Abstract. Curcumin (diferuloylmethane), a natural polyphenol present in turmeric, possesses a wide spectrum of pharmacological properties, including antioxidant and antitumor metastatic activities. However, the underlying mechanisms by which curcumin suppresses the metastasis of pancreatic cancer are still not fully elucidated. Our previous study demonstrated that a moderate amount of hydrogen peroxide (H$_2$O$_2$) is able to promote pancreatic cancer invasion. The aim of this study was to determine whether curcumin can suppress H$_2$O$_2$-induced tumor invasive and migratory abilities.

Human pancreatic cancer BxPC-3 and Panc-1 cells were exposed to H$_2$O$_2$ with or without curcumin or N-acetylcysteine (NAC; a scavenger of free radicals). The effects of curcumin on pancreatic cancer cell proliferation was analyzed using MTT assay. The intracellular reactive oxygen species (ROS) was determined using 2,7-dichlorodihydrofluorecein diacetate. The cellular invasive and migratory abilities were analyzed using Transwell Matrigel invasion assay and wound healing assay, respectively. The expressions of matrix metalloproteinase (MMP)-2 and MMP-9 were determined using qT-PCR and western blotting at mRNA and protein level. The activation of p-extracellular signal-regulated kinase (ERK) and p-nuclear factor-κB (NF-κB) were measured by western blotting.

Our data showed that curcumin inhibited cancer cell proliferation in a dose-dependent manner. Curcumin and NAC were able to inhibit H$_2$O$_2$-induced ROS production, reduce the migration and invasion, and decrease the expression of MMP-2 and MMP-9 in pancreatic cancer cells. In addition, the H$_2$O$_2$-induced elevation of p-ERK and p-NF-κB in BxPC-3 and Panc-1 cells were reduced by curcumin, NAC and PD 98059 (an ERK inhibitor). These data indicate that curcumin suppresses pancreatic cancer migration and invasion through the inhibition of the ROS/ERK/NF-κB signaling pathway. This study suggests that curcumin may be a potential anticancer agent for pancreatic cancer.

Introduction

As the fourth leading cause of cancer death worldwide, pancreatic cancer is an aggressive malignant and lethal disease with a 5-year survival rate of less than 5% (1). Although the incidence of most other cancers have been declining, the rate of incidence for pancreatic cancer continues to increase by 1.5% per year (1). Surgery is the selected treatment option; however, it is only applicable to less than 20% of pancreatic cancer patients because of the late presentation and rapid progression of pancreatic tumors (2). Current treatments for inoperable patients are still limited to chemotherapy, radiation or both (3). Thus, more constructive and effective therapeutic strategies are urgently needed. In recent years, a variety of studies have shown that many natural agents are able to inhibit the progression of pancreatic cancer, but the underlying mechanisms of these agents are still not fully understood (4,5).

Curcumin (diferuloylmethane), a natural polyphenol present in turmeric, has many biological effects, including anti-infectious, anti-inflammatory, and antioxidant, as well as chemopreventive activities (6). More recently, curcumin has also been found to possess anticancer properties that affect a variety of biological pathways involved in tumorigenesis, cell cycle regulation, apoptosis, angiogenesis, immune activity regulation and metastasis (7). Extensive studies have verified that curcumin is involved in the regulation of multiple cellular signaling pathways, including the mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB), Akt, Wnt/β-catenin and other pathways (6).
Reactive oxygen species (ROS) generated by the mitochondrial respiratory chain are a number of chemically reactive molecules derived from oxygen, such as superoxide anion, hydrogen peroxide (H₂O₂) and others. Accumulating evidence indicates that the intracellular redox state plays an important role in cellular signaling transduction and regulates multiple events (8). On one hand, an excessive amount of ROS production can kill cancer cells, whereas sublethal concentrations of ROS can stimulate tumor progression by promoting cell proliferation, survival, invasion and metastasis (9). Additionally, our previous study showed that H₂O₂ (0-200 µM) could promote pancreatic cancer progression in a dose-dependent manner, while H₂O₂ was cytotoxic at concentrations above 200 µM (10). A recent study showed that curcumin possesses a protective effect against the epithelial-mesenchymal transition (EMT) process in the prostate tumor-stroma interaction, which is dependent on its ability to ameliorate cancer-associated fibroblast-induced ROS production through the MAOA/mTOR/HIF-1α signaling pathway (11).

The MAPK signaling pathways are important signaling cascades downstream of ROS that are involved in tumor progression (12). Members of the MAPK family include extracellular signal-regulated kinase (ERK), c-Jun NH-2 terminal kinase (JNK) and p38 MAPK. Our previous study demonstrated that a moderate amount of H₂O₂ is able to promote pancreatic cancer invasion via the activation of the ERK and p38 MAPK signaling pathways (10). However, the ability of curcumin to inhibit the H₂O₂-induced progression of pancreatic cancer and the mechanisms related to this process have not been elucidated.

