Journal of Cellular Biochemistry 96:155–169 (2005)

Garcinol Modulates Tyrosine Phosphorylation of FAK and Subsequently Induces Apoptosis Through Down-Regulation of Src, ERK, and Akt Survival Signaling in Human Colon Cancer Cells

Chiung-Ho Liao,[1] Shengmin Sang,[2] Chi-Tang Ho,[2] and Jen-Kun Lin[1]*

[1]Institute of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, Taipei, Taiwan
[2]Department of Food Science and Center for Advanced Food Technology, Rutgers University, New Brunswick, New Jersey 08901-8520

Abstract Garcinol, from the fruit rind of *Garcinia indica* and other species, has been reported to suppress colonic aberrant crypt foci (ACF) formation in rats. In this study, we investigate the beneficial effects of tumor prevention by garcinol on the human colorectal cancer cell line, HT-29. Focal adhesion kinase (FAK) is the major signaling mediator of integrin-mediated cell–matrix contact-regulated cellular proliferation, migration, and apoptosis in adherent cells. Results of Matrigel analysis show that exposure of HT-29 cells to 10 μM garcinol inhibited cell invasion, and decreased the dose-dependent tyrosine phosphorylation of FAK. We further demonstrate by Western blot analysis that garcinol inhibited activation of the Src, MAPK/ERK, and PI3K/Akt signaling pathways. To investigate whether the loss of integrin-mediated cell–matrix contact can induce apoptosis, we demonstrate that garcinol induced it in HT-29 cells. The apoptotic dose of garcinol (20 μM) changed the ratio of the anti-apoptotic Bcl-2 and proapoptotic BAX proteins within 12 h, which correlated with a release of cytochrome c from the mitochondria to the cytosol, and with PARP cleavage. Additionally, we demonstrate that a decreasing MMP-7 protein level in HT-29 cells results in sensitization to garcinol. Garcinol also significantly inhibited the expression of MMP-7 in IL-1β-induced HT-29 cells. These results suggest that garcinol reduces cell invasion and survival through the inhibition of FAK's downstream signaling. J. Cell. Biochem. 96: 155–169, 2005.

Key words: FAK; apoptosis; HT-29 human colorectal cancer cell line; ERK; Akt

Colorectal cancer (CRC) is one of the most common human cancers, and more than 945,000 new cases were diagnosed worldwide in 2000 [Edwin, 2003]. Overall survival for CRC at 5 years is about 50%, and most of these patients will develop liver metastases [Parkin et al., 2001]. Reducing tumor development and preventing metastases should improve the prognosis of most colorectal cancers [Hasegawa et al., 1998; Yang et al., 2003]. Recently, a standard chemotherapeutic agent, 5-fluorouracil, has been improved to induce apoptosis in colorectal cancer with biochemical modification [Bunz et al., 1999]. Non-steroidal anti-inflammatory drugs (NSAID), like sulindac, are prototypic chemopreventive agents and inhibit cyclooxygenases through reducing cell proliferation, inducing apoptosis, or modulating angiogenesis in CRC [Janne and Mayer, 2000; Smith et al., 2000].

Focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase located in the focal adhesion complex, has been implicated in the integration of signals from integrins and neuropeptides and plays an anti-apoptotic role in anchorage-dependent cells [Sonoda et al., 2000]. The carboxy-terminal tyrosine residue (Tyr^{397}) of FAK, constitutes a major site of

Grant sponsor: National Science Council; Grant numbers: NSC93-2311-B-002-001, NSC93-2320-B-002-111, NSC93-2320-B-002-127.

*Correspondence to: Dr. Jen-Kun Lin, Institute of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-ai Road, Taipei 10018, Taiwan. E-mail: jklin@ha.mc.ntu.edu.tw

Received 27 December 2004; Accepted 30 March 2005
DOI 10.1002/jcb.20540

phosphorylation, appears important for the tyrosine phosphorylation of focal complex associated proteins, and creates a high-affinity binding site recognized by the SH-2 domain of the Src family. The recruitment and activation of Src through the formation of a bipartite kinase complex result in activation of the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway [Parsons et al., 2000]. In addition, Akt is serine-phosphorylated following the tyrosine phosphorylation of FAK. Recent studies have indicated that many colon cancer cell lines overexpress both Src and FAK, and the subsequent inhibition of FAK phosphorylation causes apoptosis in colon cancer cell lines through blocking the Akt survival pathway and activating caspase-3 [Golubovskaya et al., 2003]. Several downstream targets of Akt have also been identified as components of the cell's anti-apoptotic machinery, including Bcl-2 family members and the cell death enzyme caspase 9 [Cardone et al., 1998; Asnaghi et al., 2004]. The phenolic antioxidant caffeic acid phenethyl ester (CAPE) has been demonstrated to affect the tyrosine phosphorylation of FAK in human colorectal cancer cells, a change which reduced cell invasion and induced apoptosis [Weyant et al., 2000]. Thus, inhibition of FAK tyrosine-phosphorylation was expected to regulate Akt/caspase-9 pathway and decrease cell survival in human colorectal cancer cell line, HT-29. The cell line was chosen for this study because of its APC-null phenotype, the typically found in both sporadic and heritable forms of colon cancer.

In anchorage-dependent cells, growth factor-deprivation-induced apoptosis implies association with the proteolytic cleavage of FAK by caspase-3, which is thought to be fundamental to successful cancer treatment. Accordingly, the mitochondria play an essential role in the apoptotic death of mammalian cells, which includes the release of proteins normally stored in the intermembrane space, like cytochrome c, into the cytoplasm, which catalyses the activation of caspase-9 to subsequently cleave the effector caspase-3 and -7 [Lawen, 2003]. It was subsequently reported that the anti-apoptotic subfamily, with members such as Bcl-2 and Bcl-x_L, inhibits the cell death induced by various stimuli [Adams and Cory, 1998]. Otherwise, the multi-domain proapoptotic subfamily, with members such as Bax and Bak, is also needed to induce cell death and mediates cytochrome c release [Tsujimoto, 2003].

Garcinol (camboginol), a polyisoprenylated benzophenone derivative, is present in Guttiferae (*Garcinia indica*, *Garcinia huillkensis*, and *Garcinia cambogia*). *Garcinia* is a rich source of secondary metabolites including xanthones, flavanoids, benzophenones, lactones, and phenolic acids. Garcinol, containing both phenolic hydroxyl groups and a β-diketone moiety, is a potent anti-oxidant [Yamaguchi et al., 2000a; Sang et al., 2002]. Recently, garcinol has attracted considerable interest because of its health promoting properties, including antiulcer activities [Yamaguchi et al., 2000b] and suppression of colonic aberrant crypt foci (ACF) formation [Tanaka et al., 2000]. In our previous data, garcinol displayed the ability to inhibit nitric oxide (NO) generation in macrophages [Sang et al., 2001; Liao et al., 2004]. To further characterize the chemopreventive efficacy of garcinol, we treated HT-29 cells with it. Several observations made thereafter suggested that some chemopreventive compounds modulated the loss of integrin-mediated cell–matrix contact and induced apoptosis in human colon carcinoma cells [Weyant et al., 2000]. Here, we use human colon carcinoma cells (HT-29) to examine the effects of garcinol on FAK tyrosine phosphorylation in the pathway of the survival signal.

We found that garcinol can reduce FAK tyrosine-phosphorylation and induce apoptosis in HT-29 cells through the mitochondria-mediated pathway. Upon the tyrosine phosphorylation inhibition of FAK, garcinol down-regulated the p42/p44 MAPK and PI3K/Akt signaling pathway. In addition, the proteolytic degradation of extracellular matrix by matrix metalloproteinase (MMP) has been considered an essential element in tumor invasion in human colorectal cancers. We have also demonstrated that reduction of proteolytically active MMP-7 is involved in garcinol mediated-cell invasion and apoptosis. Our results suggest that garcinol, a chemopreventive agent, moderately prevents colon cancer cell invasion and induces apoptosis.

MATERIALS AND METHODS

Cells and Reagents

HT-29 human colorectal cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan,

UT). Cells were grown in monolayers and incubated at 37°C in 10% CO_2. Garcinol was synthesized as described [Sang et al., 2002], and its structure is shown in this reference. Recombinant human IL-1β was purchased from R & D (R & D Systems Inc., Minneapolis, MN).

