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ORIGINAL ARTICLE

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

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Abstract
Purpose Noscapine plays an important role in the regulation of cell growth and death. It has been reported to potentiate the anti-tumor effect by inducing apoptosis in various malignant cells. However, the mechanism of inducing apoptosis in gastric cancer cells by this agent remains to be clarified.
Methods In the study, we investigated the signaling pathways by which noscapine induces apoptosis in gastric cancer cell lines. Apoptosis of four human gastric cancer cell lines was induced by treatment with noscapine.
Results Our results indicate that noscapine induced a dose-dependent apoptosis of these cells. The treatment with noscapine upregulated Bax and Cytochrome *c* (Cyt-*c*) protein, downregulated Bcl-2 protein. Caspase-3 and caspase-9 were activated, suggesting that the apoptosis is mediated by mitochondrial pathways. Moreover, in xenograft tumor mouse model, noscapine injection successfully inhibited the tumor growth via apoptosis induction which was demonstrated by TUNEL assay.
Conclusions These data of the study suggest that noscapine induces apoptosis in gastric cancer cells via mitochondrial pathways.

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Keywords Noscapine · Apoptosis · Gastric cancer · Mitochondria

Introduction

Gastric cancer remains one of the most common forms of cancer worldwide [1]. Around nine million cases of gastric cancer are diagnosed worldwide each year, with the highest incidence occurring in Eastern Asia, Eastern Europe, and the Andean regions of South America [2, 3]. Furthermore, most patients with gastric cancer have tumor recurrence after treatment [4] In order to prevent recurrence after treatment, it is important to develop medicines which are high efficacy and new mechanisms of action.

Noscapine is a phthalide isoquinoline non-narcotic alkaloid derived from opium [5, 6]; for more than 75 years, noscapine has traditionally been used as an oral cough suppressant with no know toxic side effects in human [7]. Noscapine's chemical structure is similar to the known microtubule-binding agent, colchicines, and its binding activity to microtubules has been extensively characterized [5, 6, 8]. Noscapine hydrochloride is in phase I/II clinical trials for the treatment of chronic lymphocytic leukemia or low-grade non-Hodgkin's lymphoma refractory to chemotherapy and hematological malignancies. Noscapine binds stoichiometrically to tubulin and promotes microtubule polymerization, which causes growth arrest of tumor cells during mitosis [9]. Moreover, noscapine anti-cancer activity involves induction of apoptosis via mitochondrial pathways was demonstrated in various cancers [10, 11].

So far, there is no available information about the anti-tumor effects of noscapine on human gastric cancer cells. In this study, we addressed the hypothesis that noscapine

plays an important role in mitochondria-mediated apoptosis in gastric cancer cells. To test our hypothesis, we investigated the mechanism of action, by which noscapine induces apoptosis in gastric cancer cell lines. We demonstrated that mitochondrial pathways involved noscapine-mediated apoptosis in gastric cancer cell lines.

Materials and methods

Cell culture and reagents

Four human gastric cancer cell lines (BGC823, SGC7901, MGC803, HGC27) were purchased from the China center for type culture collection (Wuhan, China) and cultured in a 5% CO_2 and 95% humidified air atmosphere at 37°C in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS). Cells were split every 3 days to ensure logarithmic growth. Noscapine hydrochloride was obtained from Sigma (Sigma–Aldrich, St. Louis, MO, USA). Noscapine was dissolved in dimethyl sulfoxide (DMSO), and stock solution (100 mM) was stored at −20°C.

MTT assay

Cell proliferation was assessed with MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (Sigma, St. Louis, MO, USA) colorimetric method: The gastric cancer cells were cultured in a 96-well plate in the presence of 1–150 μM noscapine for 12–36 h. MTT reagent was added into each well and incubated for 2–4 h at 37°C. After the purple precipitate was visible, the cells were added with 150 μl isopropanol/0.04 M HCl and incubated at room temperature in the dark for 2 h. The absorbance was recorded at 570 nm. For each detection, the total procedure was repeated three times.

