The proliferative and apoptotic effects of garlicin on mouse myeloma cell line SP2/0

Hui Wang[1,2], Xiangchen Li[1], Guangbin Wang[1], Xiaohong He[1], Weijun Guan[1],Yuehui Ma[1]

[1.] Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, China
[2.] College of Wildlife Resources, Northeast Forestry University, Harbin 150040, China
Hui Wang and Xiangchen Li contributed equally to this work
Yuehui_Ma@hotmail.com;weijunguan301@gmail.com.

Abstract: In order to investigate the proliferative and apoptotic effects of garlicin on mouse myeloma cell line SP2/0, and to explore the mechanisms of garlicin induced apoptosis, mouse myeloma cell line SP2/0 was used as research material. morphological events and calcium dynamics were observed with phase contrast and confocal microscopy, mitochondrial transmembrane potential, cell cycle progression and apoptotic rates were detected using flow cytometry, and TUNEL staining and agarose gel electrophoresis were performed upon exposure to the drug. Garlicin exerts a strong inhibitory effect on SP2/0 cell line, which is dosage and duration dependent. There occured typical apoptotic morphological alterations at 48h upon exposure. Calcium dynamics were disturbed, cell cycle was arrested at G0/G1 checkpoint, and the ratio of cells in G0/G1 phase was gradually increased while the proportion in S phase was reduced with elevated concentration. Mitochondrial transmembrane potential dropped. The viable-apoptotic and non-viable apoptotic rates were both dose-dependent. Agarose gel electrophoresis revealed that "DNA ladders", a symptom of DNA fragmentation, had taken place. Garlicin could effectively inhibit the proliferation of SP2/0 cell line, influence cell cycle progression and induce apoptosis.

[Wang H, Li XC, Wang GB. **The proliferative and apoptotic effects of garlicin on mouse myeloma cell line SP2/0**. *Life Sci J* 2012;9(4):3587-3594] (ISSN:1097-8135). http://www.lifesciencesite.com. 531

Keywords: Garlicin; Mouse myeloma cell line SP2/0; Apoptosis

1. Introduction

Garlicin, or allitrid, whose chemical name is diallyldisulfide, is a sulfocompound extracted from garlic bulb. It has a unique pharmacological effect against infection, DNA damage, arteriosclerosis, plays a protective role in cell viability improvement, antioxidation and detoxification (Lv et al., 2006), and functions as a strong inhibitor of leukemia, hepatoma, oophoroma and gastric carcinoma. Evidence is accumulating that correlates daily intake of garlicin with defense against carcinogenesis (especially cancers in gastrointestinal tract and oral cavity). Garlicin can block the synthesis of carcinogens, inhibit their activation and reduce the risks of teratogenesis, as a result of which extensive attention has been attracted in regard to its therapeutic prospect (Velmurugan et al., 2003; Leist et al., 1996).

Statistics have revealed that the incidence of myeloma, a common type of carcinoma in mice, is increasing year by year, seriously threatening the robustness, and even survival, of rodents. Despite the fact that chemotherapeutic drugs have some effects in the prevention and treatment of myeloma, the concomitant side effects and development of chemoresistance have made them far from being applicable in clinical trials. A new concept is to extract active ingredient from medicinal herb for administration. In this paper, mouse myeloma cell line SP2/0 was used as the research object, to investigate the antitumor effects of garlicin and to unravel the underlying mechanism to restore the apoptotic sensitivity, therefore providing important insights in therapeutic strategies.

2. Material and Methods

2.1 Drug solution preparation

Garlicin (National institute for the control of pharmaceutical and biological products) of 0.5 ml, was dissolved with DMSO, filtered for sterilization, adjusted to a concentration of 1.5 mg/ml, subpackaged, and then preserved at -20℃ in the refrigerator. It should be diluted to the required concentration with DMEM medium prior to treatment, and the final concentration of DMSO should be no more than 0.1% in the experiments.

2.2 Cell culture

Mouse myeloma cell line SP2/0 was purchased from Shanghai cell bank, Chinese Academy of Sciences, The cells were cultured using DMEM medium (Gibico, USA) that contains 10% fetal calf serum in a humidified atmosphere at 37℃, 5% CO_2 (Freshney, 2000; Guan et al., 2005; Zhou et al., 2004). The culture medium was refreshed when its color become yellow; and the cells were passaged when 70%-80% confluent (The cells are semi-adherent and semi-suspended without the need of trypsinization when subcultured).

2.3 Growth kinetics

Following the method of Gu et al (2006). and Kong et al (2007), the SP2/0 cells were plated onto 24-well microplates at the concentration of 2.5×10^4 cells/well and cultured up to 7 days. Cells were harvested from three wells per day and counted. The mean values were used to plot a growth curve and to calculate the population doubling time (PDT).

2.4 Morphological observation

2.4.1 inverted phase contrast microscope observation

The cells were plated on each well of 6-well microplates at the concentration of 4×10^5 cells/well, and treated with garlicin 24 h later. They were observed under a phase-contrast microscope at 48 h upon treatment.

