A Sphingosine Kinase-1 Inhibitor, SKI-II, Induces Growth Inhibition and Apoptosis in Human Gastric Cancer Cells

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Abstract

SKI-II has been reported as an inhibitor of sphingosine kinase 1 and has been extensively used to prove the involvement of sphingosine kinase and sphingosine-1-phosphate (Sphk1) in cellular processes. In the current study, we investigated the effects of SKI-II and its potential mechanisms in human gastric cancer SGC7901 cells. After treatment with SKI-II, cell growth, cell cycle distribution, apoptosis, expression of Sphk1, NF-κB, Bcl-2, Bax and p27 were assessed by MTT assay, flow cytometry, electron microscopy, immunocytochemistry and Western-blot assay, respectively. Our results showed that SKI-II markedly inhibited SGC7901 cell survival in a dose-dependent manner, reduced cell proliferation with accumulation of cells in the G0/G1 phase and induced apoptosis in the tumor cells. Furthermore, Western blotting and immunocytochemistry showed that the expression of p27 and Bax was increased significantly, but the expression of NF-κB, Bcl-2 and Sphk1 decreased by different degrees. These results indicate that SKI-II induced cell growth arrest and apoptosis. The increased apoptotic sensitivity of SGC7901 was correlated with NF-κB or Bcl-2/Bax activation.

Keywords: Sphingosine kinase 1 - SKI-II - proliferation - apoptosis - SGC7901 gastric cancer cells
Materials and Methods

Cell line and reagents

The human gastric cancer SGC7901 cell line was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen, Shanghai, China) and maintained in incubators at 37°C, 5% CO₂. SKI-II (S5696), PI, RNase A and MTT assay kits were purchased from Sigma. Antibodies against NF-κB (sc-20112), Bcl-2 (sc-783), Bax (sc-70406), p27 (sc-56338), Sphk1 (sc-48825) were purchased from Santa Cruz Biotechnology, Inc.

MTT assay

SGC7901 cells were seeded into 96-well plates at 2×10³ cells/well and treated with SKI-II (0.625, 1.25, 2.5, 5, 10, 20 μmol/L) for 24, 48, 72h respectively. 20 μl of 5 mg/ml MTT solution was added to each well and cells were further incubated at 37°C for 4h. Then, the media were removed, 150μl DMSO were added to each well and pipette up and down to dissolve crystals. At last the plate was put into the incubator for 5min to dissolve air bubbles and was transferred to plate reader and measure absorbance at wavelength of 490 nm. The inhibitory rate (IR) of cells was calculated according to the equation as follows: IR (%)=[1-(A490nm absorbance in treated group/A490nm absorbance in control group)]×100%. The experiment was repeated three times.

Cell cycle analysis

SGC7901 cells were treated with SKI-II (5 μmol/L, 10 μmol/L) for 48h. Cells were harvested using 0.25% trypsin, washed with phosphate-buffered saline (PBS), counted and adjusted to 1×10⁶ cells/ml. Fixing it in 70% ethanol for one night, then treated with 100μg/L RNase A and 50mg/L PI at 37°C for 30 min. The results were analyzed using Flow cytometry.

Electron microscope analysis

SGC7901 cells were seeded in 6 well plates with 1×10⁶ cells and incubated overnight in 5ml of culture media. Then the cells were treated with SKI-II (5μmol/L, 10 μmol/L) for 48h. After being fixed and stained, changes of the ultrastructure were visualized under electron microscope.

Detection of apoptosis

SGC7901 cells were incubated with SKI-II (5μmol/L, 10μmol/L) for 48h. Cells were harvested using 0.25% trypsin (without EDTA), washed with PBS for two times, then counted and adjusted to 1×10⁶ cells/ml. After treated with Annexin V-FITC and Propidium Iodide for 15min, the apoptosis rates were detected using Flow cytometry.

