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Noscapine induces mitochondria-mediated apoptosis in human colon cancer cells in vivo and in vitro

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ABSTRACT

Noscapine, a phthalide isoquinoline alkaloid derived from opium, has been widely used as a cough suppressant for decades. Noscapine has recently been shown to potentiate the anti-cancer effects of several therapies by inducing apoptosis in various malignant cells without any detectable toxicity in cells or tissues. However, the mechanism by which noscapine induces apoptosis in colon cancer cells remains unclear. The signaling pathways by which noscapine induces apoptosis were investigated in colon cancer cell lines treated with various noscapine concentrations for 72 h, and a dose-dependent inhibition of cell viability was observed. Noscapine effectively inhibited the proliferation of LoVo cells in vitro (IC_{50} = 75 μM). This cytotoxicity was reflected by cell cycle arrest at G_2/M and subsequent apoptosis, as indicated by increased chromatin condensation and fragmentation, the upregulation of Bax and cytochrome c (Cyt-c), the downregulation of survivin and Bcl-2, and the activation of caspase-3 and caspase-9. Moreover, in a xenograft tumor model in mice, noscapine injection clearly inhibited tumor growth via the induction of apoptosis, which was demonstrated using a TUNEL assay. These results suggest that noscapine induces apoptosis in colon cancer cells via mitochondrial pathways. Noscapine may be a safe and effective chemotherapeutic agent for the treatment of human colon cancer.

1. Introduction

Colorectal cancer is diagnosed in approximately 146,940 patients per year and is the second leading cause of cancer-related death in the United States [1–3]. Among patients with colorectal cancer (CRC) in the United States, 37.2% are diagnosed with stage III and 27.9% are diagnosed with stage II disease [3]. Adequate surgical resection is the only curative treatment, with overall survival rates of just under 50% at 5 years [4]. The survival depends on the pathologic stage and varies from 30%–60% for stage III to 60%–80% for stage II [3]. However, as many as 40%–50% of patients will relapse and require additional treatment, but the absolute benefit for survival obtained with adjuvant therapy compared with controls was approximately 6% [3]. Therefore, it is of great importance to discover and develop novel agents that have high efficacy and low toxicity, can overcome drug resistance and have improved pharmacologic profiles.

Noscapine, a phthalide isoquinoline alkaloid derived from opium, has been used as an oral antitussive agent and has shown very few toxic effects in animals and humans [5,6]. Noscapine showed little or no toxicity in the kidney, liver, heart, bone marrow, spleen, and small intestine at tumor-suppressive doses [7]. Noscapine binds stoichiometrically to tubulin, alters its conformation, affects microtubule assembly, and arrests mammalian cells in mitosis [6]. Furthermore, noscapine induces apoptosis in many cell types and has potent antitumor activity against a variety of solid tumors, including murine lymphoid tumors and human breast and bladder tumors implanted in nude mice [6]. Recently, the anti-cancer activity of noscapine was found to involve the induction of apoptosis via mitochondrial pathways in various cancers [7–9].

To date, there is no information available about the anti-cancer effects of noscapine on human colon cancer cells except that p21 plays a proapoptotic role and that p53 activity is necessary but not sufficient for noscapine-mediated apoptosis [10]. In this study, we addressed the hypothesis that noscapine plays an important role in mitochondria-mediated apoptosis in colon cancer cells. To examine this hypothesis, we investigated the mechanism of action by which noscapine induces apoptosis in colon cancer cells.

2. Materials and methods

2.1. Chemicals and cell culture

Noscapine (97% purity) was purchased from Sigma (Sigma–Aldrich, St. Louis, MO, USA). The noscapine stock solution was prepared at 100 mM in dimethyl sulfoxide (DMSO) and stored at

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−20 °C. Three human colon cancer cell lines (HT-29, LoVo and SW480) were obtained from the China Center for Type Culture Collection (Wuhan, China). The cells were cultured in DMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified incubator at 37 °C with a 5% CO_2/95% air atmosphere.

