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Original Article

Role of an autophagy/lysosome pathway in NF-κB pathway blocked pancreatic cancer Panc-1 cells

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Abstract: Purpose: To investigate the role of an autophagy/lysosome pathway in NF-κB pathway blocked pancreatic cancer Panc-1 cells. Methods: The inhibitory effects of SN50 on pancreatic cancer cell line Panc-1 were detected by MTT assay. After SN50 treatment, autophagy activation was observed by MDC staining and transmission electron microscope (TEM). The expression of light chain 3 (LC3) was detected by immunofluorescence staining. Western blotting analyses were used to detect the expression of apoptosis-related protein p53 and autophagy-related proteins LC3, p62, and Beclin1. Results: Panc-1 cell activity was inhibited after SN50 treatment. The inhibition ratios of Panc-1 cells were (25.76±5.53)%, (34.35±4.49)% and (45.22±1.76)% after treatment of SN50 for 6 h, 12 h, and 24 h, and all changes were significant ($P<0.05$). Western blotting analysis showed that expressions of apoptotic protein p53, autophagic protein LC3, and Beclin 1 were increased, but the expression of p62 was down-regulated in Panc-1 cells. After SN50 treatment, immunofluorescence showed staining of microtubule-related protein 1 LC3, and MDC fluorescence staining showed increased autophagy bubbles labeled with MDC. Transmission electron microscope (TEM) was used to observe ultrastructure of Panc-1 cells that underwent autophagy after SN50 treatment. Conclusion: The activation of NF-κB was blocked by the inhibitor of p65 nuclear translocation, which activated autophagy and induced autophagic cell death in pancreatic cancer Panc-1 cell line.

Keywords: Nuclear factor-κB, SN50, autophagy, P53

Introduction

The fifth leading cause of cancer death in western countries currently is pancreatic ductal adenocarcinoma (PDAC), and it is second in incidence among gastrointestinal tumors [1]. By 2030, it is expected to be the second leading cause of cancer death [2]. PDAC is considered to have the worst prognosis among all gastrointestinal malignancies, with a 5-year survival rate of less than 5% [1, 3]. Tumors are highly invasive with potential for early metastasis, and therapeutic options are limited [4]. Although extensive efforts have been made in recent decades to improve the detection rate and survival rate of pancreatic cancer and progress has been made, the 5-year survival rate of pancreatic cancer has not changed significantly and new treatment methods are urgently needed.

NF-κB is a transcription factor, involved in many cellular signaling pathways involved in inflammation and stress-induced responses (Senftleben and Karin, 2002). Dysregulation of the NF-κB pathway is associated with the occurrence, progression, and drug resistance of cancer, in addition to other human conditions, such as inflammatory diseases [5]. In mammals, NF-κB is a family consisting of five members: p65 (RelA), p105/p50, RelB, p100/p52 and c-Rel. NF-κB can be regulated by two distinct pathways. The first is the canonical pathway, usually mediated by κB kinase complex inhibitors (IKKα/β/γ), which leads to the phosphorylation and degradation of κBs inhibitors (IκBs). Subsequently, RelA and p50 heterodimers are released into the nucleus for gene transcription. Another less well-studied pathway is the non-canonical pathway, in which p100 and p52 are processed by NIK and IKKα. Both kinases

phosphorylate p100, resulting in the partial proteolysis of p100. The resulting protein p52 heterodimerizes with RelB to mediate gene expression in the non-canonical arm of NF-κB [6].

Programmed cell death (PCD) includes three types: apoptosis, autophagy and necrosis, which are called PCDI, PCDII and PCDIII [7]. Autophagy (PCDII) is an evolutionarily conserved and highly regulated process, in which long-lived proteins, macromolecules, ribosomes, and organelles are large-scale lysosomal degradated, such as the endoplasmic reticulum, Golgi apparatus, and mitochondria [8]. Reports suggest that NF-κB controls several cellular processes and autophagy plays a role in several cellular functions regulated by NF-κB [9-11]. Inhibition of autophagy can increase IKK activity since this kinase is normally degraded by the autophagic process [12]. There are relationships between NF-κB and autophagy, so we speculate that NF-κB inhibitors activate autophagy and exert anti-tumor biologic effects by blocking the signaling pathway of NF-κB. In this study, we use SN50, a specific nuclear import inhibitor, to investigate the effect of blocking the NF-κB signaling pathway on pancreatic cancer Panc-1 cell lines. The results indicated that blocking NF-κB signaling pathway could activate the apoptosis and autophagy signaling pathway, and autophagy activation played an important role in the death of cancer cells induced by SN50.

