Abstract. Cinnamaldehyde (CA) is a bioactive compound isolated from the stem bark of *Cinnamomum cassia*, that has been identified as an antiproliferative substance with pro-apoptotic effects on various cancer cell lines *in vitro*. In the present study, the effects of CA on human colon cancer cells were investigated at both the molecular and cellular levels. Three types of colorectal cancer cells at various stages of differentiation and invasive ability (SW480, HCT116 and LoVo) were treated with CA at final concentrations of 20, 40 and 80 µg/ml for 24 h. Compared with the control group, the proliferation inhibition rate of the human colorectal cancer cells following treatment with CA increased in a dose- and time-dependent manner. The invasion and adhesion abilities of the cells were significantly inhibited as indicated by Transwell and cell-matrix adhesion assays. Meanwhile, CA also upregulated the expression of E-cadherin and down-regulated the expression of matrix metalloproteinase-2 (MMP-2) and MMP-9. CA also elevated the apoptotic rate. The levels of pro-apoptotic genes were upregulated while the levels of apoptosis inhibitory genes were decreased which further confirmed the pro-apoptotic effect of CA. In order to explore the mechanism of CA-induced apoptosis, insulin-like growth factor-1 (IGF-1) and PI3K inhibitor (LY294002) were used to regulate the phosphoinositide 3-kinase (PI3K)/AKT pathway. The transcription activity of PI3K/AKT was markedly inhibited by CA, as well as IGF-1 which functions as an anti-apoptotic factor. In conclusion, CA has the potential to be developed as a new antitumor drug. The mechanisms of action involve the regulation of expression of genes involved in apoptosis, invasion and adhesion via inhibition of the PI3K/Akt signaling pathway.

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors of the digestive system and is also one of the leading causes of cancer-related deaths worldwide (1). Early symptoms of CRC are usually not detected. Once diagnosed, CRC is often in the advanced stage, including metastasis to liver in the 20% of cases (2). The principle therapeutic method is surgery, as well as radiotherapy and chemotherapy (3). Although surgical techniques have been rapidly developed during the past few years, the 5-year survival rate of CRC patients has not significantly increased. Non-surgical therapies, such as chemotherapy and radiotherapy, lack efficacy. Currently, with advantages and achievements in the clinic, more and more active antitumor ingredients derived from Traditional Chinese medicine (TCM) are being developed, studied and used.

Cinnamaldehyde (CA), as shown in Fig. 1A, is a bioactive compound isolated from the stem bark of *Cinnamomum cassia*, and has been used as a TCM herb. Studies have demonstrated that CA displays various biological activities, including antibacterial, immunomodulatory, cytotoxic and anti-angiogenic activities (4-7). It is also known to possess marked antitumor effects *in vitro* and *in vivo* (8-11). However, the signaling mechanisms involved in the inhibition of CRC cell growth by CA are poorly understood.

The molecular mechanisms involved in the tumorigenesis and metastasis of CRC are not entirely clear. Carcinogenesis of CRC is complex and requires the accumulated alteration of multiple genes and pathways. The phosphoinositide-3-kinase (PI3K)/AKT signaling pathway is indispensable to intracellular signal transduction which involves cell metabolism, apoptosis, survival, differentiation and proliferation processes.
Therefore, it is closely related to the occurrence, development and metastasis of many types of tumors (12). AKT and p-AKT are expressed in CRC tissues at a significantly higher level than those noted in normal tissues (13). This causes not only cell malignant transformation, but also tumor cell migration, adhesion and extracellular matrix degradation (14,15). Therefore, the PI3K/Akt pathway is considered to be a potential target for cancer treatment and needs further research. Compounds that inhibit PI3K/AKT-related genes with drug-like properties blocking the activation of multiple downstream anti-apoptotic effector molecules and promoting cell apoptosis are highly anticipated.