2.4.2 confocal microscopy observation

To investigate their chromatinic alterations, the cells from a single well were harvested, pelleted, resupended and stained with 6 μl AO and EB solution (both 2 mg/ml in ethanol). Incubated at room temperature in the dark for 5 min and then observed using confocal microscopy (Nikon TE-2000-E, Japan) immediately.

2.5 Annexin V-FITC/PI double staining

Cells upon garlicin treatment were collected, and then spun at 1200 rpm, 4℃ for 10min. With supernatant discarded, they were washed with precooled PBS twice, 10^6 cells of which were then resuspended with 500 μl Binding Buffer and stained with 5 μl AnnexinV-FITC (20mg/L) and 5 μl PI (20mg/L) in the dark for 15min at room temperature. The samples were analysed with FCM (BD FACSCalibur, USA) within 1 h.

2.6 TUNEL staining

TUNEL microslides were prepared using In Situ Cell Death Detection Kit (Roche, UK). Apoptotic SP2/0 cells emitted green fluorescence. Count 1000 cells from each of 10 large visual fields randomly selected for subsequent calculation. The positive rates were regarded as apoptosis rate.

2.7 Agarose gel electrophoresis

SP2/0 cells treated for 48 h were harvested, washed twice with PBS and then mixed with lysis buffer. After proteinase K added in, the cells were digested for about 12 h at 56℃. The products were extracted using phenol-chloroform, deposited with cold ethanol, and dissolved in TE buffer. DNA samples were detected by 20 g/L agarose gel electroresis at 150 v for 40min. The results were photographed and observed for the formation of DNA ladders.

2.8 Calcium dynamics

Cell suspension was plated into 6-well microplates at the concentration of 4×10^5 cells/well, cultured for 24 h, and then treated with garlicin. The cells were harvested, washed twice with PBS and stained with 200 μl Fluo-3/AM solution (15 μmol/L in 30 mmol/L HEPES) for 30 min. Then, they were washed thrice with PBS and observed under a confocal microscope (Nikon TE-2000-E, Japan) immediately.

2.9 Cell cycle analysis

Cells upon garlicin treatment were collected, and then spun at 1200 rpm, 4℃ for 10min. With supernatant discarded, they were washed with precooled PBS twice, resuspended in ice-cold 70% ethanol (v/v), and kept at 4℃ overnight.Pelleted and washed twice with PBS, the samples were stained with PI solution (PI 0.05 mg/ml, RNase 0.02 mg/ml, NaCl 0.585 g/ml, sodium citrate 1 mg/ml, pH 7.2-7.6) at 4℃ for 30min(Zhang et al., 2007). The cells were filtered using 400-mesh sieve, and then detected with FCM (BD FACSCalibur, USA) immediately.

2.10 Detection of mitochondrial transmembrane potential

Cells upon garlicin treatment were collected, and then spun at 1200 rpm, 4℃ for 10min. With supernatant discarded, they were washed with prewarmed PBS twice. The cells were stained with Rhodamine 123 (5 μg/ml in PBS, pH 7.4) and incubated at 37 ℃ in the dark for 1 h. Washed thrice with prewarmed PBS (Zhang et al., 2006), they were resuspended and analysed using FCM (BD FACSCalibur, USA) immediately.

3 Results

3.1 Growth kinetics

The cells were in the latent period in the first to second days, then entered into the logarithmic growth phase in the second to fourth days, and presented a typical S-shaped growth curve. The concentration reached its peak in the fourth day; Cell entered the plateau phase in the fifth day, followed by an overall degeneration thenceforth (Figure 1).

3.2 Morphological observation

3.2.1 inverted phase contrast microscope observation

Cells upon garlicin treatment for 48 h were observed, and the results suggested that typical apoptosis morphological changes, for example, cell shrinkage, membrane blebbing, cytoplasmic condensation, the generation of apoptotic bodies and so on (Figure 2). The treatment produced a dose-dependent increase in apoptotic rate.

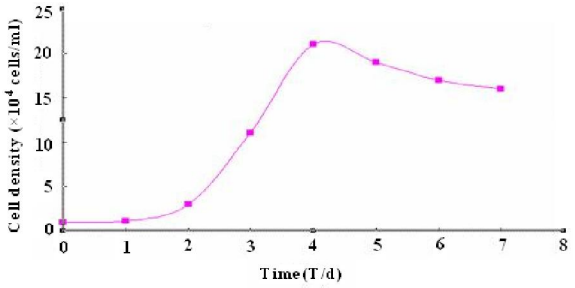

Figure 1. The growth curve of SP2/0 cell line

3.2.2 confocal microscopy observation

Apoptotic Necrotic cells gradually increased upontreated with by garlicin treatment at the concentrations of 2μg/ml, 4μg/ml and 6μg/ml with elevated drug dose. In a sharp contrast, most of the population switch to viable-apoptotic state treated with 8μg/ml, 10 μg/ml garlicin, from which it could be inferred that apoptotic effects of 8μg/ml and 10 μg/ml garlicin were optimal on SP2/0 cells (Figure 3).

