HAO XU^{A–D}, RUIHUA MI^{A, C, E}, RUIHUA FAN^{B, C, E}, QINGSONG YIN^{A, C, E},
XUDONG WEI^{A, C, D, F}

Effects of Thalidomide Combined with Interferon on Inhibiting Kasumi-1 Cell Proliferation^{*}

Department of Hematology, Affiliated Cancer Hospital of Zhengzhou University, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article

Abstract

Background. Our previous clinical observations proved that the combination of thalidomide and interferon (IFN) had certain effects in relapsed or refractory AML.

Objectives. The aim of this study was to investigate the effects and its mechanism of thalidomide and IFN on inhibiting the proliferation of Kasumi-1 cells.

Material and Methods. Thalidomide, IFN and a combination of both drugs were used to treat Kasumi-1 cells. The inhibition of cell proliferation and the apoptosis rate were measured. Vascular endothelial growth factor levels and the expression of apoptosis-related proteins were detected by ELISA and Western blotting, respectively.

Results. Thalidomide and IFN could both inhibit Kasumi-1 cell proliferation in a dose-dependent manner. When Kasumi-1 cells were treated with thalidomide 350 µg/mL or IFN 1400 U/mL for 48 h, the proliferation inhibition rates were $(48.8 \pm 4.64)\%$ and $(50.19 \pm 2.59)\%$ and the rates of apoptosis were $(14.68 \pm 2.61)\%$ and $(21.71 \pm 0.71)\%$, respectively; when treated with a combination, the cell proliferation inhibition rate and apoptotic rate were statistically significantly higher than both the control group and the groups treated with a single drug. The ELISA assay revealed that both 350 µg/mL of thalidomide and 1400 U/mL of IFN could reduce the VEGF levels in cell culture supernatants; the two-drug combination group had a further decreased VEGF concentration. Forty-eight-hour treatment of thalidomide 350 µg/mL and IFN 1400 U/mL could significantly decrease Bcl-2 expression and increase the expression levels of phosphor-P38, BAX, cytochrome c, and cleaved caspase-3, -8, and -9 as compared to the control group. The combination group exhibited significantly greater extents of reduction in Bcl-2 protein and increases in p-P38, BAX, and cytochrome c, and cleaved caspase-3, -8, and -9 protein expression as compared to the single drug groups.

Conclusions. Thalidomide and IFN can synergistically inhibit Kasumi-1 cell proliferation, which is possibly achieved through the mitochondrial and death receptor pathways and through the activation of the P38 signaling pathway to induce apoptosis and by inhibiting Kasumi-1 cell autocrine VEGF secretion (*Adv Clin Exp Med* 2016, 25, 3, 403–408).

Key words: acute myeloid leukemia, interferon, thalidomide, Kasumi-1 cells.

With improvements in treatment, the complete remission (CR) rate of young patients with acute myeloid leukemia (AML) can be as high as 70% to 80%, and after subsequent consolidation chemotherapy or hematopoietic stem cell transplantation (HSCT), the 5-year overall survival (OS) rate is approximately 40% to 45%. However, the treatment effect is poor in high-risk and elderly patients, and approximately half of patients

with CR will relapse, with a long-term survival of less than 10%. In addition, treatment-related side effects are among the main factors limiting the clinical efficacy for AML treatment. It is of great practical significance to explore safe and effective new treatment options. Steins et al. [1] reported that the effective rate of thalidomide monotherapy in AML was 25% and that this treatment could improve transfusion dependence in patients.

^{*} The study was funded by the Natural Science Foundation of China (No. 81170520).

Interferon (IFN) was used as early as the 1960s for AML treatment and showed some effects in both remission induction and salvage treatment for relapse after HSCT [2–4]. Our previous clinical observations found that the combination of thalidomide and IFN had certain effects for the treatment of relapsed or refractory AML [5]. To further explore the mechanisms of action of these two drugs, we used the Kasumi-1 AML cell line to observe the effects of thalidomide combined with IFN on Kasumi-1 cell proliferation, apoptosis and angiogenesis, providing some experimental evidence for the clinical application of thalidomide and IFN combination therapy.