In the present study, we investigated the effect of CA on cell proliferation, invasion and adhesion, and apoptosis of CRC cells. In addition, we aimed to study the mechanisms behind the effect of CA on the PI3K/AKT signaling pathway in order to provide an experimental foundation for the present research and a possible treatment of CRC.

Materials and methods

Drugs and antibodies. CA was purchased from the China National Institute for the Control of Pharmaceutical and Biological Products (purity 99%) and dissolved in dimethyl-sulfoxide (DMSO). It was dissolved in DMSO at a stock solution (200 µg/ml) and stored at -80˚C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Annexin V conjugated to fluorescein-isothiocyanate (Annexin V-FITC) apoptosis detection kit was purchased from KeyGen Biotech. Co. Ltd. (Nanjing, China). Insulin-like growth factor-I (IGF-I) was obtained from PeproTech China (Suzhou, China). Rabbit anti-human antibodies against MMP-9, MMP-2, E-cadherin, Bax, Bcl-2, PARP, cleaved-PARP, PI3K p-PI3K, AKT, p-AKT and LY294002 (the PI3K inhibitor) were purchased from Cell Signaling Technology (Beverly, MA, USA). Fluorescein-conjugated secondary antibodies were purchased from Odyssey (LI-COR, Lincoln, NE, USA). All other chemicals used in the experiment were of the highest purity grade available.

Cell lines and culture. Human CRC cell lines LoVo, SW480 and HCT116 were obtained from the Type Culture Collection, Chinese Academy of Sciences (Shanghai, China). All the cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) (both from Gibco-BRL, Gaithersburg, MD, USA) in a humidified incubator with 5% CO₂ at 37˚C.

MTT assay. Cells in the logarithmic growth phase were firstly seeded into 96-well culture plates at 3x10⁴ cells/well and then incubated overnight following treatment of CA at concentrations of 0, 20, 40, 60 and 80 µg/ml for 24, 48 and 72 h. Afterwards, 20 µl of MTT solution (5 mg/ml) was added to each well to incubate the cells at 37˚C for 4 h. In order to dissolve the resultant formazan crystals, 100 µl DMSO was added to each well. Absorbance was detected at 490 nm using an ELx800 microplate reader (BioTek, Winooski, VT, USA).

Cell growth inhibition rate was calculated using the following formula: 1 - ODexperiment/ODcontrol.

Cell apoptosis assay. Apoptosis was monitored using the Annexin V/propidium iodide (PI) method as previously described (16), and its rate was detected by flow cytometry with an Annexin V-FITC apoptosis detection kit. The cells were seeded in 6-well plates and treated with CA (0, 20, 40 and 80 µg/l) for 24 h, and then harvested by trypsinization. After washing twice with cold phosphate-buffered saline (PBS), the cells were re-suspended in 500 µl binding buffer at 1x10⁶ cells/ml. Annexin V-FITC (5 µl) PI was subsequently added for incubation at room temperature for 15 min.

Hoechst 33258 staining. Hoechst 33258 staining was performed as previously described (17). HCT116, LoVo and SW480 cells were fixed with 4% paraformaldehyde for 30 min after treatment with CA for 24 h at room temperature and washed once with PBS. Cells were incubated in Hoechst 33258 (50 ng/ml) for 30 min at room temperature and then washed with PBS. Apoptotic cells were identified by the condensation and fragmentation of their nuclei and photographed by a Zeiss Axioplan 2 fluorescence microscope (Jena, Germany).

Invasion assay. The cell migration assay was performed using Transwell membrane filter inserts (pore size, 8-µm; Costar, Corning, NY, USA) in 24-well dishes. The cells were pretreated for 24 h with different concentrations of CA. Approximately 1x10⁴ cells in 200 µl of serum-free medium were placed in the upper chamber, and 300 µl of the medium containing 10% bovine serum was placed in the lower chamber. The plates were incubated for 24 h at 37˚C in 5% CO₂, and the cells were then fixed in 4% paraformaldehyde for 5 min and stained with 0.05% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and those on the underside of the filters were examined and counted under a microscope as previously described (18).