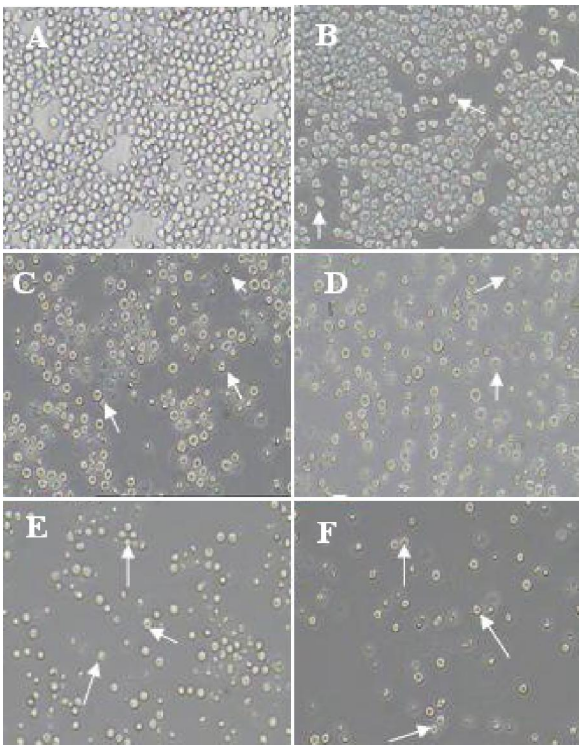

Figure 2. Morphological alterations of SP2/0 cell with garlicin treatement for 48 h (10×) (Arrows point to apoptotic cells) A. control; B. 2 μg/ml; C. 4 μg/ml; D. 6 μg/ml; E. 8 ug/ml; F. 10 μg/ml.

Normal cells possess brown fluorescent cytoplasm and slightly green nuclei. Yellow fluorescent cells with condensed nuclei were viable-apoptotic ones, and those with only red fluorescence or and condensed nuclei were in non-viable-apoptotic phase. In order to investigate the apoptotic effects with protracted time, SP2/0 cells treated with garlicin were at 60 h were stained with EB and observed with confocal microscopy. There were a large number of apoptotic cells upon the treatment with 2 μg/ml garlicin, whereas in other groups mass necrosis had taken place,giving rise to the speculation that garlicin could lead the treated cells to a flexible cell death, in other words, to apoptosis at lower concentration and to necrosis at higher concentration.

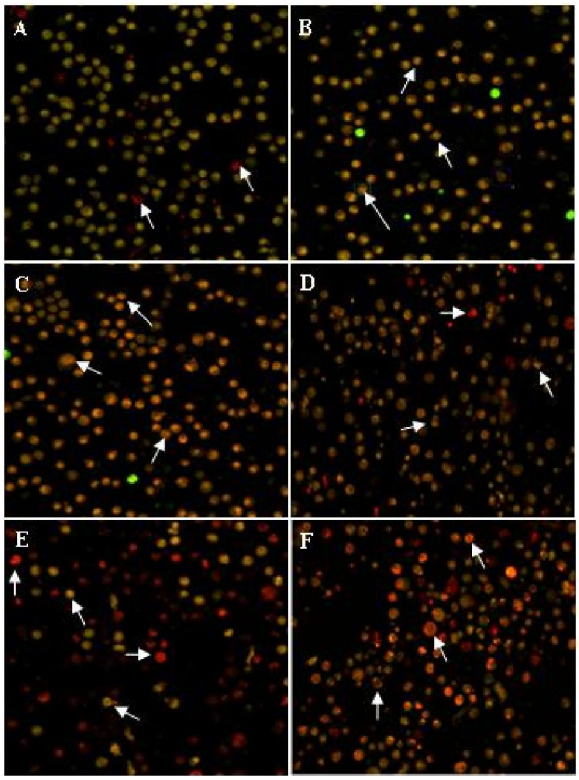

Figure 3. Morphological alterations of SP2/0 cell with garlicin treatement for 48 h (40×) (Arrows point to apoptotic cells). A. control; B. 2 μg/ml; C. 4 μg/ml; D. 6 μg/ml; E. 8 μg/ml; F. 10 μg/ml.

3.3 AnnexinV-FITC/PI double staining

The apoptotic rate increased with elevated concentration upon garlicin treatment at 48 h, indicating that concentrations of 4 μg/ml, 6 μg/ml, 8 μg/ml were already adequate for apoptosis inducing of SP2/0 cells. To the contrary of those of control, viable-apoptotic and necrotic rates in treated samples were considerably high, therefore supporting the concept that garlicin was a potent antitumor drug by means of apoptosis (Figure 4 and Table 1).

