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Antiproliferative activity of the total saponin of *Solanum lyratum* Thunb in Hela cells by inducing apoptosis

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Total saponin of *Solanum lyratum* Thunb (TSSLT), a species of natural biologically active substances isolated from *Solanum lyratum* Thunb, possesses various bioactivities. It has been proposed that the induction of apoptosis may be the basis of its antitumor activity. However, the molecular mechanism underlying the total saponin-induced apoptotic process remains unknown. In the present study, we describe the anti-proliferative effect of TSSLT on human cervical cancer cells (Hela). The TSSLT induced apoptosis of Hela in a time-dependent manner with an IC_{50} for cell viability of 6 μg/ml. The TSSLT-induced cell death was characterized by changes in cell morphology, DNA fragmentation, activation of caspase-like activities, poly (ADP-ribose) polymerase (PARP) cleavage and release of cytochrome *c* (cyt *c*) into cytosol. TSSLT activated various caspases such as caspase-3, -8, and -9 (like) activities but not caspase-1 like activity. The cell death was completely prevented by the pan caspase inhibitor benzyloxy carbonyl-Val-Ala-Asp- fluoromethyl-ketone (Z-VAD-FMK). More than 80% cell survival was observed in the presence of a caspase-3 inhibitor. In addition, treatment with TSSLT induced the increase of Bax:Bcl-2 ratio in Hela cells. These results suggest that the induction of apoptosis by TSSLT involves multiple pathways antigen including death receptor and mitochondrial pathway and strongly suggest that the mitochondrial pathway was mediated by low expression of Bcl-2 and upregulation of Bax, release of cyt *c* and subsequent activation of caspase-3 followed by down stream events leading to apoptotic cell death.

1. Introduction

Human cervical cancer is the most prevalent malignancy in women in many developing countries. It is generally accepted that radical surgery or radiotherapy can be curative for the majority of patients with early-stage cervical cancer, while chemotherapy or neoadjuvant chemotherapy are always the first choice for those patients with advanced cervical cancer, where the prognosis remains very poor (Benedetti et al.1988; Thomas 1999). However, with the currently available chemotherapeutics, only a modest increase in five-year survival rate can be achieved in the patients with advanced cervical cancer due to the little chemosensitivity of cervical cancer cells to chemotherapeutics (Xiao 2007). More effective chemotherapeutics are apparently needed for those patients to improve five-year survival rate.

As one of Chinese traditional medicines, *Solanum lyratum* Thunb (Solanaceae) (SLT) is well known as "*Hedrba Solani Lyrati*" in mainland of China, with bioactivities of the regulation of the immune function (Sun et al. 2005), antitumor, anti-anaphylaxis and hepatoprotective effect (Sun et al. 2006). This herb contains glycoside, organic acid, triterpenoid, coumarins, and so on (Xu et al. 2006). In recent years, some work has been carried out on specific active properties of various extracts from SLT (Wu et al. 2005). Total saponin of SLT (TSSLT), is an unusual class of compounds with a wide variety of biological activities

Abbreviations: cyt *c* – cytochrome *c*, DAPI – 4′,6′-diamidino-2-phenylindole hydrochloride, DMSO – dimethyl sulfoxide, FBS – fetal bovine serum, ICE – interleukin-1-converting enzyme, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide, PBS – phosphate buffered saline, PI – propidium iodide, Z-VAD-FMK – benzyloxy, Z-VAD-FMK – carbonyl-Val-Ala-Asp-fluoromethyl-ketone

including anti-tumor, anti-anaphylaxis activity, etc. In a program aimed at the discovery of cytotoxic metabolites from SLT, we found the TSSLT has significant proliferative inhibition activity against Hela cell lines. Several studies have shown that TSSLT possesses various bioactivities such as anti-anaphylaxis and hepatoprotective activity, anti-tumor activity against various tumor cells *in vitro* or *in vivo* (Ren et al. 2006a, b). However, the molecular mechanism underlying the TSSLT-induced apoptotic process remains unclear.

With becoming clear that caspases (cysteine aspartic acid-specific protease) play a central role in the execution of apoptosis (Los et al. 2001), they are found in cells as inactive precursors, being converted into their active form dismantle key cellular structure by generating proteolytic torrent (Earnshaw et al. 1999). There is evidence that caspase activation occurs in cell death pathway through the initiation of a certain tumor necrosis factor. However, more recently an alternative route to caspase activation involving mitochondria has emerged. This process is initiated by the interaction of mitochondria with one or more of the Bcl-2 family proteins. Thus, a number of Bcl-2 family proteins have been considered to be the major regulators of the apoptotic process, and represent a critical checkpoint within apoptotic pathways, acting upstream of such irreversible damage to cellular constituent. Bcl-2 family of homologous proteins comprise pro-, as well as, anti-apoptotic molecules. For instance, anti-apoptotic proteins Bcl-2 has been shown to inhibit apoptosis, whereas proapoptotic Bax has been reported to enhance apoptosis. Furthermore, the ratio of Bcl-2 to Bax also determines, at least in part, how a cell responds to apoptotic or survival signals (Farrow and Brown 1996).