Material and Methods

Main Reagents and Equipment

Standard thalidomide samples were provided by Changzhou Pharmaceutical Factory, Jiangsu province. RPMI 1640 medium and fetal bovine serum were purchased from Gibco. The Cell Counting Kit-8 (CCK-8) cell proliferation and cytotoxicity assay kit was purchased from Shanghai BestBio. The Annexin V-FITC (fluorescein isothiocyanate) apoptosis detection kit and total protein extraction kit was purchased from Nanjing KeyGEN Biotech. The bicinchoninic acid (BCA) protein assay kit was purchased from Shanghai Beyotime Institute of Biotechnology. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH), P38, phosphorylated (p)-P38, Bcl-2, BAX, cytochrome C, cleaved caspase-3, cleaved caspase-8, and cleaved caspase-9 rabbit anti-human monoclonal antibodies (mAb) were purchased from Cell Signaling Technology (USA); and the horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody was purchased from Beijing Zhongshan Golden Bridge biotechnology. The human vascular endothelial growth factor (VEGF) enzyme-linked immunosorbent assay (ELISA) kit was purchased from Shanghai Sangon Biotech. The Fluorescence-Activated Cell Sorting instrument was a FACSCalibur™ previously acquired from Becton-Dickinson (USA).

Cell Culture

Cells were cultured using RPMI 1640 medium with 15% fetal bovine serum and were placed in a 37°C and 5% CO₂ humidified incubator. Cells were passaged once every 48–72 h, and cells at the logarithmic growth phase were used for experiments.

CCK-8 Method for Detection of Cell Proliferation Levels

Cells at the logarithmic growth phase were taken and seeded in 96-well plates at a density of 1×10^5 cells/mL with 100 μ L in each well. Each group had 5 technical replicates. Cells were added with different concentrations of thalidomide (with final concentrations of 50, 100, 200, 300, 400, and 500 μ g/mL) and IFN (with final concentrations of 500, 1000, 2000, 5000, and 10000 U/mL). After either 24 or 48 h of treatment, 10 μ L of CCK-8 solution was added to each well. After incubation at 37°C for 4 h, we used a microplate reader to measure the absorbance (A) values at the wavelength of 450 nm and calculated the proliferation rate according to the formula below. The experiment was repeated three times.

$$\begin{aligned} \text{Cell proliferation inhibition rate (\%)} &= \\ &= \left(1 - \frac{A_{\text{experimental group}} - A_{\text{control group}}}{A_{\text{control group}} - A_{\text{blank group}}}\right) \times 100\% \end{aligned}$$

We used the various proliferation inhibition rates of cells treated with different concentrations of thalidomide or IFN to plot the proliferation inhibition curve. The thalidomide or IFN concentrations leading to an inhibition rate of 50% were the IC₅₀ values.

We used 350 μ g/mL of thalidomide, 1400 U/mL of IFN or a combination of the two drugs to treat Kasumi-1 cells and applied the same method to detect proliferation inhibition rates. The interaction between the two drugs was determined based on Jin's formula: $Q = E(a + b) / [Ea + Eb - (Ea \times Eb)]$, wherein Ea and Eb are the inhibition rates of thalidomide or IFN monotherapy groups and $E(a + b)$ is the inhibition rate of the combination therapy group. When $Q < 0.85$, the two drugs show antagonism; when $0.85 \leq Q < 1.15$, the two drugs have simple addition effects, and when $Q \geq 1.15$, the two drugs show synergy. The experiment was repeated three times.

Flow Cytometry Detection of Cell Apoptosis

A total of 5×10^5 cells were seeded in 12-well plates and were supplemented with 350 μ g/mL of thalidomide, 1400 U/mL of IFN, or a combination of two drugs. Each group had 3 technical replicates. After 48 h of treatment, cells were collected and resuspended by adding 500 μ L of binding buffer. Annexin V-FITC (5 μ L) was added to the suspension, and after mixing well, 5 μ L of propidium iodide (PI) was then added. After 15 min of incubation in the dark, cells were analyzed in the flow cytometry instrument. The above experiment was repeated three times.