Materials and methods

Reagents

Pancreatic carcinoma cells Panc-1 were provided by Dr. Fei Shen at Blood Institute of Soochow University. DMEM medium (Cat. No.12100-038) was from Gibco. Fetal bovine serum (Cat. #10020.01) was from Hangzhou Saile Biotechnology Co. Ltd. MTT (St. Louis, MO, USA) was from Sigma.

Drug preparation

Dilute SN50 (BIOMOL, Cat. No.BML-P-600-0005) in distilled water to make the stock solution and store it as recommended by the manufacturer. Through our preliminary experiments on Panc-1 cells, the drug concentration of SN50 was finally screened to be 18 μmol/L.

Cell culture and MTT assay

The cell culture medium was prepared by adding 10% heat-inactivated fetal bovine serum into DMEM medium, and Panc-1 cells were incubated at 37°C under 5% CO_2. We used logarithmic growth phase cells for MTT assay to determine cell growth activity. Panc-1 cells were cultured in 96-well plates at a concentration of 7×10^4 cells/well, then added SN50 (18 μmol/L) to the culture medium for 6, 12 and 24 h. Before the end of the experiment, MTT solution with a final concentration of 500 mg/L was added to the culture medium for 4 h. Then 100 μL 10% acidification of SDS was added to stop the reaction. The absorbance value (A) of 570 nm was determined by automatic spectrophotometer (Bio-Rad, Richmond, CA, USA). Tumor cell growth inhibition rate (IR) was calculated according to the following formula: IR (%) = [(A value of positive control group-A value of experimental group)/A value of positive control group] × 100%.

MDC fluorescence staining was used to detect the effect of SN50 on autophagy of Panc-1 cells

The cells in logarithmic growth stage were cultured on 24-well plates for 24 h, and then 10% FCS/DMEM containing drugs was added for 6 to 24 h. Monodansylcadaverine (MDC) is used to label autophagy vesicles [13]. MDC powder (Sigma, St Louis, MO, USA D4008-107K1562) was dissolved in DMSO with a concentration of 0.1 M. We diluted the concentration with DMEM medium to 0.05 mmol/L before use, and incubated cells for 10 min at 37°C. Phosphate buffered saline (PBS) was used to wash the cells twice and they were observed under inverted fluorescence microscope (Nikon Eclipse TE 2000, Japan).

Effect of SN50 on the distribution and expression of LC3 in Panc-1 cells by immunofluorescence labeling

The logarithmic Panc-1 cells were inoculated on a 24 well cell culture plate and treated with SN50 (18 μmol/L) for 6 h, 12 h and 24 h. After incubation, we rinse with PBS twice and fix with -20°C precooled methanol for 10 min. We add 1% BSA blocking solution containing 0.1% Triton X-100 to seal the cells at 4°C for 1 h. The anti-LC3 antibody from Cell Signaling

Technology, at a dilution of 1:500 was added and incubated overnight at 4°C. Cy3 secondary antibody (Sigma) at a 1:1000 dilution was incubated for 1 h, then we observed cells under a laser confocal microscope (Leica, Germany).

Effect of SN50 on ultrastructure of Panc-1 cells by transmission electron microscope (TEM)

Transmission Electron Microscope (TEM) was used to observe ultrastructure of Panc-1 cells that underwent autophagy after SN50 treatment. Panc-1 cells were collected 6 h, 12 h, and 24 h after treatment by SN50 (18 μmol/L) and washed with precooled PBS, then centrifuged at 1500 rpm for 10 min. Following that, cells were resuspended in 2.5% glutaraldehyde fixative (Sigma). Cell pellets were embedded in 2% agarose, post-fixed in 1% osmium tetroxide, and we used different concentrations of ethanol solution and acetone solution to dehydrate. Then the samples were soaked in acetone at 37°C for 24 h. Ultrathin sections of 70 nm were generated with a ultramicrotome (LKB-1) and placed on copper grids. Sections were examined using transmission electron microscope (Philips CM-120) at 100 kV. Transmission electron microscopy services were performed by the Fudan University in Shanghai for Advanced Microscopy.