Immunoprecipitation and Western Blot

Total cellular extracts were prepared using lysis buffer containing 10% glycerol, 1.0% Triton X-100, 1.0 mM $NaHPO_3$, 137 mM NaCl, 10 mM NaF, 1.0 mM EGTA, 5.0 mM EDTA, 20 mM Tris-HCl, 100 μM β-glycerophosphate, 1.0% sodium dodecyl sulfate, 1.0 mM Na_3VO_4, and protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). For immunoprecipitation, the cell lysates (500 μg protein) were precleared with protein-A/G agarose bead at 4°C for 10 min followed by incubations with 5 μg anti-FAK or anti-phosphotyrosine (4G10) antibodies for 2 h and 6 μg rabbit anti-mouse IgG for 30 min. The immune complexes were recovered by a 16 h incubation with protein-A/G agarose bead and then washed twice in wash buffer and boiling in reducing SDS sample buffer. For Western blotting analysis, equal amounts of total cellular protein (30–50 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto Immobilon polyvinylidene difluoride membranes (Millipore, MA). The indicated primary antibodies containing anti-FAK and anti-Phospho-FAK (Y379) were purchased from Transduction Laboratories (San Diego, CA). The anti-caspase-3, anti-caspase-9, anti-PARP, anti-cytochrome c antibodies, and anti-MMP-7 (matrilysin) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Oncogene (Boston, MA). The anti-phospho-ERK1/2 (Thr202/ Tyr204) and anti-phospho-Akt (Ser473) antibodies from Cell Signaling Technology (Beverly, MA) were used to determine the levels of phosphorylated proteins. The blots were revealed by chemiluminescence (ECL kits, Amersham Pharmacia Biotech, Taiwan). Band intensities were quantitated by densitometry (IS-1000 Digital Imaging System). Each blot was performed in duplicate or triplicate. The ratios of phosphorylated protein to total protein were determined and normalized to 1.0 for control cells. Two-tailed *t*-tests were used to assess statistical significance.

DNA Fragmentation Analysis

Cells were harvested, washed with ice-cold PBS, and lysed in 50 μl lysis buffer (5 mM Tris-HCl pH 8.0, 10 mM EDTA, and 0.5% Triton X-100) on ice for 20 min. Then, the cell lysate was centrifuged at 12,000 rpm for 20 min. The supernatant was treated with RNase A (100 μg/ml) at 37°C for 60 min and Proteinase K (200 μg/ml) at 50°C for 120 min. Then, the DNA was extracted by phenol/chloroform before loading and analysis by 1.8% agarose gel electrophoresis. The gels were run at 50 V for 120 min in TBE (Tris-borate/EDTA) buffer. Approximately 20 μl DNA was loaded, stained by ethidium bromide, and visualized under UV light.

Flow Cytometry

The cells were harvested and fixed in 75% ethanol at −20°C for at least 1 h. After centrifugation at 1,000 rpm for 5 min, cell pellets were resuspensed in 0.5 ml PBS buffer with 0.5% Triton X-100 and 100 μg/ml RNase A and incubated at 37°C for 30 min. Propidium iodide (50 μg/ml) was added, and the mixture was allowed to stand on ice. Fluorescence emitted from the PI–DNA complex (564–606 nm) was examined using argon laser excitation at 488 nm by a FACScan flow cytometer (Becton-Dickinson, Franklin Lake, NJ). The effect of various modulators on garcinol and its derivative-triggered apoptosis was analyzed and quantified by FACS in sub-G_1 phase. Annexin V-FITC and propidium iodide double-staining of HT-29 cells was also measured using an annexin V-FITC apoptosis kit (Boehringer, Mannheim, Germany) by a FACScan flow cytometer (Becton-Dickinson). For each sample, ~10,000 cells were analyzed, and results were presented as dot plots where the FL1 and FL3 values indicated the intensity of annexin-V-FITC and propidium iodide staining, respectively. The CellQuest (Becton-Dickinson) software was used to analyze results.

Measurement of Caspase Activity

Caspase activities of cell lysate with various indicated times (0, 1, 3, 6, 12, 24 h) were determined by a fluorogenic assay. Cells, triggered by testing compounds, were lysed with buffer containing 25 mM HEPES pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 0.1M NaCl, and 5 mM dithiothioine. Then 50 μg of total protein was incubated with 25 μM substrate acetyl-Asp-Glu-

Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) of caspase-3 at 30°C for 1 h. The fluorescence of released 7-amino-4-methylcoumarin (AMC) was measured by excitation at 360 nm and emission at 460 nm using a fluorescence spectrophotometer (Hitachi F4500).

Invasion Assay

HT-29 cells were subjected to the standard treatment conditions (see above), incubated for 24 h, and then trypsinized. The invasive ability of the cells was assayed in transwell chambers (Costar, Cambridge, MA). The cells (5×10^4) in low serum DMEM medium were placed on the Matrigel-coated polycarbonate filters of the invasion assay plates (8-mm wells, 8-mm pores). Conditioned medium from NIH3T3 cells, supplemented with 10% fetal calf serum, was placed in the bottom chambers as a chemoattractant. Plates were incubated at 37°C for 24 h. Cells that migrated to the bottom compartment were stained with hematoxylin and counted under a light microscope. Each assay was performed in triplicate.

RESULTS

Garcinol Caused Morphological Change and Inhibited Invasion of Human Colon Cancer Cells

The effect of garcinol on cell survivals was investigated in human colorectal cancer cell line HT-29. As shown in Figure 1A, these cells were placed in medium with 5% serum containing 10 μM garcinol and incubated for 24 h. The morphological change upon garcinol treatment was visualized by phase-contrast microscopy, in which cells became round. The propidium iodide staining that is sensitive to DNA was used to assess changes in nuclear morphology following garcinol treatment. The nuclei in garcinol-treated cells, similar to those in normal cells, exhibited diffused staining of the chromatin. Figure 2B shows the flow cytometric analysis of garcinol-treated (10 μM) HT-29 cells within 48 h. The M1 area was quantitated as a sub-G_1 peak. The sub-G_1 peak area was 0.56% at 30 min, 6.28% at 24 h, and 21.2% at 48 h. Garcinol did not cause apparent cytocity at the concentration of 10 μM for 24 h. However, after exposure to 10 μM garcinol for 48 h, a marked increase of the sub-G_1 peak was observed in HT-29 cells. To further characterize the effects of garcinol in human colorectal cancer cells, we measured the migration of HT-29 cells through Matrigel. HT-29 cells treated with garcinol (2.5, 5, 10 μM) for 24 h were placed on top of Matrigel-coated polycarbonate filters, with conditioned medium from NIH3T3 cells (supplemented with 10% calf serum) in the chambers below. After a further incubation of 24 h, cells that succeeded in migrating to the bottom compartment were stained with hematoxylin and counted under a light microscope (Fig. 1C). In the study, garcinol displays an inhibition of cell growth and migration in HT-29 colon cancer cells.

Garcinol Decreased the Tyrosine Phosphorylation of FAK and Inhibited the Formation of Src Family PTK Signaling Complex

Due to the dramatic inhibitory effect of garcinol on cell invasion, we attempted to determine whether garcinol regulates focal adhesion-associated signaling protein. As shown in Figure 2A, cells were treated with garcinol (2.5, 5, 10 μM) in the medium containing 5% serum. Immunoprecipitation (IP) was performed using an anti-phosphotyrosine antibody (clone 4G10) and anti-FAK antibody, followed by immunoblotting with an anti-FAK antibody and anti-phosphotyrosine antibody, respectively. The phospho-tyrosyl protein level of 125 kDa FAK was elevated by serum addition for 30 min, and a dose-dependent reduction was observed after treatment with 10 μM garcinol in HT-29 cells. Moreover, garcinol (10 μM) blocked the phosphorylation at Tyr^{397} of FAK protein in a time-course manner (Fig. 2B). Following the serum addition, FAK was auto-phosphorylated at Tyr^{397} within 1 h. As expected, exposure of HT-29 cells to garcinol decreased FAK phosphorylation for 30 min. Garcinol was found to significantly reduce the amount of phospho-FAK at time points ranging from 30 min to 12 h, and the reduction percentage reached 49% at the dose of 10 μM for 1 h. Recent study has indicated that integrin-induced auto-phosphorylation of tyrosine 397 creates a high-affinity binding site for the Src family kinases via their SH2 domains and also for the p85 regulatory subunit of PI 3-kinase [Jones et al., 2000]. To examine events downstream of FAK, we next determined the effect of garcinol on the steady-state levels of FAK and other proteins that associate with this kinase. As shown in Figure 2C, cell lysates were immunoprecipitated by anti-FAK antibody and immunobloted