Cell-matrix adhesion assay. The cell adhesion assay was performed as previously described (19). Briefly, CRC cells were treated with or without CA (20, 40 or 80 µg/ml) for 24 h, and then harvested and resuspended in RPMI-1640 medium. Approximately 2x10⁴ live cells were seeded into precoated 96-well plates with 2.5 µg/ml fibronectin (FN). Each concentration group contained 12-wells and every 3-wells were washed twice after 30, 60, 90 and 120 min to remove the non-adherent cells. After washing with PBS, the adherent cells were measured by an MTT assay. Similar to the MTT assay, optical density (OD) values were measured and the cell adhesion inhibition rates were calculated based on the means of three wells.

Western blot analysis. Western blot analysis was performed according to the method reported by Li et al with slight modification (20). The cells were lysed in RIPA buffer in an ice bath for 20 min, and centrifuged at 12,000 x g for 10 min at 4˚C. The supernatant was stored at -80˚C until analyses. The protein concentration was measured using the BCA method (Beyotime). An equal amount of proteins was loaded onto 10% SDS-polyacrylamide gel for electrophoresis and transferred by electroblotting to a polyvinylidene difluoride membrane (Millipore, Boston, MA, USA), which was blocked with 5%...
bSA, and then incubated with the indicated primary antibodies against MMP-9, MMP-2, E-cadherin, Bax, Bcl-2, PARP, cleaved-PARP, PI3K AKT, p-PI3K and p-AKT (1:500) at 4˚C overnight. After washing with Tris-buffered saline with Tween-20 (TbST) for three times (5 min/time), secondary fluorescent antibody (1:2,000 dilutions) was added to the membrane at room temperature for 1 h. The signal intensity of the OD of each band on the membrane was detected by Odyssey (LI-COR) ImageJ analyzer software; β-actin was used as loading control and for normalization.

Statistical analysis. All data are presented as means ± standard deviation (SD). Statistical analysis was performed by SPSS 19.0 software with one-way ANOVA followed by Dunnet’s test to compare the treatment and the control groups. A p-value of ≤0.05 was considered to indicate a statistically significant result.

Results

Effects of CA on the cell inhibition rate of human CRC cells. It is well-known that uncontrolled proliferation is the major malignant characteristic of cancer cells. In the present study, we first tested whether CA inhibits CRC cell proliferation in vitro. After treatment with 0, 10, 20, 30, 40, 60 and 80 µg/ml of CA for 12, 24 and 48 h, the effect of different concentrations on the inhibition rate of the cells was observed using the MTT assay. As shown in Fig. 1B-D, the inhibition rates of the cells were significantly higher compared with the control group at the same time points (p<0.01), and the inhibition by CA was exhibited in an approximate dose- and time-dependent manner. Twenty-four hours were found to be the optimal administration time for the next study. The IC50 values of CA inhibition of HCT116, LoVo and SW480 cell growth at 24 h were 30.7, 30.6 and 35.69 µg/ml, respectively.

CA induces the apoptosis of human CRC cells. Apoptosis is a highly regulated process and is regulated by a series of genes and cell-signaling pathways. To further confirm whether CA induces apoptosis in CRC cells, we evaluated the effects of CA on CRC cells using Annexin V-FITC and observed the nuclear morphological changes in cells using Hoechst 33258 staining. The results (Fig. 2) indicated that the rates of early and late apoptosis in the cells were increase by CA, and the apoptotic rates following treatment with 20, 40 and 80 µg/ml of CA were significantly higher than the rates in the control group. Cell morphological change was assessed by fluorescence microscopy after staining with Hoechst 33258. The apoptotic cells (Fig. 3, indicated with arrows) exhibited highly fluorescent condensed chromatin. In the treated cells, we observed small, fragmented and condensed nuclei with typical apoptotic morphology in contrast with normal symmetrical, blue nuclei (Fig. 3).