Effect of SN50 on the expression of apoptotic and autophagic proteins

Panc-1 cells were collected 6 h, 12 h, and 24 h after treatment of SN50 (18 μmol/L) and washed in precooled PBS. RIPA lysate buffer was added to extract cell proteins, which contains 10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% TritonX-100, 1% sodium deoxycholate, 1% SDS, 1 mmol/L phenylmethysulfonyl fluoride (PMSF), 0.28 u/mL Aprotinin, 50 μg/mL leupeptin, 7 μg/mL Pepstatin A, then centrifuge for 10 min at 12000 rpm. After electrophoresis, proteins were transferred to a PVDF membrane. Membranes were blocked in TBST buffer for 1 h and then incubated with primary antibodies for 2 h at 4°C overnight. The primary antibodies used in this study were against p65 (Cat. #3034, Cell Signaling Technology), IκBα (Cat. #07-1483, Millipore), p53 (Cat. #2524, Cell Signaling Technology), LC3 (ab62721, abCAM), Beclin1 (Cat. Sc-11427, Santa Cruz Biotechnology), and p62- (PW9860, Enzo life science). Membranes were washed and incubated with horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies for 2 h at room temperature. Antibody binding was visualized by chemiluminescence (ECL) before exposure of the membrane to a photosensitive film. Sigma Pro5 software was used to collect the images, and GraphPad Prism 4 software was used for image analysis.

Statistical analysis

All data are presented as mean ± SD. Statistical software SAS 8.1 was used for data processing. Univariate analysis (ANOVA) was used for statistical analysis, and $P<0.05$ was considered significant.

Results

SN50 inhibited the growth of Panc-1 cells

SN50 could inhibit the growth of Panc-1 cells and the survival of SN50 on Panc-1 cells was time-dependent. MTT results showed that the inhibition rate of SN50 reached 25.76±5.53% after 6 h of treatment. After 12 h, the inhibition rate reached 34.35±4.49%, increasing to 45.22±1.76% when extended to 24 h total time.

Blocking NF-κB nuclear translocation activates autophagy

MDC is a specific autophagy marker, that can be absorbed by cells and selectively aggregated in autophagic vesicles. The foci of autophagy vesicles infected with MDC that are distributed in the cytoplasm and around the nucleus can be observed by fluorescence microscopy. Therefore, the activation of autophagy can be inferred from the changes of intracellular fluorescent particles [14]. After treatment with SN50 for 6 h to 24 h, autophagy vesicles gradually increased, indicating that SN50 could activate autophagy and induce the death of tumor cells (**Figure 1**).

Microtubule-associated protein 1 light chain 3 (LC3), the counterpart of yeast Atg8, is a 16 kDa soluble protein present ubiquitously in mammalian cells and plays a critical role in the macroautophagic formation. It is a common marker for autophagy [15]. In this study, LC3 expression and localization were detected by

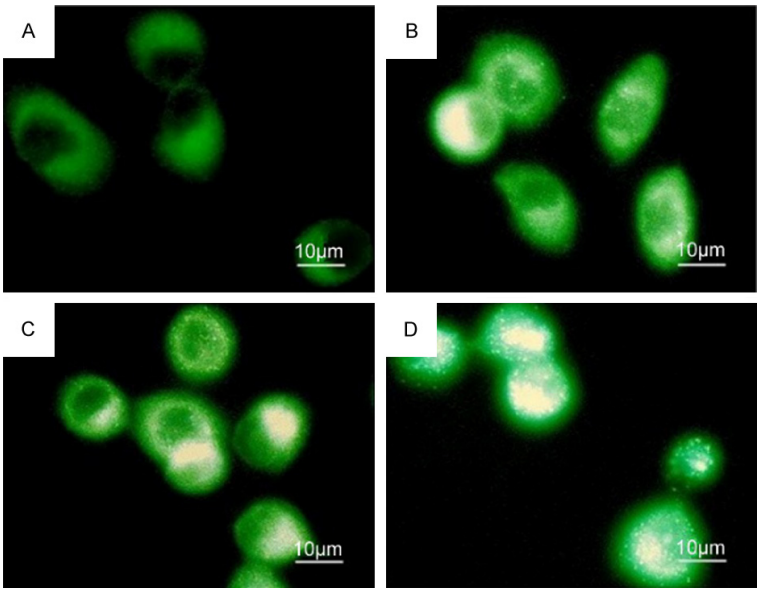

Figure 1. SN50 induced autophagy cell death in Panc-1, observed by using MDC fluorescence staining. Results showed that the Panc-1 cells had a strong autophagy ability, and dotted fluorescent particles could be seen scattered in the cytoplasm of the cells. After the treatment with SN50, autophagic vesicles gradually increased, indicating that blocking the NF-κB signaling pathway could activate autophagy and induce cell death. A. Untreated Panc-1 control cells; B. SN50 (6 h); C. SN50 (12 h); D. SN50 (24 h). (× 1000) (n = 3).

