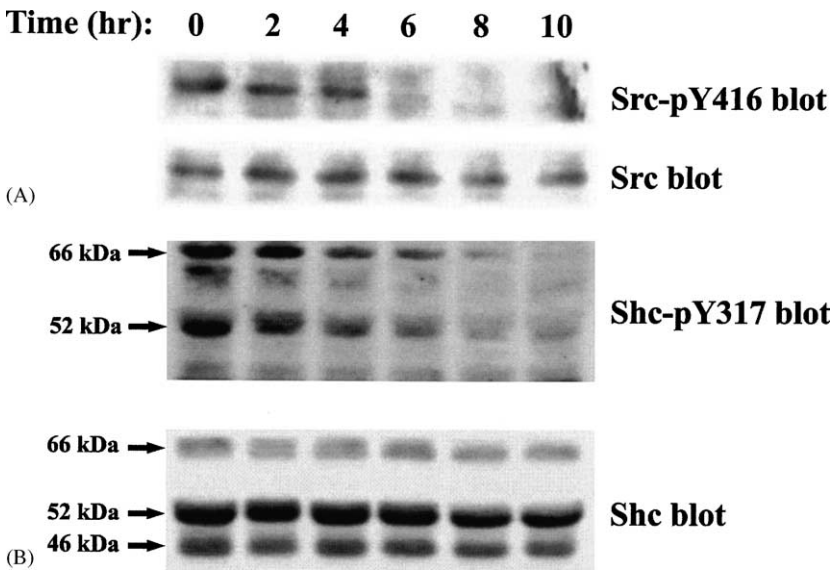

Fig. 4. Reduced Tyr-317 phosphorylated Shc in curcumin-treated IV5 cells. IV5 cells were incubated with curcumin (100 μM) for various times as indicated. (A) The content of Tyr-416 phosphorylated Src and Src was analyzed by direct Western blot analysis with anti-Src Pi-Tyr-416 Ab and anti-Src Ab, respectively. (B) At the meantime, the content of Shc Pi-Tyr-317 and Shc was also analyzed. Similar results were repeated three times and the representative was demonstrated.

the former possibility, Src immunoprecipitates prepared from IV5 cells were incubated with enolase as an exogenous substrate and [γ-^{32}P]-ATP in the presence or absence of curcumin. As shown in Fig. 3C, compared to the control, significantly reduced ^{32}P-labeled Src and enolase were detected in curcumin-incubated samples ($IC_{50} = \sim 50$ μM). This finding indicated that curcumin could directly inhibit the enzymatic activity of Src.

3.3. Curcumin treatment led to the reduction of ERK phosphorylation

It is well documented that tyrosyl-phosphorylated Shc associates with Grb2/SOS complex and activates Ras, which in turn triggers the Raf-MEK-ERK cascade. Since curcumin diminished the level of Shc Pi-Tyr-317, thereby we addressed the point whether the reduced Shc Pi-Tyr-317 might affect ERK activation by determining the ERK activities in control and curcumin-treated IV5 cells. Because MEK-mediated ERK phosphorylation on residues Thr-202 and Tyr-204 increases the enzymatic activity of ERK [24,25], we therefore, applied monoclonal antibody specifically recognized these phosphorylated residues of ERK in Western immunoblotting. As demonstrated in Fig. 5, a significant dose- and time-dependent reduction of phosphorylated ERK was detected in curcumin-treated IV5 cells as compared to control when the expression of ERK in these cells was normalized.