Immunocytochemical analysis

SGC7901 cells were seeded in 24 well plates with 1×10⁶ cells/ml and incubated overnight in 1ml of culture media. The cells were added with SKI-II (10μmol/L, 20 μmol/L) for 48h.

The following antibodies were used for immunostaining: anti-Sphk1, anti-Bax, anti-NF-κB, anti-Bcl-2 and anti-p27. Detection of immunostaining was performed using the Streptavidin/Peroxidase-Plus Kits, which was purchased from Zhongshan Goldenbridge Biotechnology Co, Ltd (Beijing, China). For quantitative assessment of immuno-labeling, a total of 1×10³ tumor cells were evaluated in each specimen in fields showing the highest density of immuno-positive cells.

Western-blot analysis

After treatment with SKI-II (10μmol/L, 20μmol/L) for 48h, cells were harvested and total protein was extracted, measured before subjected to SDS-PAGE. Sphk1, Bax, NF-κB, Bcl-2 and p27 were detected at dilution of 1:500, followed by HRP-conjugated anti-mouse IgG or anti-rabbit IgG at a dilution of 1:2000. Immunocomplexes were visualized using DAB. β-actin protein (1:5000, Sigma) was used as a loading control.

Statistical analysis

All values are expressed as means±SD. One-way analysis of the variance (ANOVA) or Pearson correlation analysis was performed for determination of P values with SPSS16.0 software (SPSS). All experiments were performed at least three times unless otherwise indicated. p-value<0.05 was considered as statistically significant.

Results

Sphk1 inhibitor SKI-II induce growth inhibition of SGC7901 cells

We first examined the effect of SKI-II on the growth of human gastric cancer cells SGC7901. Results in Table 1 demonstrate that significant and dose-dependent inhibition of cell proliferation existed in all three time, and the inhibition rate increased with time gradually extending. The lowest concentration of SKI-II being able to affect cell growth was 1.25 μmol/L.

Table 1. Effects of Sphk1 Inhibitor SKI-II on the Proliferation of SGC7901 Cell Line

| Group        | 24h Inhibition | 48h Inhibition | 72h Inhibition |
|--------------|---------------|----------------|---------------|
| Control      | 0             | 0              | 0             |
| 0.625_mol/L  | 3.62±1.83%    | 3.93±1.36%     | 3.73±1.20%    |
| 1.25_mol/L   | 7.35±1.64%*   | 12.96±2.55%*   | 15.05±2.07%*  |
| 2.5_mol/L    | 10.27±1.77%*  | 14.84±1.33%*   | 22.77±4.40%*  |
| 5_mol/L      | 17.34±3.08%*  | 22.82±2.65%*   | 29.95±2.88%*  |
| 10_mol/L     | 25.05±2.30%*  | 31.57±2.12%*   | 38.70±3.99%*  |
| 20_mol/L     | 33.57±3.64%*  | 38.72±1.84%*   | 46.24±2.01%*  |

SKI-II could markedly inhibit the survival of SGC7901 cells at 24, 48 and 72h and the inhibition rate increased with time gradually extending. Data are presented as Mean±SD (n=10); *p<0.05 vs control group.
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Cell cycle control, a key process in normal cell growth and proliferation, is precisely regulated by cyclins and cyclin-dependent kinases (CDKs), and the activity of these enzymes is restricted by CDK-inhibitory proteins (CDKIs) (Kim et al., 2014). p27, a member of the CDKIs, acts as a tumor suppressor because it binds and inactivates the cyclin E/CDK2 complex that is required for S phase onset. To investigate whether p27 involved in the regulation of SKI-II arresting the cell cycle, Immunoblotting and Western-blot analysis were performed after SKI-II treatment in SGC7901 cells (Figure 2). Results showed that the expression of p27 was increased by the using of SKI-II, but the expression of Sphk1 decreased. Pearson correlation analysis showed that the expression of Sphk1 and p27 were significantly correlated (r=-0.914, p<0.01).