Detection of apoptotic morphological feature by Hoechst 33258

Apoptotic cells were detected by Hoechst 33258 staining as follows: BGC823 cells were treated with 0, 50, 100, and 150 μM noscapine for 24 h, followed by incubation with 20 μM Hoechst 33258 (Sigma–Aldrich, St. Louis, MO, USA) for 10 min at room temperature. The cells were then washed twice with PBS and examined under a fluorescence microscope (Nikon, Japan). Apoptotic feature was assessed by observing chromatin condensation and fragments staining by Hoechst 33258. In each case, ten random fields were counted.

Annexin V staining

After treatment with noscapine for 24 h, cells were harvested and washed twice with cold PBS and resuspended in binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl and 2.5 nM $CaCl_2$) at a concentration of 1×10^6 cells/ml cells were stained with 5-μl ready-made Annexin V-FITC and 5-μl propidium iodide(PI) (BioVision, Mountain New, CA, USA) and incubated for 10 min at room temperature in the dark according to the manufacturer's instructions. At the end of incubation, 200 μl of binding buffer was added, and the cells were analyzed immediately by flow cytometry. Flow cytometric analysis was performed with a FACSCaliber using the CellQuest software (BDIS). The analyses of cells stained with V/PI were presented: Annexin V^+/PI^- = apoptotic cells; Annexin V^-/PI^+ = necrotic cells [12]. For each detection, the total procedure was repeated three times.

Western blot assay

Protein expression levels were analyzed by Western blot analysis. About 6.0×10^5 BGC823 cells were cultured on a 6-well dish and allowed to attach overnight followed by treatment with noscapine. Briefly, cells were washed with PBS and lysed in lysis buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 50 μg/ml leupeptin, 30 μg/ml aprotinin, and 1 mM PMSF). A total volume of 40 μg of protein was loaded per lane and separated on 12.5% sodium dodecyl sulfate polyacrylamide (SDS–PAGE) gels and then transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA) by wet transfer system (Bio-Rad, Hercules, CA, USA). After the membrane was blocked with 10% non-fat dry milk in TBST and then incubated with primary antibodies and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The following dilutions were used: Bcl-2, Bax, Cyt-*c*, Caspase-3 and Caspase-9 both 1:1000, and actin 1:3000. After three washes with TBST, the membranes were incubated with biotinylated goat anti-rabbit IgG secondary antibody (Promega, Madison, WI, USA, 1:5000 dilution) for 2 h at room temperature and then developed with the use of 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 video imaging CMIASWIN system (Bio-Rad, Hercules, CA, USA).

Xenograft tumor model

All animal procedures were approved by the Committee on Animal Experimentation of Wuhan University, and the procedures were complied with the NIH Guide for the Care

and Use of Laboratory Animals. Male BALB/c nude mice, 4–5 weeks of age, obtained from the Center of Experimental Animals of Wuhan University, were used in all of the experiments. Nude mice were divided into four groups: control group, low-dose group (10 mg/kg), mid-dose group (20 mg/kg), and high-dose group (40 mg/kg). There were no statistical differences among the sizes of all the groups. BGC823 cells, 5.0×10^6, suspended in 100-μl PBS, were subcutaneously inoculated into the lower right flank of the nude mice ($n = 6$ in each group). When the tumors were 100–150 mm^3 in size, noscapine was administrated via intratumoral injection every 3 days. Tumor growth was monitored using calipers every 3 days. There was no statistical difference between these groups at the start of treatment. Tumor volume (V) was calculated by using the formula: tumor volume V (mm^3) = $\pi/6 \times$ width (mm^2) $\times$ length (mm). At the end of the experiment, tumors were harvested for additional analyses as described below. Differences in tumor growth were tested for statistical significance.

HE staining and TUNEL assay

Tumor tissues were fixed in 4% formaldehyde, dehydrated with gradient ethanol, and embedded in paraffin. Tissue sections (4 μm) were then dewaxed and rehydrated according to a standard protocol. For histologic analysis, sections were stained with hematoxylin and eosin (HE). For TUNEL assay, an in situ apoptosis detection kit (Roche Diagnostics, Branchburg, NJ, USA) was used to detect apoptotic cells in tumor tissue sections. After incubation with proteinase K and rinsing with ddH_2O, tumor sections were dewaxed with dimethylbenzene and rehydrated with gradient ethanol. Endogenous peroxidase was blocked with 3% H_2O_2, and sections were incubated with equilibration buffer and terminal deoxynucleotidyl transferase (TdT) enzyme. Finally, the sections were incubated with anti-digoxigenin–peroxidase conjugate. Peroxidase activity in each section was shown by the application of DAB. Sections were counterstained with hematoxylin. The positive cells were identified, counted (three random fields per slides), and analyzed under the light microscopy (CarlZeiss, Thornwood, NY, USA).