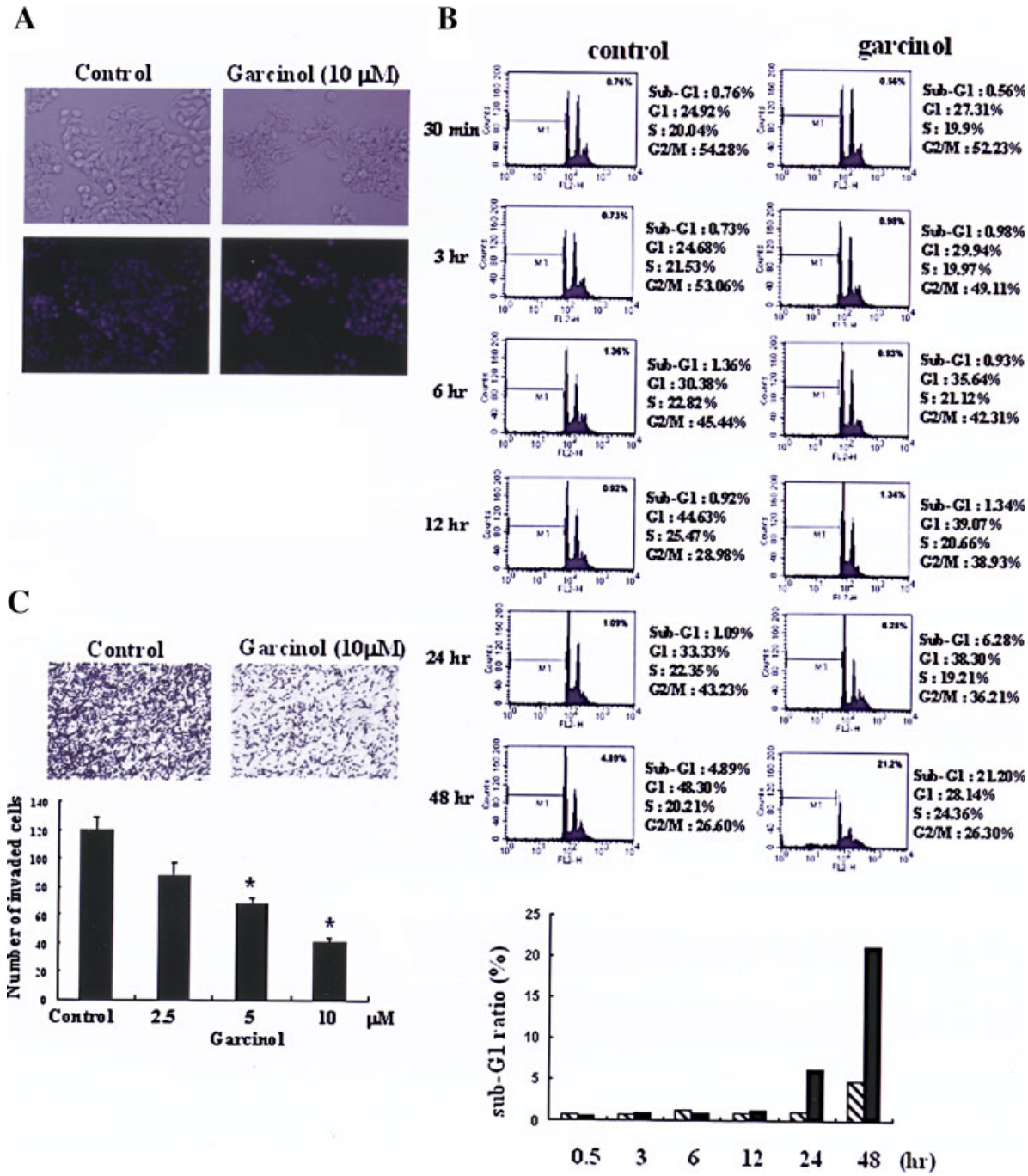

Fig. 1. Garcinol induces changes in cell morphology and inhibits Matrigel invasion in HT-29 cells for 24 h. HT-29 cells were subjected to treatment with garcinol in 5% serum conditions. **A**: Garcinol (10 μM) was used to treat HT-29 cells for 24 h. The cells were observed by light-microscopy, and the nuclei of HT-29 cells were stained by propidium iodide. **B**: Determination of sub-G_1 cells in control and garcinol treated HT-29 cells were quantitated by flow cytometry. HT-29 cells were exposed to 10 μM garcinol for the indicated times (0.5, 3, 6, 12, 24, and 48 h, **right panel**) and were treated with DMSO only as control (**left panel**). The method of flow cytometry used is described in ''Materials and Methods.'' **C**: After treatment with garcinol for 24 h, the invasive capacity of HT-29 cells was analyzed in transwell chambers. Matrigel invasion assay procedure was described in ''Materials and Methods.'' Results shown are from a representative experiment and were reproduced in three other identical experiments. Data were mean ± SD. $^*P < 0.001$ as compared to control cells, maintained in the absence of garcinol. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

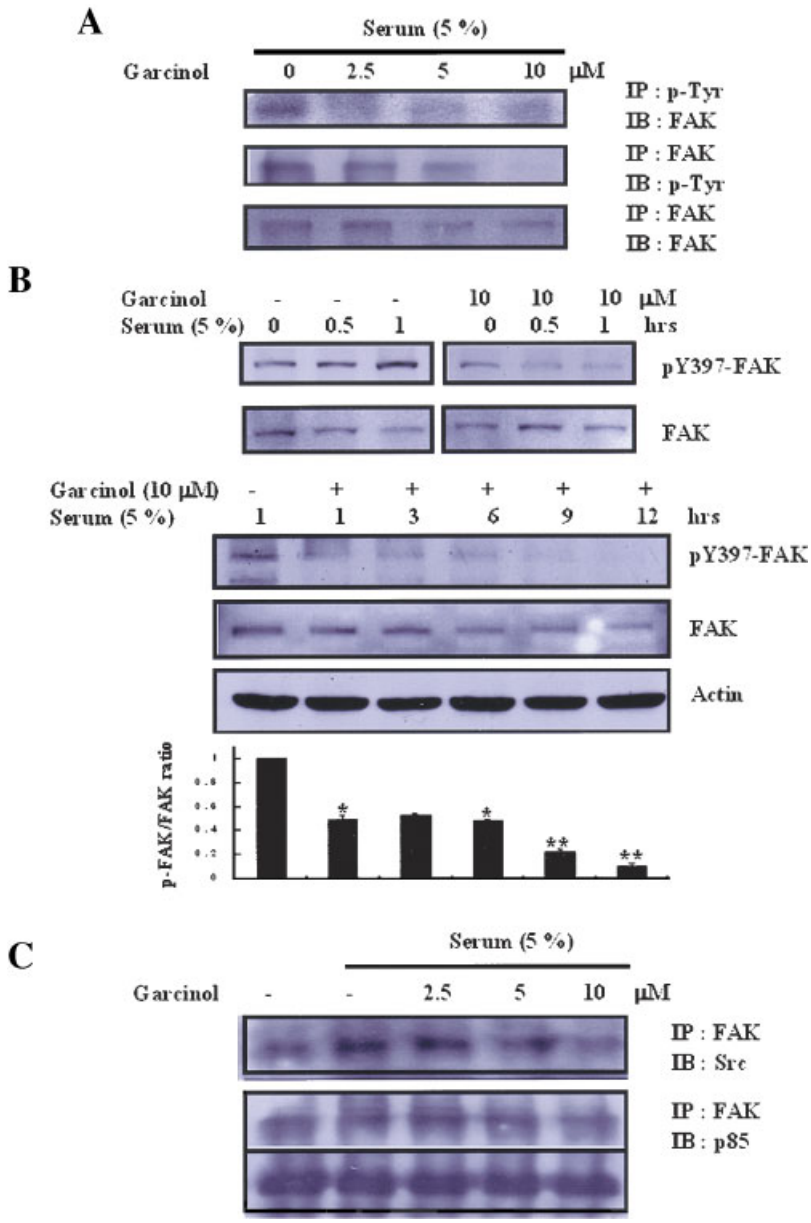

Fig. 2. Garcinol inhibits tyrosine phosphorylation of focal adhesion kinase (FAK), and disrupted PTK signaling complex in colorectal cancer cells. **A**: HT-29 cells were treated with garcinol for 30 min in a dose-dependent manner (2.5, 5, 10 μM), and cell lysates were prepared for Western blot analysis. The amount of protein (500 μg) from each treatment was immunoprecipitated using the anti-phosphotyrosine beads or anti-FAK antibody and immunobloted using the anti-FAK or anti-phosphotyrosine antibody. **B**: Subsequently, the eluted proteins from the time-dependent treatment (0.5, 1, 3, 6, 9, and 12 h) of 10 μM garcinol were immunobloted to determine the level of phosphorylated-FAK protein on Tyr^{397} residue. The tyrosine phosphorylation of FAK was resolved by SDS–PAGE for immunobloting analysis by anti-phospho-FAK (pY397), and detection of FAK was used as a standard of protein quantities analyzed. A significant association was observed between the time-dependent treatment of garcinol and a decrease in HT-29 cells (*$P < 0.001$). **C**: Cells, serum-starved for 24 h, were treated with garcinol in a dose-dependent manner (2.5, 5, 10 μM) in medium supplemented with 5% FCS. The amount of protein (500 μg) from each treatment was immunoprecipitated using the anti-FAK antibody and immunobloted using the anti-p85 or anti-Src antibody. The experiments were performed three times with similar results. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