Figure 1. The Effect of SKI-II on the Cell Cycle of SGC7901 Cell Line. SGC7901 cells were treated with SKI-II (5 μmol/L, 10 μmol/L) for 48h. Cells were treated with 100 mg/L RNase A and 50 mg/L PI for 30 min. The cell cycle was analyzed using Flow cytometry.

Figure 2. The Expression of Sphk1 and p27 in SGC7901 Cells. Cells were treated with SKI-II (10 μmol/L, 20 μmol/L) for 48h; (A) The expression of Sphk1 and p27 was investigated by Immunocytochemical analysis, and the results were showed at 200× magnification. Cells were collected and total protein was extracted; (B) Western-blot assay was used to detect the expression of Sphk1 and p27. Immunocomplexes were visualized using DAB. β-actin protein was used as a loading control treatment for 48 h. Results showed that SKI-II halted the cell cycle of SGC7901 cells at the G1 phases after 48h treatment. The results indicated that SKI-II induced cell cycle arrest of SGC7901 cell line (Figure 1).

Figure 3. The Apoptosis of SGC7901 Cells after SKI-II Treatment. Cells were treated with SKI-II (5 μmol/L, 10 μmol/L) for 48h; (A) Morphological changes were detected by electron microscope analysis. The changes were showed at 3000x magnification; (B) Cells were stained with Annexin V-FITC, the apoptosis rates were detected using Flow cytometry. There was a significant difference between the SKI-II groups and the control group.

Cell cycle control could include apoptosis of SGC7901

We next demonstrated whether SKI-II could affect cell apoptosis in SGC7901 cell lines. Electron microscope analysis revealed that the apoptotic body existed in SGC7901 cells after SKI-II treatment (Figure 3A). And we detected the apoptosis of SGC7901 by Flow cytometry after 48h exposure. The results indicated that SKI-II could induce apoptosis of SGC7901. There was a significant difference between the SKI-II groups and the control group (Figure 3B).

In order to explore the mechanism of SKI-II inducing apoptosis of SGC7901, we detected the expression of NF-κB, Bcl-2 and Bax by Immunoblotting and Western-blot analysis. The results showed the expression of...
NF-κB, Bcl-2 decreased with the increase of SKI-II, but the expression of Bax increased (Figure 4). In addition, Pearson correlation analysis showed that there was a significant correlation between Sphk1 and NF-κB \((r=0.958, p<0.01)\), NF-κB and Bcl-2 \((r=0.968, p<0.01)\).

**Discussion**

Numerous evidence shows that Sphk1 is an oncogenic enzyme and that activation of Sphk1 is closely associated with proliferation and survival of cancer cells (Herr and Chun, 2007; Cuvillier, 2008; Yang et al., 2012). Here we demonstrated that SKI-II, an inhibitor of Sphk1, could inhibit proliferation of SGC7901 cells significantly in a dose-dependent manner, and the lowest concentration of SKI-II being able to affect cell growth was 1.25µmol/L.

Eukaryotic nuclear transcription factor B (NF-κB) was first extracted from B lymphocytes by Sen and Baltimore in 1986, and it widely distributes in the cytoplasm of quiescent. An abundance of research has unraveled that NF-κB is involved in the process of immune stress, inflammation, cell proliferation and apoptosis (Esteban et al., 2004; Gao et al., 2007). Recent evidence shows that NF-κB was activated in many cancers, including gastric cancer (Sasaki et al., 2001), breast cancer (Cao and Karin, 2003), and its continuous activation could be a sign of a variety of solid tumors (Shishodia and Aggarwal, 2004). Results of Immunocytochemistry and Western-blot showed that NF-κB expression decreased gradually after the exposure of SKI-II. In contrast, there was no significant difference in the negative control group. Pearson correlation analysis showed that Sphk1 and NF-κB expression in SGC7901 cells was positively correlated \((r=0.978, p<0.01)\). These results suggested that apoptosis of SGC7901 cells induced by SKI-II may be attributed to inhibition the expression of NF-κB.