2.2. Cell proliferation assay

Cell proliferation was determined using the WST-8 tetrazolium salt assay (Cell Counting Kit-8, Beyotime Inst. of Biotech, China), which quantifies the amount of formazan dye formed when a tetrazolium salt is cleaved by cellular mitochondrial dehydrogenase present in viable cells. Cells were seeded in 96-well plates at a density of 5×10^3/well in 0.1 mL of culture medium. They were allowed to adhere for 12 h and were then incubated with 0.01, 0.1, 1, 10, 100, or 1000 μM noscapine for 0, 12, 24, 36, 48, or 72 h. At two hours before the end of the specified incubation periods, 10 μL of WST-8 reagent were added to the cells. At the end of the incubation, cell density was estimated by measuring the absorbance of the colored formazan reaction product at 450 nm using an iMark Microplate Absorbance Reader (Bio-Rad, USA). The percentage of cell survival as a function of drug concentration was then plotted to determine the IC_{50} value.

2.3. Cell cycle analysis

Cell cycle status was determined by measuring cellular DNA content after staining with propidium iodide (PI) by flow cytometry. Cells were seeded in culture dishes and grown to 70% confluence before the addition of the IC_{50} dose of noscapine for 0, 12, 24, 36, 48, or 72 h. After drug incubation, cells were centrifuged, washed twice with ice-cold PBS, and fixed in 70% ethanol at 4 °C for 24 h. Cells were then centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. The pellets were then washed twice with 4 mL PBS and then stained with 0.5 mL RNase A (2 mg/mL) and 0.5 mL of PI (0.1% in 0.6% Triton-X in PBS) for 30 min in the dark. Samples were then analyzed with a FACSCalibur flow cytometer (Beckman Colter, Inc., Fullerton, CA).

2.4. Annexin V staining for apoptosis

Cells were grown in culture dishes, and 24 h after seeding, they were exposed to the IC_{50} of noscapine for 0, 12, 24, 36, 48, or 72 h. After the incubation period, adherent cells were harvested by mild trypsinization, washed twice with cold PBS and pooled together with detached cells in 500 μL of binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). The cells were then incubated with 5 μL of Annexin V-FITC and 10 μL of PI at room temperature for 5 min in the dark. Flow cytometric analysis was performed with a FACSCalibur using the CellQuest software (BDIS). The density plots illustrate four cell populations (live, apoptotic, necrotic, and late apoptotic/dead), defined by their fluorescence characteristics. Live cells are Annexin V- and PI-negative, early apoptotic cells are Annexin V-positive and PI-negative and late apoptotic and dead cells are both Annexin V-positive and PI-positive.

2.5. Western Blot analysis

Proteins were resolved by SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA) using a wet transfer system (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 10% non-fat dry milk in TBST and incubated first with primary antibodies at 4 °C overnight and then with horseradish peroxidase-conjugated anti-mouse secondary antibody for 2 h at room temperature. The dilutions used were: survivin, Bax, Bcl-2, Cyt-c (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:1000; Caspase-9 (Abcam, USA) 1:1000; Caspase-3 (Abcam, USA), 1:250; and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:2000. Specific proteins were visualized using an enhanced chemiluminescence (ECL) system (Millipore, Bedford, MA, USA) and then exposed with Kodak X-ray film. Protein band intensities were determined densitometrically using the CMIASWIN video imaging system (Bio-Rad, Hercules, CA, USA).

2.6. Xenograft tumor model

All experiments were conducted according to the NIH Guide for the Care and Use of Laboratory Animals, and the study protocol was approved by the Ethics Committee for Animal Research of Wuhan University, China. Male BALB/c-nu/nu nude mice, 4–6 weeks old, were purchased from the Center for Animal Experiment of Wuhan University and used in the experiments. LoVo cells, suspended in 100 μL PBS, were subcutaneously inoculated into the lower right flank of the nude mice. When the tumors reached approximately 100 mm^3 in size, the nude mice were divided into four groups (6 in each group): a control group, a low-dose group (10 mg/kg), a mid-dose group, (20 mg/kg), and a high-dose group (40 mg/kg), and noscapine was administered via intratumoral injection every 3 days [8]. There was no significant difference among these groups at the beginning of treatment. Tumor growth was measured using calipers every 3 days. Tumor volume (TV) was calculated using the formula: TV (mm^3) = $d^2 \times D/2$, where d and D are the shortest and the longest diameters, respectively. At the end of the experiment, tumors were harvested for additional analyses as described below.