In this study, we attempted to confirm whether the pathways mentioned above were linked. We hypothesized that TSSLT might actively induce apoptosis and reduce proliferation of Hela cells, which may be mediated by an activated caspase pathway and elevated Bax/Bcl-2 ratio.

2. Investigations and results

2.1. Effect of TSSLT on cell proliferation

To assess the effect of TSSLT on cell proliferation, MTT assay was used. As the results showed, TSSLT inhibited proliferation of HeLa cell in a dose- and time-dependent manner (Fig. 1). Proliferation inhibition was observed as early as 12 h when cells were treated with TSSLT at a concentration higher than 10 μg/ml, and the strongest inhibition was detected when cells were treated with TSSL at a concentration higher than 250 μg/ml for 72 h. About 6 μg/ml of TSSLT inhibited 50% of cell growth after 48 h,

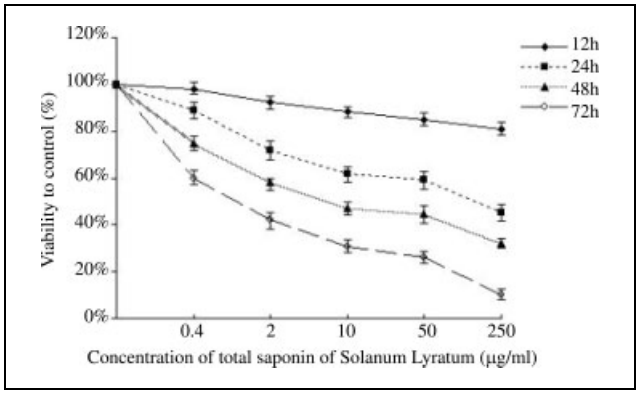

Fig. 1: The cells (1×10^4/well) treated with different concentrations of TSSLT were cultured for 12, 24, 48 and 72 h, respectively. The percentage of cell viability was determined by MTT assay (C = 0.4 μg/ml, 2 μg/ml, 10 μg/ml, 50 μg/ml, 250 μg/ml). Data are shown as the mean ± SD (n = 6)

and for all subsequent experiments, the same concentration was used.

2.2. Effect of TSSLT on Hela cell apoptotic induction

The translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the cell surface is characteristic for early apoptotic cells (Wang et al. 2006). To confirm that TSSLT induces cell apoptosis, annexin V-FITC/PI assay, based on flow cytometry, was used to examine the early apoptotic cells. Cells undergoing apoptosis will first express phosphatidylserine on the outer leaflet of the cell membrane, marked by annexin-V-FITC binding, followed by the membrane becoming compromised, marked by PI intercalation in the cellular DNA and RNA. As indicated in Fig. 2, in untreated cells, 1.75% of cells were annexin V-positive/PI-negative, whereas 2.24% of cells were annexin V/PI double positive. After treatment with TSSLT for 8 h, the corresponding quantities were 9.79% and 28.45%, respectively. This result suggests that these cells are expressing phosphatidylserine on the outer leaflet of the membrane and that these cells may be undergoing apoptosis.

2.3. Effect of TSSLT on DNA fragmentation and DNA laddering

DNA fragmentation is a typical biochemical feature of apoptosis. To further confirm the occurrence of TSSLT-induced apoptosis, a time dependent cellular DNA fragmentation was studied by gel electrophoresis, and a ladder like