immunofluorescence assay. After 6 h of treatment, LC3 red fluorescence was scattered around the cell membrane and lasted until 24 h, with the strongest fluorescence at 6 h and 12 h after treatment, while the negative control group had extremely weak fluorescence (**Figure 2**).

Transmission electron microscopy can demonstrate an independent double-layer membrane and autophagosomes, which is still widely recognized as the most reliable means of autophagy detection. Autophagy was first detected by TEM in the 1950s (reviewed in ref. [16]). After SN50 treatment, some independent double-layer membrane structures appeared in the cytoplasm, which gradually extended and curved, and some cytoplasmic components formed autophagosomes with double-layer and multi-layer membrane structures. With time, the formation of autophagosomes and their independent double-layer membrane structures gradually increased, and a large number of vacuoles appeared in the cytoplasm, representing a relatively typical form of autophagy. TEM can be used to monitor both selective and nonselective autophagy. Our results indicate that SN50 induced autophagic cell death in Panc-1 cells (**Figure 3**).

SN50 inhibited nuclear translocation of p65 and blocked activation of the NF-κB signaling pathway in Panc-1 cells

In order to confirm the inhibitory effect of SN50 on the nuclear transposition of NF-κB p65 in cells, we measured the expression levels of p65 and IκBα in the nucleus 6 h to 24 h after the effect of SN50 by western blotting analysis. The results showed that nuclear p65 expression was down-regulated, but the expression of IκBα was increased by SN50 (**Figure 4A**, **4B**). SN50 inhibited nuclear transposition of p65 in Panc-1 cells, decreased phosphorylation and degradation of IκBα protein in the cytoplasm, and blocked activation of the NF-κB signaling pathway.

SN50 induced the expression of apoptosis-related protein p53 and autophagy-associated proteins

The tumor suppressor gene p53 is a key regulatory gene of cell death, that is often mutated in human tumors, and a proven role in apoptosis. Recent studies have shown that p53 is also an important gene in regulating autophagy, which regulates different biologic processes mainly through the activation of target genes in the downstream region. To investigate the molecular mechanism of SN50-induced tumor cell death and whether p53 is involved in the process of SN50-induced tumor cell apoptosis, we used western blotting to analyze the expression of p53 at 6 h to 24 h after SN50 treatment. The results showed that the expression level of p53 in the control group was low. After 6 h, the expression of p53 was increased, with the highest expression at 12 h, and the expression at 24 h was slightly decreased but still higher than that of the normal group (**Figure 4C**).