by anti-Src or anti-p85 antibody. At 1 h following serum treatment, Src and p85 associate with FAK (Fig. 2C, lane 2). The association between FAK and Src was disrupted when garcinol was applied in a dose-dependent manner (2.5, 5, 10 μM) for 1 h to HT-29 cells. This demonstrated that garcinol produced a decrease in FAK tyrosine phosphorylation, and

suppressed the downstream pathways for proteins associated with the focal adhesion complex.

Garcinol Suppressed the Src Family Kinases and the Phosphorylation of ERK and Akt Signaling Pathway

To determine whether garcinol suppressed Src kinase signaling, we examined the effect of garcinol on the phosphorylation of Src, ERK, and Akt. FAK tyrosine phosphorylation was affected by garcinol treatment in HT-29 cells (Fig. 2). For the experiments, HT-29 cells were serum-starved for 24 h and then treated with garcinol at various concentrations (2.5, 5, 10 μM) in the medium containing 5% serum for 30 min. Cell lysates were immunoprecipitated with anti-phosphotyrosine (4G10) antibody, and phosphorylation of Src was assessed using an anti-Src antibody by Western analysis (Fig. 3A). To further address the molecular mechanisms through which garcinol reduced tyrosine phosphorylation, blotting comparisons of serum-starved and garcinol-treated HT-29 cells in the presence of serum were performed. As shown in Figure 3A, both tyrosine phosphorylations of Src (M_r 60,000) and the 85 subunit of PI3K (M_r 85,000) were immunoblotted by anti-phosphotyrosine antibody. Our results suggested that activations of Src and PI3K were suppressed by garcinol in HT-29 cells. These data support the concept that garcinol-reduced FAK phosphorylation may down-regulate Src-family PTK and PI3K/Akt survival signaling in HT-29 cells. As shown in Figure 3B, the phosphorylation of p42/p44 MAPK was induced by serum addition for 30 min. Immunoblotting with anti-phospho-ERK antibody indicated that garcinol reduced the activation of ERK in a dose-dependent manner (2.5, 5, 10 μM) for 30 min (Fig. 3B). The data also shows that the survival signal, activated-Akt, was blocked by garcinol in a dose-dependent manner. We examined, by means of immunoblotting, the phosphorylation of Akt at Ser^{473} in HT-29 cells, and the result shows that it was inhibited during garcinol (2.5, 5, 10 μM) treatment for 30 min (Fig. 3B).

Garcinol Induced Apoptosis in a Dose-Dependent Manner and Activated Caspase-3 in Colon Cancer Cells

The interaction of FAK with several proteins might lead to survival signals from FAK, suggesting that the binding of PI3-kinase and Src with FAK is required for the anti-apoptotic function of FAK. Previous study has confirmed the notion that the general features of FAK play a role in the lack of apoptosis in various cells [Sonoda et al., 2000]. As demonstrated in Figure 2A, a dose-dependent reduction in the tyrosine phosphorylation of FAK protein was observed in HT-29 cells treated with garcinol (10 μM). To further characterize the apoptosis triggered by garcinol, the HT-29 cells were treated with various concentrations (5, 10, 20, 40 μM) of it for 24 h, and the apoptotic action was confirmed by measuring the presence of phosphatidylserine on the outer cell membrane. Figure 4A shows the results of annexin-V binding experiments to measure the presence of phosphatidylserine on the outer cell membrane revealed approximately 8% and 32% of cells with apoptosis after 20 and 40 μM garcinol treatment, respectively. Our data indicate that 20 μM garcinol has significant effects on HT-29 cell death within 24 h: cells exhibit significant morphological changes and chromosomal condensation (data not shown). Pronounced DNA laddering also occurred (Fig. 4B). The appearance of DNA fragmentation under garcinol treatment in a dose-dependent manner (5, 10, 20, 40 μM) also displayed its ability to induce apoptosis.

To further examine the regulation of caspase activity in the apoptotic process, we analyzed the effects on cleavage of the pro-caspase-3 protein of garcinol in HT-29 cancer cells. Caspase-3 is synthesized as a 32-kDa inactive precursor (pro-caspase-3), which is proteolytically cleaved to produce a mature enzyme composed of 17-kDa fragments. As shown in Figure 5A, pro-caspase-3 protein disappeared concomitantly with apoptosis by garcinol (20 μM) treatment in a time-dependent manner (3, 6, 9 h), and caspase-3 in cleaved form was generated. We also measured caspase-3 protease activity using specific peptide substrates in cells treated with 20 μM garcinol in a time-course manner (Fig. 5B). In contrast to DMSO only as control, caspase-3 activity in HT-29 cells was markedly elevated by garcinol (20 μM) treatment within 24 h. Activation of caspase-3 leads to the cleavage of the effector caspase substrate PARP. Our data shows that garcinol (20 μM) was able to trigger cleavage of PARP (as judged by the appearance of the p85 PARP cleavage product) in a time-dependent manner (Fig. 5C).

