

Reversal of Apoptotic Resistance by *Lycium barbarum* Glycopeptide 3 in Aged T Cells¹

LONG-GUO YUAN*, HONG-BIN DENG*, LI-HUI CHEN, DIAN-DONG LI, AND QI-YANG HE²

Institute of Medicinal Biotechnology, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100050, China

Objective To study whether *Lycium barbarum* glycopeptide 3 (LBGP3) affects T cell apoptosis in aged mice. **Methods** LBGP3 was purified with DEAE cellulose and Sephadex columns. Apoptotic "sub-G1 peak" was detected by flow cytometry and DNA ladder was resolved by agarose gel electrophoresis. Levels of IFN- γ and IL-10 were measured with specific kits and mRNA expression was detected by RT-PCR. Apoptosis-related proteins of FLIP, FasL, and Bcl-2 were determined by Western blotting. **Results** LBGP3 was purified from *Fructus Lycii* water extracts and identified as a 41 kD glycopeptide. Treatment with 200 μ g/mL LBGP3 increased the apoptotic rate of T cells from aged mice and showed a similar DNA ladder pattern to that in young T cells. The reversal of apoptotic resistance was involved in down-regulating the expression of Bcl-2 and FLIP, and up-regulating the expression of FasL. **Conclusion** *Lycium barbarum* glycopeptide 3 reverses apoptotic resistance of aged T cells by modulating the expression of apoptosis-related molecules.

Key words: *Lycium barbarum* glycopeptide 3; Aged T cells; Cytokines; Apoptosis; Senescence

INTRODUCTION

Apoptosis plays an important role in maintaining the integrity and homeostasis of metazoan. In immune system, development and activity of T cells are closely correlated with cellular apoptosis, through which the aged, dysfunctional T cells are selectively destroyed and prevented from accumulating in the body^[1]. However, apoptotic resistance occurs in the aging processes. In an IL-2 and ConA-induced apoptosis model, for instance, its mechanism in aged T cells is closely associated with the functional regulation of apoptosis proteins, such as FasL, FLIP, survivin, and T cell type transition from Th1 to Th2^[2-3]. Up to the present, there are only a few studies about how to regulate apoptosis by drugs in aged T cells.

Lycium barbarum polysaccharides, the main active components of *Fructus lycii*, have been proved to enhance the response of SAMP aged mouse T splenic cells to SRBC, in which concentration-dependent increase in free calcium ion was detected in splenic cells and celiac macrophages^[4]. *Lycium barbarum* glycopeptide 3 (LBGP3), one of the polysaccharides, plays a critical role in the

above-mentioned response. It was reported that LBGP3 enhances the proliferation of splenic cells in aged mice^[5] and activates the function of monocytes by increasing the expressions of IL-2 and TNF- α mRNA and protein levels in a dose-dependent manner. However, little is known about the effect of LBGP3 on aged T cells from the viewpoint of apoptosis.

In the present research, the effect of LBGP3 on apoptosis of T cells in aged mice was examined and the expression of certain apoptosis-related proteins was detected. The results show that LBGP3 could efficiently reverse apoptotic resistance of T cells in aged mice and is thus a promising drug to modulate the aging process.

MATERIALS AND METHODS

Animals

C57BL/6J mice were purchased from Laboratory Animal Center, Chinese Academy of Medical Sciences. The 2-month old young mice and 26-month old mice were used in the experiment. The mice were sacrificed at the end of treatment. Their tissues and

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*Those authors contributed equally to this work.

²Correspondence should be addressed to Qi-Yang HE, Tel: 86-10-63131856. Fax: 86-10-63017302. E-mail: qyh2000bj@yahoo.com.cn

Biographical note of the first author: Long-Guo YUAN, male, born in 1977, M. D. candidate, majoring in microbial and biochemical pharmacy; Hong-Bin DENG, male, born in 1975, M. D., majoring in molecular pharmacology.

organs were immediately collected and stored at -70°C for experiments.