2.7. HE staining and TUNEL assay

For histologic analysis, tumor tissues were fixed in 4% formaldehyde, dehydrated with an ethanol gradient, and embedded in paraffin, and the paraffin tumor tissue sections were stained with hematoxylin and eosin (HE). Then, an in situ apoptosis detection kit (Roche Diagnostics, Branchburg, NJ, USA) was used to detect apoptotic cells in paraffin tumor tissue sections. The positive cells were identified, counted (six random fields per slides), and analyzed by light microscopy (Olympus, Japan).

2.8. Statistical analysis

All data were expressed as the mean ± standard error of the mean. The data were analyzed with the unpaired Student's t-test, and differences were considered significant at a p value of less than 0.05.

3. Results

3.1. Noscapine inhibits colon cancer cell proliferation

Using the tetrazolium salt (WST-8) cell viability assay (see "Section 2"), we generated a dose-response curve by incubating cultures of HT-29, LoVo, and SW480 cells with various concentrations of noscapine for 72 h and observed a dose-dependent inhibition of cell viability (Fig. 1A). Of the three colon cancer cell lines, LoVo was the most sensitive to noscapine. Noscapine inhibited the viability of LoVo cells with an IC_{50} of 75 μM at 72 h (Fig. 1A). Because exposure to 75 μM noscapine resulted in a significant inhibition of LoVo cell growth, we selected this dose to examine how this concentration affects cell viability at various timepoints

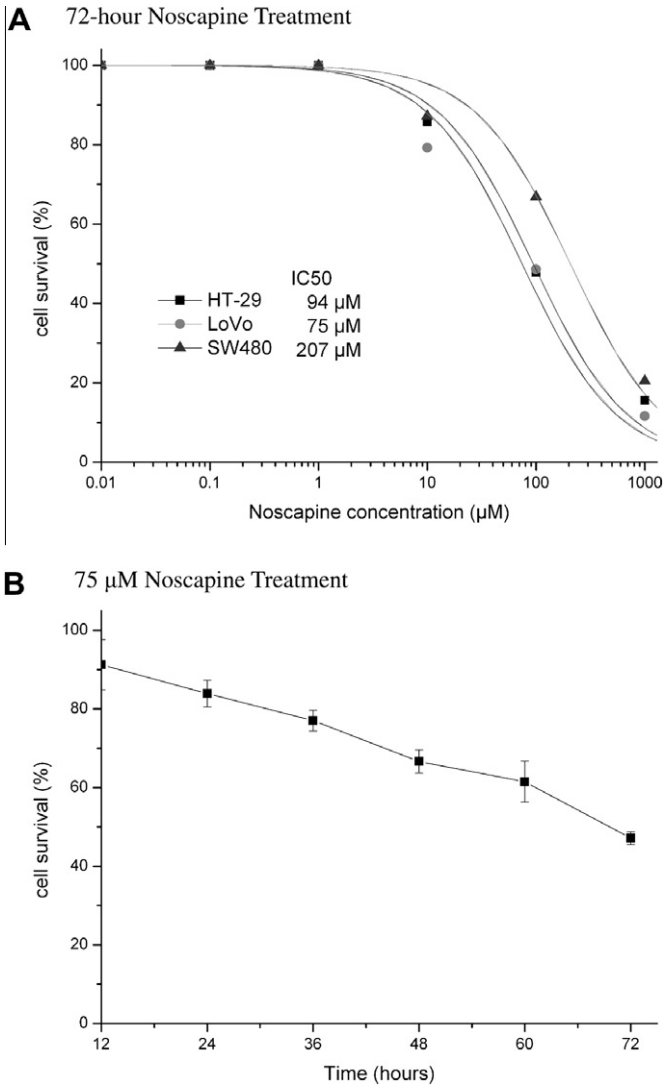

Fig. 1. Dose- and time-dependent effects of noscapine on colon cancer cell viability. (A) Noscapine inhibits the viability of colon cancer cells in a dose-dependent manner; (B) LoVo cells incubated with 75 μM noscapine for 12, 24, 36, 48 and 72 h.

(12–72 h; Fig. 1B). We conclude that exposure to 75 μM noscapine for 72 h is the optimal treatment for inhibiting LoVo cell viability.