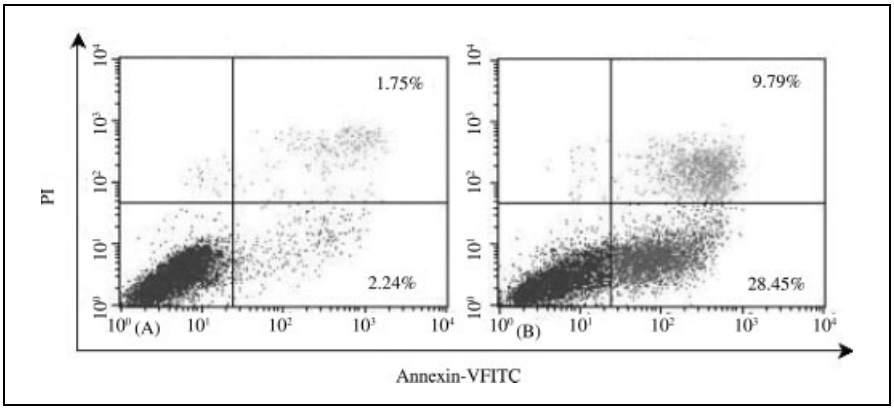

Fig. 2:
Flow cytometric analysis of HeLa cells treated with TSSLT (6 μg/ml). (A) Control; (B) HeLa cells treated with TSSLT for 8 h. The dual-parameter fluorescent dot plots showed the viable cell population in the lower left quadrant (annexin V^-PI^-), the cells at the early apoptosis are in the lower right quadrant (annexin V^+PI^-), and the ones at the late apoptosis are in the upper right quadrant (annexin V^+PI^+)

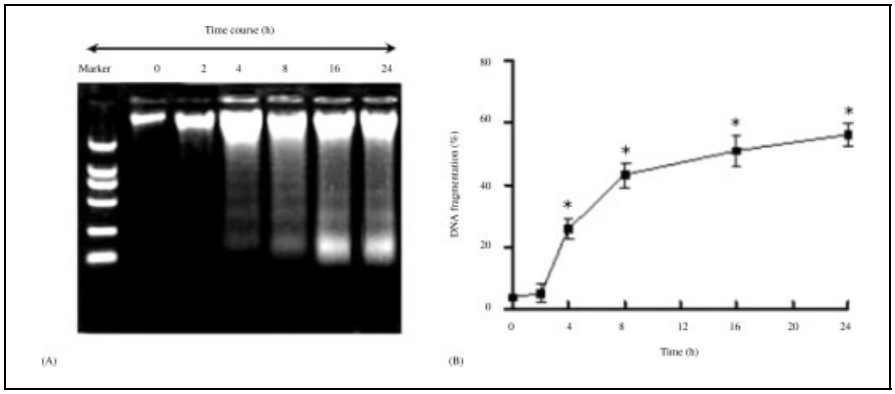

Fig. 3:
Time course effect of TSSLT (6 μg/ml) on DNA fragmentation. (A) DNA agarose gel electrophoresis of TSSLT-treated Hela cells (6 μg/ml). Lane 1 marker, lane 2 control, lanes 3–7 clitocinetreated cells for 2, 4, 8, 16, 24 h, respectively. (B) % DNA fragmentation tested by DAPI. Data are shown as the mean ± SD ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs control group

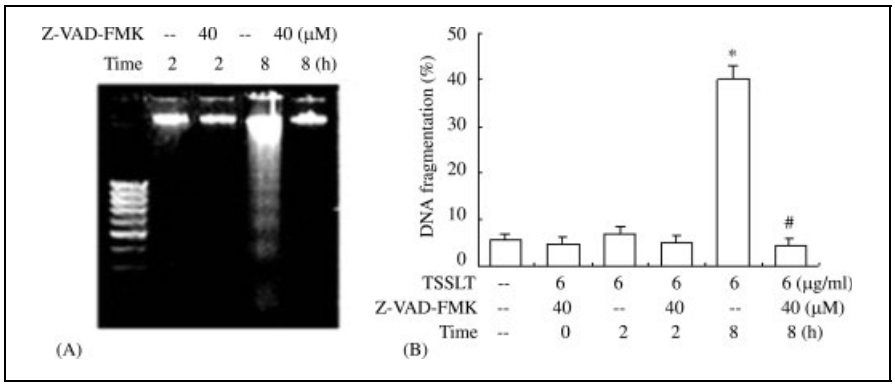

Fig. 4:
Effect of a pan caspase inhibitor on TSSLT-induced DNA fragmentation. Cells were pretreated with or without pan caspase inhibitor (Z-VAD-FMK) for 2 h, and then incubated with TSSLT (6 μg/ml) for 2 and 8 h. DNA fragmentation was visualized by agarose gel electrophoresis (A) or estimated by DAPI method (B). Data are shown as the mean ± SD ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs TSSLT group alone

pattern, typical of DNA cleavage between nucleosomes was visible during 4–24 h after incubation with 6 μg/ml TSSLT (Fig. 3A). The intensity of banding was more prominent at 16 and 24 h compared to earlier time points. In addition to qualitative DNA fragmentation quantitative fragmentation analysis was carried out by a method involving separation of low molecular weight DNA by centrifugation ($27{,}000 \times g$) and staining with DAPI. Hela cells treated with 6 μg/ml TSSLT after 4 h resulted in remarkable increase in DNA fragmentation (Fig. 3B).