Reagents

Rabbit anti-FasL polyclonal antibody, rabbit anti-Bcl-2 polyclonal antibody, and rabbit anti-actin polyclonal antibody were purchased from Santa Cruz. Rabbit anti-FLIP polyclonal antibody and rabbit anti-Bax polyclonal antibody were bought from NeoMarkers. CellTiter 96 aqueous non-radioactive and SV total RNA isolation system were purchased from Promega. Cell proliferation assay and SuperscriptTM one-step RT-PCR were bought from Gibco BRL. IFN- γ ELISA kit and IL-10 irradiation-immune kit were from Beijing Jingmei Co. Ltd. DEAE-cellulose, Sephadex G-75, and CM-Sephadex C-50 were from Pharmacia. ConA and IL-2 were from Sigma.

Purification of LBGP3

Lycium (~500 g) was minced and soaked in a three-fold volume of distilled water for 24 h at room temperature and filtered, aqueous solution was collected. The pellets were again soaked in a 1.5-fold volume of H_2O for additional 6 h, and the filtered solution was kept. The pooled mixture from this procedure was further concentrated by rotary evaporation at room temperature. After centrifugation at 3000 rpm for 5 minutes, the supernatant was deposited with a 4-fold volume of absolute ethanol. The deposition was dissolved with H_2O , and repeatedly extracted seven times with a 1/5-fold volume of savage agent ($\text{CHCl}_3:\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{OH} = 4:1$). Solid crude LBP was made by dialyzing the final aqueous mixture for 48 h, and vacuum-dried at 4°C . LBP was purified by column chromatography with DEAE-cellulose (0.15 mol/L NaHCO_3 as an elution buffer), and the third peak component (designated as LBP3) was collected, and further purified by Sephadex G-75 (washed with 0.15 mol/L NaCl) and CM-Sephadex C-50 (washed with 0.25 mol/L phosphate buffer). The purity of LBGP3 was determined by HPLC Shimadzu LC-10A (column: TSK-200SW), using 50 mmol/L $\text{NaH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ (pH 7.0) as an elution buffer at the flow rate of 1 mL/min (monitored at the wavelength 280 nm).

Determination of Molecular Weight

SDS-PAGE electrophoresis (12%) was performed to measure the LBGP3 molecular weight following standard electrophoresis protocol. The gel was stained with Comassie brilliant blue R250. The protein standard markers used included rabbit phosphorylase (97.4 kD), bovine serum albumin

(66.2 kD), rabbit actin (43.0 kD), bovine carbonylase (31.0 kD), trypsin inhibitor (20.1 kD), and egg white bacteriolysin (14.4 kD). The measured Rfs of the proteins were used to plot a standard curve with which the LBGP3 molecular weight was calculated.

Isolation and Stimulation of T Cells

The animals were sacrificed with their spleens removed aseptically and rinsed with RPMI-1640 medium. Single cell suspension was prepared by rubbing the tissue against sterile stainless steel wire meshes (100 μm) in 10 mL PBS and filtered through an aseptic fiberglass. Erythrocytes were lysed in 5 mL Tris- NH_4Cl lysis buffer. The cell suspension was centrifuged at 800 rpm, and the pellets were resuspended in 2 mL RPMI-1640 medium. The cells were loaded onto a nylon fiberglass column and incubated at 37°C for 45 min. After the column was washed with RPMI-1640 medium, T cell fractions were collected and counted. The purified T cells were stimulated with ConA (6 $\mu\text{g}/\text{mL}$) and IL-2 (10 ng/mL) for 18 h to induce apoptosis. The cells were washed with RPMI-1640 medium, and cultured in the same medium at 37°C in an atmosphere containing 5% CO_2 .

Determination of Cell Proliferation

Proliferation of T cells was detected by CellTiter 96 aqueous non-radioactive cell proliferation assay. The cells were exposed to LBGP3 at 50, 100, 200, 500, and 1000 $\mu\text{g}/\text{mL}$ before 20 $\mu\text{L}/\text{well}$ MTS/PMS mixture solution was added to the wells. The cells were incubated for an additional 4 h at 37°C in an atmosphere containing 5% CO_2 and read at OD_{490} .