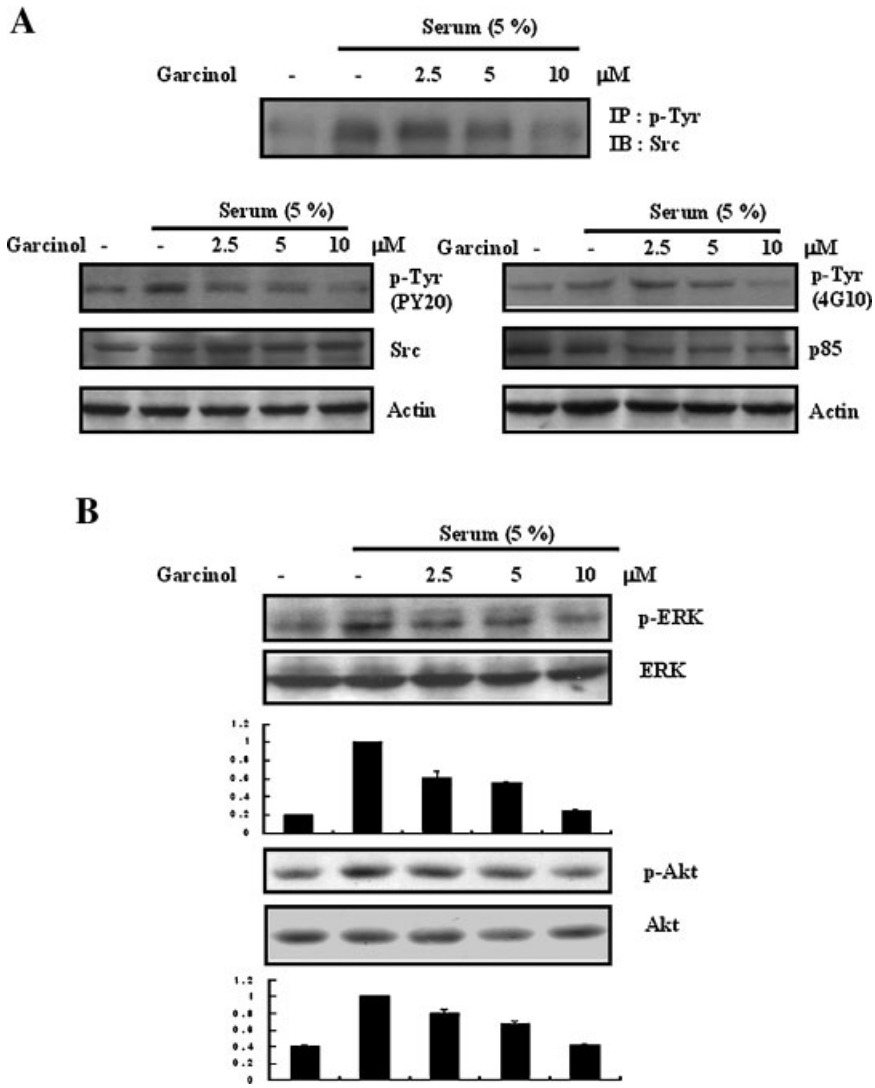

Fig. 3. Reduced tyrosine phosphorylation of FAK associated protein, Src and p85, by garcinol treatment results in decreased serum-stimulated ERK and Akt activation. **A**: HT-29 cells were treated with 2.5, 5, 10 μM garcinol (**lanes 3–5**) for 30 min in medium supplemented with 5% FCS or serum-starved (**lane 1**) for 16 h. Total cell lysates were immunoprecipitated with anti-phosphotyrosine (PY20) antibody and immunobloted using the anti-Src antibody. The effects of garcinol on tyrosine phosphorylation of Src and 85 subunit of PI3K were further assessed by SDS–PAGE for immunobloting analysis using anti-phosphotyrosine (PY20) and anti-phosphotyrosine (4G10) antibody, respectively. The blots were stripped and reprobed with antibody against Src and p85, respectively. **B**: HT-29 cells were treated with 2.5, 5, 10 μM garcinol (**lanes 3–5**) for 30 min in medium supplemented with 5% FCS, or serum-starved (**lane 1**) for 16 h. Whole cell lysates were prepared and analyzed for p42/p44 MAPK and Akt phosphorylation by Western blot using phospho-p42/p44 MAPK and phospho-Akt (Ser473) antibodies, respectively. The blots were stripped and reprobed with antibody against total p42/44 MAPK and Akt, respectively. Values shown were the mean ± SD of at least three independent experiments and were expressed as the percentage of the maximal decrease in ERK, Akt phosphorylation value.

Garcinol Influenced the Mitochondria-Dependent Apoptosis Signaling

In order to further investigate the activation of mitochondria-disruption processes under garcinol treatment in HT-29 human colon cancer cells, the caspase-8 substrate Bid was analyzed by Western blot. As shown in Figure 6A, the full-length Bid protein had decreased at the analyzed time point (0.5, 1, 2, 4, and 6 h) during garcinol treatment (20 μM). Thus, treatment of HT-29 cells with 20 μM garcinol for 9 h caused cytochrome c to be released from mitochondria to cytoplasm (Fig. 6B). We next examined the cleavage of pro-caspase-9 during treatment of HT-29 cells

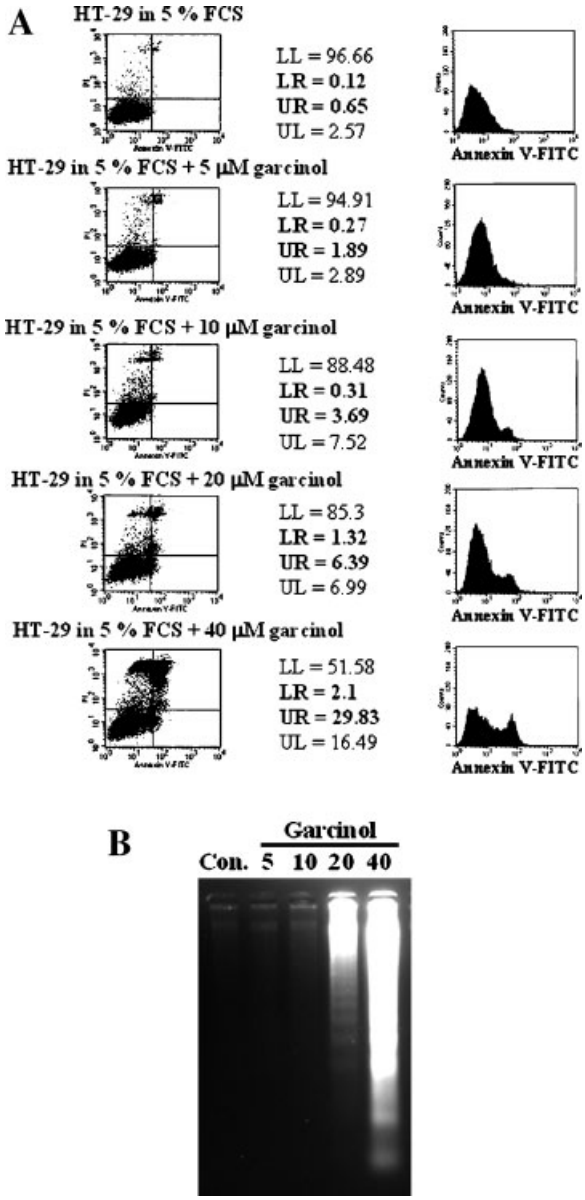

Fig. 4. Determining cell viability of garcinol-treated HT-29 cells by annexin V-FITC and propidium iodide double-staining and detection DNA fragmentation by gel electrophoresis. **A**: Flow cytometric histograms of HT-29 cells were examined at different concentrations of garcinol (5, 10, 20, 40 μM) for 24 h. Healthy cells are in the bottom left quadrant, apoptotic cells are in the bottom right quadrant, and necrotic and late apoptotic cells are in the upper right. Numbers in each quadrant are percentage of cells they contain. Annexin V-FITC was used to bind phosphatidylserine and was recorded on FL1-H filters (**right panel**). **B**: Cells were incubated with different doses (5, 10, 20, and 40 μM) of garcinol for 24 h. The DNA fragmentation was analyzed by agarose gel. The experiments were performed three times with similar results.

with garcinol (20 μM). A time-dependent proteolytic cleavage of pro-caspase-9 was observed (Fig. 6C). These data suggest that mitochondrial dysfunction causes cytochrome c to be released into the cytosol and then induces the activations of caspase-9 and caspase-3. To further identify changes in protein levels of the Bcl-2 family in the mitochondria-dependent apoptosis signaling, we treated the cells with garcinol (20 μM) at various time points. As shown in Figure 6D, the Bcl-2 level decreased following a 0–12 h incubation with garcinol (20 μM). The protein level of pro-apoptotic Bax did not change during garcinol treatment, and remained constant throughout the experiments at the analyzed time point. Our data supports the notion that garcinol induces HT-29 cell death through the mitochondria-dependent pathway.

Garcinol Decreased the Protein Levels of Proteolytically Active MMP-7 to Regulate Cell Survival in Colon Cancer Cells

Cell–extracellular matrix attachment has been shown to play a primary role in the regulation of cell invasion and survival in different cellular models. Migration of malignant cancer cells requires the serial assembly and disassembly of focal adhesion complexes and the expression of matrix metalloproteinases. Recent evidence suggests that matrilysin (MMP-7) also plays a critical role in cell survival and the metastatic pathway of human colorectal cancers [Adachi et al., 1999; Mitsiades et al., 2001]. To determine whether garcinol down-regulates the MMP-7 protein, the HT-29 cells were treated with it in a dose-dependent manner for 24 h. As shown in Figure 7A, treatment of cells with 10 μM garcinol reduced the protein level of MMP-7 up to 50%. Interestingly, HT-29 cells were treated with IL-1β (20 ng/ml) in serum-withdrawal conditions, and the expression of MMP-7 protein increased under treatment within 24 h (Fig. 7B, lane 2). In the study, garcinol markedly reduced the expression of MMP-7 upon IL-1β treatment in a concentration-dependent manner, and it shrank to undetectable levels under 5 μM garcinol treatment for 24 h (Fig. 7B, lane 5). To further examine whether the tyrosine phosphorylation of FAK can be induced by IL-1β and the induction of phospho-FAK can be blocked by garcinol, IL-1β treated-HT-29 cells were exposed to garcinol for 30 min at 1.25, 2.5, 5 μM (Fig. 7C). The result indicates that garcinol inhibits the activation of FAK and reduces protein levels of MMP-7 in IL-1β-activated HT-29 cells.