3.2. Noscapine induces G_2/M arrest in LoVo cells

To investigate the precise mechanisms through which cell viability is decreased in LoVo cells exposed to noscapine, we analyzed the cell cycle distribution profile by flow cytometry with PI (Fig. 2A). These data revealed that the cell cycle progressively slowed from time 0 of treatment to 72 h. There was an abnormal accumulation of cells in a polyploid state at early timepoints, replaced by sub-G_1 amounts of DNA at later timepoints, indicating apoptosis. Untreated LoVo cells had normal cell cycle profiles, with approximately 50% of cells in the G_0/G_1 phase with 2 N DNA content and 15% of cells in the G_2/M phase containing 4 N DNA content (Fig. 2A, 0 h). Noscapine exposure significantly perturbed the cell cycle progression in LoVo cells at 12 h, resulting in a clear decrease (4%) in G_0/G_1 phase cells with 2 N DNA content and an increase in the proportion of cells in G_2/M with 4 N DNA, to 63% (Fig. 2A, 12 h). At 24 h, most cells were arrested in G_2/M with 4 N DNA content (84%; Fig. 2A, 24 h). The increase in the population of cells with 4 N DNA was concomitant with a decrease in the G_0/G_1 population.

3.3. Noscapine induces apoptosis and chromatin condensation

To quantitate the populations of both early and late apoptotic cells, the cells were co-stained with annexin V and PI, a membrane-impermeant red DNA-binding dye, and analyzed with flow cytometry. Fig. 2B shows the density plots of PI versus Annexin/Alexa-Fluor 488 fluorescence obtained from untreated control LoVo cells. The untreated cell cultures contained very few apoptotic cells (~0.4%), which we assigned as the background cell death. LoVo cells treated with noscapine for 36 h contained both early and late apoptotic cells (21.2% and 0.9%, respectively), suggesting continued initiation and execution of the apoptotic program. The morphological changes in the apoptotic LoVo cells were detected by Hoechst 33258 staining (data not shown). In the untreated LoVo cells, the nuclei were stained a weak homogeneous blue, while in the groups treated with noscapine, bright chromatin condensation and nuclear fragmentation could be observed.

3.4. Noscapine induces apoptosis through the activation of the mitochondrial pathway

To further investigate the detailed mechanism underlying noscapine-induced apoptosis, we examined the effect of noscapine on the mitochondrial pathway. As shown in Fig. 3A, 75 μM noscapine treatment caused a decline in the levels of survivin, a member of the inhibitor of apoptosis proteins (IAP) family, which is known to block apoptosis by inhibiting caspase activity and mitochondria-mediated apoptosis [11]. Noscapine also induced an increase in Bax protein levels and a decrease in Bcl-2 levels in LoVo cells, which led to a decrease in the antiapoptotic/proapoptotic (Bcl-2/Bax) protein ratio. In addition, the expression of cytosolic cytochrome c, which was suggested to be involved in mitochondrial damage, activated caspase-3 and activated caspase-9 were significantly increased with noscapine treatment.

3.5. Anti-tumor effects in vivo

Following the investigation of apoptosis in LoVo cells in vitro, the anti-tumor effect of noscapine was evaluated in human xenograft tumor models. As shown in Fig. 4A, noscapine significantly inhibited tumor growth in vivo ($P < 0.05$). Tumors grew progressively and reached approximately 1000 mm^3 within 30 days in the control group. However, tumor growth was markedly suppressed in the treatment groups in a dose-dependent manner. At the end of 36 days, noscapine at 10, 20, and 40 mg/kg inhibited tumor growth by 32%, 44%, and 68%, respectively, compared with the control group (1480 mm^3) (Fig. 4A). The mean tumor weight in the high-dose group was only 0.267 g at the end of the experiment (Fig. 4B). HE staining (not shown) and TUNEL assays of the subcutaneous tumor tissue sections demonstrated that noscapine produced obvious cell apoptosis in the tumor mass (Fig. 4C), whereas little apoptosis was observed in the control group ($P < 0.05$).

