In vitro Growth Inhibition and Apoptotic Effects of Hang-baek-Tang on HL-60 Cells

Jun Ho Park, Sung Min Ju[1], Kun Jung Kim[1], Byung Hoon Jeon[1], Jung Mi Oh[2], Chae Ho Lee[2], Dong Min Han, Won Sin Kim*

Division of Biological Science, Wonkwang University, Iksan, Chonbuk,
1 : Department of Pathology, College of Oriental Medicine, Wonkwang University, Iksan, Chonbuk,
2 : Division of Bio-Nano Chemistry Wonkwang University, Iksan, Chonbuk

To develop novel anti-leukemic medicine, we have prepared a Korean traditional medicine, named Hang-baek-Tang, which is composed of 8 kinds of anti-leukemic medicinal plants. The water extracts was examined anti-leukemic activity using the human leukemia cell line, HL-60 cells. HL-60 cells showed the growth inhibition and several apoptotic features, including DNA ladders, morphological changes, by treatment of the cells with Hang-baek-Tang. We have observed that Hang-baek-Tang induced the activation of caspase-3, caspase-8 and caspase-9. Further molecular analysis demonstrated that Hang-baek-Tang induced cleavage of PARP and increase of hypodiploid (Sub-G1) population in flow cytometric analysis. These results indicate that Hang-baek-Tang has been considered to exert anti-leukemic activity through the procaspase-3 activation pathway.

Key words : Apoptosis, caspase-3, PARP, HL-60 cells, leukemia

Introduction

Various forms of cancer are one of the leading causes of human death. In Korean traditional medicinnes, many herbal plants have been widely employed in reducing the mortality caused by cancers or in controlling them. Recently, a number of investgators have concentrated their efforts to understand various activities of the oriental herbal plants and found that many herbal plantscontained beneficial properties to treat the cancer patients. For example, Juzen-taiho-to, which is Chinese traditional medicine, was prevented malignant progression and metastasis oftumor cells (Saiki, 2000). Also, Bojung-tang and Gagamleejung-tang showed both *in vitro* and *in vivo* anti-tumor activities on Sacoma 180 cells and CT-26 tumor cells, respectively (Ha *et al.*, 1998). We have investigated 31 kinds of anti-leukemic medicinal plants, which its phamacological action was already reported through many experimental articles and oriental medical book (Pae *et al.* 2003). In this study, to develop novel anti-leukemic medicine, named Hang-baek-Tang, we have chosen the 8 herbs among the anti-leukemic plants. The herb mixture was extracted with water and examined anti-leukemic activity using the human leukemia cell line, HL-60 cells.

* To whom correspondence should be addressed at : Won Sin Kim, Division of Biological Science, Wonkwang University, Iksan, Chonbuk
· E-mail : wsnkim@wonkwang.ac.kr, · Tel : 063-850-6578
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Materials and Methods

1. Constituents of Hang-baek-Tang

Hang-baek-tang was prepared as follows: A mixture of Angelica dahurica (50 g), Fritillaria ussuriensis (50 g), Ailanthus altissima (50 g), Visocum coloratun (50 g), scutellaria Radix (50 g), Ginseng Radix (50 g), Astragali Radix (50 g), Glycyrrhiza Radix (50 g) is added to 1,000㎖ of water and extracted at 100℃ for 2h.

2. Cell culture

HL-60 was cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 1 ml/100 ml antibiotics at 37℃ in 5% CO_2 incubator.

3. MTT assay

To assess the cytotoxicity of Hang-baek-Tang, cells were treated with Hang-baek-Tang at concentration of 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, and 200 ㎍/㎖. After cells were seeded at 1×10^5 cells/well in 500 ㎕ of medium in 24 well plates, those were incubated for 24 h and 125 ㎕ of MTT were added to each well, and plates were gently shaken and

incubated at 37℃ for 4 h. The resulting blue formazan crystals were dissolved with 625 ㎕ of DMSO. The plates were read by ELISA reader at 575nm.