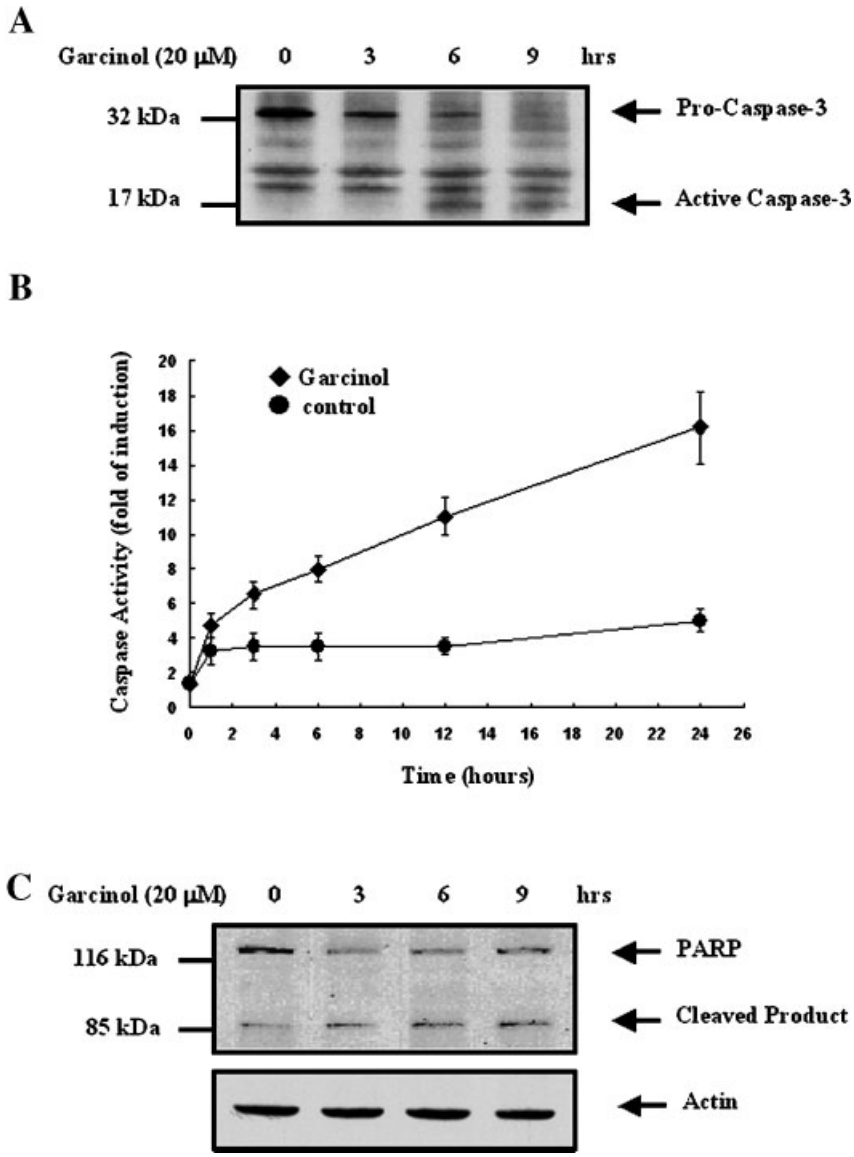

Fig. 5. Garcinol induced the activation of caspase-3 and cleavage of FAK and poly-(ADP-ribose) polymerase (PARP). **A**: Cell lysates, prepared from garcinol (20 μM) treatment for various periods (3, 6, and 9 h), were analyzed by Western blot. The samples were probed with antibody against caspase-3. **B**: Kinetics of caspase-3 were determined by incubation of 100 μg of total protein with fluorogenic substrate, Ac-DEVD-AMC, respectively, for 1 h at 30°C. The release of AMC was measured by emission at 460 nm using a fluorescence spectrophotometer. Results show the mean ± SD of three experiments. **C**: After treatment of cells with 20 μM garcinol for various periods (3, 6, and 9 h), PARP cleavage was analyzed by Western blot. The experiments were performed three times with similar results.

DISCUSSION

Epidemiological studies of colorectal cancer incidence suggest that tumor development can be modulated by dietary factors, with a high intake of fruits and vegetables providing a protective effect [Block et al., 1992]. Here, we are interested in whether garcinol, a polyisoprenylated benzophenone, can reduce the risk of colon carcinogenesis. Our data suggest that FAK plays an important role in protecting against apoptosis and promoting invasion in HT-29 colorectal cancer cells. Garcinol (10 μM) treatment induces morphological changes in HT-29 cells and reduces cell migration according the results of Matrigel analysis. We demonstrate that garcinol has the potential to reduce the activation of FAK and to induce

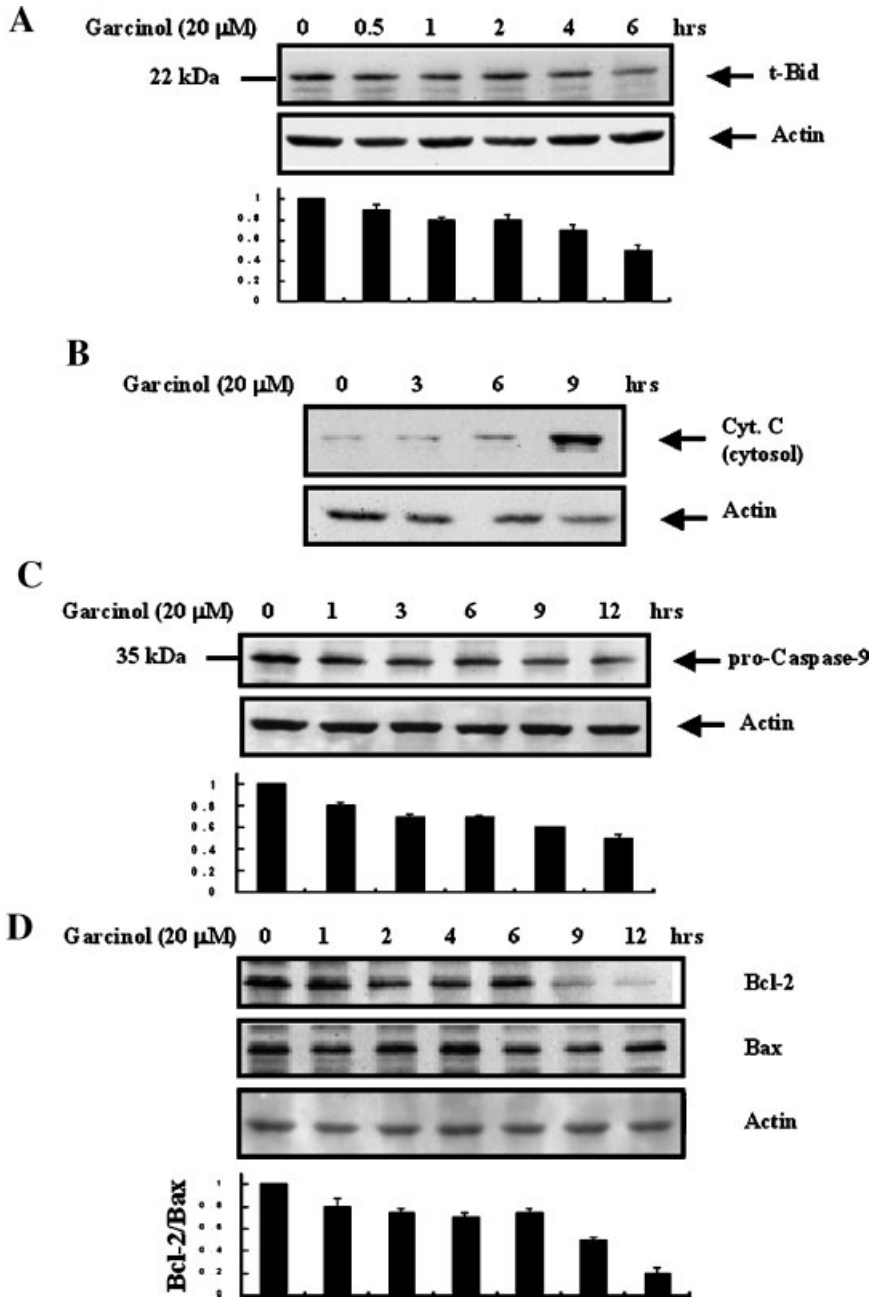

Fig. 6. Induction of cell death by garcinol regulated the Bcl-2 family in mitochondria. **A**: Cells for each timepoint following 20 μM garcinol treatment were analyzed by Western blot. The cell lysates were probed with antibodies against full-length t-Bid. **B**: HT-29 cells were treated with 20 μM garcinol and then cytochrome c was released from mitochondria into the cytosol. The fraction of cytosol protein was detected by anti-cytochrome c antibody. **C**: Caspase-9 processing was induced by garcinol treatment. Total cell lysates were prepared from HT-29 cells and analyzed by Western blot using anti-pro-caspase-9 antibody. **D**: Cells from each time point following 20 μM garcinol were prepared. Total lysates were separated by SDS–PAGE, electro-transferred on nitrocellulous membranes, and then probed with antibodies for the detection of Bcl-2 or Bax. Values shown were the mean ± SD of at least three independent experiments and were expressed as the percentage of the maximal decrease in t-Bid, procaspase-9 protein levels, and the ratios of Bcl-2/Bax.