In the present study, we tested the hypothesis that curcumin is able to inhibit pancreatic cancer cell proliferation and suppress the H₂O₂-induced invasion and migration of pancreatic cancer cells. We also investigated the effect of curcumin on H₂O₂-induced activation of the ERK/NF-κB signaling pathway. The results from this study suggest that curcumin may be a novel therapy option for pancreatic cancer via the inhibition of the ERK/NF-κB signaling pathway.

Materials and methods

Cell culture and reagents. The human pancreatic cancer cell lines BxPC-3 and Panc-1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% dialyzed heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ in a humidified atmosphere of 5% CO₂. The cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% dialyzed heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. DMEM and FBS were purchased from Gibco (Grand Island, NY, USA). Curcumin, N-acetylcysteine (NAC) and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Millicell Transwells used in the invasion assays were obtained from Millipore Corp. (Billerica, MA, USA). Matrigel was purchased from BD Biosciences (Bedford, MA, USA). The primary antibodies against (MMP)-2 and MMP-9 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The anti-ERK, anti-phospho-ERK (Thr202/Tyr204), anti-NF-κB and anti-phospho-NF-κB (Ser468) antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). The nitrocellulose membranes used were from Millipore Corp.

The BCA assay kit and the chemiluminescence kit were purchased from Pierce (Rockford, IL, USA). Other reagents were purchased from common commercial sources. All drug solutions were freshly prepared on the day of testing.

MTT assay. BxPC-3 and Panc-1 cells were seeded in 96-well plates at a density of 1x10⁴ cells/well and incubated overnight in 10% FBS medium. The cells were then treated with curcumin (0, 5, 10, 20 and 40 µM). After incubation for 24, 48 and 72 h at 37°C, 15 µl of the MTT solution [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well, and the cells were incubated for 4 h at 37°C. Then, 100 µl of DMSO were added to each well. The optical density (OD) value at 490 nm was determined using a spectrophotometer (Bio-Rad, Berkeley, CA, USA). The results are presented as the percentage relative to the control. The proliferation inhibition rate was calculated as (1-ODsample/ODcontrol) x 100%.

Measurement of intracellular ROS. The level of intracellular ROS was measured using a ROS assay kit. In brief, the cells were incubated with 2,7-dichlorodihydrofluorescein diacetate (DCFDA) for 30 min and washed with PBS three times, and their fluorescence intensity was measured using a fluorometer (Becton-Dickinson, San Diego, CA, USA) with an excitation of 488 nm and an emission of 525 nm.

Wound healing assay. The migratory ability of the cells was detected by a wound healing assay. Pancreatic cancer cells were seeded in 24-well plates (1.0x10⁵ cells/500 µl). After the cells grew to 90-100% confluence, a sterile pipette tip was used to produce a wound line between the cells. The cellular debris was removed by washing with PBS, and then, the cells were allowed to migrate for 24 h. Images were taken at 0 and 24 h post-wounding using a Nikon Diaphot TMD inverted microscope (magnification, x10). The relative distance traveled by the leading edge from 0 to 24 h was assessed using the Photoshop software (n=5).

Transwell Matrigel invasion assay. The invasion of pancreatic cancer cells was tested using Transwell chambers. The 8.0-µm pore inserts were coated with 25 µl of Matrigel. The cell suspensions (5x10⁴) were added to the upper chambers containing DMEM with 1% FBS. Simultaneously, 500 µl of DMEM containing 20% FBS was placed in the lower chambers. The cells were allowed to migrate for 48 h at 37°C. The non-invading cells were removed from the upper surface by a wet cotton swab. After rinsing with PBS, the filter was fixed and stained with crystal violet. The invasion ability was determined by counting the stained cells on the bottom surface. Three random fields were captured at x20 magnification (n=3).

Real-time quantitative PCR (qT-PCR). The total RNA was extracted from the pancreatic cancer cells using the Fastgen 200 RNA isolation system (Fastgen, Shanghai, China) according to the manufacturer's instructions. The total RNA was reverse-transcribed into cDNA using a PrimeScript RT reagent kit (Takara, Dalian, China). The primer sequences were as follows: MMP-2 forward, 5'-GAT GAT GCC TTT GCT CGT GC-3' and reverse, 5'-CAA AGG GGT ATC CAT CGC CA-3'; MMP-9 forward, 5'-TGG TCC TGG TGC TCC TGG TG-3'
and reverse, 5'-GCT GCC TGT CGG TGAG ATT GG-3'; β-actin forward, 5'-GAC TTA GTT GCG TTA CAC CCT TTC T-3' and reverse, 5'-GAA CGG TGA AGG TGA CAG CAG T-3'. The PCR reactions were subjected to a thermocycler program consisting of 30 sec at 95˚C followed by 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec and 72˚C for 30 sec. For each qT-PCR data set, dissociation curve analysis was conducted. The relative gene expression was calculated using the 2-ΔΔCt method as previously reported (13).