4. Discussion

The prevalence of colon cancer is increasing in Asia. Many Asian countries, including China, Japan, South Korea, and Singapore, have experienced a two- to fourfold increase in the incidence of colorectal cancer (CRC) over the past few decades [12]. Even in the United States, colorectal cancer is the third most commonly diagnosed cancer and the second leading cause of cancer deaths among cancers that affect both men and women [1,2]. The incidence rates of colon cancer were greater in men than in women and in blacks than in whites and other races; the incidence rates among

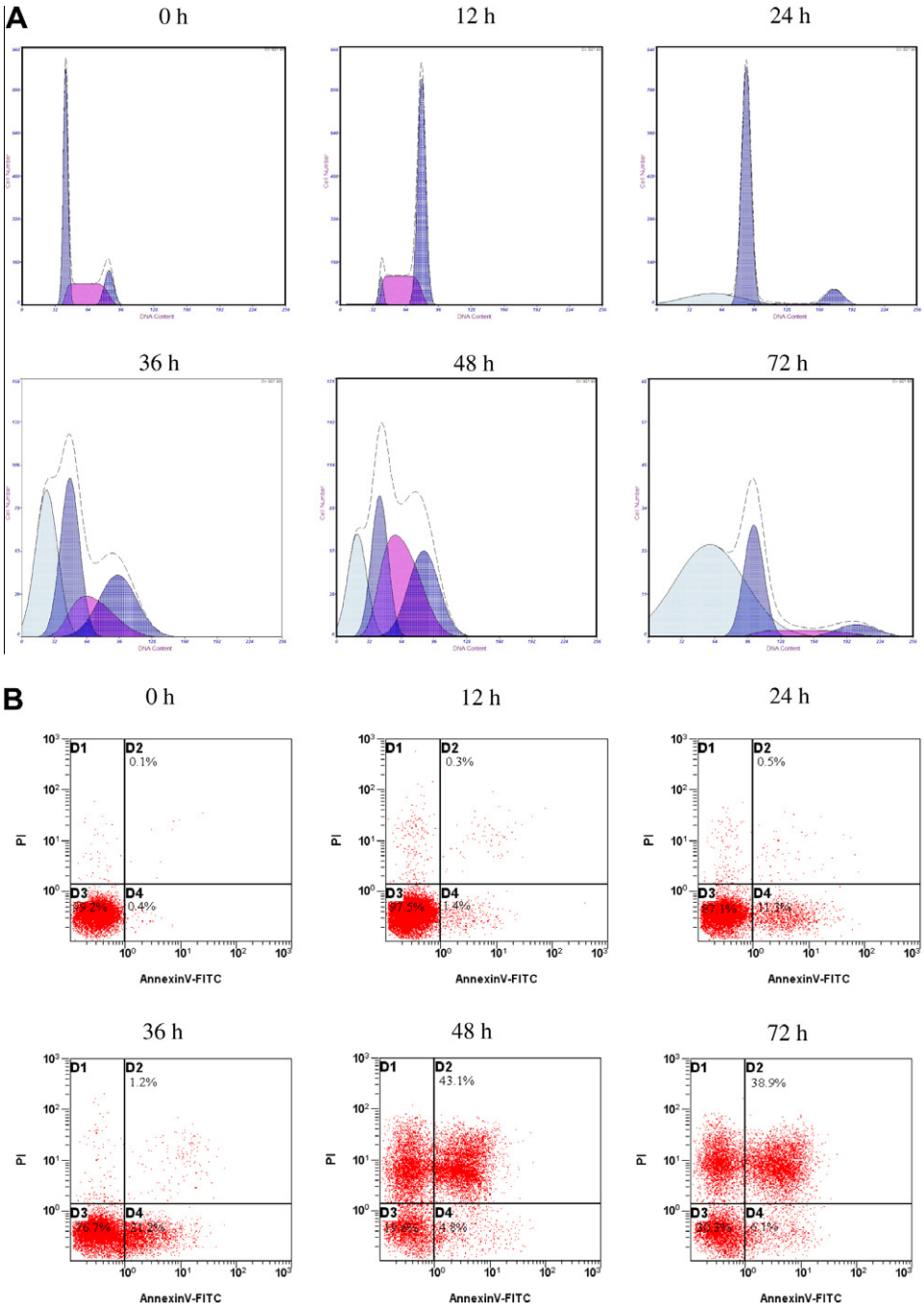

Fig. 2. Quantitative flow cytometric measurements of G_2/M arrest and apoptosis in LoVo cells treated with noscapine. (A) Noscapine induces significant G_2/M arrest followed by the appearance of a sub-G1 population indicative of apoptosis. (B) The density plots illustrate four cell populations (live, apoptotic, necrotic, and late apoptotic/dead) defined by their fluorescence profiles. Live cells are Annexin V negative and PI negative, early apoptotic cells are Annexin V positive and PI negative, necrotic cells are Annexin V negative and PI positive, and late apoptotic and dead cells are both Annexin V positive and PI positive.