4. DAPI staining

Cells were treated with Hang-baek-Tang in a concentration-dependent manner, and collected by centrifugation. The pellet was resuspended and stained with the methanol solution containing 1 ㎍/㎖ DAPI for 15 min at 37℃.

5. Analysis DNA fragmentation

The cells were treated with Hang-baek-Tang at various concentrations. After exposure to Hang-baek-Tang, DNA was isolated and analysed on a 1% agarose gel.

6. Flow Cytometric Analysis

To determine DNA contents, cells were stained with propidium iodide(PI). Cell suspensions prepared as described above in material and method were centrifuged, and the resuspended cell pellets(1×10^6) were washed twice with phosphate-buffered saline and fixed in 70% ethanol at 4℃ for 15min. Cellular DNA was stained with 25㎍/㎖ PI and 100 ㎍/㎖ RNase in PBS containing 0.05% NP-40. The stained cells were analytic flow cytometric measurements for relative DNA contents (Becton Dickinson).

7. Capase activity

The activity of caspase-3 was measured by using a fluorogenic substrate from Alexis. Hang-baek-Tang treated cells were incubated with various concentrations for 24 h at 37℃. After measurement of caspase-3 activity, cells were cultured at indicated concentrations for 24 h to confirm the caspase-8, and caspase-9 activities. Untreated and Hang-baek-Tang treated cells (1×10^6) were harvested and washed with ice-cold PBS and lysed with lysis buffer(50 mM Tris-HCl, pH 7.5 containing 0.5 mM EDTA, 0.5% Igepal and 150mM NaCl) for 30 min on ice before centrifugation at 14,000 rpm for 10 min at 4℃. Cell lysate were mixed with fluogenic substrate; caspase-3 (Ac-DEVD-AMC), caspase-8 (Ac-IETD-AMC), caspase-9 (Ac-LEHD-AMC), and incubated at 37℃ for 2 h. The release of 7-amino-4-metyl-coumarin(AMC) was measured at excitation/ emission wavelength of 360/460nm.

8. western blot analysis

After treatment with the different concentrations of Hang-baek-Tang for 24 h, HL-60 cells were collected and lysed in ice-cold 80㎕ lysis buffer (50mM Tris-HCl, pH 7.5, containing 0.5 mM EDTA, 0.5% Igepal, 150 mM NaCl and 1% protease inhibitor) for 30min. Cell lysates were separated on 12% SDS-polyacrylamide gel and analysed polyclonal anti-human anti-caspase-3 and monoclonal anti-human anti-PARP.

Results

1. Growth inhibition of Hang-baek-Tang on HL-60 cells

HL-60 cells were incubated with 0, 20, 40, 80, 100, 120, 140, 160, 180, and 200 ㎍/㎖ of Hang-baek-Tang, for 24 h and carried out MTT assay. As shown in Fig. 1A, viabilities of cells incubated with Hang-baek-Tang at the various concentrations were determined to be 100±7.1%, 95.7±6.1%, 104.2±3.5%, 104.9±4.6%, 94.7±4%, 87.3±3.9%, 78.6±4.7%, 68.9±3.9%, 70±2.5%, 65.7±2.1% of the control value, respectively. This data indicated that the growth inhibition of Hang-baek-Tang on HL-60 cells was increased in a dose-dependent manner (Table 1). Cells were investigated by a fluorescence microscope after DAPI staining. It was shown that the rounding, cytoplasmic blebbing, and irregularities in Hang-baek-Tang treated cells. It was thought that the Hang-baek-Tang induced morphological changes of cells as shown Fig. 1B. Also, DNA fragmentation were observed in Hang-baek-Tang treated cells (Fig. 1C).