Statistical analysis

The Statistical Package for the Social Science (SPSS) for Microsoft Windows 13.0 (SPSS Inc., IL, USA) was used to complete the analysis of the collected data. All data were expressed as means ± SEM. The means of the different groups were compared using one-way analysis of variance (ANOVA) test. Significant differences were accepted when $P < 0.05$.

Results

Growth inhibitory effect

After treatment of the four gastric cancer cell lines (BGC823, SGC7901, MGC803, HGC27) with 10 μM noscapine, cell viability was determined by MTT assay. As known in Fig. 1a, noscapine significantly inhibited the proliferation of all cell lines tested. Especially, noscapine at 12, 24, and 36 h inhibited BGC823 cells viability by 10, 22, and 76% of control group, respectively. Furthermore, the number of BGC823 cells was greatly decreased by incubation 100 and 150 μM noscapine, while a minimal decrease was shown in 10 μM noscapine after treatment for 24 h (Fig. 1b). These data indicated that noscapine has a potential not only to prevent gastric cancer cell growth, but also to reduce the number of gastric cancer cells.

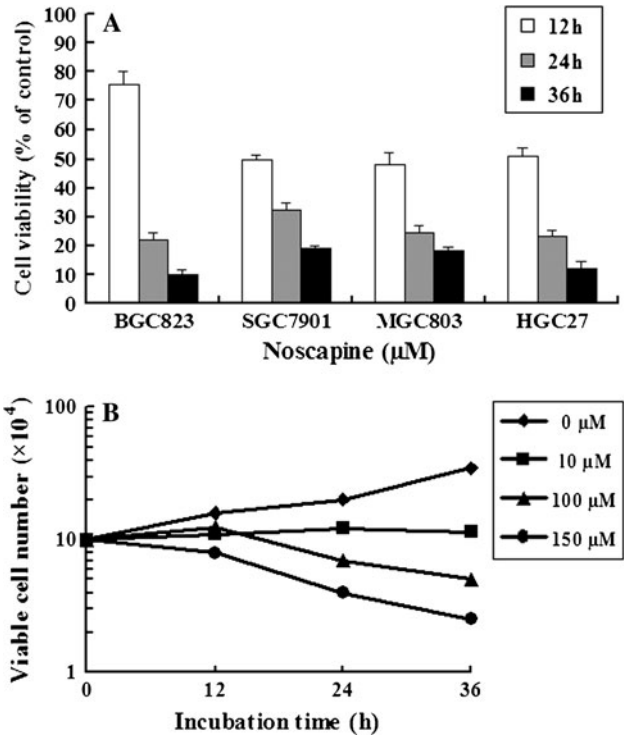

Fig. 1 Noscapine inhibited cell growth of gastric cancer cell lines. **a** Growth inhibition of gastric cancer cell lines by noscapine. Four gastric cancer cell lines were cultured in the presence of 10 μM noscapine for 12, 24, and 36 h. Cell viabilities relative to control group at each time point were determined by MTT assay. Data are shown by means ± SEM ($n = 3$). **b** Kill curve for BGC823 cell growth by noscapine. BGC823 cells were incubated with 0, 50, 100, 150 μM of noscapine for the indicated periods. The number of cells was determined by trypan blue exclusion test. Data are shown by means ± SEM ($n = 3$)

Induction of apoptosis

In addition, the morphological changes of the apoptotic BGC823 cells were detected by Hoechst 33258 staining. Chromatin condensation and nuclear fragmentation are the classic characteristics of apoptotic cells. In the control cells, the nuclei were stained a weak homogeneous blue, while in treatment groups, bright chromatin condensation and nuclear fragmentation could be found (Fig. 2).