3.4 TUNEL staining

Postive cells occurred in all the samples (Figure 5), in particular evident in the treated groups with 6 μg/ml, 8 μg/ml and 10 μg/ml garlicin, which concentrations seems to have satisfactory apoptotic effects on SP2/0 cells. Positive cells were those with green fluorescences.

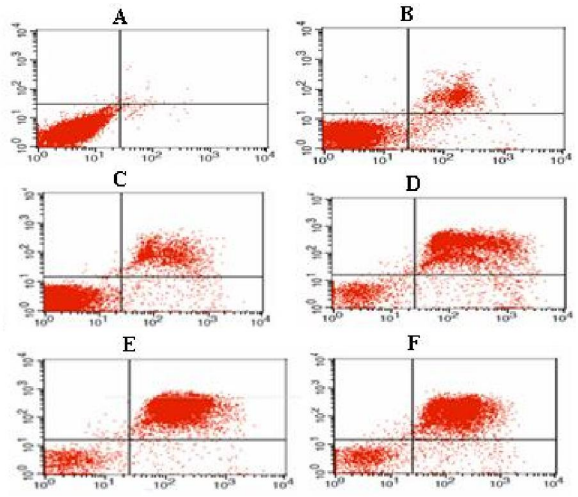

Figure 4. Apoptosis rate of SP2/0 cell with garlicin treatment for 48 h. A. control; B. 2 μg/ml; C. 4 μg/ml; D. 6 μg/ml; E. 8 μg/ml; F. 10 μg/ml.

Table 1 Apoptosis rates of SP2/0 cell line upon garlicin treatment for 48 h ($\overline{X} \pm SD$, n=3)

group	apoptotic rate(%)
control	1.3±0.11
2 μg/ml	12.23±0.94**
4 μg/ml	33.84±3.33**
6 μg/ml	44.77±4.27**
8 μg/ml	59.7±5.19**
10 μg/ml	62.4±5.66**

Statistical significance to control is marked with (*) (P<0.05) and (**) (P<0.01)

3.5 DNA alterations

After the initiation of apoptotic signaling, chromatinic DNA is cleaved by several families of nuclease that is specifically activated in programmed cell death. As it was shown in Figure 6, DNA ladders appeared in DNA samples extracted from garlicin treated SP2/0 cells, indicating that chromatin fragmentation had taken place.

3.6 Calcium dynamics

The cellular distribution of free calcium in SP2/0 cells suggested that there was a dose-dependent calcium release upon garlicin treatment at 48 h (Figure 7). The strongest positive signals occurred in the cells treated with 6 μg/ml, 8 μg/ml and 10 μg/ml garlicin, which gave rise to the hypothesis that garlicin induced apoptotic cascade were transducted in a large part by the accumulation of free calcium.

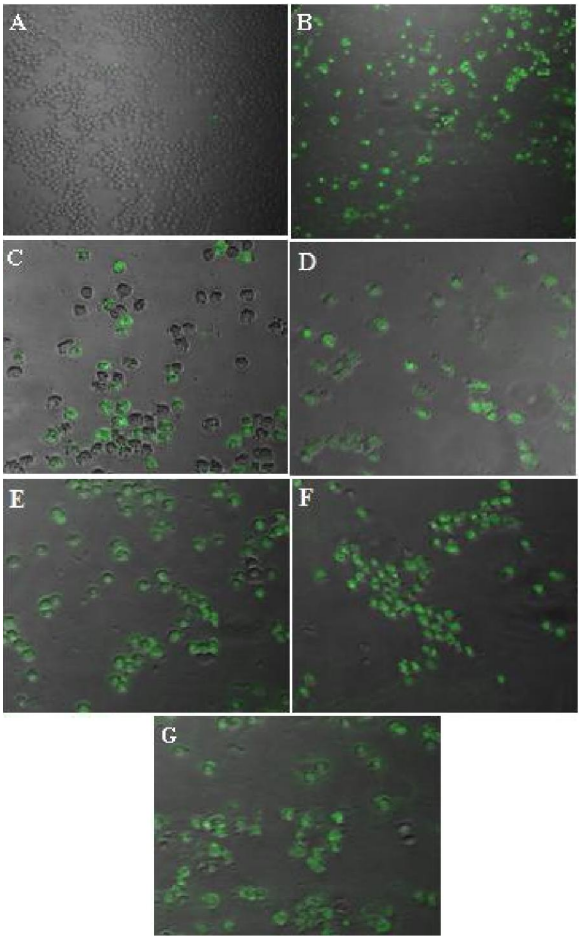

Figure 5. TUNEL staining of garlicin treated SP2/0 cells at 48 h. A. Negative control (10×); B. Positive control (10×); C. 2 μg/ml (40×); D. 4 μg/ml (40×); E. 6 μg/ml (40×); F. 8 μg/ml(40×); G. 10 μg/ml (40×)

3.7 Cell cycle progression

At 48 h upon garlicin treatment, there were increasing proportions of populations in G1 phase and decreasing proportions of those in G2 phase with elevated garlicin concentration (Figure 8 and Table 2), indicating that cell cycle progression was likely to have been blocked at S/G2 checkpoint.