ELISA Detection of VEGF Levels

Kasumi-1 cells were seeded in 96-well plates at 1×10^5 cells/well. After 24 h of incubation, cells were supplemented with 350 $\mu\text{g/mL}$ of thalidomide, 1400 U/mL of IFN, or a combination of the two drugs with 3 technical replicates for each group. After 48 h of treatment, cell culture supernatants were collected. We used untreated cells as the control and conducted the procedures according to the manual for the human VEGF ELISA kit. The experiment was repeated three times.

Western Blot Detection of the Expression Levels of the Apoptosis-Related Proteins

A total protein extraction kit was used to isolate total cellular proteins after the cells were treated with thalidomide, IFN or thalidomide combined with IFN, and a BCA assay was used to determine protein concentrations. The various samples were adjusted to the same concentration and were then aliquoted and stored at -80°C . GAPDH was used as the endogenous control, and the protein samples were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were then transferred to a polyvinylidene difluoride (PVDF) membrane under 300 mA constant current for 1~2 h. The membrane was blocked in 5% non-fat milk or bovine serum albumin (BSA), and antibodies against Bcl-2, BAX, cytochrome c, cleaved capase-3, -8, and -9, p-P38 and P38 at 1:200 to 1:1000 dilutions were added. The membranes were then incubated overnight at 4°C . Subsequently, 1:5000 diluted horseradish peroxidase (HRP)-conjugated secondary antibody was added for 1 h of reaction, followed by exposure and development on X-ray film. The results were subjected to grayscale analysis using an image-processing instrument. The experiment was repeated three times.

Statistical Analysis

SPSS 18.0 software was used for data analysis. The results are presented as mean $\pm$ standard deviation, the independent sample *t*-test was used for analysis, and $p < 0.05$ was considered to be statistically significant.

Results

1. The effects of thalidomide and IFN on inhibiting the proliferation of Kasumi-1 cells: Tha-

lidomide and IFN could both inhibit Kasumi-1 cell proliferation in a dose-dependent manner ($p < 0.001$) (Fig. 1 and 2). The respective IC_{50} values of thalidomide after 24 and 48 h of treatment were $(451.13 \pm 6.92) \mu\text{g/mL}$ and $(362.50 \pm 14.52) \mu\text{g/mL}$, and the IC_{50} values of IFN after 24 and 48 h of treatment were $(2209 \pm 127.19) \text{U/mL}$ and $(1393.33 \pm 63.07) \text{U/mL}$. It was also noted that the proliferation inhibition effects of IFN plateaued after 24 h at the concentrations of 5000 and 10000 U/mL. The Kasumi-1 cell proliferation inhibition rates of the 350 $\mu\text{g/mL}$ thalidomide group, the 1400 U/mL of IFN group and the two-drug combination group were $(48.8 \pm 4.64)\%$, $(50.19 \pm 2.59)\%$, and $(88.50 \pm 2.40)\%$, respectively, which were statistically significant differences compared to the control group at $(1.55 \pm 0.37)\%$ ($p < 0.001$). The proliferation inhibition rate of the two-drug combination group was also significantly different compared to each monotherapy group ($p < 0.001$), with $Q = 1.19 (\geq 1.15)$, suggesting that 350 $\mu\text{g/mL}$ of thalidomide and 1400 U/mL of IFN can synergistically inhibit Kasumi-1 cell proliferation.

2. The effects of thalidomide and IFN on Kasumi-1 cell apoptosis: After the Kasumi-1 cells were treated with 350 $\mu\text{g/mL}$ of thalidomide and 1400 U/mL of IFN for 48 h, the apoptosis rates

Fig. 1. Proliferation inhibition effects of different concentrations of thalidomide on Kasumi-1 cells

Fig. 2. Proliferation inhibition effects of different concentrations of IFN on Kasumi-1 cells

were ($14.68 \pm 2.61\%$) and ($21.71 \pm 0.71\%$), respectively, which were statistically significant differences compared to the control group at ($0.89 \pm 0.38\%$) ($p < 0.001$). The apoptosis rate of the two-drug combination group was ($41.95 \pm 3.41\%$), which was significantly higher compared to the control and to each of the monotherapy groups ($p < 0.001$).