Apoptosis is characterized by distinct morphological features such as nuclear fragmentation and chromatin condensation. Noscapine has been reported to have an anti-tumor effect by apoptosis induction in various solid tumors [10, 11]. Thus, we examined here whether it resulted in gastric cancer cell death via apoptosis. BGC823 cells were treated with noscapine (50–150 μM) for 24 h followed by annexin V/PI staining to examine the proportion of apoptotic cells. As shown in Fig. 3a–d, it revealed that noscapine caused cell apoptosis in a dose-dependent manner.

Caspase activity in BGC823 cells treated with noscapine

The apoptosis is initiated by a caspase cascade. We therefore addressed whether noscapine-induced apoptosis by caspase signaling. BGC823 cells were treated with 50, 100, and 150 μM noscapine for 24 h, followed by Western blot to assess the caspase activation. In Fig. 4a, cleaved forms of caspase-3 and caspase-9 were detected, indicating that noscapine-induced apoptosis via caspase cascade. Caspase-9 plays an important role as a downstream effecter of mitochondrial pathways, then, activating caspase-3, which results in a proteolytic cascade that gives rise to the cell death [13]. The results suggest that noscapine induces mitochondrial dysfunction in gastric cancer cells.

Bcl-2 family proteins, such as Bcl-2 and Bax, play another important role in apoptosis. Bax protein was upregulated and Bcl-2 protein was downregulated after noscapine treatment (Fig. 4b). It is established that Bcl-2 inhibits Bax activity, which reduces mitochondrial membrane potential, leading to Cyt-*c* upregulation and cell apoptosis.

Anti-tumor effect in vivo

Following the investigation of apoptosis induction in BGC823 cell in vitro, the anti-tumor effect of noscapine was evaluated. The in vivo study was investigated in xenograft model. After growing for 6 days, the tumor xenografts reached a mean size of 100 mm^3. We chose 18 mice with tumor xenografts of around 100 mm^3 in size and randomly divided them into three groups. There were no statistical differences among the sizes of all the groups. The nude mice were given different concentrations of noscapine via intratumoral injection every 3 days. As shown in Fig. 5a, b, noscapine causes the inhibitory effects on the growth of tumor in vivo. Control group, in which tumors grew progressively and reached 1,000 mm^3 within 18 days. However, the treatment groups were significantly suppressed. Treatment with noscapine reduced tumor growth in a dose-dependent manner. At the end of 33 days, noscapine at 10, 20, and 40 mg/kg inhibited tumor growth by 45, 60, and 81% of control group (2,178 mm^3), respectively (Fig. 5a). Compare with control group, tumor weight was only 0.206 g in high-dose group at the end of the experiment (Fig. 5b).

HE staining (no data) and TUNEL assay of the subcutaneous tumor sections demonstrated that noscapine caused obvious cell death in tumor mass via apoptosis (Fig. 6), whereas less apoptosis was found in control group ($P < 0.05$). These results proved that noscapine has significant anti-tumoral potential in vivo.

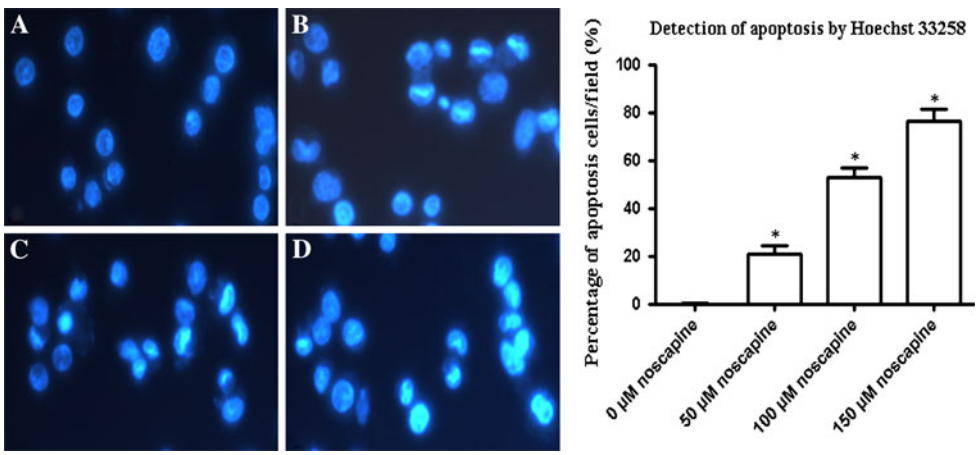

Fig. 2 Detection of apoptosis by Hoechst 33258. BGC823 cells were incubated with 0, 50, 100, 150 μM of noscapine for 24 h (**a**–**d**) and fixed and stained with 20 μM Hoechst 33258 at room temperature, and apoptotic feature is assessed by observing chromatin condensation and fragments staining. *$P < 0.05$ versus control cells