3.8 Mitochondrial transmembrane potential

Mitochondrial transmembrane potential of SP2/0 exhibited a dose-dependent decrease upon garlicin treatement at 48 h, which was regarded as a

symbolic event in apoptotic signalling cascade (Figure 9 and Table 3).

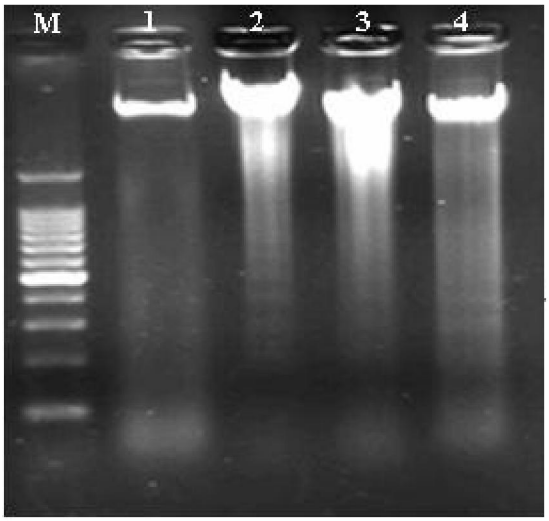

Figure 6. Electrophoresis image of sp2/0 cells treated with garlicin for 48 h. M. marker; 1. control; 2. 6 μg/ml; 3. 8 μg/ml; 4. 10 μg/ml.

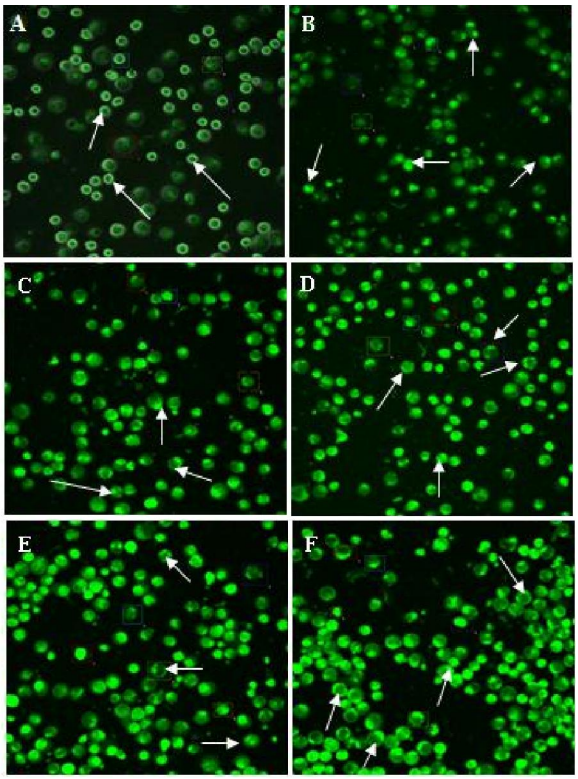

Figure 7. Calcium dynamics of SP2/0 cells treated with garlicin at 48 h (40×). (Arrow point to apoptotic cells) A. control; B. 2 μg/ml; C. 4 μg/ml; D. 6 μg/ml; E. 8 μg/ml; F. 10 μg/ml.

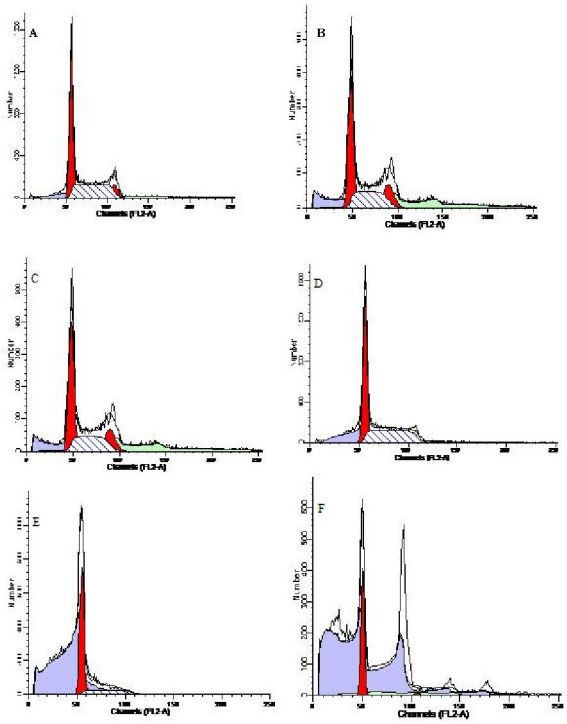

Figure 8. Cell cycle analysis of SP2/0 cells treated with garlicin for 48 h. A. control; B. 2 μg/ml; C. 4 μg/ml; D. 6 μg/ml; E. 8 μg/ml; F. 10 μg/ml.