3. VEGF levels in the cell culture supernatants 48 h after thalidomide and IFN treatment: The ELISA assay revealed that the respective VEGF levels in the cell culture supernatants of the 350 $\mu\text{g/mL}$ of thalidomide group, the 1400 U/mL of IFN group and two-drug combination group were (141.11 ± 3.70) ng/L, (119.90 ± 2.00) ng/L and (94.61 ± 5.46) ng/L, all lower than the control group level at (156.38 ± 5.48) ng/L ($p = 0.012$, $p < 0.001$ and $p < 0.001$, respectively). The VEGF concentration of the two-drug combination group was significantly lower than both of the monotherapy groups ($p < 0.001$).

4. The effects of thalidomide and IFN on the expression of apoptosis-related proteins in Kasumi-1 cells: Western blotting revealed that 350 $\mu\text{g/mL}$ of thalidomide and 1400 U/mL of IFN could decrease Bcl-2 expression to varying degrees and could increase the expression levels of p-P38 (with simultaneously reduced P38 expression), BAX, cytochrome c, and activated (cleaved) caspase-3, -8, and -9, with statistically significant differences compared to the control group ($p < 0.001$). When Kasumi-1 cells were treated with 350 $\mu\text{g/mL}$ of thalidomide combined with 1400 U/mL of IFN for 48 h, the degrees of reduction in Bcl-2 protein expression and increases in p-P38, BAX, and cytochrome c, and cleaved caspase-3, -8, and -9 protein expression were all significantly greater compared to the single-drug groups ($p < 0.001$) (Fig. 3).

Discussion

In this study, we used the Kasumi-1 AML cell line to demonstrate that, within a certain range of concentrations, both thalidomide and IFN could inhibit Kasumi-1 cell proliferation in a dose-dependent manner. We found that after 48 h of treatment with 350 $\mu\text{g/mL}$ of thalidomide and 1400 U/mL of IFN, thalidomide and IFN had a synergistic effect in inhibiting Kasumi-1 cell proliferation. To further investigate the mechanism of synergy between the two drugs, we used Annexin V-FITC/PI double staining to conduct flow cytometry analysis of the cells before and after treatment with thalidomide, IFN and a combination of the two drugs. The combination of the two drugs could increase the rate of apoptosis of Kasumi-1 cells and significantly inhibited VEGF autocrine secretion, as detected by ELISA assay.

Fig. 3. Western blot assay detection of the effects of thalidomide and IFN on the expression of proteins related to apoptosis in Kasumi-1 cells.

1 – untreated group; 2 – thalidomide 350 $\mu\text{g/mL}$; 3 – IFN 1400 U/mL group; 4 – thalidomide combined with IFN

The Bcl-2 family of proteins is closely associated with cell apoptosis. Among the family members, Bcl-2 is an anti-apoptotic protein, BAX is a pro-apoptotic protein, and they both play important roles in the mitochondrial apoptotic pathway. Leukemia patients with high Bcl-2 expression have poor responses to chemotherapy, low complete remission rates, and short survival times [6]. During the apoptosis process, Bcl-2 protein expression is reduced and BAX protein is increased, thereby promoting the release of cytochrome c. Subsequently, cytochrome c, apoptosis-promoting factor-1 (Apaf-1) and caspase-9 form complexes, leading to the activation of the caspase-3 apoptotic cascade [7, 8]. The activation of caspase-3 is a marker indicating the entry into an irreversible stage of apoptosis [9]. This study found that 48-h treatments of 350 $\mu\text{g/mL}$ of thalidomide or 1400 U/mL of IFN could both inhibit Bcl-2, while a combination of the two drugs led to significantly enhanced inhibition of Bcl-2 expression and significantly increased expression levels of BAX, cytochrome c and activated caspase-3 and -9. These results suggest that thalidomide combined with IFN can induce Kasumi-1 cell apoptosis through

the caspase-dependent mitochondrial pathway. In addition, thalidomide combined with IFN could also significantly stimulate the expression of activated caspase-8. Caspase-8 can directly activate caspase-3 by the death receptor pathway and may also be involved in the thalidomide- and IFN-induced apoptosis in Kasumi-1 cells.