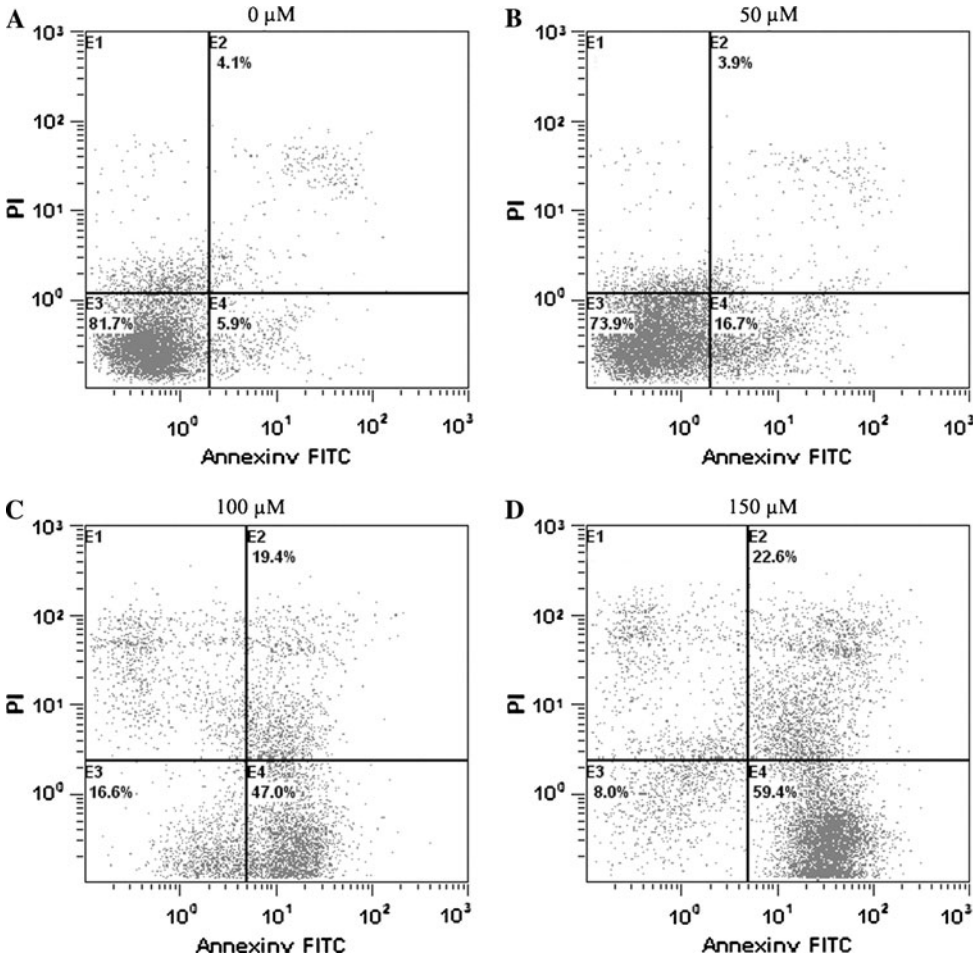

Fig. 3 Induction of apoptosis in BGC823 cell. BGC823 cell was treated with various concentrations (0–150 μM) of noscapine for 24 h, and annexin V/PI staining was performed (*X* axis: annexin V; *Y* axis: PI). Percentage value indicates the proportion of apoptotic cells

Discussion

Gastric cancer is the second most common cause of cancer-related death in the world, with around 700,000 deaths a year. Gastric cancer is common in China, and its survival of patients with gastric cancer is substantially worse than the patients with most other solid malignancies [1]. *Helicobacter pylori* cause gastric cancer with high frequency. This updated summary of the relationship between *Helicobacter pylori* and gastric cancer shows the strong link between the organism and the development of gastric cancer and suggests eradication of this bacterial infection as a possible prophylactic measure against the development of this malignancy [14]. Gastric cancer is one of the malignancies, that is, difficult to cure by conventional drug-therapies when the disease has progressed to the unresectable stage, moreover, many patients develop tumor recurrence after the first treatment. To reduce the mortality of gastric cancer, developing medicines with high efficacy, low toxicity, and new mechanisms of action is required [15].