2.4. Effect of caspases inhibitor on TSSLT-induced DNA fragmentation

Caspases play a central role in the execution phase of apoptosis and are responsible for many of the morphological features normally associated with this form of cell death (Bratton 2000). Morphological and biochemical assay of apoptosis led us to hypothesize that TSSLT might activate the caspase-dependent cell death pathway. Thus, the activation of caspase induced by TSSLT was evaluated in Hela cells. Z-VAD-FMK, a pan caspase inhibitor was used to investigate the possible role of caspases on TSSLT-induced apoptosis. As indicated in Fig. 4, DNA fragmentation induced by TSSLT was effectively suppressed when cells were pretreated with 100 μg/ml of as analyzed using agarose gel electrophoresis and DAPI method. The results indicate that activation of caspases were crucial for TSSLT-induced apoptosis in Hela cells.

2.5. Effect of caspases inhibitor on TSSLT-induced cell death

To further demonstrate the involvement of caspase activation in the apoptotic effect, the effects of a pan caspase inhibitor (Z-VAD-FMK), and two specific caspase inhibitors, Z-DEVD-FMK for caspase-3 and Z-LEHD-FMK for caspase-9 on TSSLT-induced apoptosis was investigated. Z-VAD-FMK completely prevented TSSLT-induced cell death (Fig. 5). Among the specific caspase inhibitors, Z-DEVD-FMK was found to be most effective in preventing cell viability loses. The cell survival reduced to 48.7% at 48 h after 6 μg/ml of TSSLT treatment was increased up to 87% when the cells were pretreated with a caspase-3 inhibitor (100 μM) however at the same condition a caspase-9 inhibitor increased cell viability up to 75%. These findings allow us to conclude the involvement of caspase-dependent pathway(s) in TSSLT-induced apoptotic death of Hela cells.

2.6. Effect of TSSLT on caspase-like activity

In view of the inhibitory effect of pan caspase inhibitor on the fragmentation of DNA in TSSLT-induced apoptosis,

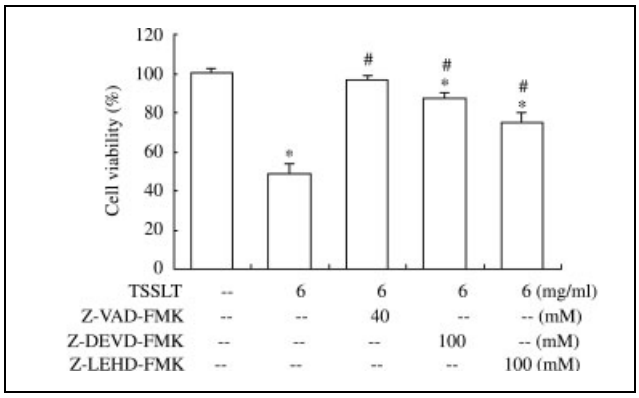

Fig. 5: Inhibition of TSSLT-induced cell death by caspase inhibitors. HeLa cells were pretreated with or without Z-VAD-FMK (pan caspase inhibitor), Z-DEVD-FMK (caspase-3 inhibitor), Z-LEHD-FMK (caspase-9 inhibitor) for 30 min and exposed to TSSLT (6 μg/ml) for 48 h. Cell viability was measured by MTT assay method. Data are shown as the mean ± SD ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs TSSLT group alone