Table 2 Cell cycle analysis of SP2/0 cells treated with garlicin for 48 h ($\overline{X} \pm SD$, n=3)

phase	G1(%)	S(%)	G2 (%)
control	41.24±5.66	44.17±4.78	8.45±1.34
2 μg/ml	49.76±6.48*	34.25±3.13*	15.80±2.39**
4 μg/ml	49.89±5.87*	32.12±3.39**	15.43±1.98**
6 μg/ml	55.70±5.92**	44.15±4.36	1.15±0.08**
8 μg/ml	79.68±6.48**	20.67±3.01**	0.06±0.01**
10 μg/ml	96.56±7.34**	0.09±0.01**	2.54±0.14**

Statistical significance to control is marked with (*) (P<0.05) and (**) (P<0.01)

4. Discussion

Tumorigenesis, a multi-step process evolves, rests gradually to a large extent on the deregulated tempo of cell division. It has been noted that drug elicited apoptosis of malignant cells is one of the efficacious therapies in clinical trials (Hino et al., 1996; Hirsch et al., 2000). This study adopted a variety of metheods to detect cell proliferation and apoptosis, and the results we obtained proved that garlicin at a certain concentration inhibited the proliferation of SP2/0 cells and induced apoptosis, playing a outstanding role in anti-tumor.

4.1 Morphological observation

Apoptosis, which is defined as programmed cell death, has unique morphological characteristics, such as cell plasma membrane "blebbing", concentration of cytoplasm and nuclear material and chromatin, emergence of apoptotic bodies (Nagata, 1997). SP2/0 cells took on typical apoptotic morphology after treatment by garlicin.

4.2 AnnexinV-FITC/PI double staining

In normal cells, phosphatidylserine (PS) is only found in the cytoplasmic surface of cell membrane. While in early apoptosis, PS is transferred to the extrocytoplasmic surface by the invertion of cell membrane. Annexin V is a phospholipid binding protein with a high affinity for PS. Thus Annexin V is used frequently to detect early apoptosis. Annexin V-FITC/PI double staining is a better method on detection of apoptosis and distinction of necrotic cells (Shu et al., 2009). For PI can not go through the cell membrane in normal cells to stain the nuclei, but for necrotic cells. Compared with PI single staining, Annexin V-FITC/PI double staining can detect the early apoptotic cells more sensitively. Quantitative detection of apoptotic rate by flow cytometry showed that garlicin could induce apoptosis of SP2/0 cells with concentration-dependency.

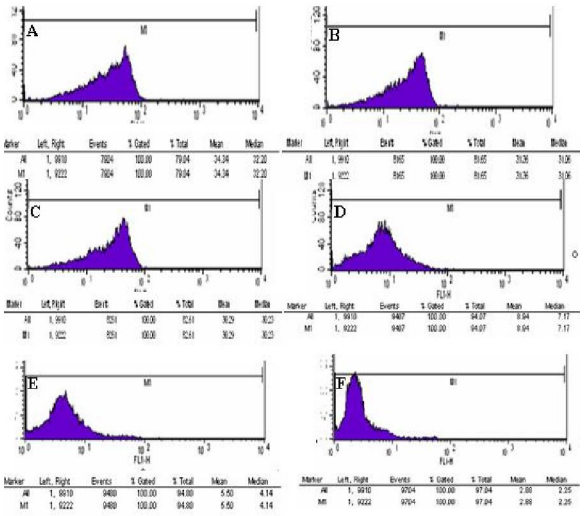

Figure 9. Mitochondrial transmembrane potential of SP2/0 cell with garlicin treatement for 48 h. A. control; B. 2 μg/ml; C. 4 μg/ml; D. 6 μg/ml; E. 8 μg/ml; F. 10 μg/ml.

Table 3 Mitochondrial transmembrane potential of SP2/0 cell with garlicin treatement for 48 h ($\bar{X} \pm SD$, n=3)

group	control	2 μg/ml	4 μg/ml	6 μg/ml	8 μg/ml	10 μg/ml
mitochondrial transmembrane potential (ΔΨ)	32.20±4.59	31.06±4.48	30.23±4.36	7.17±1.13**	4.14±1.12**	2.25±0.09**

Statistical significance to control is marked with (*) (P<0.05) and (**) (P<0.01)

4.3 DNA alterations

Cells experience specific cascade biochemical reactions during the process of apoptosis. The chromatin DNA is degraded, by activated endonuclease which is dependent on Ca^{2+} and Mg^{2+}, into 180bp - 200bp or multiple fragments that are called DNA ladders (Srivastava et al., 2009). DNA fracture is a landmark event in the late period of apoptosis. TUNEL method is considered one of the basic method to study apoptosis. Electrophoresis recorded the emergence of typical DNA ladders, and proved that garlicin can degrade chromatin DNA of SP2/0 cells into 180bp - 200bp. The results of TUNEL assay suggested that the treated cells, to the contrary of the control, exhibited higher apoptotic rate.