Bcl-2 family is the key mediator of cellular sensitivity to apoptosis and it can be divided into two categories: pro-apoptotic class, such as Bid, Bax, Bak, etc; anti-apoptotic classes, such as Bcl-2, Bcl-x1 and so on. The mechanism of Bcl-2 regulating cell apoptosis is mainly: first, Bcl-2 inhibited cell apoptosis by binding to other protein. Second, Bcl-2 inhibited apoptosis by participating in antioxidant pathway. Third, high expression of Bcl-2 could inhibit the release of Ca2+ in the endoplasmic reticulum during the occurrence of apoptosis. Fourth, Bcl-2 inhibited the activity of pro-apoptotic proteins Bax and Bak (Fan et al., 2005; Lin et al., 2005). Bax does not directly induce cell death, but it can significantly speed up the death signal which induced apoptosis. Bax and Bcl-2 may form Bax/Bcl-2 heterodimer, antagonizing biological effects of Bcl-2. The ultimate cellular outcome depends on the ratio of Bax to Bcl-2 (Xie et al., 2001). Our results showed that SKI-II reduced Bcl-2 expression, while increased the expression of Bax in SGC7901 cells, making the ratio of Bax/Bcl-2 increased. Compared to the negative control group, the difference was significantly \((p<0.05)\). These data suggested that SKI-II inhibited cell growth by down-regulating Bcl-2 expression and increasing Bax/Bcl-2 ratio. Recent studies showed that NF-κB can regulate the expression of Bcl-2, Bax and other genes. These gene products may be synergistic with each other, inhibiting apoptosis in the early, thereby promoting unlimited cell proliferation and accelerating tumor formation (Lee et al., 2008). In the present study, Pearson correlation analysis furthermore confirmed expression of NF-κB and Bcl-2 in SGC7901 cells was positively correlated \((r=0.986, P <0.01)\). These data suggested that apoptosis was regulated by the interaction of the two ways in SGC7901 cells, which was consistent with studies by Zhu and colleagues indicated that the increased apoptotic sensitivity of SGC7901/DDP to cisplatin was due to the decreasing proportion of Bcl-2/Bax via down-regulating NF-κB (Zhu et al., 2012). Dysregulation of cell cycle is a strong initiating factor and an early event in the tumorigenesis of many
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P27 protein, a cyclin-dependent kinases (CDKs) inhibitor protein, has effect on negative regulation of cell cycle. Activation of P27 can inhibit the transition from the G1 phase of the cell cycle to the S phase and induce G1/S phase arrest (Abukhdeir and Park, 2008). Correspondingly, down-regulation of P27 is closely related with the occurrence, development and invasion of gastrointestinal tumors. In this study, flow cytometry demonstrated that the G0/G1 phase of the cell cycle increased after SKI-II treatment, but the S phase of the cell cycle decreased compared with the controls. Consistent with this finding, Immunocytochemistry and Western-blot analysis showed that the expression of p27 protein increased, and the expression of Sphk1 and P27 in SGC7901 cells was negatively correlated (r=-0.952, p<0.01), which speculated that SKI-II up-regulated expression of p27 protein through the inhibition of Sphk1, to induce G1 to S phase block, thus inhibited SGC7901 cells growth.

In conclusion, our data provides evidence that Sphk1 inhibitor SKI-II can inhibit SGC7901 cells growth by reducing the expression of NF-κB and increasing the ratio of Bax/Bcl-2. Furthermore, SKI-II promoted expression of p27, a negative regulation of cell cycle, extended the cell doubling time, blocked cells in the G0/G1 phase. However, specific cytokines and mechanisms of SKI-II inhibiting the proliferation, the existence of other apoptosis-inducing factors and combination of other drugs to reduce the adverse drug reaction are not yet fully clear, which are all worth further study.

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