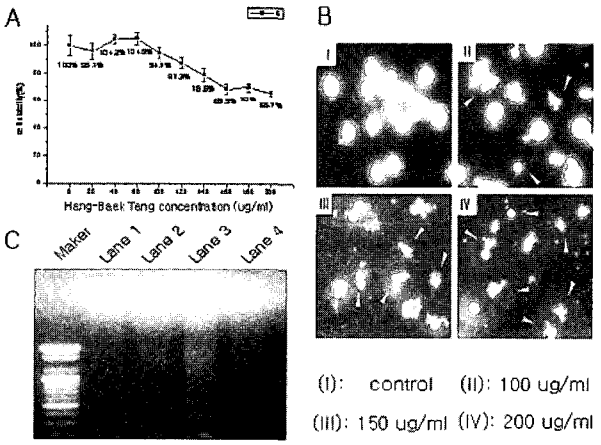

Fig. 1. Apoptotic effects Hang-baek-Tang on HL-60 cells. (A) Cells were cultured with various concentration of Hang-baek-Tang for 24 h. Then cell death was assessed by the MTT assay. Each value represents the mean±S.D. of three experiments. (B) The cells were investigated by a fluorescence microscope after DAPI staining. 0, 100, 150, and 200 ㎍/ml treatment of Hang-baek-Tang shows that the morphological changes of cells indicated an apparent apoptotic cell death pattern (arrows). DNA ladder formation following exposure of HL-60 cells to Hang-baek-Tang for 24 h. Genomic DNA was extracted and analyzed by electrophoresis on 1% agarose gels electrophoresis; molecular-weight markers, control cells (Lane 1), cell treated with 100, 150, 200 ㎍/ml (Lane 2-4).

2. Hang-baek-Tang indued caspase-3, -8, -9 activation

Subsequently, we have examined whether caspase activation is induced by treatment of HL-60 cells with Hang-baek-Tang. Hang-baek-Tang markedly increased the caspase-3 activity in a dose-dependent manner for 24 h (Fig.

2A). It is also observed that the activities of caspase-8 and -9 was increased dose-dependently in Hang-baek-Tang treated HL-60 cells (Fig. 2B, 2C).

Table 1. Growth inhibition effect of Hang-baek-Tang on HL-60 cells.

Hang baek-Tang concentration(㎍/㎖)	Cell viability(%)
Um-treated(Control)	100 ± 7.1
20	95.7 ± 6.1
40	104.2 ± 3.5
80	104.9 ± 4.6
100	94.7 ± 4
120	87.3 ± 3.9
140	78.6 ± 4.7
160	68.9 ± 3.9
180	70.0 ± 2.5
200	65.7 ± 2.1

Values are represented of three or more ndependent experiment.

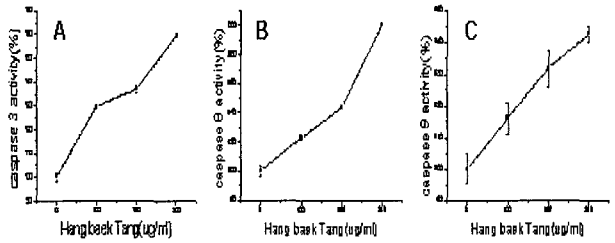

Fig. 2. Caspase activity analysis. HL-60 cells were incubated with increasing concentration of Hang-baek-Tang for 24 h. The caspase activities were measured by cleavage of its substrates, caspase-3 (Ac-DEVD-AMC), caspase-8 (Ac-IETD-AMC), caspase-9. Values were the mean±SD error of three experiments. (A) caspase-3, (B) caspase-8, (C) caspase-9.

3. Hang-baek-Tang caused degradation of PARP

Activation of caspase-3 leads to cleave a number of proteins, such as PARP. Full-length PARP(116 kDa) is cleaved to create 85 kDa fragment is a typical evidence during apoptosis (Tewari et al., 1995). Therefore, we observed the proteolytic activation of the effects of Hang-baek-Tang on cleavage of pro-caspase-3 (35 kDa) by western blot analysis in HL-60 cells. Hang-baek-Tang treated cells were exhibited the decrease of the pro-caspase-3 (Fig. 3). Next, we assessed the degradation PARP protein, the known substrate of caspase-3 (Ferrer et al., 2003; Kothakota S. et al., 1997). As shown in Fig. 3, Hang-baek-Tang caused degradation of PARP, a typically 85 kDa band, which was almost completely degraded at the concentration of 150 and 200 ㎍/㎖. It may be supposed that the activation of caspase-3 by treatment of HL-60 with Hang-baek-Tang induced cleavage of PARP.