Noscapine is considered as safe anti-tumor agent, which was demonstrated anti-tumor activity both in vitro and in vivo in various cancer cells that are resistant to the conventional anti-tumor drugs. More importantly, noscapine did not exhibit severe side effects that are commonly seen with many chemotherapeutic drugs [11, 16–18]. Noscapine is in phase I/II clinical trials for the treatment of hematological malignancies and chronic lymphoma. Noscapine has been determined to have significant effect on different types of lethal tumors [7, 9, 19]. However, its effect on gastric cancer has not yet been addressed. In the study, we demonstrate that noscapine inhibits BGC823, SGC7901, MGC803, and HGC27 cells growth by inducing the cell death, especially in BGC823 cells. Noscapine treatment resulted in caspase-3 and caspase-9 activation in BGC823 cells, suggesting that a mitochondria-mediated mechanism plays a role in apoptosis induction. These results led us to propose that the mitochondrial dysfunction is the main pathway in noscapine-induced apoptosis in gastric cancer cells.

We examined the anti-proliferation activity of noscapine against four gastric cancer cell lines. Interestingly, the

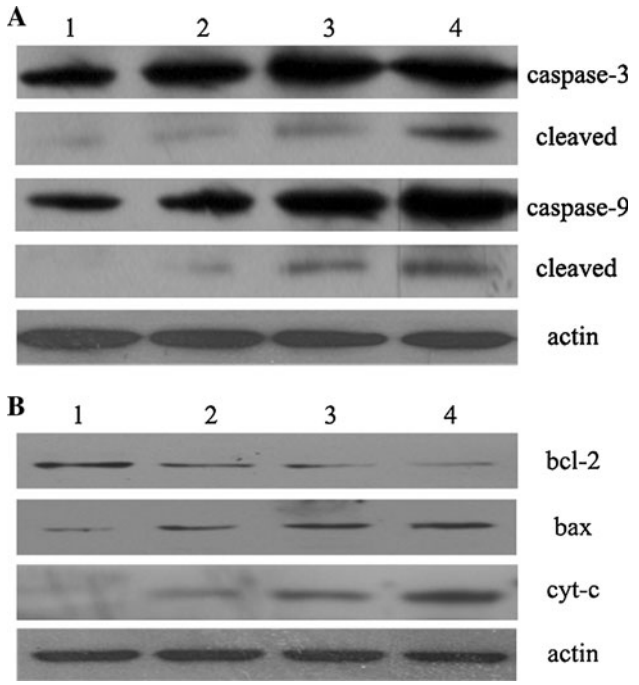

Fig. 4 Immunoblot analysis of untreated and noscapine-treated BGC823 cell (0–150 μM) for 24 h. *Lane 1* control, *Lane 2* noscapine 50 μM, *Lane 3* noscapine 100 μM, and *Lane 4* noscapine 150 μM. **a** Noscapine-induced caspases activation. Expression of activation of caspase-3 and caspase-9 detected by Western blot assay. Actin was used as the internal control. **b** Western blot analysis was performed with anti-bcl-2, anti-bax, and anti-Cyt-*c* antibodies. Actin was shown as an internal control