Mayer et al. [10] found that IFN could inhibit the proliferation of the K562 chronic myelogenous leukemia cell line, whereas the application of P38 pathway inhibitors could reverse IFN-mediated inhibition of K562 cell proliferation, indicating that IFN inhibits K562 cell proliferation *via* the P38MAPK pathway. Lu M et al. [11] found that IFN can act through the P38 pathway to inhibit CD34+ cell proliferation in the bone marrow or peripheral blood of patients with Janus kinase 2 (JAK-2)-positive polycythemia vera and induce CD34+ cell apoptosis. Further, the P38MAPK inhibitor SB203580 can reverse IFN-mediated proliferation inhibition and pro-apoptotic effects. Using Western blotting, the present study showed that after Kasumi-1 cells were treated with 1400 U/mL of IFN for 48 h, p-P38 expression was increased. The P38MAPK pathway may also mediate IFN-induced apoptosis in Kasumi-1 cells. Forty-eight hours of treatment with 350 µg/mL of thalidomide could also enhance p-P38 expression, and when the two drugs were combined together, p-P38 expression was further increased, indicating that the two drugs could possibly synergistically activate the P38MAPK pathway and thereby inhibit proliferation and induce apoptosis in Kasumi-1 cells.

Studies have confirmed that the microvessel density in the bone marrow of patients with leukemia is significantly increased and that high levels of VEGF can be detected in the peripheral blood or bone marrow of patients with AML. VEGF can influence and regulate the malignant behavior of leukemic cells *via* paracrine and autocrine pathways [12], and high levels of plasma VEGF in AML patients indicate a poor prognosis [13]. A high VEGF level in childhood AML is also an independent factor indicating poor prognosis [14], suggesting that VEGF inhibition can

have potential therapeutic effects on AML. Thalidomide is an anti-angiogenesis drug and can inhibit VEGF production. The present study found that 350 µg/mL of thalidomide can suppress autocrine VEGF secretion in Kasumi-1 cells and that 1400 U/mL of IFN can mildly inhibit VEGF expression, while a combination of the two can further enhance the inhibition of autocrine VEGF secretion in Kasumi-1 cells. These results suggest that thalidomide and IFN may synergistically suppress VEGF secretion to change the tumor microenvironment, therefore inhibiting Kasumi-1 cell proliferation.

We previously used thalidomide combined with IFN for the treatment of relapsed or refractory non-Hodgkin's lymphoma and achieved considerable effects. We later used a regimen based on thalidomide combined with IFN to treat two cases of relapsed/refractory AML. One case achieved complete remission, and in the other case, the proportion of leukemia cells in the bone marrow was reduced from 48.6% to 2%, suggesting that thalidomide and IFN have a synergistic effect in immune regulation and the promotion of immune cell activities [5, 15, 16]. This study further found that thalidomide and IFN have synergistic effects in inhibiting cell proliferation, inducing apoptosis through the mitochondrial, death receptor and P38MAPK pathways and inhibiting VEGF secretion, which may have an important significance in the treatment of relapsed/refractory AML. There is no standard salvage therapy for relapsed/refractory AML. In particular, elderly patients often cannot tolerate high-dose chemotherapy and stem cell transplantation. Therapies based on thalidomide combined with IFN are safe and effective in clinical applications and can inhibit Kasumi-1 cell proliferation *in vitro* through a variety of mechanisms and induce their apoptosis. We are now conducting a multi-center phase III clinical study utilizing a regimen that is mainly based on thalidomide combined with IFN to treat relapsed/refractory AML and AML in elderly patients. This regimen may become an effective means of treatment for relapsed or refractory AML, especially AML in elderly patients.

Acknowledgments. The Kasumi-1 leukemia cell line was a gift from Professor Jianxiang Wang at the Institute of Hematology at the Chinese Academy of Medical Sciences.

References

- [1] Steins MB, Padró T, Bieker R, Ruiz S, Kropff M, Kienast J, Kessler T, Buechner T, Berdel WE, Mesters RM: Efficacy and safety of thalidomide in patients with acute myeloid leukemia. *Blood* 2002, 99, 834–839.
- [2] Hill NO, Pardue A, Khan A, Aleman C, Hilario R, Hill JM: Clinical trials of human leukocyte interferon in malignancy. *Tex Rep Biol Med* 1981–1982, 41, 634–640.
- [3] Arellano ML, Langston A, Winton E, Flowers CR, Waller EK: Treatment of relapsed acute leukemia after allogeneic transplantation: A single center experience. *Biol Blood Marrow Transplant* 2007, 13, 116–123.