Asians/Pacific Islanders (APIs), American Indians/Alaska Natives (AI/ANs), and Hispanics were consistently lower than those in whites and non-Hispanics. Sex disparities were greatest in the population aged 65 years, whereas racial disparities were more pronounced in the population aged <65 years [2]. When classified by cancer subsite, blacks had a higher incidence of proximal and distal colon cancer compared with whites and other races; the reverse was true in the rectum; by stage, whites had higher incidence rates for localized disease than blacks and other races, while blacks had higher incidence of regional disease than whites [2]. The routine use of adjuvant chemotherapy for medically fit patients with stage II colon cancer is not recommended except in patients with inadequately sampled nodes, T4 lesions, perforation, or poorly differentiated histology. Patients and oncologists who accept the relative benefit of adjuvant chemotherapy in stage III disease as adequate indirect evidence of benefit for stage II disease are justified in considering its use [2]. To reduce the mortality of colon cancer, the development of medicines with high efficacy and low toxicity is required.

Noscapine, a phthalide isoquinoline alkaloid derived from opium, is a nontoxic ingredient in cough medicine and is currently in clinical trials to assess its antitumor efficacy in patients with

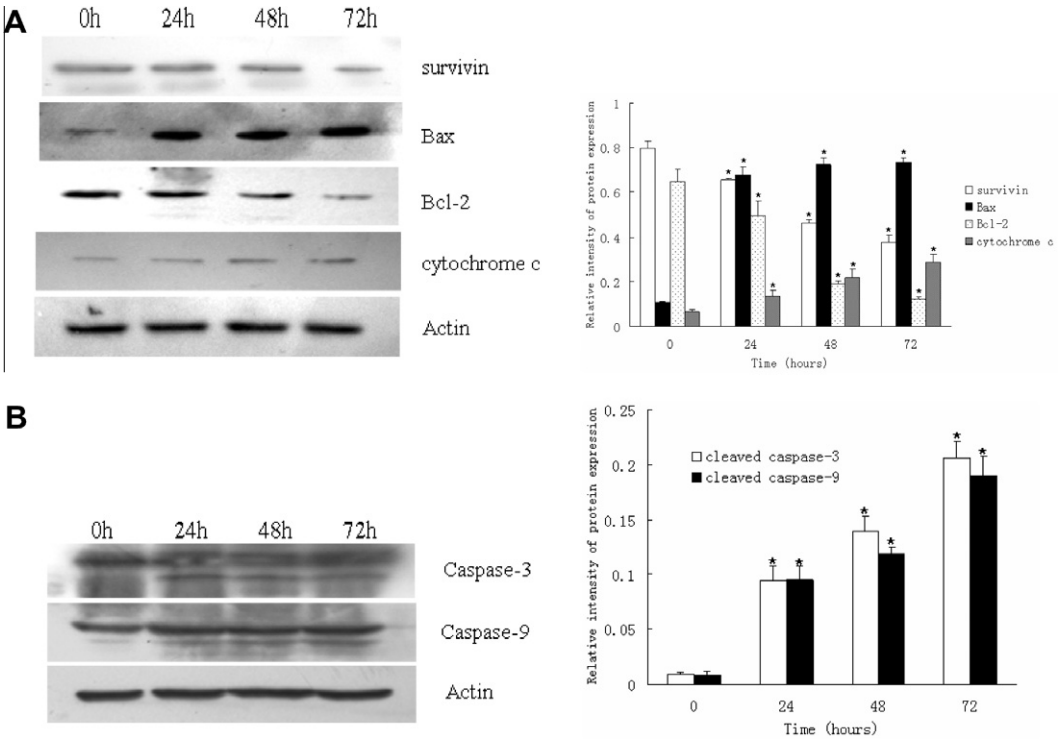

Fig. 3. Noscapine induces apoptosis in LoVo cells through the mitochondrial pathway. (A) Western Blot analysis of survivin, Bcl-2, Bax and cytochrome c protein upon treatment of cells with noscapine for the indicated times (*$P < 0.05$ versus control cells). (B) Noscapine induced the activation (cleavage) of caspase-3 and caspase-9, as determined by Western Blot assay (*$P < 0.05$ versus control cells). Actin was used as a loading control.