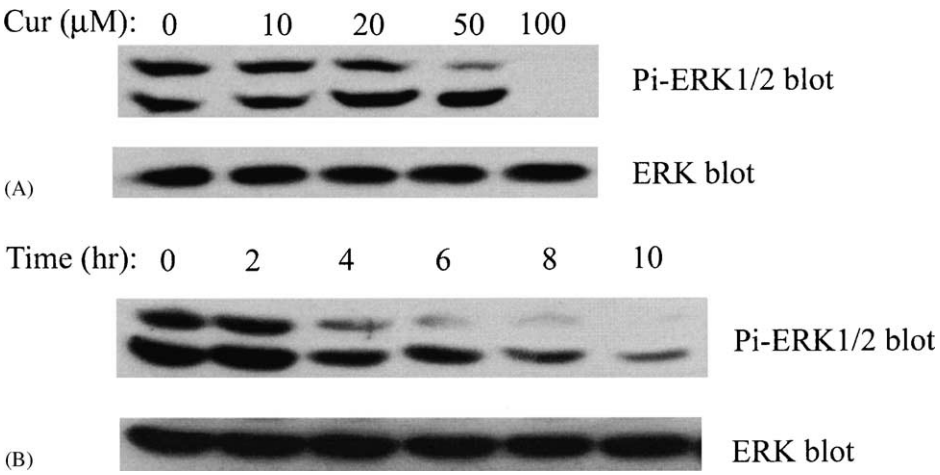

Fig. 5. Curcumin inhibited ERK activation in IV5 cells. (A) IV5 cells were incubated with indicated concentration of curcumin for 24 hr. Their content of phosphorylated ERK and ERK was analyzed by direct Western blot analysis with phosphorylated ERK antibody (E10) and anti-ERK Ab, respectively. (B) Similar experiment was performed with lysates from cells treated with 100 μM curcumin for various time as indicated.

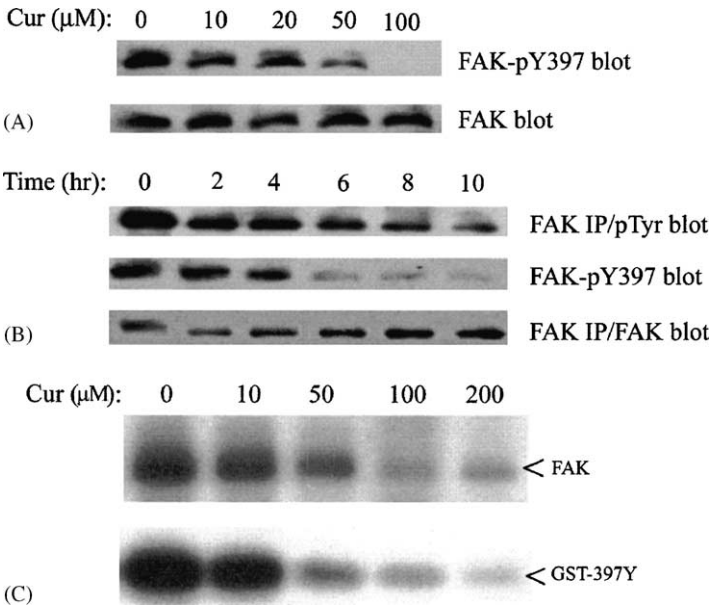

Fig. 6. Curcumin reduced FAK tyrosyl phosphorylation in IV5 cells and could directly inhibit its kinase activity *in vitro*. (A) IV5 cells were incubated with various concentration of curcumin for 24 hr. Their content of Tyr-397 phosphorylated FAK and FAK was analyzed by direct Western blot analysis with anti-FAK Pi-Tyr-317 Ab and anti-FAK Ab, respectively. (B) Cellular lysates prepared from untreated or 24 hr, 100 μM curcumin-treated IV5 cells were immunoprecipitated with anti-FAK antibody. One-third of immunoprecipitate was Western immunoblotted with anti-pTyr Ab (upper), anti-FAK Pi-Tyr-397 Ab (middle), or anti-FAK Ab (lower). (C) The FAK immunocomplexes derived from IV5 cells were aliquot and incubated with various concentration of curcumin at 4° for 10 min. Then, FAK immunocomplexes were subjected to *in vitro* kinase reactions in the presence of GST-397Y. The ^{32}P-incorporated proteins were analyzed by SDS–PAGE and detected by autoradiography. For all these experiments, similar results were repeated three times and the representative was demonstrated.