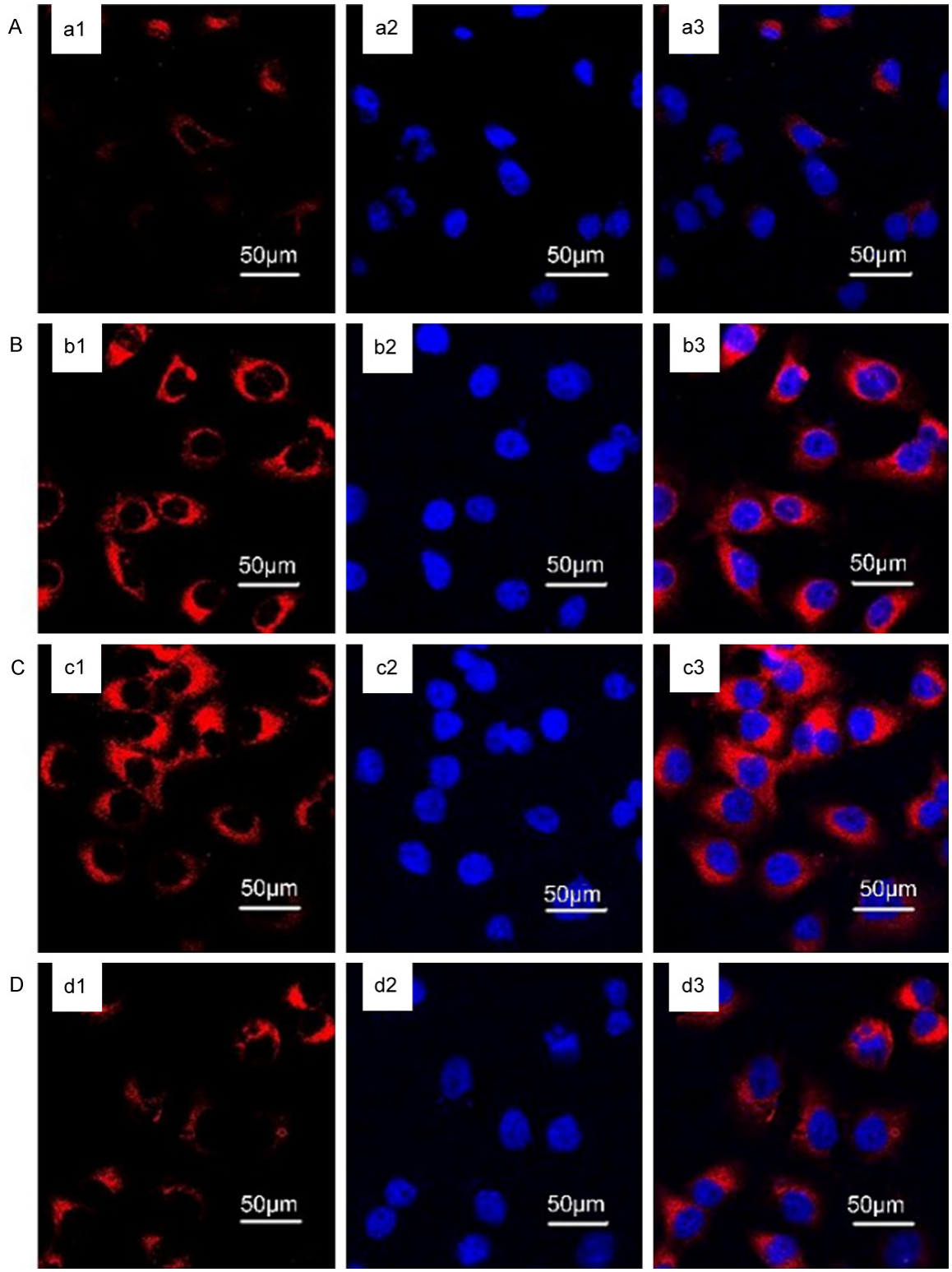

Figure 2. Immunofluorescence staining showed changes in the distribution and expression of autophagy-specific protein LC3 in Panc-1 cells induced by SN50. After 6 h of treatment, LC3 red fluorescence was scattered around the cell membrane and lasted until 24 h, with the strongest fluorescence at 6 h and 12 h after treatment, while the negative control group had extremely weak fluorescence. A. Untreated Panc-1 control cells; B. SN50 (6 h); C. SN50 (12 h); D. SN50 (24 h). (× 1000) (n = 3).