Western blotting. The proteins in the samples were electrophoretically resolved on a denaturing SDS-polyacrylamide gel and electrotransferred onto nitrocellulose membranes. The membranes were initially blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 2 h and then probed with each primary antibody. After co-incubation with the primary antibodies at 4˚C overnight, the membranes were blotted with the secondary antibody for 2 h at 37˚C. The results were visualized using an ECL western blotting substrate and photographed using GeneBox (Syngene, Frederick, MD, USA).

Statistical analysis. Statistical analysis was performed using SPSS software (version 17.0; SPSS Inc., Chicago, IL, USA). The data are presented as the means ± SEM of three replicate assays. Differences between the groups were assessed by an analysis of variance (ANOVA). Statistical significance was set at P<0.05. All experiments were repeated independently at least three times.

Results

Effect of curcumin on the proliferation of pancreatic cancer cells. In a previous study, curcumin (Fig. 1A) was shown to possess anti-proliferative activity against many tumor types (6). To investigate the cytotoxicity of curcumin on pancreatic cancer cells, BxPC-3 and Panc-1 cells were treated with curcumin at various concentrations (5, 10, 20 or 40 µM) for 24, 48 and 72 h. The results demonstrated that the proliferative abilities of both BxPC-3 and Panc-1 cells decreased in response to the treatment of curcumin in a time- and dose-dependent manner. Curcumin showed a 50% inhibitory concentration (IC50) of approximately 20 µM, and this concentration exhibited no cytotoxic effects in BxPC-3 or Panc-1 cells (Fig. 1B). Therefore, the treatment concentrations of 5, 10 and 20 µM of curcumin were used for the subsequent experiments.

Curcumin attenuates H2O2-induced oxidative stress in pancreatic cancer cells. Accumulating evidence indicates that ROS may play dual roles in cancer in a dose-dependent manner (9). Additionally, our previous study demonstrated that H2O2 could induce pancreatic cancer cell proliferation in a dose-dependent manner from 0 to 200 µM, while H2O2 was cytotoxic at concentrations above 200 µM (10). Therefore, 200 µM of H2O2 was used to treat the cells in the current experiments.

To explore the possible relationship between curcumin and oxidative stress, we first examined the effects of curcumin on H2O2-induced ROS production in BxPC-3 and Panc-1 cells using the cell-permeable and redox-sensitive compound DCFDA by flow cytometry. Our results showed that H2O2 significantly increased intracellular levels of ROS, while curcumin suppressed these effects in a dose-dependent manner. NAC, a scavenger of free radicals, also efficiently reduced the H2O2-induced ROS level in both BxPC-3 and Panc-1 cells (Fig. 2).

Curcumin inhibits the H2O2-induced invasion and migration of pancreatic cancer cells. A vital step of cancer metastasis is the invasion of cancer cells through the basement membrane. To examine the potential anti-invasive effects of curcumin, the invasion ability of BxPC-3 and Panc-1 cells treated with curcumin were analyzed. As shown in Fig. 3, H2O2 exposure significantly increased the invasive ability of pancreatic cancer cells, but the average cell number that invaded into
the lower chamber decreased as the curcumin concentration increased from 5 to 20 µM. NAC also efficiently reduced the H$_2$O$_2$-induced invasive ability of the cancer cells.

The effect of curcumin on pancreatic cancer cell motility was determined using a wound healing assay. Our results showed that H$_2$O$_2$ exposure for 24 h caused a significant increase in the migration of both BxPC-3 and Panc-1 cells, whereas the cells treated with NAC and different concentrations (5, 10 and 20 µM) of curcumin showed dose-related delays in wound closure (Fig. 4). These findings indicate that curcumin may be an effective inhibitor of H$_2$O$_2$-induced migration and invasion of pancreatic cancer cells.

Curcumin downregulates the H$_2$O$_2$-induced expression of MMP-2 and MMP-9 in pancreatic cancer cells. Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that degrade extracellular matrix components. Specifically, MMP-9 and MMP-2 are thought to facilitate cancer invasion and metastasis. As shown in Fig. 5, H$_2$O$_2$ exposure significantly increased the mRNA and protein expression levels of MMP-2 and MMP-9 in both BxPC-3 and Panc-1 cancer cells, while curcumin counterbalanced these effects of H$_2$O$_2$. The H$_2$O$_2$-induced expression of MMP-2 and MMP-9 could also be downregulated by NAC treatment.