4.4 Calcium dynamics

In normal cells, Ca^{2+} is major combind with proteins that store in the endoplasmic reticulums and mitochondria. Generally there is little dissociative Ca^{2+}, only when receiving external stimuli to release as signal molecule. Almost all the cell responses, involve contraction, exocytosis, gene expression and apoptosis, are controlled by the change of partial or total dissociative Ca^{2+} concentration. Research has shown that the excessive accumulation of intracellular free Ca^{2+} can lead to occurrence of apoptosis (Jiang et al., 1994). Accumulating evidence has linked intracellular Ca^{2+} signaling system closely with apoptosis. As a second message, cellular Ca^{2+} plays a pivotal role in apoptotic signaling cascade (Sakamoto et al., 1997). Intensified Ca^{2+} signal in garlicin treated cells at 48 h indicated that garlicin could induce apoptosis through the perturbation of Ca^{2+} homeostasis.

4.5 Cell cycle progression

It is generally believed that physiological and pathological apoptotic stimuli are also correlated with cell cycle progression (Siegers et al., 1999). Unscheduled proliferation constitutes a key step in

canceration, and an altered division speed would eventually lead to malignant transformation and neoplastic growth (Frantz et al., 2000). In current consideration, cell cycle arrest would induce apoptosis which occurs at the same stage, and regulation of cell cycle can affect cell apoptosis as well as cell proliferation. Many apoptotic stimulation signals affects apoptosis and cell cycle at the same time and in the same mechanism. Therefore, detection of cell cycle is a important apoptotic evaluation. On the other hand, through blocking cell cycle to induce apoptosis is a new target for anticancer drugs. DNA histograms of SP2/0 cells treated with garlicin were detected and the results showed that garlicin influenced cell cycle progression in a dose-dependent manner. The percentage of population in G_1 and S phase increased and that in G2 phase decreased, indicating that cell cycle was arrested at G1/S checkpoint. It is herein speculated that garlicin could exert a disruption effect on the DNA replication of SP2/0 cells, affect distribution of cell cycle, and promote apoptosis finally. This study will provide important experimental basis for the future mechanism study of garlicin induced apoptosis in SP2/0 cells.

4.6 Mitochondrial transmembrane potential

In the apoptosis process, many important events are closely related with the mitochondria (Bouchier-Hayes et al., 2005). The study of apoptosis mechanism shows that mitochondria play a key role in the process of apoptosis. Concretely, mitochondrial transmembrane potential decreases when cell apoptosis is induced (Armstrong, 2006), leading to the increase of membrane permeability and open of mitochondrial permeability transition pores (Dias et al., 2005; Lucken-Ardjomande et al., 2005), where after, the cytochrome C (Mohamad et al., 2005), apoptosis inducing factor (AIF) (Liu et al., 1997), etc. enter the cytoplasm, causing the apoptosis. The decline of mitochondrial transmembrane potential is considered to be the first event during apoptotic cascade reactions, it occurs before occurrence of apoptotic characteristics of cell nuclei. Flow cytometry is commonly used to detect the change of mitochondrial transmembrane potential.

In this study, cells were labeled with Rh-123 to detect the change of mitochondrial transmembrane potential using flow cytometry. Decrease of mitochondrial transmembrane potential took place when the SP2/0 cells were treated with garlicin. Consonantly membrane permeability increase, cytochrome C enter the cytoplasm from mitochondria, and combine with apoptotic protease activating factor-1, activate Caspase-9, then activate Caspase-3, start Caspase cascade reaction, promote apoptosis. Our finding that garlicin induces apoptosis of SP2/0 cells relate to mitochondrial pathway further supports the important role of mitochondria in apoptotic cascade reactions.

Acknowledgements:

The work was supported by the “863” National Major Research Program (2007AA10Z170), the central level, scientific research institutes for R & D special fund business (2011cj-9), and the project National Infrastructure of Animal Germplasm Resources (2012 year).

Corresponding Author:
Pro. Weijun Guan and Pro.Yuehui Ma
Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, China
E-mail:Yuehui_Ma@hotmail.com;
weijunguan301@gmail.com