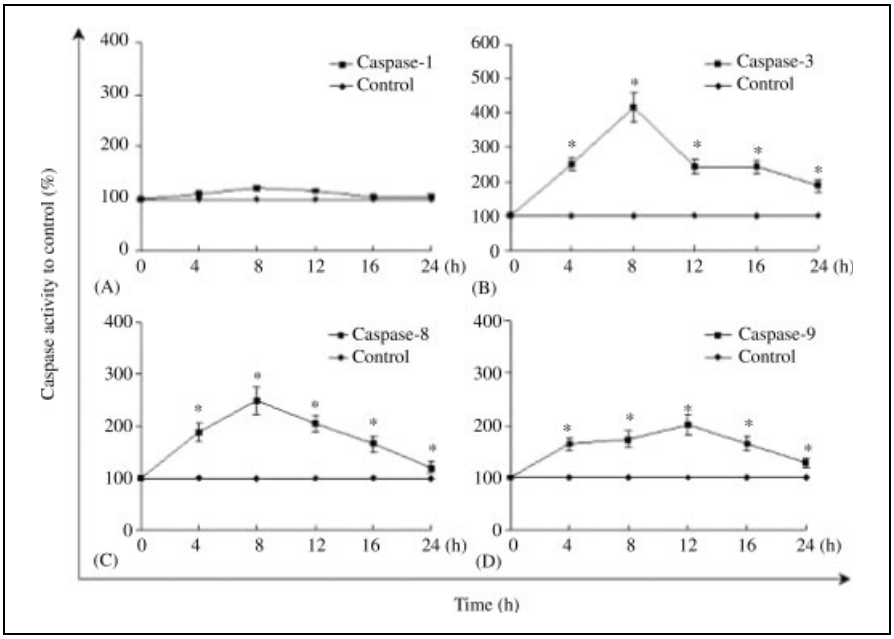

Fig. 6:
Time course activation of various caspase-like enzymes in TSSLT-treated HeLa cells. HeLa cells were incubated with TSSLT (6 μg/ml) for various time periods (0, 4, 8, 12, 16, 24 h). Cytosolic fraction of cells was analyzed for changes in the activity of caspase-1 (A), caspase-3 (B), caspase-8 (C), caspase-9 (D). Data are shown as the mean ± SD ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs control group

we examined the effect of TSSLT on the activation of caspase-1, -3, -8, -9-like activities in Hela cells. For this purpose, cytosolic extract of control cells and TSSLT-treated cells were incubated with specific caspase substrates for each caspase. The results showed that caspase-3 and caspase-8 activities were rapidly elevated and peaked at 8 h of TSSLT treatment (Fig. 6B, C), whereas caspase-9 activity peaked at 12 h (Fig. 6D). In addition, we found the activity of caspase-3 remained at high level after the peak time (Fig. 7B), which may be associated with the increased level of caspase-9 activity. The activity of caspase-1 was almost unaffected by TSSLT (Fig. 6A).

2.7. Effect of TSSLT on the cleavage of PARP

Cleavage of PARP is an important indicator of caspase-3 activation during apoptosis (Tewari et al. 1995). To gain the additional evidence of caspase-3 activation, the cleavage of PARP was investigated by Western blot analysis using a mouse monoclonal antibody. Incubation of cells with TSSLT resulted in the formation of 85 kDa protein fragments compared to the intact protein (116 kDa). As showed in Fig. 7A, the cleavage of PARP in Hela cells started at 4 h after treatment with TSSLT (6 μg/ml) and became clearer when caspase-3 activation increased significantly.

2.8. Release of cyt c induced by TSSLT

Cyt *c* is localized in the intermembrane space and loosely attached to the surface of the inner mitochondrial membrane. Release of cyt *c* from the intermembrane spaces of the mitochondria into the cytosol is a key event in apoptosis. To examine this step in the apoptotic cell death pathway initiated by TSSLL, we examined the cyt *c* content in cytosol of Hela cells treated with TSSLT (6 μg/ml) through 0–16 h. In Fig. 7B, progressive accumulation of cyt *c* in cytosolic compartment is demonstrated. The release of cyt *c* was observed as early as 2 h of TSSLT treatment. The release of cyt *c* preceded the activation of caspase-3 and PARP cleavage thus suggesting that it might be important in the execution of TSSLT-induced apoptosis.

2.9. Effect of TSSLT on level of Bax and Bcl-2 expression

The release of cyt *c* from mitochondria is tightly regulated by a variety of factors. Among these, Bcl-2 family proteins, including anti-apoptotic members (such as Bcl-2) and proapoptotic members (such as Bax), play a pivotal role (Burlacu 2003). To further unravel the possible me-

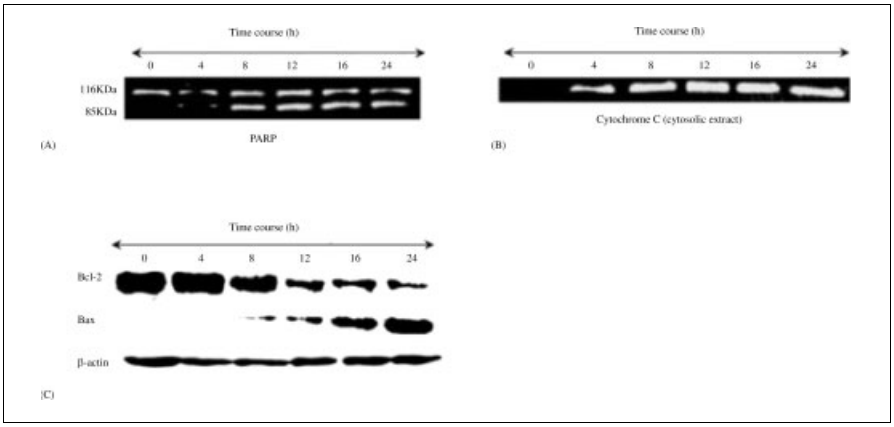

Fig. 7:
Time-dependent cleavage of PARP, cytosolic accumulation of cyt *c*, and expression of Bcl-2 and Bax in TSSLT-induced apoptosis. The cells were treated with TSSLT (6 μg/ml) for various time periods. Total cell lysates were prepared and analyzed by immunoblotting for PARP analysis as described under Section 2 (A). Cytosolic fraction (about 30 mg) was analyzed to detect release of cyt *c* in cytosol by immunoblotting with an anti-cyt *c* antibody (B). Cell lysates were prepared and protein level of Bcl-2 and Bax was determined by Western blotting. β-actin was used for normalization and verification of protein loading