3.4. Curcumin inhibits the kinase activity of FAK

As demonstrated in Fig. 2, curcumin treatment decreased tyrosyl phosphorylation of a number of proteins, especially a 125-kDa protein, in IV5 cells. Since FAK is a putative Src substrate with a similar molecular weight, it is likely that curcumin reduces tyrosyl phosphorylation of FAK. To prove this hypothesis, lysates prepared from curcumin-treated and untreated IV5 cells were immunoprecipitated with anti-FAK antibody and analyzed by SDS–PAGE with either anti-pTyr or anti-FAK immunoblotting. As expected, while a similar amount of FAK was present in each FAK immunoprecipitate analyzed, a significant decrease of tyrosyl phosphorylation of FAK was observed when the concentration of curcumin reached 20 μM ($P < 0.01$) (Fig. 6B). And consistent with what has been described previously (i.e. that Src-mediated phosphorylation results in FAK activation), FAK activity measured by the level of FAK Tyr-397 phosphorylation was greatly reduced in curcumin-treated IV5 cells (Fig. 6B). Again, this curcumin-mediated downregulation of FAK activity was not only time-dependent, but also

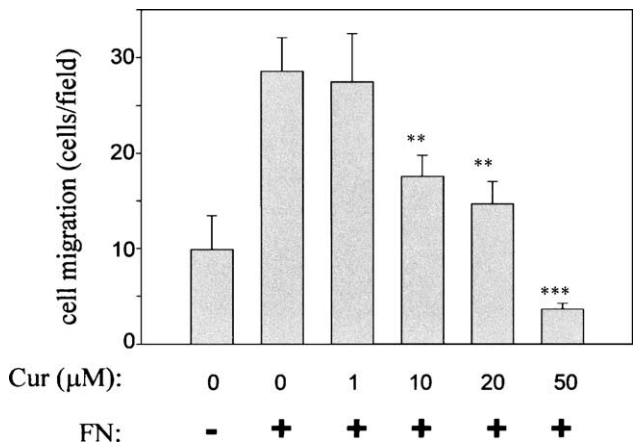

Fig. 7. Curcumin reduced cell motility in IV5 cells. Curcumin-treated or untreated IV5 cells were allowed to migrate for 6 hr through polycarbonate filters in the presence of fibronectin (10 μg/mL) that acts as a chemoattractant. The migrated cells were determined as described in Section 2. Results were the means ± SD of three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$ as compared to the untreated fibronectin-induced cell migration.

dose-dependent (Fig. 6A). To further study whether curcumin can directly affect FAK enzymatic activity, FAK immunoprecipitates prepared from IV5 cells were incubated with GST-397Y, which contains FAK Tyr-397 and its neighboring sequence as an exogenous substrate, and [γ-^{32}P]-ATP in the presence or absence of curcumin. As demonstrated in Fig. 6C, the ^{32}P-incorporation of FAK and GST-397Y was greatly diminished in curcumin-treated samples in a dose-dependent manner ($IC_{50} = \sim 34$ μM). This finding suggests that curcumin can directly abrogate FAK kinase activity.

3.5. Curcumin abolished fibronectin-mediated cell migration in IV5 cells

FAK is a component promoting focal-contact turnover, and its interaction with Src is crucial for integrin-induced cell motility [12]. Since curcumin could inhibit the enzymatic activity of both Src and FAK, thereby its effect on integrin-mediated cell mobility was attempted. To address this question, a modified Boyden chamber assay was performed to determine the fibronectin-induced chemotaxis in the presence or absence of curcumin. As demonstrated in Fig. 7, curcumin could reduce the migration of IV5 cells toward fibronectin in a dose-dependent manner. This observation implies that curcumin can inhibit integrin-mediated cell migration.

4. Discussion

By suppressing, retarding, or reversing the process of carcinogenesis in cell culture and animal models, curcumin has been well established as a dietary constituent with chemopreventive properties. Although a variety of target proteins have been reported, we are the first to report on the inhibitory effect of curcumin on both Src and FAK that results in reduced cellular growth and movement in v-Src-transformed cells.