References

1. Lv YX, Li W, Yan YQ. The effect of allicin on inducing tumor cells apoptosis. Journal of Modern Oncology 2006;14 (1):117-118.
2. Velmurugan B, Bhuvaneswari V, Nagini S. Effect of S-allylcysteine on oxidant-antioxidant status during N-methyl-N'-nitro-N-nitrosoguanidine and saturated sodium chloride-induced gastric carcinogenesis in Wistar rats. Asia Pac J Clin Nutr 2003;12(4):488-94.
3. Leist M, Raab B, Maurer S, Rösick U, Brigelius-Flohé R. Conventional cell culture media do not adequately supply cells with antioxidants and thus facilitate peroxide-induced genotoxicity. Free Radical Biology and Medicine 1996;21(3):297-306.
4. Freshney RI. Culture of Animal Cells: A Manual of Basic Technique, 4th ed. 2000.Wiley-Liss, New York.
5. Guan WJ, Ma YH, Zhou XY, Liu GL, Liu XD. The establishment of fibroblast cell line and its biological characteristic research in Taihang black goat. Review of China Agricultural Science and Technology 2005;7:25-33.
6. Zhou XM, Ma YH, Guan WJ, Zhao DM. Establishment and identification of Debao pony ear marginal tissue fibroblast cell line. Asian-Australian Journal of Animal Science 2004;17(10):1338-1343.
7. Gu Y, Li H, Miki J, Kim KH, Furusato B, Sesterhenn IA, Chu WS, McLeod DG, Srivastava S, Ewing CM, Isaacs WB, Rhim

JS. Phenotypic characterization of telomerase-immortalized primary non-malignant and malignant tumor-derived human prostate epithelial cell lines. Exp Cell Res. 2006;312(6):831-843.
8. Kong D, Nishino N, Shibusawa M, Kusano M. Establishment and characterization of human pancreatic adenocarcinoma cell line in tissue culture and the nude mouse .Tissue and Cell 2007; 39(4):217-223.
9. Zhang HT, Feng ZL, Wu J, Wang YJ, Guo X, Liang NC, Zhu ZY, Ma JQ. Sodium butyrate-induced death-associated protein kinase expression promote Raji cell morphological change and apoptosis by reducing FAK protein levels. Acta Pharmacologica Sinica 2007; 28(11):1783-1790.
10. Zhang HT, Wu J, Zhang HF, Zhu QF. Efflux of potassium ion is an important reason of HL-60 cells apoptosis induced by tachyplesin. Acta Pharmacologica Sinica 2006;27(10):1367-1374.
11. Hino N, Higashi T, Nouso K, Nakatsukasa H, Tsuji T. Apoptosis and proliferation of human hepatocellular carcinoma. Liver 1996;16(2):123-129.
12. Hirsch K, Danilenko M, Giat J, Miron T, Rabinkov A, Wilchek M, Mirelman D, Levy J, Sharoni Y. Effect of purified allicin, the major ingredient of freshly crushed garlic, on cancer cell proliferation. Nutr Cancer 2000;38(2):245-254.
13. Nagata S. Apoptosis by death factor. Cell. 1997;88(3):355-365.
14. Shu B, Duan W, Yao J, Huang J, Jiang Z, Zhang L. Caspase 3 is involved in the apoptosis induced by triptolide in HK-2 cells. Toxicol In Vitro 2009;23(4):598-602.
15. Srivastava V, Rawall S, Vijayan VK, Khanna M. Influenza a virus induced apoptosis: inhibition of DNA laddering & caspase-3 activity by zinc supplementation in cultured HeLa cells. Indian J Med Res 2009;129(5):579-586.
16. Jiang S, Chow SC, Nicotera P, Orrenius S. Intracellular Ca^{2+} signals activate apoptosis in thymocytes: studies using the Ca(2+)-ATPase inhibitor thapsigargin. Exp Cell Res 1994;212(1):84-92.
17. Sakamoto K, Lawson LD, Milner JA. Allyl sulfides from garlic suppress the in vitro proliferation of human A549 lung tumor cells. Nutrition and Cancer 1997;29(2):152-156.
18. Siegers CP, Steffen B, Röbke A, Pentz R. The effects of garlic preparations against human tumor cell proliferation. Phytomedicine. 1999;6(1):7-11.
19. Frantz DJ, Hughes BG, Nelson DR, Murray BK, Christensen MJ. Cell cycle arrest and differential gene expression in HT-29 cells exposed to an aqueous garlic extract. Nutrition and Cancer. 2000;38(2):255-264.
20. Bouchier-Hayes L, Lartigue L, Newmeyer DD. Mitochondria: pharmacological manipulation of cell death. J Clin Invest 2005;115(10):2640-2647.
21. Armstrong J S. Mitochondrial membrane permeabilization: the sine qua non for cell death. BioEssays 2006;28(3):253-260.
22. Dias N, Bailly C. Drugs targeting mitochondrial functions to control tumor cell growth. Biochem Pharmacol 2005;70(1):1-12.
23. Lucken-Ardjomande S, Montessuit S, Martinou JC. Changes in the outer mitochondrial membranes during apoptosis. J Soc Biol 2005;199(3):207-210.
24. Mohamad N, Gutiérrez A, Núñez M, Cocca C, Martín G, Cricco G, Medina V, Rivera E, Bergoc R. Mitochondrial apoptotic pathways. Biocell 2005;29(2): 149-161.
25. Liu X, Zou H, Slaughter C, Wang X. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell 1997;89(2):175-184.

11/21/2012