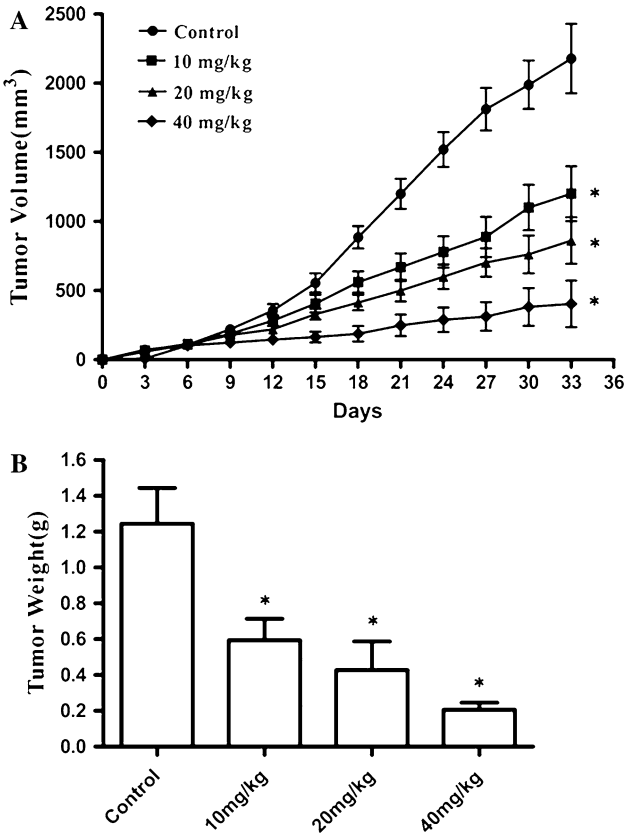

Fig. 5 In vivo anti-tumor effect of different doses of noscapine compared to untreated group in gastric tumor xenograft model. **a** Each time point represents the mean tumor volume for each group. *Error bars* represent the standard error of the mean (SEM). **b** Tumor weight was obtained at the end of the experiment

number of BGC823 cells was greatly decreased in the same treatment times and drug concentration. Compare with other cell lines, these result suggested that BGC823 cells have a better sensitive to noscapine. These data (Fig. 1b) mean that noscapine exerts a cytostatic effect at low concentrations, so that BGC823 cells do not proliferate but they are metabolically impaired (and therefore they do not metabolize MTT).

It also reveals that noscapine resulted in apoptosis (Hoechst 33258 and annexin V^+/PI^-) of treated cells in a dose-dependent manner (Figs. 2, 3). The percentage of necrosis is much lower with respect to apoptotic death. One part (annexin V^+/PI^+) might be undergoing secondary necrosis from the part of early apoptotic versus necrotic (annexin V^-/PI^+) cells. Theses morphological features in BGC823 cells of apoptotic versus necrotic cell death can be distinguished under microscopy [20]. It suggests that if early apoptotic cells are not ingested by phagocytes in time, then secondary necrosis would proceed [21]. Therefore, pooling these secondary necrosis cells with annexin V^+/PI^+, the extent of apoptosis resulted by noscapine is apparently much greater.

The Bcl-2 gene was originally found as a proto-oncogene of human follicular lymphoma [22]. Bcl-2 is the prototype of a large family of proteins, which share a high degree of homology, but they exert different functions including anti-apoptotic and proapoptotic [23]. Compare with many oncogenes, Bcl-2 gene does not trigger cell proliferation but promotes survival under deleterious growth conditions [24]. Bcl family proteins are always involved in mitochondria-mediated apoptosis as exemplified by the apoptosis accelerator [25, 26]. Anti-apoptotic proteins have usually four Bcl-2 homology (BH) domains, whereas proapoptotic proteins display either three BH domains. The latter proteins are called BH3-only proteins and represent by far the most numerous proteins [27–29]. In BGC823 cells treated with noscapine, expression of Bcl-2 protein was downregulated while that of Bax was upregulated (Fig. 4b). It has been reported that the ratio of Bcl-2 and Bax determines the response to a death signal via modulating membrane permeability transition (MPT) pore opening [30]. Subsequently, noscapine may modulate the death-receptor-associated extrinsic caspase-8 pathway, leading to activation of the mitochondria-associated intrinsic caspase-9 pathway in BGC823 cells, the result