Analysis of T Cell Apoptosis by Flow Cytometry^[6]

The purified aged T cells at $2 \times 10^6/\text{mL}$ were divided into 2 groups. One group was treated with ConA/IL-2, the other group with ConA/IL-2 plus 200 $\mu\text{g}/\text{mL}$ LBGP3. After cultured for 0, 6, 24 h, the cells were washed 2 times with cold phosphate buffer and fixed for 24 h in 70% ethanol. The cells were washed thrice with cold phosphate buffer, incubated with 100 $\mu\text{g}/\text{mL}$ RNase for 30 min at 37°C and stained with 50 $\mu\text{g}/\text{mL}$ PI for 30 min at 4°C . After filtration through nylon membrane meshes, cell apoptosis was analyzed with a flow cytometer FACS420. The data were analyzed using Cellplus2.0 software and the ratio of apoptosis was calculated.

DNA Extract and Agarose Gel Electrophoresis

T cells (2×10^6) were plated in a 6-well plate and divided into 3 groups: young cell group, control group, and LBGP3 treatment group. After stimulated

with IL-2/ConA for 18 h, the cells were collected by centrifugation at 800×g for 5 min, 100 µL lytic buffer containing 100 µg/mL RNase was then added to the cell pellets. After incubated at 37°C for 0.5 h, 100 µL lytic buffer containing 100 µg/mL protease K was added to the solution and incubated at 50°C for an additional 2.5 h. The same volume of phenol: chloroform: isopropanol (25:24:1) was used to extract protein. The aqueous phase was transferred to a new tube and 1/10 volume of 3 mol/L sodium citrate (pH 5.2) was added. DNA was precipitated with a 2.5-fold volume of cold ethanol in dry ice for 30 min. After centrifugation, DNA was washed once with 70% ethanol, air-dried, and redissolved in 20 µL TE (pH 8.0), 10 µg DNA sample was loaded onto the 1.8% agarose gel and run at 30 volt for 2.5 h in TAE buffer and stained in 1% EB TAE buffer. The result was recorded and analyzed with the UVP gel imaging system.

Influence of LBGP3 on Expression of IFN-γ and IL-10 in Aged T Cells

T cells (1×10^6) were plated in a 6-well plate and divided into 3 groups: young cell group, control group, and LBGP3 treatment group. After treated for indicated times, culture medium was collected for measuring the concentration of IL-10 with liquid scintillation counting^[7]. Reagent PR was added and mixed, the admixture was allowed to stand for 20 min at room temperature. IL-10 marked with I^{125} and IL-10 for competitive test was combined with antibodies. After centrifugation at 3500 rpm for 25 min, radioactivity of the pellet was measured with an automatic γ -arithmometer. Referring to the standard curve, the concentration of IL-10 was then measured.

The concentration of IFN- γ was measured by double antibody sandwich ELISA^[8-9]. The test samples were added to a 96-well plate, in which mouse anti-IFN monoclonal antibody was embodied. After incubated for 120 min at room temperature, the plate was washed four times and 100 µL per well working reagent was added, the plate was then sealed and incubated for 60 min at room temperature. After washed four times, developing and ending were carried out consequently. The value of OD₄₅₀ was read and the concentration of IFN- γ was measured based on the standard curve.

Influence of LBGP3 on Expression of IL-10 and IFN-γ mRNA Detected by RT-PCR

After treated with LBGP3, aged T cells were washed twice with PBS. The total RNA was purified with an extraction kit (Promega), following its standard protocol. The extracted RNA was stored at -70°C.

The *Sibaisheng* Primer 5.0 software was used to

design the primers of IL-10 and IFN- γ and β -actin housekeeping gene as an internal control.

IL-10 (496 bp):

sense 5' AACAACAAGACGCTTGACTTGA3'
antisense 5' GTCCCACCACATCCTGACTACT3'.

IFN- γ (256 bp):

sense 5' ACTTCTTGGAGGCAAGATGAAG3'
antisense 5' ATGTTTACAGGACAGAGGTGTGTG3'.

β -Actin (733 bp):

sense 5' GAACCCTAAGGCCAACCGTGAA3'
antisense 5' CTGCTGGAAGGTGGACAGTGAG3'.