apoptosis in HT-29 cells. According to the general view, binding of the SH2 domains of Src family kinases and the p85 subunit of PI3K to auto-phosphorylation of FAK on Tyr^{397} residue results in the activation of these kinases, and mediates anti-apoptotic signalings [Jones et al., 2000]. It has been demonstrated that garcinol inhibits tyrosine phosphorylation

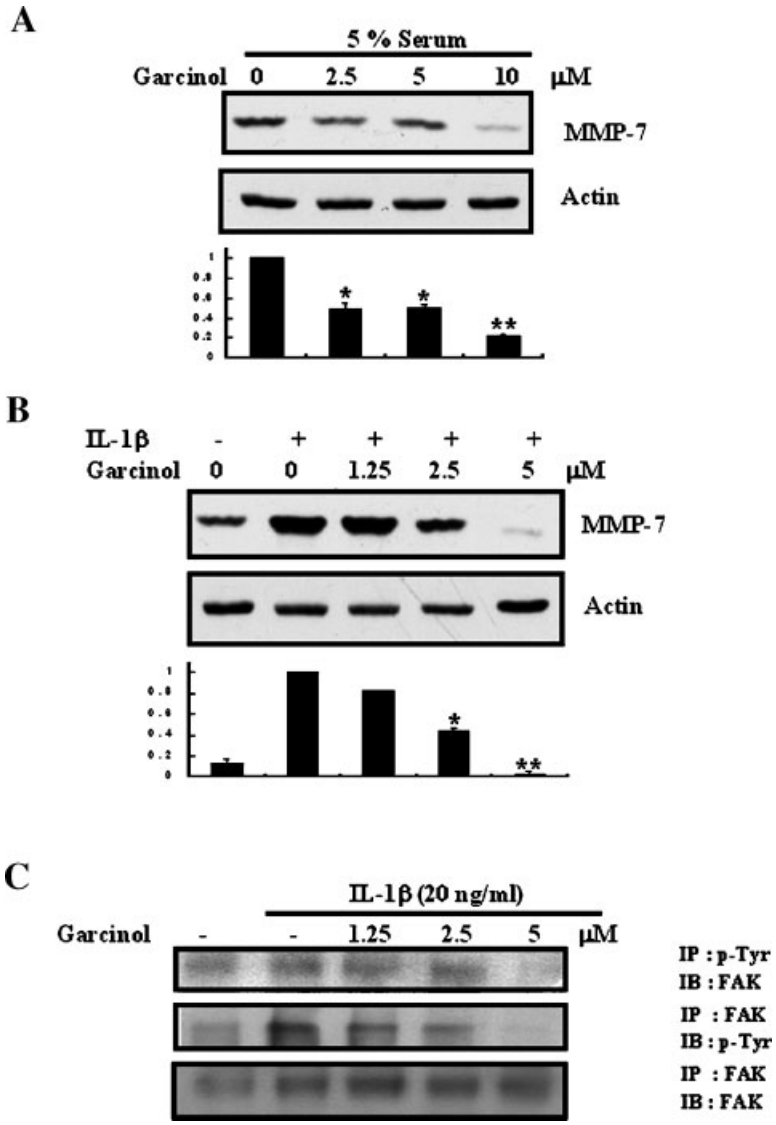

Fig. 7. Garcinol significantly reduced the expression of MMP-7. **A**: Cells were incubated with different doses (2.5, 5, and 10 μM) of garcinol. Effect of garcinol on MMP-7 was examined by Western blot. Garcinol caused a decrease in the protein levels of MMP-7 for the 24-h treatment in the medium containing 5% serum. $^*P < 0.001$ as compared to control cells, maintained in the absence of garcinol. **B**: After treatment with IL-1β (20 ng/ml) for 24 h in serum-withdrawal conditions, the level of MMP-7 protein increased in HT-29 cells. Garcinol (5 μM) showed strong inhibition of the expression of MMP-7. The samples were probed with antibody against MMP-7, and detection of Actin was used as a standard of protein quantities analyzed. Immunoreactive bands were semi-quantified, and the relative protein levels of MMP-7 were established with the ratios MMP-7/Actin. Data were mean ± SD of at least three independent experiments. $^*P < 0.001$ as compared to IL-1β-treated cells, maintained in the absence of garcinol. **C**: HT-29 cells were treated with 20 ng/ml IL-1β and garcinol at various concentrations as indicated for 30 min. The amount of protein (500 μg) from each treatment was immunoprecipitated using the anti-phosphotyrosine beads or anti-FAK antibody and immunobloted by anti-FAK or anti-phosphotyrosine antibodies. The experiments were reproduced in three other identical experiments.

of Src family kinases and the p85 subunit of PI3K (Fig. 3A). Garcinol also inhibits the activation of ERK and Akt, the downstream effectors of FAK, in HT-29 cells (Fig. 3B). Additionally, matrilysin (MMP-7) has also contributed to the in vivo invasive and anti-apoptotic ability of colorectal cancer cells [Mitsiades et al., 2001]. Our data indicate that the level of proteolytically active MMP-7 in the colorectal cancer cell line HT-29 is reduced under garcinol treatment. We have observed that IL-1β-induced overexpression of MMP-7 in

HT-29 cells is completely suppressed by 5 μM garcinol.

To determine the effect of garcinol on the prevention of cancer, we chose various cancer cell lines and normally primary cultured cells to examine cell viability. We found that garcinol had more potential to disrupt colon cancer cells ($IC_{50} = 22$ μM) than other derivatives, like GDPPH-1, GDPPH-2, and cambogin. Garcinol showed a slight effect on rat cortical neuron from primary culture ($IC_{50} = 34$ μM) and on NIH3T3 mouse fibroblast cells ($IC_{50} = 69$ μM). However, other oxidative compounds derived from garcinol, such as GDPPH-1 and cambogin, significantly induced cell death in rat primary cortical neuron and fibroblast ($IC_{50} = 6$ μM). Our data indicate that garcinol has more potential as a chemopreventive agent than either its derivatives or curcumin.

Recent study has asserted that some chemopreventive drugs, such as CAPE and sulindac, reduce the risk of colorectal cancer development and invasion through the inhibition of FAK-mediated signaling pathways [Weyant et al., 2000]. The tyrosine 397 of FAK is in an autophosphorylation position, a high-affinity binding site for Src homology 2 (SH2) domains of Src family kinases and also for the p85 regulatory subunit of PI3K [Jones et al., 2000]. Consequently, downstream signaling is stimulated by the creation of additional phosphotyrosine binding, including that of Grb2, which links integrin engagement to the Ras/MAPK pathway and the Akt/PKB pathway [Liu et al., 2002]. In our study, garcinol displays inhibitive effects on the survival of the Ras/MAPK and Akt signaling pathways following FAK autophosphorylation, and activation of caspase-3 leads to apoptosis in HT-29 colon carcinoma cells.

Additionally, we demonstrated that garcinol treatment had not only down-regulated the anti-apoptotic Bcl-2 protein and pro-apoptotic Bax protein, but had also cleaved the substrate of caspase-8, the pro-apoptotic Bid protein (Fig. 6) that resulted in cell apoptosis (Fig. 4). Accordingly, mitochondria play an important role in cytochrome c being released to the cytosol and inducing caspase-9-dependent activation of caspase-3 in activation of the cell death stage. Our data show that both caspase-9 activation and cytochrome c release happened within 9 h of 20 μM garcinol treatment (Fig. 6). Caspase-3 activation and PARP cleavage then occurred in garcinol-induced apoptosis. The induction of apoptosis by garcinol in HT-29 colorectal cancer cells takes place through the mitochondrial-disruption process.

The ability to inhibit tumor formation during both the initiation and post-initiation phases of carcinogenesis may be required for a chemopreventive agent to be effective. In the mouse model, previous studies indicate that effective agents modulate certain stroma–enterocyte interactions early in the promotion or progression phases of tumorigenesis and decrease FAK protein levels and the relative tyrosine phosphorylation of this kinase [Xu et al., 1996; Reddy et al., 1999]. We further examined MMP-7 because ECM–enterocyte interactions play an important role in early stages of intestinal carcinogenesis. The tumor formation process was prevented by elimination of the matrix metalloproteinase gene, MMP-7 [Hasegawa et al., 1998; Masaki et al., 2001]. MMP-7 is produced from malignant tumor cells such as colorectal, breast, lung, prostate, and liver cancer cells at their earliest stage. MMP inhibitors have been shown to inhibit tumor growth and spread and to decrease the liver metastatic potential of colorectal cancer cells [Wang et al., 1994]. We have established that garcinol hindered the invasive ability of HT-29 cells at the concentration of 5 μM for 24 h. MMP-7 protein levels in the human colorectal cancer cell line HT-29 were down-regulated by garcinol treatment (10 μM). To further examine the protein level of MMP-7 in HT-29 cells, we utilized IL-1β to induce the over-expression of MMP-7 protein while garcinol suppressed it. These findings lead us to conclude that garcinol decreases the malignant potential of colorectal cancer cells by reducing their capability to bind to ECM, with a consequent decrease in the proliferative activity and synthesis of MMP-7. A decrease in the protein level of MMP-7 by garcinol may also contribute to the cell death of human colon carcinoma cells.