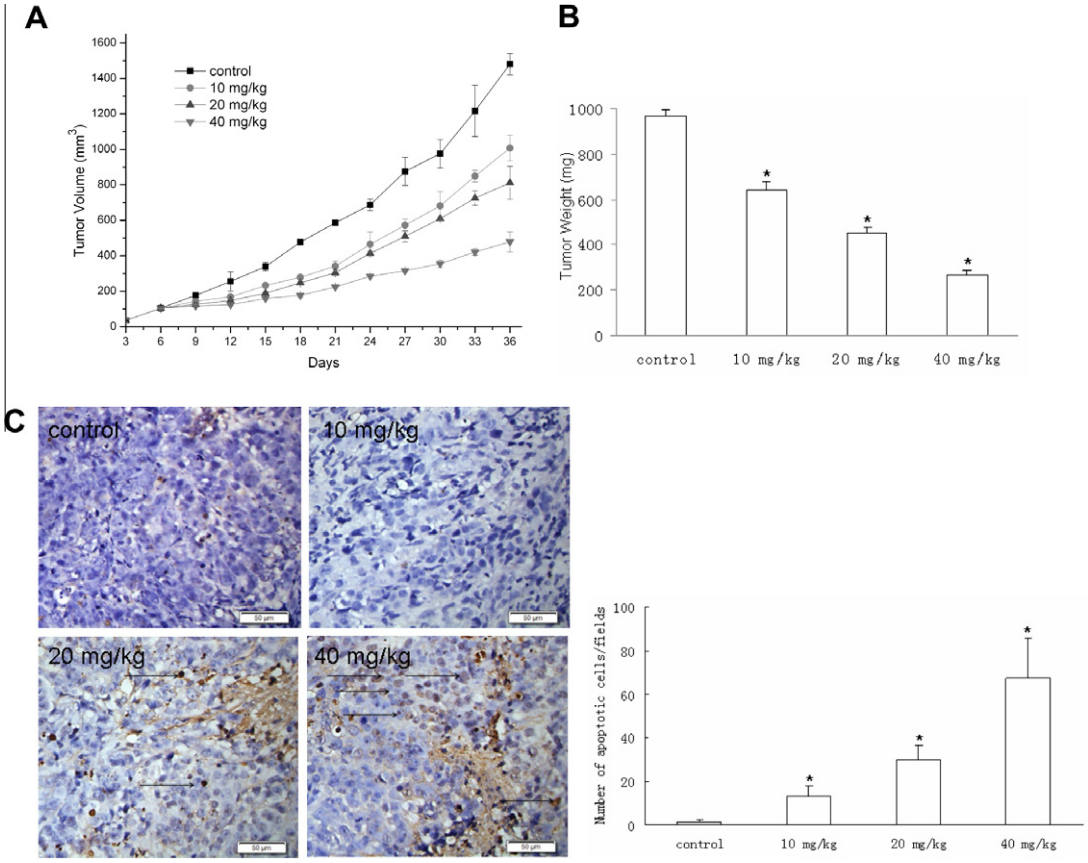

Fig. 4. Tumorigenicity was inhibited by noscapine. (A) Tumor sizes of nude mice in each group. The data are presented as the mean ± SD ($n = 6$). (B) Tumor weight was obtained at the end of the experiment (*$P < 0.05$ versus control). (C) Apoptotic cells detected in xenograft tumor tissue using the TUNEL assay. The apoptotic cells appear brown and are indicated by the arrows. The data are presented as the mean ± SD, *$P < 0.05$ versus control.

non-Hodgkin's lymphoma or chronic lymphocytic leukemia [7,13,14]. Noscapine is considered to be a safe anti-tumor agent and has demonstrated anti-tumor activity both in vitro and in vivo in various cancer cells that are resistant to conventional anti-tumor drugs [8,15]. However, its effects on colon cancer have not been clarified. In the study, we demonstrate that noscapine inhibits HT-29, LoVo and SW480 cell proliferation by inducing cell death, especially in LoVo cells. Noscapine treatment resulted in a significant increase in activated caspase-3 and activated caspase-9, suggesting that a mitochondria-mediated mechanism may play an important role in apoptosis induction. It is proposed that mitochondrial dysfunction might be the major mechanism of noscapine-induced apoptosis in colon cancer cells.