Despite that phosphorylation mediated by TK is a crucial post-translational modification regulating proliferation, transformation, and migration, only EGFR and Neu are reported to be downregulated in response to curcumin [19,20]. Korutla *et al*. have demonstrated that curcumin inhibits the ligand-induced activation, but not the expression, of EGFR. By contrast, curcumin not only dose dependently inhibits Neu auto- and transphosphorylation *in vitro*, but also enhances its depletion *in vivo* [20]. Interestingly, we observed that curcumin reduces v-Src kinase activity, causing decreased tyrosyl phosphorylation of Shc, cortactin, and FAK, the putative substrates for Src with ~20 μM curcumin upon 24 hr exposure (Figs. 3, 4 and 6). Remarkably, we demonstrated that curcumin could directly exert its negative effect on Src (Fig. 3C).

As a substrate for Src, it is well established that Src-mediated Tyr-576/577 and -863 phosphorylation upregulates the enzymatic activity of FAK [15–17]. Thus, concurrent to the reduced Src-mediated FAK phosphorylation in curcumin-treated IV5 cells, we did observe the abrogation of FAK kinase activity as evidenced by its decreased Tyr-397 autophosphorylation (Fig. 6). Notably, in addition to this indirect effect, curcumin could also directly impair FAK activity *in vitro* (Fig. 6C). However, the mechanism by which curcumin exerts its negative effect on both Src and FAK remains unknown. Accumulated evidence indicates that the biological functions of curcumin are attributable to its antioxidant activity [2,26–28]. Further investigation is required to address this issue and dissect the mechanism(s) involved.

In agreement with curcumin-mediated reduction of Src kinase activity was the finding of a decrease in Shc Tyr-317 phosphorylation, which resulted in inhibition of ERK activation. Considering the role of ERK activation in mitogenesis [29], this curcumin-initiated effect could partly provide the molecular mechanism depicting the growth inhibition in curcumin-treated cells. And our preliminary data demonstrated that 50 μM PD98059 (the MEK inhibitor) could abolish approximately 55% cellular growth of IV5 cells albeit phosphorylation of ERK was completely abrogated (data not shown). This implicated that signaling protein(s) other than ERK downstream of Shc might also play an important role in v-Src-mediated proliferation. In addition to Shc, accompanied with the decrease of Src activity was the reduced tyrosyl phosphorylation of cortactin. Cortactin Tyr-421, -466, and -482 have been defined as the Src-mediated sites whose phosphorylation can result in reduced F-actin cross-linking activity leading to cytoskeleton reorganization and cell migration [30]. Since both FAK activation and tyrosyl phosphorylation of cortactin are critical players modulating cell motility, curcumin could effectively abolish fibronectin-mediated cell migration in IV5 cells by interfering with these two Src substrates. Consistent with what we observed in this report, Santibanez *et al*. demonstrated that curcumin could block TK-dependent TGF-β1-induced migration [31].

Recently, by means of cDNA microarrays, researchers have successfully and simultaneously examined expression levels of thousands of genes [32]. Genes that are upregulated or downregulated in response to drug treatment can be identified to help to clarify drug–response mechanisms. In spite of being a potential and popular tool for investigating the mechanism of drug action, the cDNA microarray technology has its own limitation in that proteins with altered activity before and after drug application cannot be identified. Indeed, such approach did not pinpoint Src and FAK as the molecular targets of curcumin [33]. Undoubtedly, integration of these complementary results derived from different methodology provides a foundation on which a more through understanding of curcumin action can be built.

Curcumin inhibits a variety of cellular protein activities and exhibits minimal systemic cytotoxicity in animal and human studies [34,35]. Since overexpression and/or

enhanced activity of both Src and FAK have been implicated in human tumors [4,36,37], the therapeutic potential of curcumin in the treatment of advanced metastatic tumors merits further investigation.

Acknowledgments

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