Recent evidence has indicated that modulation of integrin-mediated signaling, reflected by FAK phosphorylation status and FAK protein levels, by plant phenolics can trigger apoptosis in colorectal cancer cells [Weyant et al., 2000]. Garcinol has been reported as a chemopreventive agent and potential antioxidant [Tanaka et al., 2000]. We have observed its modulation activity on FAK signaling and cell survival in the human colon carcinoma cell line HT-29. Our

data also indicate that garcinol attenuates the tumor cell expression of MMP-7, which may reduce the proteotic cleavage of FasL and enhance the involution of HT-29. The specific inhibition of MMP-7 by garcinol may also have potential in chemotherapy. In summary, our findings suggest that garcinol treatment reduces the risk of colorectal cancer proliferation and invasion.

REFERENCES

Adachi Y, Yamamoto H, Itoh F, Hinoda Y, Okada Y, Imai K. 1999. Contribution of matrilysin (MMP-7) to the metastatic pathway of human colorectal cancers. Gut 45:252–258.

Adams JM, Cory S. 1998. The Bcl-2 protein family: Arbiters of cell survival. Science 281:1322–1326.

Asnaghi L, Calastretti A, Bevilacqua A, D'Agnano I, Gatti G, Canti G, Delia D, Capaccioli S, Nicolin A. 2004. Bcl-2 phosphorylation and apoptosis activated by damaged microtubules require mTOR and are regulated by Akt. Oncogene 23(34):5781–5791.

Block G, Patterson B, Subar A. 1992. Fruit, vegetables, and cancer prevention: A review of the epidemiological evidence. Nutr Cancer 18:1–29.

Bunz F, Hwang PM, Torrance C, Waldman T, Zhang Y, Dillehay L, Williams J, Lengauer C, Linzler KW, Vogelstein B. 1999. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. J Clin Invest 104:263–269.

Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. 1998. Regulation of cell death protease caspase-9 by phosphorylation. Science 282(5392):1318–1321.

Edwin CD. 2003. Development of ZD1839 in colorectal cancer. Semin Oncol 30(3, Suppl 6):17–22.

Golubovskaya VM, Gross S, Kaur AS, Wilson RI, Xu LH, Yang XH, Cance WG. 2003. Simultaneous inhibition of focal adhesion kinase and src enhances detachment and apoptosis in colon cancer cell lines. Mol Cancer Res 1: 755–764.

Hasegawa S, Koshikawa N, Momiyama N, Moriyama K, Ichikawa Y, Ishikawa T, Mitsuhashi M, Shimada H, Miyazaki K. 1998. Matrilysin-specific antisense oligonucleotide inhibits liver metastasis of human colon cancer cells in a nude mouse model. Int J Cancer 76:812–816.

Janne PA, Mayer RJ. 2000. Chemoprevention of colorectal cancer. Engl J Med 342:1960–1968.

Jones RJ, Brunton VG, Frame MC. 2000. Adhesion-linked kinases in cancer; emphasis on Src, focal adhesion kinase and PI 3-kinase. Eur J Cancer 36:1595–1606.

Lawen A. 2003. Apoptosis—An introduction. BioEssays 25: 888–896.

Liao CH, Sang S, Liang YC, Ho CT, Lin JK. 2004. Suppression of inducible nitric oxide synthase and cyclooxygenase-2 in downregulating nuclear factor-kappa B pathway by Garcinol. Mol Carcinogenesis 40: 140–149.

Liu ZX, Yu CF, Nickel C, Thomas S, Cantley LG. 2002. Hepatocyte growth factor induces erk-dependent paxillin phosphorylation and regulates paxillin-focal adhesion kinase association. J Biol Chem 277:10452–10458.

Masaki T, Matsuoka H, Sugiyama M, Abe N, Goto A, Sakamoto A, Atomi Y. 2001. Matrilysin (MMP-7) as a significant determinant of malignant potential of early invasive colorectal carcinomas. Br J Cancer 84:1317–1321.

Mitsiades N, Yu WH, Poulaki V, Tsokos M, Stamenkovic I. 2001. Matrix Metalloproteinase-7-mediated cleavage of Fas ligand protects tumor cells from chemotherapeutic drug cytotoxicity. Cancer Res 61:577–581.

Parkin DM, Bray FI, Devesa SS. 2001. Cancer burden in the year 2000. The global picture. Eur J Cancer 37(Suppl 8): S4–S66.

Parsons JT, Martin KH, Slack JK, Taylor JM, Weed SA. 2000. Focal adhesion kinase: A regulator of focal adhesion dynamics and cell movement. Oncogene 19: 5606–5613.

Reddy BS, Kawamori T, Lubet RA, Steele VE, Kelloff GJ, Rao CV. 1999. Chemopreventive efficacy of sulindac sulfone against colon cancer depends on time of administration during carcinogenic process. Cancer Res 59: 3387–3391.

Sang S, Pan MH, Cheng X, Bai N, Stark RE, Rosen RT, Lin-Shiau SY, Lin JK, Ho CT. 2001. Chemical studies on antioxidant mechanism of garcinol: Analysis of radical reaction products of garcinol and their antitumor activities. Tetrahedron 57:9931–9938.

Sang S, Liao CH, Pan MH, Rosen RT, Lin-Shiau SY, Lin JK, Ho CT. 2002. Chemical studies on antioxidant mechanism of garcinol: Analysis of radical reaction products of garcinol with peroxyl radicals and their antitumor activities. Tetrahedron 58:10095–10102.

Smith ML, Hawcroft G, Hull MA. 2000. The effect of non-steroidal anti-inflammatory drugs on human colorectal cancer cells: Evidence of different mechanisms of action. Eur J Cancer 36:664–674.

Sonoda Y, Matsumoto Y, Funakoshi M, Yamamoto D, Hanks SK, Kasahara T. 2000. Anti-apoptotic role of focal adhesion kinase (FAK). J Biol Chem 275:16309–16315.

Tanaka T, Kohno H, Shimada R, Kagami S, Yamaguchi F, Kataoka S, Ariga T, Murakami A, Koshimizu K, Ohigashi H. 2000. Prevention of colonic aberrant crypt foci by dietary feeding of garcinol in male F344 rats. Carcinogenesis 21(6):1183–1189.

Tsujimoto Y. 2003. Cell death regulation by the bcl-2 protein family in the mitochondria. J Cell Physiol 195: 158–167.

Wang X, Fu X, Brown PD, Crimmin MJ, Hoffman RM. 1994. Matrix metalloproteinase inhibitor BB-94 (batimastat) inhibits human colon tumor growth and spread in a patient-like orthotopic model in nude mice. Cancer Res 54:4726–4728.

Weyant MJ, Carothers AM, Bertagnolli ME, Bertagnolli MM. 2000. Colon cancer chemopreventive drugs modulate integrin-mediated signaling pathways. Clin Cancer Res 6:949–956.

Xu L, Owens LV, Sturge GC, Yang X, Liu ET, Craven RJ, Cance WG. 1996. Attenuation of the expression of the focal adhesion kinase induces apoptosis in tumor cells. Cell Growth Differ 7:413–418.

Yamaguchi F, Ariga T, Yoshimura Y, Nakazawa H. 2000a. Antioxidative and anti-glycation activity of garcinol from *Garcinia indica* fruit rind. J Agric Food Chem 48:180–185.

Yamaguchi F, Saito M, Ariga T, Yoshimura Y, Nakazawa H. 2000b. Free radical scavenging activity and antiulcer activity of Garcinol from *Garcinia indica* fruit rind. J Agric Food Chem 48:2320–2325.

Yang WL, Addona T, Nair DG, Qi L, Ravikumar TS. 2003. Apoptosis induced by cryo-injury in human colorectal cancer cells is associated with mitochondrial dysfunction. Int J Cancer 103:360–369.