SuperscriptTM one-step RT-PCR kit was employed to analyze the expression of the above cytokines.

Western Blotting

After treated with IL-2/ConA, young and aged T cells were washed twice with cold PBS. The cells were lysed in a lysis buffer (Tris 50 mmol/L, NaCl 150 mmol/L, NaN₃ 0.02%, SDS 0.1%, NP-40 1%, PMSF 1 mmol/L, leupeptin 10 µg/mL, aprotinin 1 µg/mL, pepstatin A 1 µg/mL, DTT 1 mmol/L, glycerol 10%, pH 8.0). The protein concentration was measured with a Bio-Rad protein assay kit. SDS-PAGE was performed with a 12% gel and 40 µg per sample was loaded onto the gel. A PVDF membrane was transferred for 2 h at a constant current of 0.8 mA/cm² gel area. The first-antibody (1:200) and the second-antibody (1:1000 horseradish peroxidase) were used. After an illuminant substrate was added, UV gel imaging was performed to obtain the results.

Data Analysis

Data from the various groups were compared by Student's *t*-test. In each case, $P < 0.05$ was considered statistically significant. All data listed in the figures or the tables were expressed as ($\bar{x} \pm s$).

RESULTS

Purification of LBGP3

LBGP3 was analyzed by HPLC with a TSK-2000SW column, and the retention time of LBGP3 was 10.282 min, indicating that the purification procedure yielded a high purity of LBGP3. LBGP3 was further analyzed by SDS-PAGE, and a single band was observed (data not shown). Based on the flow rates of protein standard markers with the bromophenol blue front as 1.0, the calculated molecular weight of LBGP3 was 41 kD.

Effective Concentration of LBGP3 for T Cells

When aged T cells were exposed to different concentrations of LBGP3, the proliferation of T cells was more effective at 200 µg/mL LBGP3 (Fig. 1),

which was used in the subsequent experiments.

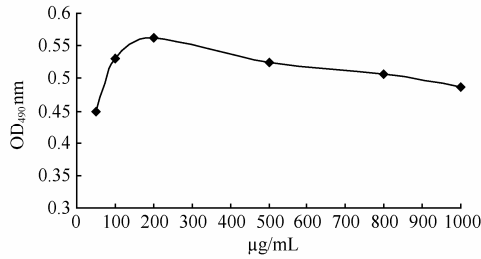


FIG. 1. Effect of LBGP3 on the proliferation of aged T cells. The cell growth was determined by CellTiter 96 aqueous non-radioactive cell proliferation assay.

Effect of LBGP3 on Apoptotic Rate of Aged T Cells

In the classical apoptotic process, after fixation and incubation in PBS, aggregated chromatin partially flows out from the cells and forms the sub-G1 peak, named "apoptotic peak", which can be detected by flow cytometry^[10]. When the aged cells were cultured for 6 and 24 h with LBGP3, the apoptotic cells constituted 29.8%±4.9% and 63.1%±8.5%, respectively, higher than those in the control groups (23.5%±3.8% and 42.0%±6.7%, respectively), similar to those in the young mice group (32.6%±4.1% and 67.2%±9.6%, respectively, $n=3$, $P<0.05$), suggesting that LBGP3 could recover the apoptotic rate of T cells from the spleen of aged mice up to that in the young group (Fig. 2).

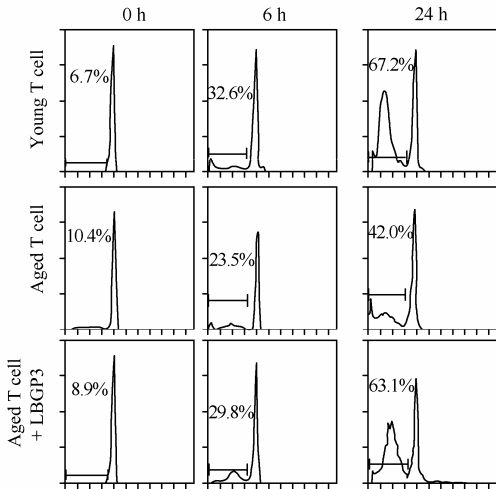


FIG. 2. Percentage of splenic T cell apoptosis after treatment with 200 µg/mL LBGP3 in aged T cells determined by flow cytometry.