chanism underlying the TSSLT-induced apoptosis, we examined the expression of Bcl-2 and Bax in Hela cells after TSSLT treatment. As shown in Fig. 7C, exposure of Hela cells to 6 μg/ml TSSLT for 0–24 h resulted in a marked decrease of Bcl-2 protein expression, but a drastic increase of Bax protein expression in a time-dependent manner.

3. Discussion

During the past decade, the evidence is gradually accumulating that many cancer chemotherapeutic agents induce cell death by a process known as programmed cell death, or apoptosis. Although, the precise pathways by which chemotherapeutic agents kill tumors have been controversially discussed, the killing of tumors through the induction of apoptosis has been demonstrated as one of important pathway and now recognized as a novel strategy for the identification of anti-cancer drugs. Thus, much effort has been directed towards the search for compounds that influence apoptosis and understand their mechanism of action. In our study for the anti-tumor activity *in vitro*, TSSLT showed significant proliferation inhibition activity against Hela cells. In the context, much effort devoting to assess the mechanism (s) of apoptosis induced by TSSLT in Hela appears of remarkable importance.

The present study showed that TSSLT exerted a significant proliferation inhibitory activity against Hela cells in a dose- and time-dependent manner (Fig. 1). Further cellular and biochemical analysis indicated that the proliferation inhibitory activity of TSSLT was related to the induction of apoptosis. A landmark of cellular self-destruction by apoptosis is the degradation of higher order chromatin structure of DNA into fragments of 50–300 kbp. These DNA fragments result in the appearance of ladder like pattern when analyzed by agarose gel electrophoresis. In the present study, the time-dependent formation of DNA fragment was determined by means of DAPI method and agarose gel electrophoresis (Fig. 3). Similar to previous work (Roy et al. 2004), morphological changes of nuclear chromatin were observed earlier than the formation of fragmented DNA. In addition, apoptosis evoked by TSSLT was also confirmed by the observed translocation of phosphatidylserine (Fig. 2).

It is believed that the activation of caspase cascade induces the cleavage of several specific cellular substrates and therefore results in biochemical and morphological changes associated with the apoptotic cell death. In the present work, TSSLT-induced DNA fragmentation was completely prevented by the pretreatment of cells with Z-VAD-FMK (40 μM), a cell permeable inhibitor of cysteineproteases as determined by agarose gel electrophoresis and DAPI method (Fig. 4). The results suggested the involvement of caspases in TSSLT-induced cell death. Furthermore, an important role for caspases in TSSLT-induced apoptosis was also strongly confirmed by the observation that Z-VAD-FMK (40 μM) reversed the apoptotic effect exerted by TSSLT in Hela cells (Fig. 5).

There are several caspases that play an important role in the regulation of apoptosis. They are broadly grouped into initiator or effector caspases. The initiator caspases including caspase-1, 8, 9, typically caspase-8 and caspase-9, are activated by two alternative pathways. The first involves cell death receptormediated apoptosis through caspase-8. It is characterized by binding cell death ligands and cell death receptors, and subsequently activates caspase-8 and caspase-3 (Schempp et al. 2001). The second involves mitochondria mediated apoptosis through caspase-9. The key element in the pathway is the liberation of the cyt *c* from mitochondria to cytosol. Once cyt *c* is in the cytosol, cyt *c* together with Apaf-1 activates caspase-9, and the latter then activates caspase-3 (Li et al. 1997). In both pathways activation of caspase-3 plays the central role in the initiation of apoptosis. Our study revealed that TSSLT induced the elevation of some caspases including caspase-3, -8 and -9 but not caspase-1 as shown in Fig. 7. During the investigation, from 4 to 24 h, caspase-3 activation was dominant and reflected in the cleavage of PARP, a well-known caspase-3 substrate. Treatment of Hela cells with 6 μg/ml TSSLT induced proteolytic cleavage of PARP (116 kDa) with the accumulation of 85 kDa cleaved products (Fig. 7A). PARP cleavage was apparent at 4 h after TSSLT (6 μg/ml) treatment with similar time course analysis of evaluating caspase-3 like activity. In TSSLT-induced apoptosis, involvement of caspase-3, 9 was further confirmed by the fact that the TSSLT-induced cell death were significantly prevented by pretreatment with their specific inhibitior, Z-DEVD-FMK for caspase-3 and Z-LEHD-FMK for caspase-9 (Fig. 5). The activation of caspase-9 suggested the involvement of mitochondria. Many studies reported that the release of mitochondrial cyt *c* is an important event in caspase activation and apoptotic process. Our data showed that TSSLT accumulation of cyt *c* occurred at 2 h after treatment with TSSLT, which resulted in the increased level in the activity of caspase-9 2 h latter (Fig. 7B). Therefore, our study indicated that mitochondrial pathway and death receptor signaling pathway were both involved in apoptosis induced by TSSLT in Hela cells. A cross-talk exists between the death receptor and mitochondrial pathways, which also leads to cyt *c* release (Tanel and Averill-Bates 2005). Thus, it deserved further investigation on the role of death receptor and cross-talk pathways in TSSLT-induced apoptosis.