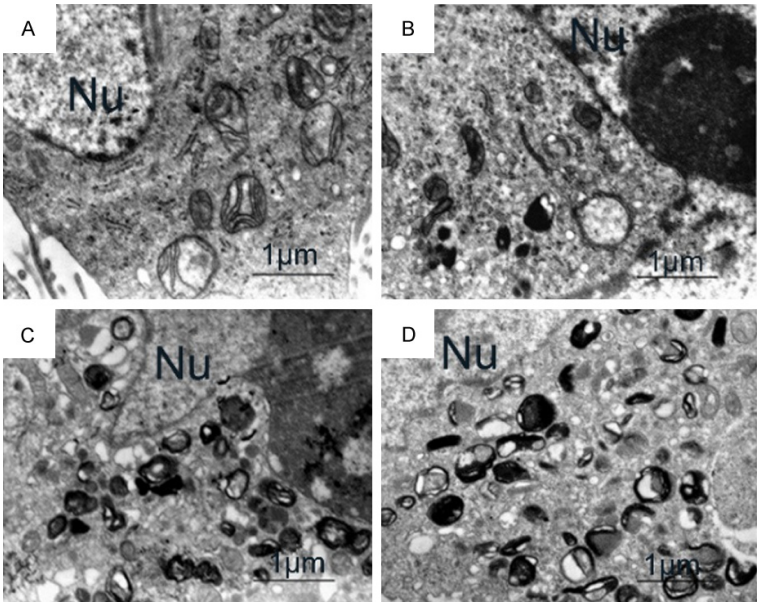

Figure 3. The effects of SN50 on the ultrastructure of Panc-1 cells were observed by transmission electron microscopy (TEM). After treatment with SN50, some independent double-layer membrane structures appeared in the cytoplasm, which gradually extended and curved, and some cytoplasmic components formed the double-layer and multi-layer membrane structure of autophagosomes. A. Untreated Panc-1 control cells; B. SN50 (6 h); C. SN50 (12 h); D. SN50 (24 h). Nu: nucleus.

LC3, a mammalian analogue of Atg8, is the only credible autophagy marker currently identified. There are two types of LC3 in wild-type cells: 18 kDa of LC3-I and 16 kDa of LC3-II [17, 18]. When autophagy is induced, the newly synthesized LC3 precursor is firstly cleaved by human cysteine protease Atg4B to generate LC3-I in the cytoplasm, and then phosphatidyl ethanolamine (PE) is added to glycine residue 120 at its c-terminal to convert it into membrane-bound LC3-II. LC3-II is firmly bound to the membrane of autophagosome and is, thus, regarded as a specific marker for autophagy [15]. Therefore, the level of LC3-II/LC3-I can be measured to reflect the level of autophagy. The results showed that the ratio of LC3-II/LC3-I increased after SN50 treatment for 6 h, and lasting up to 24 h, showing time-dependence (**Figure 4D**).

Beclin-1 is a protein encoded by the Becn1 gene, which is a mammalian ortholog of the yeast autophagy-related gene 6 (Atg6). Beclin-1 was initially thought to be a Bcl-2 interacting protein, and this binding inhibited the binding of beclin-1 to PI3K, thus preventing the autophagy nucleation stage. Subsequent studies mapped the Becn1 gene in close proximity to the BRCA1 region on chromosome 17, a locus frequently deleted in breast, ovarian, and prostate cancers [19]. Beclin1 is required for the activation of autophagy and is under the control of different transcription factors. After SN-50 treatment, Beclin1 protein expression was increased and showed time dependence at 6 h to 24 h (**Figure 4E**).

P62, also known as sequestosome-1 (SQSTM1), A170, and zeta-interacting proteins (ZIP), is a scaffold protein with multiple domains that plays a role in signal transduction, cell proliferation, cell survival, cell death, inflammation, tumorigenesis, and the oxidative stress response [10, 20]. Recent autophagy studies have revealed that p62 is an autophagy substrate that also serves as an autophagy receptor for protein aggregates and damaged or excess organelles as well as for invading microbes [21-23]. Moreover, p62 functions as a signaling hub for various signaling pathways, including NF-κB, Nrf2 and mTOR [24]. We used western blotting analysis to detect the protein levels of p62 after SN50 treatment from 6 h to 24 h. The results showed that the p62 level of the cells decreased and this continued to 24 h after administration, showing significant time dependence (**Figure 4F**).

Discussion

NF-κB is a transcript factor first identified in mature B cells in 1986, which was so named as it was found in the nucleus bound to the intronic enhancer region of the κ light chain gene in the B cells [25, 26]. Many of the genes involved in immune and inflammatory responses are regulated by NF-κB, inlcuding those that determine developmental processes, cell growth, and apoptosis [27-30]. In most cases, the activation of NF-κB causes anti-apoptotic signaling pathways, stimulating cell proliferation, invasion, metastasis, and angiogenesis in cancer