CA induces apoptosis via regulation of apoptosis-related genes in human CRC cells. It is well-known that proteins of the Bcl-2 family and PARP are influential in the apoptotic process (21). Targeting the proteins of the Bcl-2 family is a common practice for many anticancer agents inducing apoptosis and the ratio of Bax/Bcl-2 also plays a critical role (22). In order to study the molecular mechanisms behind the CA-induced apoptosis of human CRC cells, the expression levels of apoptosis-related proteins were evaluated by western blot analysis after treatment with various concentrations of
Figure 2. Cinnamaldehyde (CA) induces the apoptosis of colorectal cancer (CRC) cells. (A) Flow cytometric analysis. (B) Cell apoptosis rate. After treatment with 0, 20, 40 and 80 µg/ml of CA for 24 h, the cells were stained with FITC-conjugated Annexin V and PI for flow cytometric analysis. The results from three independent experiments are represented in the form of means ± SD. **p<0.01 compared with the control.

Figure 3. Effect of cinnamaldehyde (CA) on morphological changes in colorectal cancer (CRC) cells. After treatment with 0, 20, 40 and 80 µg/ml of CA for 24 h, CRC cells were stained with Hoechst 33258 and morphological changes were observed with fluorescent microscope. Arrowheads indicate the cells with abnormal nuclei, indicating fragmentation of nuclei/chromatin. Magnification, x200.
CA for 24 h. Western blot analysis revealed that the Bax and cleaved-PARP expression was obviously increased, whereas PARP and Bcl-2 expression was decreased, leading to an upregulation in the ratio of Bax/Bcl-2 (Fig. 4). This may be one of the molecular mechanisms by which CA induces apoptosis in CRC cells.

**CA inhibits CRC cell invasion and adhesion.** We used Transwell chamber and cell-matrix adhesion assays to test whether CA inhibits CRC cell invasion and adhesion. As shown in Fig. 5, the result of the Transwell assay showed that after treatment with different concentrations of CA (0, 20, 40 and 80 µg/ml) for 24 h, the number of the cells that invaded across the 8-µm diameter pores to the lower chamber was markedly decreased compared with the number of invasive cells in the control group, indicating that CA suppresses the invasion of CRC cells in a dose-dependent manner (Fig. 5B). The results indicated that increasing CA concentrations promoted an increased inhibition of cell invasion.

Meanwhile, similar to the results of the Transwell invasion assay, the adhesion rates of the cells treated with CA at all time points were lower than those of the control group. Further data demonstrated that the inhibitory rate of adhesion escalated as the concentrations of CA increased (Fig. 6). Consequently, CA markedly inhibited the adhesion of CRC cells to FN and the inhibition rate exhibited a dose- and time-dependent trend.

**Effects of CA on expression of invasion- and adhesion-related genes in human CRC cells.** We investigated the expression of E-cadherin and MMP-2 and MMP-9 in the CRC cells to explore whether changes in the expression were involved in the inhibition of invasion and adhesion of these cells. E-cadherin, a member of the cadherin superfamily, is involved in maintaining cell polarity and organizing the epithelium by strengthening intercellular adhesion; and is also closely associated to invasion in an MMP2-dependent manner (23). In addition to adhesion molecules, matrix metalloproteinases (MMPs) have the ability to degrade extracellular matrix (ECM) proteins and influence cell behaviors, including invasion and differentiation. Among the MMPs mentioned above, the activity of two gelatinases, MMP-2 and MMP-9, was found to be closely related with tumor metastasis in particular (24-26). Western blotting was used to analyze the levels of E-cadherin, MMP-2 and MMP-9. As shown in Fig. 7, CA significantly reduced the expression of MMP-2 and MMP-9 in a concentration-dependent manner. Expression of E-cadherin was upregulated as the dose of CA increased which may be responsible for the reduction in cell invasion and adhesion observed in the CRC cells.