Many studies reported that the release of cyt *c* is a process tightly regulated by Bcl-2 family proteins that consist of anti-apoptotic and pro-apoptotic members (Nakagawa et al. 2004). Previous reports have also documented that the ratio of anti-apoptotic Bcl-2 to pro-apoptotic Bax protein determined, at least in part, the susceptibility of cell to a death signal (Gross et al. 1999), and used as a predictive marker for therapeutic response to radiotherapy (Mackey et al. 1998). Our data clearly demonstrated that TSSLT treatment to Hela cells resulted in a time-dependent increase in the level of Bax with a concomitant decrease in Bcl-2 levels and increase in Bax/Bcl-2 ratio (Fig. 7C). Many studies have shown that there are many regulatory molecules involved in the upstream pathway, by which the expression of many apoptosis regulatory genes such as Bcl-2 and Bax are regulated. For example, wild-type p53 can down-regulate the expression of Bcl-2 and up-regulate that of Bax, altering the balance of couple genes in favor of apoptosis. Meanwhile, several studies have demonstrated that Stat3 can upregulate the expression of Bcl-2 (Grandis et al. 2000). EGCG can decrease the level of the Bcl-2 protein by inhibiting transcription3 (Masuda et al. 2001). Moreover, studies have reported that caspases can be important regulatory factors targeting Bcl-2 family members. It has been shown that the antiapoptotic proteins Bcl-2 of mammals can be converted into potent proapoptotic molecules when they are cleaved by caspases during apoptosis (Bellows et al. 2000). The correlation between the present data and information as to the involvement of these regulatory molecules in apoptosis is not known. Further study to clarify the upstream pathway is needed.

In conclusion, this study demonstrated that TSSLT might inhibit the proliferation of Hela cells by the mechanism involved the induction of apoptosis. And further proved that apoptosis of Hela cells was induced by the activation of caspase-8, caspase-9, and caspase-3, release of cyt *c* from mitochondria, decrease of the Bcl-2 level, and increase of the Bax level. The present findings suggested that TSSLT could be a potential candidate for developing anticancer drug for the treatment of human cervical cancer.

4. Experimental

4.1. Drugs and reagents

TSSLT was purchased from the Chinese National Institute for the Central of Pharmaceutical and Biological Products (Beijing, China), which was prepared with dimethyl sulfoxide (DMSO) and stored at −20 °C. The stock solution was further diluted with the appropriate assay medium immediately before use. Caspase-1, 3, 8, 9 colorimetric assay kits, caspase inhibitor Z-VAD-FMK, Z-DEVD-FMK, Z-LEHD-FMK, cyt *c* immunoassay kits, monoclonal antibodies of PARP, Bcl-2, Bax, and apoptotic DNA laddering kits were purchased from Biovision. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide, propidium iodide (PI), Hoechst 33342, and low melting point agarose were purchased from Sigma. All other chemicals were of the highest pure grade available.

4.2. Cell culture

Hela cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI 1640 medium; Gibco BRL Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% glutamine, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B (Sigma). Cells were grown in an atmosphere of 5% CO_2 at 37 °C in a humidified incubator and used for assays during exponential phase of growth.

4.3. Cell viability assay (MTT)

Cell viability was determined by a colorimetric MTT assay, as previously described (Mosmann 1983). Briefly, 1 day after exponentially growing cells were seeded at 2×10^3 cells/well in a 96-well plate, the culture medium was changed to the experimental medium supplemented with TSSLT at indicated concentrations. Following culture with TSSLT for 12, 24, 48 and 72 h, MTT (5 mg/ml) was added. After incubation for 4 h, the formazan precipitate was dissolved in 100 ml DMSO, and the absorbance at 570 nm was detected with a Benchmark microplate reader (Bio-TEK, USA). The percentage of cell survival was calculated by the following formula: %cell survival = (mean absorbency in test wells)/(mean absorbency in control wells) × 100. IC_{50} of TSSLT on cells was calculated by an IC_{50}-calculator computer program.