4. Cell cycle analysis

Fig. 4 shows the results of cell cycle analysis. To characterize whether the growth inhibitory effect induced by Hang-baek-Tang is related to induction of cell cycle arrest or apoptotic process, HL-60 cells were treated with various concentrations (Un-treated, 100, 150, 200 ㎍/㎖) of Hang-baek-Tang for 24 h, and the distribution of cells in various compartment of the cell cycle was analyzed by flow cytometry (Fig. 4A-D). Our results inform the decrease of the cells at S and G2/M phase of each cell cycles (Fig. 4E-H) after 24 h incubation, respectively. These results suggested a possibility that Hang-baek-Tang induced apoptosis happened at S and G2/M phase of cell cycle.

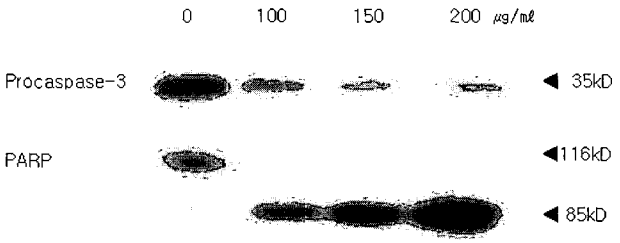

Fig. 3. Western blot analysis of procaspase-3 and PARP on HL-60 cells treated with Hang-baek-Tang. Cells were treated with Hang-baek-Tang (0, 100, 150, 200 ㎍/㎖) for 24 h. Procaspase-3 was decreased and PARP was cleaved into 85 kD fragment in a dose-dependent manner.

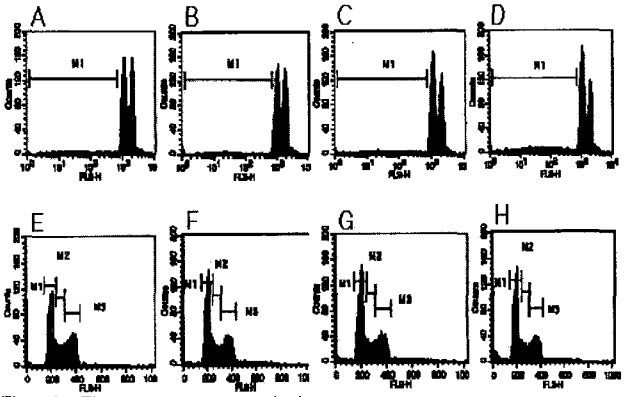

Fig. 4. Flow cytometry analysis. HL-60 cells were treated increasing concentrations of Hang-baek-Tang for inducing apoptosis during 24 h. After treatment, cells were stained with PI and analysed by flow cytometry. M1 shows quantity of hypodiploid cell (A-D) and (E-H) show each phases of cell cycle (M1=G1, M2=S, M3=G2/M).

Discussion

Recently, considerable attention has been focused on identifying naturally occurring chemo-preventive substances capable of suppressing the production of cancer (Dassonneville *et al.*, 2000). We prepared a new water extracted herbs mixture; Hang-baek-Tang, to examine whether it has a possibility to be a naturally potential substance for inducing apoptosis on HL-60 cells or not.

We have shown that Hang-baek-Tang can trigger apoptosis of HL-60 cells. After HL-60 cells were treated with

Hang-baek-Tang for 24 h, MTT assay showed that Hang-baek-Tang has a growth inhibition effect in a concentration-dependent manner. The reduction of MTT assesses mitochondrial integrity through the activity of succinate dehydrogenase (Mosmann, 1983; Melo *et al.*, 2001). The fluorescence microscopic observations by DAPI stain revealed the apoptotic features such as apoptotic bodies. In this study, we have also observed DNA fragment ladder formation, a characteristic gel electrophoretic band pattern associated with apoptosis. DNA ladder, the biochemical hallmark of apoptosis, is degradation of DNA by endogenous DNase, which cut the internucleosomal regions into double-stranded DNA fragments of 180-200 base pairs(Nagata S. et al., 2003). Mentioned above, PARP is a neclear enzyme, which is involved in DNA repair process. Recently, 116 kDa PARP protein has known to be cleaved into 85 kDa and 35 kDa fragment by the activation of a caspase-3 protease (Ferrer et al.. 2003; Kothakota *et al.*, 1997).