- [4] **McSweeney EN, Worman CP, Tsakona CP, Jewel AP, Hoffbrand AV, Milligan DW, Burnett AK, Lewis ML, Goldstone AH:** Low-dose recombinant alfa-2a-interferon: A feasible maintenance therapy in acute myeloid leukaemia in the older patient. *Acta Haematol* 1993, 89, 1–5.
- [5] **Ai H, Zhang Y, Wei X, Wang P, Mi R, Song Y:** Thalidomide in combination with interferon and interleukin 2 in the induction therapy for relapsed refractory acute myeloid leukemia: Two case report and literature review. *Chin J Hematol* 2014, 35, 954–956.
- [6] **Campos L, Rouault JP, Sabido O, Oriol P, Roubi N, Vasselon C, Archimbaud E, Magaud JP, Guyotat D:** High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. *Blood* 1993, 81, 3091–3096.
- [7] **Cory S, Huang DC, Adams JM:** The Bcl-2 family: Roles in cell survival and oncogenesis. *Oncogene* 2003, 22, 8590–8607.
- [8] **Sharpe JC, Arnoult D, Youle RJ:** Control of mitochondrial permeability by Bcl-2 family members. *Biochim Biophys Acta* 2004, 1644, 107–113.
- [9] **Cryns V, Yuan J:** Protease to die for. *Genes Dev* 1998, 12, 1551–1570.
- [10] **Mayer IA, Verma A, Grumbach IM, Uddin S, Lekmine F, Ravandi F, Majchrzak B, Fujita S, Fish EN, Platanias LC:** The p38 MAPK pathway mediates the growth inhibitory effects of interferon-alpha in BCR-ABL-expressing cells. *J Biol Chem* 2001, 276, 28570–28577.
- [11] **Lu M, Zhang W, Li Y, Berenzon D, Wang X, Wang J, Mascarenhas J, Xu M, Hoffman R:** Interferon-alpha targets JAK2V617F-positive hematopoietic progenitor cells and acts through the p38 MAPK pathway. *Exp Hematol* 2010, 38, 472–480.
- [12] **Lee CY, Tien HF, Hu CY, Chou WC, Lin LI:** Marrow angiogenesis-associated factors as prognostic biomarkers in patients with acute myelogenous leukaemia. *Br J Cancer* 2007, 97, 877–882.
- [13] **Aguayo A, Estey E, Kantarjian H, Mansouri T, Gidel C, Keating M, Giles F, Estrov Z, Barlogie B, Albitar M:** Cellular vascular endothelial growth factor is a predictor of outcome in patients with acute myeloid leukemia. *Blood* 1999, 94, 3717–3721.
- [14] **de Bont ES, Fidler V, Meeuwse T, Scherpen F, Hählen K, Kamps WA:** Vascular endothelial growth factor secretion is an independent prognostic factor for relapse-free survival in pediatric acute myeloid leukemia patients. *Clin Cancer Res* 2002, 8, 2856–2861.
- [15] **Zhao H, Wei X, Yin Q, Song Y:** Thalidomide combined with interferon in relapsed or refractory mantle cell lymphoma: Two cases report and literatures review. *Chin J Hematol* 2012, 33, 776–777.
- [16] **Hao Q, Wei X, Yin Q, Mi R, Yuan F, Ai H, Wang P, Li Y, Zhang L, Song Y:** Thalidomide combined with interferon in 12 cases of relapsed or refractory mantle cell lymphoma. *Chin J Hematol* 2014, 35, 1013–1015.

Address for correspondence:

Xudong Wei
Department of Hematology
The Affiliated Cancer Hospital of Zhengzhou University
Zhengzhou 450000
Henan
China
Tel: +86 138 37 169 301
E-mail: zyxuhao@163.com

Conflict of interest: None declared

Received: 08.12.2014

Revised: 12.03.2015

Accepted: 25.03.2015