4.4. Annexin V-FITC/PI assay of early apoptotic cells

Surface exposure of phosphatidylserine in apoptotic cells was quantitatively detected using Annexin V-FITC/PI apoptosis detection kit (BD Biosciences Clontech) (Overbeeke et al. 1998). This assay is based on the translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the cell surface in the early apoptotic cells. Briefly, cells were incubated with TSSLT at indicated concentration for 4, 8 h, respectively. The adherent and floating cells were combined and treated according to the manufacturer's instruction and measured with FITC/PI flow cytometry (FACScalibur Becton Dickinson) to differentiate apoptotic cells (annexin-positive and PI-negative) from necrotic cells (annexin- and PI-positive).

4.5. Analysis of DNA fragmentation

DNA fragmentation was qualitatively analysed by agarose gel electrophoresis, as previously described (Tong et al. 2004). Briefly, 2×10^6 cells were pelleted from the medium, washed twice with PBS. An apoptotic DNA laddering kit was used to selectively extract DNA fragmentation according to manufacturer's instructions, and then the DNA fragments in the samples were separated on 1.5% agarose gel. Quantitative analysis of DNA fragmentation was carried out by 4′, 6′-diamidino-2-phenylindole hydrochloride (DAPI) method as reported earlier (Roy et al. 2002) . Briefly, following various treatments, 1×10^5 cells were harvested by centrifugation, washed twice with PBS, and then lysed in 2 ml of ice-cold lysis buffer (1 mM EDTA, 5 mM Tris-HCl, 0.5% Triton X-100, pH 7.4) prior to centrifugation at 27,000 g for 20 min. Both pellet (intact chromatin) and supernatant (DNA fragments) fractions were assayed for DNA content with the fluorescent reagent (100 mM NaCl, 10 mM Tris, pH 7.0 containing 100 ng/ml of DAPI). The percentage of fragmented DNA was defined as the ratio of DNA content of the supernatant to that of the lysate multiplied by 100.

4.6. Assay of caspase-like activity

The activities of caspase-like protease were measured using a caspase colorimetric assay kit as described by the manufacturer. Briefly, after treatment with indicated concentration of TSSLT for various periods, the cells were harvested, pelleted and frozen on dry ice. Cell lysis buffer 50 μl was added to the cell pellets, and protein concentration was determined by a micro BCA kit. Then 100 μg protein was diluted in 50 μl cell lysis buffer for each assay, and 50 μl of 2 × reaction buffer (containing 10 mM of DTT) were added to each tube incubated at 4 °C. 5 μl of fluorogenic report substrate specific for each caspase such as YVAD-*p*NA for caspase-1, DEVD-*p*NA for caspase-3, IETD-*p*NA for caspase-8, LEHD-*p*NA for caspase-9 were added into the tubes, respectively. After the samples were incubated for 1.5 h at 37 °C, liberation of *p*NA (p-nitroanilide) was monitored by an ELISA Micro-plate Reader (Bio-tek) at 405 nm. For caspase inhibition assay following inhibitors were used. Benzyloxy carbonyl–Val–Ala–Asp–fluorome- thylketone Z-VAD-FMK for interleukin-1-converting enzyme (ICE)-like protease (pan caspase inhibitor), Z-DEVD-FMK for caspase-3, Z-LEHD-FMK for caspase-9. The inhibitors were diluted in DMSO and a 100 (g/ml (or 100(M) of inhibitors were added to the culture 30 min to 15 h before or during TSSLT-treatment. Cell viability was measured using MTT assay.

4.7. Western blot analysis of PARP cleavage, cyt c release and level of Bcl-2 family protein

Hela cells were treated with indicated concentration of TSSLT. At designated time points, cells were harvested and homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0; 5 mM EDTA; 150 mM NaCl; 0.5% NP-40; 0.5 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin; and 0.5 mM dithiothreitol) for 30 min on ice. The cell debris was pelleted by centrifugation at 10,000 g, 4 °C for 30 min. The supernatant proteins were measured by bicinchoninic acid assay. 50 μg of total cellular proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10% for PARP; 15% for Bcl-2, Bax, and cyt *c*), transferred onto a nitrocellulose membrane filter using an electro-blotting apparatus (Bio-Rad). Then the membranes were probed with primary antibody followed by adding horseradish peroxidase-labeled secondary antibody. The immunocomplexes were visualized by the ECL system (Amersham Life Science, Amersham, Bucks, UK).

4.8. Statistical analysis

Data were expressed as mean ± SD. Data were subjected to one-way analysis of variance (ANOVA) followed by multiple comparison with least significant differences (LSD) test. A value of $p < 0.05$ was considered statistically significant.

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