**Effects of CA on the PI3K/Akt signaling pathway in human CRC cells.** The PI3K/Akt signaling pathway participates in regulating cell biological behaviors. It has been reported to inhibit cellular apoptosis and promote cell survival (27). Thus, for many chemotherapeutic drugs, their antitumor effects are achieved by blocking this pathway (28,29). Phosphorylated PI3K and Akt are attractive molecular targets as they contribute to the development of human CRC and resistance to conventional therapies. Therefore, the potential role of CA on the PI3K/Akt signaling pathway was examined.

In the present study, the cells were pretreated with insulin-like growth factor-1 (IGF-1) (one of the most potent activators of the PI3K/Akt signaling pathway) for 2 h in order to activate the PI3K/Akt signaling pathway, and then treated with 40 µg/ml CA for 24 h (30). To further confirm the results, CRC cells were pretreated with IGF-I for 2 h, followed by exposure to...
the well-characterized pharmacological inhibitor LY294002 (50 µM) (a specific inhibitor of PI3K) for 24 h as a positive control (31). Western blot analysis indicated that CA inhibited IGF-1-induced expression of p-AKT protein. The total p-PI3K level was also inhibited although the total PI3K, total Akt level in each experimental group did not obviously change (Fig. 8). Taken together, CA inhibited the PI3K/Akt signaling pathway by downregulating p-PI3K and p-AKT.

To confirm whether the apoptosis of CRC cells by CA was mainly regulated through the PI3K/Akt pathway, IGF-1 and LY294002 were used to evaluate the underlying mechanisms. Recent studies indicate that IGF-1 not only acts as an insulin and mediates the growth hormone action, but also can inhibit apoptosis (32). The PI3K/Akt pathway is implicated in the entire spectrum of IGF-1-induced anti-apoptotic mechanisms (33). The results indicated that the IGF-1-induced anti-apoptotic effect was inhibited by LY294002 and CA (Fig. 9). Taken together, CA downregulate PI3K/Akt-dependent transcriptional activity, resulting in the regulation of apoptosis-related gene expression.
Figure 7. Cinnamaldehyde (CA) suppresses MMP-2 and MMP-9 expression while increasing E-cadherin expression. Colorectal cancer (CRC) cells were treated with CA at different concentrations for 24 h. The expression levels of E-cadherin, MMP-2 and MMP-9 were detected by western blot analysis, β-actin was used as a loading control.

Figure 8. Effects of cinnamaldehyde (CA) on the PI3K/Akt signaling pathway in human colorectal cancer cells. (A) Cells were treated with 40 µg/ml CA alone, and the expression of PI3K, p-PI3K, Akt and p-Akt was detected by western blot analysis. β-actin was used as a loading control. (B) Densitometric analysis was used to evaluate the p-AKT/AKT ratio. (C) Cells were pretreated with 10 ng/ml IGF-I for 2 h, and then treated with 40 µg/ml CA. The cells were also pretreated with IGF-I for 2 h, followed by exposure to LY294002 for 24 h as a positive control. The expression of PI3K, p-PI3K, Akt and p-Akt was detected by western blot analysis. β-actin was used as a loading control. (D) Densitometric analysis was used to evaluate the p-AKT/AKT ratio. The results from three independent experiments are represented in the form of means ± SD. **p<0.01 compared with the control group.
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Discussion

In recent years, with advances in treatment, the survival rate of colorectal cancer (CRC) patients has increased. However, many patients still relapse after surgery, radiotherapy and chemotherapy. Thus preventing the recurrence and metastasis of CRC is one of the focuses of the present study. Resistance of cell death, increased invasion and metastasis ability, and self-sufficiency in growth signals are intrinsic characteristics of all types of carcinomas (34), including CRC. Based on these facts, three types of CRC cells with different differentiation stage and invasive ability were selected for the present research.