Agarose Gel Electrophoresis

The DNA ladder pattern of aged mouse T cells occurred less than that of the young mouse T cells^[11].

A similar DNA pattern generated after incubation of the aged T cells with LBGP3, consistent with the results detected by flow cytometry (Fig. 3).

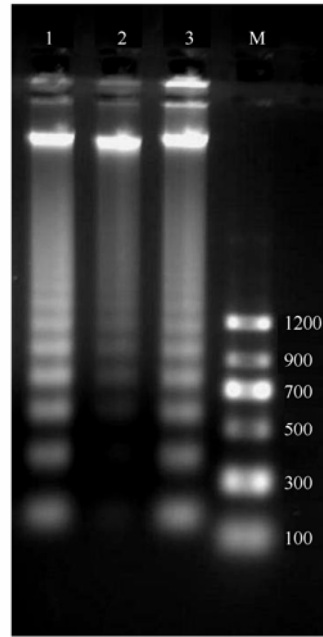


FIG. 3. DNA ladder pattern of the aged mouse splenic T cells after exposure to LBGP3. The cells were stimulated with IL-2/ConA for 18 h. Ten µg of DNA was loaded into a 2% agarose gel and run at 30 voltage for 2 h, and stained with 2% ethidium bromide. 1: young T cells; 2: aged T cells; 3: aged T cells plus LBGP3; M: DNA markers.

Effect of LBGP3 on Expression of IFN-γ and IL-10 in Aged T Cells

T cells could be divided into two kinds according to the cytokines they produce. Th1 cells could secrete IL-2, INF-γ, and TNF-α, whereas Th2 cells could generate IL-4, IL-5, and IL-10. The transition from Th1 to Th2 occurs from middle to old age and Th2 cells are resistant to apoptosis induced by CD95. Hence, we determined the expression of INF-γ and IL-10 to indicate the transition of T cells. The increased INF-γ expression and decreased IL-10 expression were shown after exposure to LBP3 treatment (Fig. 4). The changes in INF-γ and IL-10 expression were consistent to those in mRNA expression (Fig. 5).

Effect of LBGP3 on Expression of Apoptosis-related Proteins

A number of signal molecules are involved in apoptosis, such as FasL, FLIP, Bax, and Bcl-2^[12-15]. In old T cells, lower expression of FasL and higher expression of Bcl-2 resulted in apoptotic resistance in

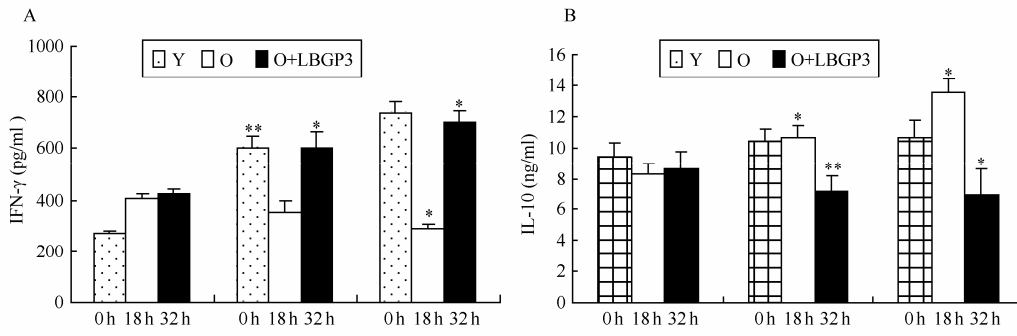


FIG. 4. Changes of IFN- γ (A) and IL-10 (B) levels in splenic aged T cells after treatment with LBGP3. * $P < 0.05$, ** $P < 0.01$ vs control.