Curcumin downregulates the activation of the ERK/NF-$\kappa$B signaling pathway. The MAPK signaling pathways are important signaling cascades downstream of ROS that are involved in tumor migration and invasion (12). Our previous study showed that exposing BxPC-3 and Panc-1 cells to H$_2$O$_2$ for 15 min caused a significant increase in the phosphorylation level of ERK (10). In this study, we observed that the H$_2$O$_2$-induced level of p-ERK was inhibited after a 24-h treatment with curcumin or NAC without affecting its total expression. In addition, the H$_2$O$_2$-induced phosphorylation of NF-$\kappa$B also strongly decreased with the addition of curcumin and NAC (Fig. 6A). Moreover, the ERK inhibitor PD 98059, could inhibit the expression of p-ERK and p-NF-$\kappa$B, indicating...
that the NF-κB transcription factor is modulated by the ERK pathway (Fig. 6B). Taken together, our results demonstrate that curcumin inhibits H₂O₂-induced cancer progression via the suppression of the ERK/NF-κB signaling pathway in both BxPC-3 and Panc-1 cells.

Discussion

The potential antioxidant and anticancer effects of curcumin and its analogues have been extensively studied over the last three to four decades. Curcumin has been found to suppress...
The phosphorylation levels of ERK and NF-κB. Curcumin can also reverse the transforming growth factor-β (TGF-β) signaling pathway (22). Shakibaei et al. (24) showed that combining curcumin with conventional chemotherapeutic agents, such as 5-FU, could provide more effective treatment against chemoresistant colon cancer cells, which may be mediated by the NF-κB/PI-3K/Src pathways. In addition, Lu et al. (25) showed that curcumin is able to suppress the proliferation and invasion of non-small cell lung cancer by modulating the MTA1-mediated Wnt/β-catenin pathway. Our present study proved that curcumin could inhibit H2O2-induced cancer invasion and migration via the suppression of the ERK/NF-κB signaling pathway.

ROS may play dual roles in cancer progression in a dose-dependent manner. On one hand, excess ROS production can cause oxidative damage to DNA and genomic instability and trigger cancer cell death; on the other hand, mild intracellular ROS can stimulate tumor progression by promoting cell proliferation, survival, invasion and metastasis (9). Our previous studies have demonstrated that both hyperglycemic conditions and superoxide dismutase (SOD)-induced mild ROS production were able to promote the invasive and migratory activity of pancreatic cancer (26,27). In the present study, our results showed that the effects of H2O2-induced intracellular ROS could be inhibited by curcumin or NAC to reduce cell invasion and migration, which also support this perspective. It has been reported that dietary supplementation of curcumin to male mice was able to increase the activities of glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase and catalase and induce glutathione S-transferase and quinine reductase, which can further neutralize ROS derived from chemical carcinogens (28). Curcumin can also induce heme oxygenase-1, an important ROS scavenging enzyme, via nuclear factor 2-related regulation (29). Recent studies have also shown that curcumin could abrogate ROS production via the MAOA/mTOR/HIF-1α signaling pathway (11). Our data showed that curcumin inhibits H2O2-induced cancer progression via the suppression of the ERK/NF-κB signaling pathway in pancreatic cancer cells.

The MAPK signaling pathways are important signaling cascades downstream of ROS that are involved in tumor migration and invasion (12). Lee et al. (30) showed that hepatocyte growth factor regulates H2O2 production, which further activates the ERK pathway and regulates uPA production, eventually increasing the invasive potential of stomach cancer cells. Our recent study showed that SOD could promote the EMT of pancreatic cancer cells via the activation of the H2O2/ERK/NF-κB axis (26). We also demonstrated that H2O2 could promote the activation of p-ERK, p-p38, p-NF-κB and p-c-Jun, and in turn, promote pancreatic cancer cell invasion (10). The depletion of H2O2 by catalase inhibits the activation of the MAPK signaling pathways and tumor invasion (10). Several studies have demonstrated that the MAPK pathways, especially that of ERK, regulate MMP expression (31,32). Our previous study demonstrated that mir-106a can induce the over-expression of MMPs, which can further promote pancreatic cancer cell invasion (33). Additionally, the expression of MMPs is downregulated when the ROS/ERK pathway is blocked (34). The present study determined that the inhibition of the ROS/ERK pathway by curcumin could
downregulate the expression of MMP-2 and MMP-9, which in turn attenuates cell invasion and migration.

In conclusion, the present study demonstrates that curcumin plays an important role in suppressing the proliferation, migration, and invasion of pancreatic cancer cells via the ROS/ERK/NF-κB signaling pathway. These results suggest that curcumin may be a potential anticancer agent for the treatment of pancreatic cancer.

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References