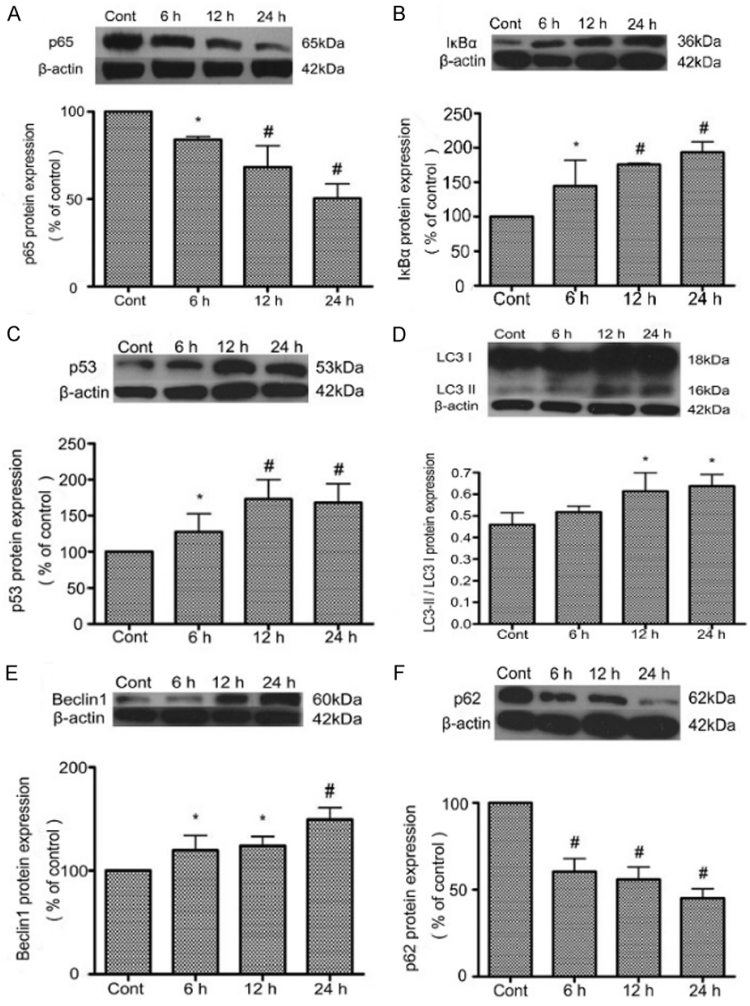

Figure 4. Western blotting analysis was used to detect the expression of proteins involved in apoptosis and the autophagy signal pathway in the control group and the experimental group, respectively. SN50 (18 μmol/L) was added to Panc-1 cells for 6 h to 24 h, and nuclear proteins were harvested from Panc-1 cells after SN50 treatment. Statistical comparisons were carried out with ANOVA then Dunnett's t-test. Values were given as mean ± SD. $^{*}P<0.05$ and $^{\#}P<0.01$ compared to control group (n = 3). A. p65 protein: expression of p65 protein decreased after SN50 treatment compared with the control group, suggesting that SN50 blocked the activation of NF-κB by inhibiting nuclear translocation of p65 in pancreatic cancer cell Panc-1. B. IκBα protein: SN50 inhibited nuclear translocation of p65, reduced phosphorylation and degradation of IκBα protein in cytoplasm, and increased IκBα expression. C. p53 protein: expression level of p53 was low in the control group, while p53 expression increased after 6 h of SN50 treatment, with the highest expression level at 12 h, and slightly decreased expression but still higher than the normal group at 24 h. D. LC3 protein: the expression of LC3I and LC3IIincreased after SN50 treatment for 6 h, especially the ratio of LC3II/LC3I, and continued to 24 h, presenting significant time dependence. E. Beclin 1 protein: the expression of Beclin 1 increased after SN50 treatment for 6 h, and continued to 24 h, presenting significant time dependence. F. p62 protein: expression of p62 in Panc-1 cells decreased after 6 h of SN50 treatment and continued until 24 h, presenting an obvious time dependence.

cells. For example, studies have shown constitutive activation of NF-κB signaling pathway in many human solid tumors [31]. In addition, constitutive activation of NF-κB is associated with poor outcome in a portion of patients with pancreatic cancer [32]. Therefore, targeting NF-κB as well as its related signaling pathways has been considered a potential therapeutic target for cancer treatment.

Current studies suggest that SN50 inhibits the intracellular transport of p65 in NF-κB signaling pathway within the effective drug dose range, thereby inhibiting the growth of tumor cells and inducing the death of tumor cells. However, the molecular mechanism of SN50-induced tumor cell death remains unclear. In this study, we investigated the inhibitory effect of SN50 on the growth of pancreatic cancer cells and the regulatory effect on autophagy and apoptosis, and discussed the related molecular mechanism. In this study, MTT assay was used to detect the inhibitory effect of SN50 on proliferation of human pancreatic cancer cell line at different times. The results showed that SN50 had a significant inhibitory effect on Panc-1 cells, and this effect was time-dependent. After 6 h, 12 h and 24 h, MTT results showed that the inhibition rate of SN50 reached 25.76± 5.53%, 34.35±4.49% and 45.22±1.76% respectively.