CA has attracted a great deal of research interest for its anticancer properties. It has been used to inhibit the growth and induce the apoptosis of cancer cells as a natural bioactive substance in several studies. Its potential in the development of an effective anticancer and chemopreventive agent has been a focus in previous studies (35-37). However, research on the mechanism of CA against colon cancer is rare. Thus, we explored the effect of CA on apoptosis, invasion and metastasis of CRC cells and related molecular mechanisms.

Resisting apoptosis is the principal mechanism by which tumor cells resist death, and it is an important point for the development of anticancer drugs. In the present study, we applied Annexin V-FITC/PI double staining and FCM (Fig. 2) and Hoechst staining (Fig. 3) to investigate the effects of CA on the apoptosis of human CRC cells. The results indicated that CA induced apoptosis at both the early and late stages.

Apoptosis can be triggered by two signaling pathways, the extrinsic pathway (death receptor pathway) and the intrinsic pathway (the mitochondrial pathway) (38). Poly(ADP-ribose) polymerase (PARP) is a key signaling nuclear protein involved in apoptosis. Activated caspase protein cuts it into cleaved poly(ADP-ribose) polymerase which is a marker of the progression of apoptosis (39,40). To further explore the molecular mechanisms, western blot analysis was used (Fig. 4). Then, we found that the protein level of PARP was decreased in a dose-dependent manner, while the c-PARP protein level was increased. The Bcl-2 family plays a significant part in apoptosis (41). Particularly, the stoichiometries of bax (pro-apoptotic gene) and bcl-2 (anti-apoptotic gene) are influential factors for the downstream activation of caspase protein (42,43). Therefore, we continued to detect the expression change in Bcl-2 family members. We found that the ratio of bax/bcl-2 increased with increasing doses of CA. These results suggest that CA can regulate the ratio of Bax/Bcl-2 to induce apoptosis in human CRC cells.

In regards to invasion, it is the one of most critical and fatal characteristics of malignant tumors. Adhesion of tumor cells is an essential and initial step for tumor invasion and...
metastasis (44,45). Research has shown that invasion and adhesion are mediated largely by MMPs, and among the MMPs, MMP-2 and MMP-9 are critical factors (46). However, no study has extensively explored the underlying mechanisms of the anti-adhesive, anti-invasive effects of CA. To test whether CA inhibits invasion and adhesion of CRC cells, Transwell chamber and cell-matrix adhesion assays were applied. We found that CA inhibited cell invasion (Fig. 5) and adhesion (Fig. 6) of human CRC cells in vitro. Meanwhile, we also found that the effectiveness of CA on cell invasion and adhesion was influenced by the inhibition of MMP-2 and MMP-9 activities in a dose-dependent manner (Fig. 7).

Self-sufficiency in growth signals is another characteristic of tumor cells. As one of the most important intracellular pathways, the PI3K/Akt pathway is frequently activated in a number of cancers and is responsible for cell growth, metabolism, proliferation, survival, motility and invasion. Furthermore, previous research found that high PI3K expression is associated with CRC metastasis (47-50). In the present study, it was discovered that CA effectively inhibited activation of the PI3K/AKT pathway (Fig. 8).

In previous research, many scholars found that IGF-1 is highly expressed in the serum of patients with CRC. Moreover, IGF-1 plays an important role in the pathogenesis and metastasis of CRC (51,52). In the present study, we found that CA effectively inhibited activation of PI3K/AKT pathway, and the efficiency of CA can basically approach the efficacy of LY294002 (Fig. 9).

In conclusion, CA induces apoptosis and inhibits invasion and adhesion in CRC cells by inhibiting activation of the PI3K/Akt pathway, and it is capable of antagonizing the activation of the PI3K/AKT signaling pathway by IGF-1. These findings provide an experimental basis for CA to be used as a drug for colon cancer; however, more basic research is needed to verify the antitumor activity of CA.

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