Here, we carried out the western blot analysis using the antibody against PARP, because the cleavage of PARP is considered to be a biochemical evidence during apoptosis (Kamesaki, 1998). Fig. 3 shows that PARP is cleaved into 85 kDa fragment suggesting that caspase-3 was activated. Moreover, we can find the decrease of the procaspase-3 protein in this study. In addition, it was also appeared that the activation of caspase-8 and -9 were increased in our results. During the apoptotic process, caspase-3 is a pivotal factor of apoptosis, and DNA fragmentation mediated by caspase-activated DNase (CAD, also known as DFF, DNA fragmentation factor) activation (Hengartner *et al.*, 2000). In this study, we compared the caspase-3 activation with the results of a flow cytometric analysis for DNA contents. The data indicated that Hang-baek-Tang could induce apoptosis at the G2/M phase of the cell cycle on HL-60 cells in a dose-dependent manner.

In conclusion, we have demonstrated that Hang-baek-Tang is able to induce apoptosis of HL-60cells through the caspase activation pathway. However, further studies should be carried out in vivo to prescribe Hang-baek-Tang for augmentation of anti-leukemic effects in combination with other treatment modalities.

Acknowledgements

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References

1. Dassonneville, L., Lansiaux, A., Wattelet, A., Wattez, N., Mahieu, C., Van Miet, S., Pieters, L., Bailly, C. Cytotoxicity and cell cycle effects of the plant alkaloids cryptolepine and neocryptolepine: relation to drug-induced apoptosis. E*ur. J. Pharmacol.* 409, pp 9-18, 2000.
2. Ferrer, I., Planas, A.M. Signaling of cell death and cell survival following focal cerebral ischemia: life and death struggle in the penumbra. *J Neuropathol. Exp. Neurol.* 62, pp 329-339, 2003.
3. Ha, J.Y., Lim, H.J. Antitumor and immune modulatory effects of Bonjungtang in ICR mice. *Kor. J. Oriental Pathology* 12, pp 90-98, 1998.
4. Ha, J.Y., Lim, HJ, Antitumor and immune modulatory effects of Gagamleejungtang in BALB/c mice. *Kor. J. Oriental Pathology* 12, pp 73-81, 1998.
5. Hengartner, M.O. The biochemistry of apoptosis. Nature 407, pp 770-776, 2000.
6. Kamesaki, H. Mechanism involved in chemotherapy-induced apoptosis and their implications in cancer chemotherapy. *Int. J..Hematol.* 68, pp 29-43, 1998.
7. Kothakota, S., Azuma, T., Reinhard, C., Klippel, A., Tang, J., Chu, K. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* 278, pp 294-298, 1997.
8. Melo, P.S., Duran, N., Haun, M. Cytotoxicity of derivatives from dehydrocrotonin on V79 cells and *Escherichia coli*. *Toxicology* 159, pp 135-141, 2001.
9. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J. Immunol. Methods* 65, pp 55-63, 1983.
10. Nagata, S., Nagase, H., Kawane, K., Mukae, N., Fukuyama, H. Degradation of chromosomal DNA during apoptosis. *Cell Death Differ.* 10, pp 108-116, 2003.
12. Pae, H.O., Lim, C.K., Jang, S.I., Han, D.M., Ah, W.G., Yoon, Y.S., Jeon, B.H., Kim, W.S. and Yun, Y.G. Review of anti-leukemia effects from medicinal plants. *Kor. J. Oriental Physiology & Pathology* 17, pp 605-610, 2003.
13. Tewari, M., Quan, L.T., O' Rourke, K., Desnoyers, S., Zeng., Beidler, D.R., Poirier, G.G., Salvesen, G.S., Dixit, V.M. Yama/CPP32 beta, a mammalian homology of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* 81, pp 801-809, 1995.