We examined the anti-proliferation ability of noscapine against three colon cancer cell lines (HT-29, LoVo and SW480). The three colon cancer cell lines were treated with various concentrations of noscapine, and the extent of cell proliferation was measured by the WST-8 assay (Fig. 1A). Noscapine effectively suppressed the proliferation of colon cancer cells in a dose- and time-dependent fashion, and LoVo cells were more sensitive to noscapine than were HT-29 and SW480 cells.

To investigate the precise mechanisms responsible for noscapine-mediated suppression of cell division, we examined the cell cycle distribution profile of noscapine-treated LoVo cells. Noscapine exposure caused 4 N DNA accumulation in LoVo cells, which indicated that noscapine-treated cells arrest in the G2-M phase prior to cell death. Because cell cycle checkpoint mechanisms in tumor cells are frequently faulty [16–18], colon cancer cells may be more susceptible to noscapine than normal cells. These results also revealed that noscapine produced apoptosis (Hoechst 33258 and annexin V /PI) in treated LoVo cells in a time-dependent manner (Fig. 2B). The morphological features of apoptotic versus necrotic cell death were distinguished via microscopy [19]. Apoptotic LoVo cells were identified based on chromatin condensation and nuclear fragment staining by Hoechst 33258. These observations suggest that if early apoptotic cells are not ingested by phagocytes, then secondary necrosis will proceed [20]. Four cell populations (live, apoptotic, necrotic, and late apoptotic/dead) were defined by their fluorescence characteristics by flow cytometric analysis. In live cells (Annexin V^-/PI^+), phosphatidylserine translocation has not occurred, and the plasma membrane is still intact; early apoptotic cells (Annexin V^+/PI^-) are not permeable to PI; necrotic cells are Annexin V-negative and PI-positive because of damaged cell membranes; and late apoptotic and dead cells are both Annexin V- and PI-positive [21].

The overexpression of antiapoptotic Bcl-2 family members can tip the delicate prosurvival/proapoptosis balance in favor of survival, thereby conferring drug resistance, at least in some tumor model systems [22–24]. On the other hand, the overexpression of proapoptotic Bax or Bak is sufficient to increase the sensitivity of malignant cancer cells to apoptosis and to overcome drug resistance [24–26]. Unphosphorylated Bcl-2 forms complexes with Bax, and its phosphorylation releases Bax from the Bcl-2-Bax complex [27–30]. Unbound Bax translocates from the cytosol to the mitochondrial membrane to trigger the events of the downstream apoptotic cascade, such as the release of cytochrome c and the activation of executioner caspases [27–30]. The activation of caspase-3 by its cleavage by upstream proteases is considered a hallmark of the apoptotic process [24]. In agreement with this hypothesis, activated caspase-3 and -9 were detected in LoVo cells treated with noscapine in each group. Our results show that noscapine decreases the Bcl-2/Bax ratio, causes the release of cytochrome c, and ultimately drives the activation of caspase-3 and caspase-9 in LoVo cells (Fig. 3). Further studies will be required to determine the exact mechanism by which noscapine induces apoptosis in human colon cancer cells, such as whether there is cross-talk-mediated activation of the death receptor pathway of apoptosis.

In human xenograft tumor-bearing nude mouse models, noscapine was shown to have significant anti-cancer effects (Fig. 4A, B). The therapeutic effect of noscapine was confirmed to be mediated at least in part through apoptosis induction by the TUNEL staining of tumor sections (Fig. 4C).

In conclusion, we demonstrated that noscapine has anti-proliferative and proapoptotic effects in colon cancer cells in vivo and in vitro. This study identifies the potential usefulness of noscapine, a non-toxic and safe anti-tumor agent, in the management of colon cancer.

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