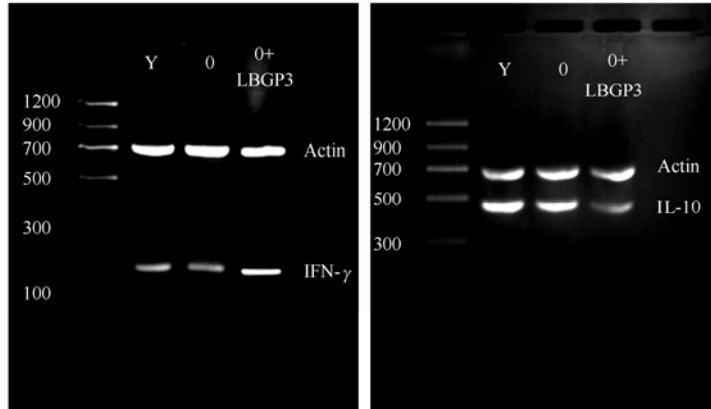


FIG. 5. Changes of IFN- γ (A) and IL-10 (B) mRNA expression in aged splenic T cells after treatment with LBGP3 for 18 h. The expression levels were detected by RT-PCR with the Actin expression as an internal control. The figures were representatives of two independent experiments.

contrast to the young T cells (Fig. 6, middle lanes). Increased FasL expression and decreased Bcl-2 expression in aged T cell were observed after treatment with LBGP3, suggesting that the

sensitivity of aged T cells to apoptosis was associated with the apoptosis-related molecules. However, the change in Bax expression was not obvious.

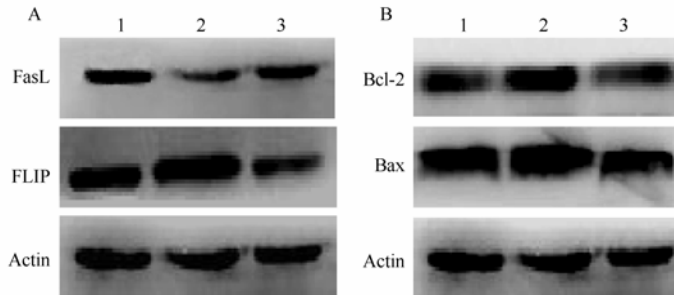


FIG. 6. Changes in expression of apoptosis-related proteins after exposure to LBGP3. The expressions of FasL and FLIP proteins (A), Bcl-2 and Bax proteins (B) were determined by Western blotting after treatment of aged T cells with LBGP3 for 18 h. 1: young T cells; 2: aged T cells; 3: aged T cells plus LBGP3.

DISCUSSION

Senescence in immune system is a progressive

and irreversible physiological decline, leading to infection, malignancy, and abnormal immune diseases. Studies showed that apoptosis is correlated

with the occurrence of these diseases, and stimulation with antigens can induce apoptosis to destroy the useless cells^[16], especially apoptosis of lymphocytes. Our previous study showed that the expressions of IL-2R in aged T cells as well as FasL and NF- κ B in young T cells are decreased, though the expression of FLIP in aged T cells is higher than that in the young T cells, suggesting that signal molecules play an important role in apoptosis^[17-18]. The present study showed that increased Bcl-2 expression and decreased FasL could lead to apoptotic resistance in aged T cells. Our previous study has proved that the apoptotic rate of T cells in aged mice is lower than that of young T cells. The apoptosis resistance in aged T cells may be related to the transition of T cells. Our previous study showed transformation from type I to type II T cells in aged mice. In this study, we have proved that expression of IFN- γ and IL-10 in T cells of aged mice is changed after treatment with LBGP3.

As a Chinese traditional medicine, *Lycium barbarum* has been used to delay aging in Asia for thousands of years. However, little is known about its biological activities. It was reported that *Lycium barbarum* can regulate apoptosis^[19]. We purified *Lycium barbarum* glycopeptide 3 by 3-step chromatography and showed that it could regulate the apoptosis of aged T cells. The results obtained by flow cytometry and agarose gel electrophoresis indicate that the apoptosis rate of aged and young T cells after exposure to LBGP3 was similar, suggesting that the apoptosis resistance of aged T cells can be reversed by treatment with LBGP3. Decreased Bcl-2 expression and increased FasL expression are correlated with apoptosis resistance and action of LBGP3. The exact molecular targets of LBGP3 on apoptosis pathways are under investigation in our laboratory.

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