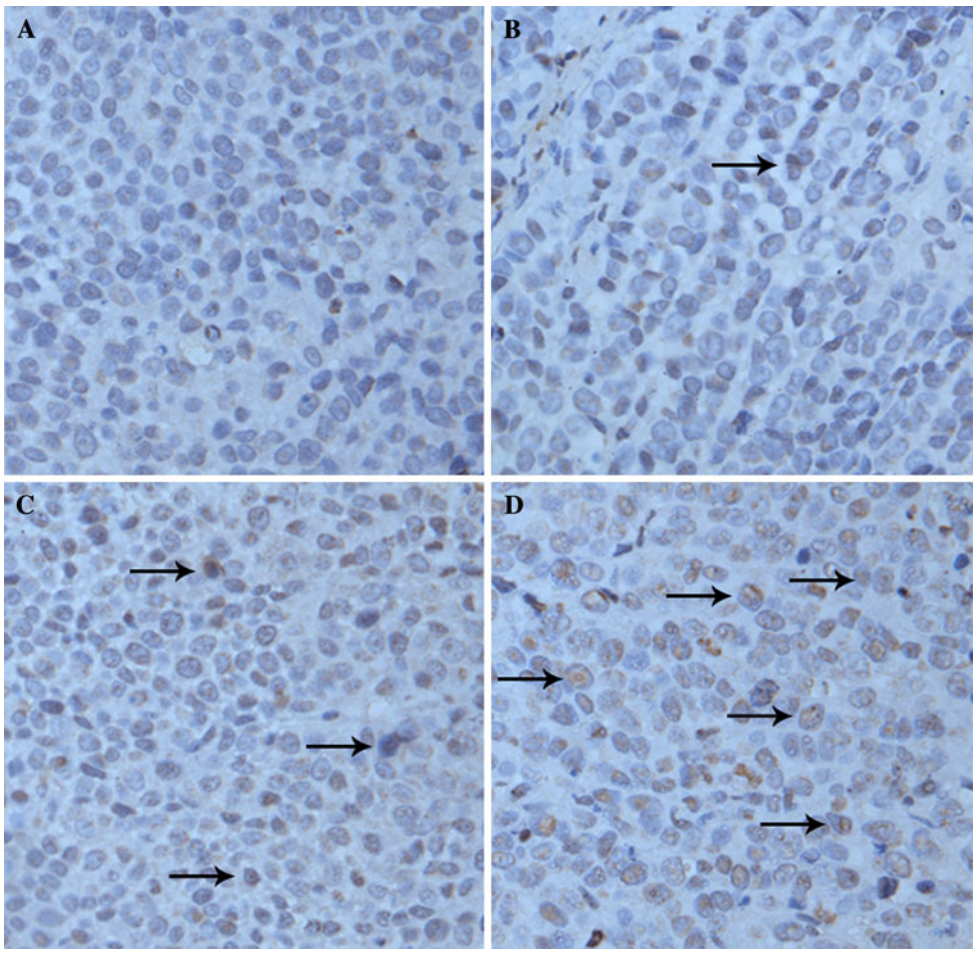

Fig. 6 Detection of apoptotic cells in tumor tissue by TUNEL assay. **a** Tumor with untreated group, **b** tumor with low-dose noscapine (10 mg/kg), **c** tumor with mid-dose noscapine (20 mg/kg), and **d** tumor with high-dose noscapine (40 mg/kg). The *brown* color of apoptotic signals is shown by the *arrows*. Original magnification: ×400

was cooperative activation of the executioner caspase-3 by both caspase pathways in the end. In agreement with the hypothesis, activated caspase-3 and -9 were detected in BGC823 cells treated with noscapine in each group (Fig. 4a).

In the xenograft tumor-bearing nude mouse model, noscapine is revealed to have significant anti-cancer effect (Fig. 5a, b). The therapeutic effect has been confirmed to occur, at least in part, through apoptosis induction, as determined by TUNEL staining of tumor sections (Fig. 6). BGC823 cell line-bearing mouse models are shown to have degrees of susceptibility to noscapine treatment; however, the therapeutic effect is exclusively correlated with caspase-3 and caspase-9 activity-induced ability by noscapine in these cancer cells (Fig. 4a). It implies that apoptosis induction is a critical function of noscapine to exert its anti-cancer effect on BGC823 cells. Our further studies are required to make clear that whether noscapine enhancing apoptosis-inducing effects on human gastric cancer cells which are cross-talking activation of death receptors pathway of apoptosis.

In conclusion, we demonstrated the mechanism of action in inducing apoptosis in gastric cancer cell lines by noscapine, in which caspase-dependent mitochondrial dysfunction is involved. Studies on noscapine provide valuable information in the development of chemotherapeutics with high-efficacy low side effects and new mechanisms of action. These results may lead to the usage of noscapine on chemoprevention in gastric cancer in the future.

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