The term autophagy is derived from the Greek roots "auto" (self) and "phagy" (eating), which is triggered by starvation (amino acid and nutrient deprivation), hypoxia, and metabolic stress to maintain temporary survival. Self-digestion provides a way to recycle macromolecules as an alternative energy source. However, if the cellular

stress leads to continuous or excessive induction of autophagy, cell death may follow. Autophagy requires the formation of double-membrane vesicles to isolate the cytoplasmic contents, and is mediated by highly organized and layered ATG proteins [33]. The initial phagocytes are formed by endoplasmic reticulum, which surrounds and envelops organelles to form autophagosomes [33, 34]. Subsequently, the autophagosome then merges with the lysosome and digests its contents (i.e., misfolded proteins and organelles), which provides energy for the synthesis of macromolecules and metabolites that ultimately lead to cell survival or death, depending on the duration and severity of the process [33, 34]. After acting on pancreatic cancer cell lines for a period of time, it was found that the expression of LC3, a specific marker of autophagy, increased, and could cause an increase of cytoplasmic autophagosomes in the middle and late stage. With the up-regulation of autophagy specific protein LC3, we found an increase in cell death, suggesting that autophagy cell death plays a key role in SN50-induced pancreatic cancer cell death.

We used western blotting to investigate the expression of another autophagy-associated protein, Beclin1. Beclin1 (BECN1) is a key autophagy regulatory gene, homologous to yeast autophagy gene Atg6/Vps30, and is a key factor mediating other autophagy proteins to locate in the proautophagosome, and participates in the regulation of mammalian autophagosome formation. When spontaneous lung and liver tumors were found to arise in Beclin-1+/- mice, a major breakthrough was made in understanding the role of autophagy in tumorigenesis [35]. Beclin1 is located on human chromosome 17q21 and plays an important role in embryonic development and tumor inhibition by regulating autophagy activity [36]. In 40-75% of cases of human sporadic ovarian, breast and prostate cancer, there was monoallelic deletion of the human homolog of Beclin-1 (BECN1) [37]. In this study, SN50 could lead to up-regulation of the expression of autophagy gene Beclin-1, suggesting that the autophagy gene Beclin-1 may also be involved in cell death.

P53 is an important tumor suppressor gene that is mutated or deleted in 50% of all human tumors [38]. P53 is activated in response to different forms of cellular stress, leading to various effects including cell cycle arrest and induction of cell death [39]. Studies have shown that p53 can respond to and regulate metabolic stress, so it is not surprising that p53 can regulate autophagy [40-42]. In Panc-1 cells, p53 expression is at a low level, but the expression of p53 is up-regulated after treatment with SN50. The increased expression level of p53 may play a key role in SN50-induced tumor cell death.

P62/SQSTM1 is a multifunctional protein involved in a variety of cellular functions, including signal transduction and degradation of proteins and organelles (Johansen and Lamark 2011; Nezis and Stenmark 2012). Johansen's group and Komatsu's group independently showed that p62 directly binds to LC3 through a conserved LIR (LC3 interacting region) sequence, which is a motif required for autophagic degradation of p62 [43, 44]. In addition, p62/SQSTM1 can also mediate autophagic clearance of non-ubiquitinated protein aggregates by binding to the PB1 domain (Watanabe and Tanaka 2011). Normal, efficient autophagy maintains a low level of p62, whereas autophagy deficiency leads to aggregation of p62 and p62-positive ubiquitinated proteins [45]. Therefore, the detection of p62 protein level can reflect the autophagy level to a certain extent.

In conclusion, by blocking the NF-κB signaling pathway with SN50, the expression of apoptosis-related protein p53 and autophagy-related proteins LC3 and Beclin1 were increased, and the expression of p62 was down-regulated. The inhibitor of NF-κB p65 nuclear translocation activated autophagy and induced autophagic cell death in pancreatic cancer cell line Panc-1, which indicates that the NF-κB signal transduction pathway plays an important role in occurrence and development of pancreatic carcinoma. This study elucidates a new molecular mechanism of anti-tumor effect of NF-κB inhibitor. Further studies on the role of NF-κB signaling pathway in regulating cell proliferation and death may provide a new strategy for tumor therapy.

Disclosure of conflict of interest

None.

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