ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

EFFECT OF L-ASPARIGINASE ON EXPRESSION CD8, CD95 FROM LEUKEMIA LYMPHOCYTE CULTURE

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Manuscript Info

Manuscript History:

Received: 16 August 2013
Final Accepted: 22 August 2013
Published Online: September 2013

Key words:
acute lymphocyte leukemia ALL, chronic lymphocyte leukemia CLL, L-aspariginase, Immunocytochemistry technique

Abstract

This work aimed to study effect L-aspariginase isolated from local *Withania somnifera* plant on expression CD8 and CD95 on surface of leukemia lymphocyte cells (acute lymphocyte leukemia ALL; chronic lymphocyte leukemia CLL, and control), to achieve this goal, blood samples were collected from 20 leukemic cases (10 ALL and 10 CLL), and isolation of lymphocyte by lymphoprep method and L-asparaginase has been purified from *Withania somnifera* fruits by two purification steps, ion-exchange chromatography using DEAE-cellulose and gel filtration chromatography using Sephadex G-150, There two purification steps raised the specific activity from 1.73 U/mg in crude extract to 2.29 U/mg after Ion-exchange and 10.5 U/mg after gel filtration, The purification fold was 1.32 after Ion-exchange and 6.06 after gel filtration. The enzyme recovery was 56 % after two purification steps, than study expression of CD8 and CD95 by Immunocytochemistry technique after and before add of L-aspariginase compare with control and examine of slides by light of microscope, the results, appeared elevated levels of CD8 and CD95 after add L-aspariginase, however, lower levels before add L-aspariginase as compared to control. The conclusion is that there is enough evidence to support the claim that there is L-asparagines are known as chemotherapeutic agent against cancer.

Introduction

Leukemia is a cancer originating in any of hematopoietic cell that tends to proliferate as single cells within bone marrow and often circulate in the blood stream. Myelogenous leukemia's are derived from granulocyte or monocyte precursors and erythroid leukemia's are derived from red blood cell precursors [1; 2]. Four types of leukemia are classified; chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL), and acute myelogenous leukemia (AML). Acute leukemia is characterized by the rapid increase of immature blood cells. This crowding makes the bone marrow unable to produce healthy blood cells [3]. Immediate treatment is required in acute leukemia due to the rapid progression and accumulation of the malignant cells, then spill over into the bloodstream and spread to other organs of the body. acute leukemia must be treated immediately, chronic forms are sometimes monitored for some time before treatment to ensure maximum effectiveness of therapy [4; 5]. Tumour cells may evade immune responses by losing expression of antigens and major histocompatibility complex (MHC) molecules by producing immunosuppressive cytokines. The development of a malignant cell clone is due to the dysregulation of the balance between cell proliferation and the programmed cell death–apoptosis [6]. L-asparaginase is an enzyme that destroys asparagine external to the cell. Normal cells are able to make all the asparagine they need internally whereas tumor cells become depleted rapidly and die. The enzyme converts asparagine in the blood into aspartic acid by a deamination reaction; the leukemia cells are thus deprived of their supply of asparagine and will die [7]. When asparaginase breaks down asparagine it is

broken down into 2 chemicals, aspartic acid and ammonia, the neurologic side effects seen with asparaginase (such as, confusion, excessive sleepiness, agitation, disorientation or coma) are related to increased levels of these chemicals circulating in the body. L-Asparagines are known as chemotherapeutic agent against cancer, such as acute lymphoblastic leukemia and lymphosarcoma, which are used mainly in the treatment of children [8]. The present study aimed to investigate the molecular immunological profile of ALL and CLL through the following parameters: Cellular expression of lymphocyte for CD8 and CD95 markers through adding enzyme L-aspariginase.

MATERIALS AND METHODS

Sample collection of plant

The fresh leaves, unripe and ripe fruits of *Withania somnifera* plant collected from the garden of plants in Baghdad University/ Science College, Biotechnology were included in this study. The plant parts were cleaned from the dust and other particles and stored in the freeze until use.

Extraction of L-aspariginase from plant tissues

After cleaning the plant tissues with distilled water, the plant tissues (leaves, unripe fruits and ripe fruits) were homogenized using liquid nitrogen and approximately 3 grams from each sample were ground with two volumes of potassium phosphate buffer 0.1M (pH 8.6) in a pestle and mortar, left on magnetic stirrer for 10 minutes, the extract filtered to get rid of the cell debris, centrifuged at 12000 rpm for 10 minutes and the supernatant was taken to determine the L-aspariginase activity and protein concentration as explained in [9; 10].

Extraction of L-aspariginase using liquid nitrogen

The plant tissues (leaves, unripe fruits and ripe fruits) were homogenized in liquid nitrogen, then the same of above.

Determination of L-aspariginase activity [9]

0.5 ml of crude extract, 0.5 ml of 50 mM asparagine and 1ml potassium phosphate buffer (0.02 M and pH 8.6) were mixed well, the mixture was incubated in water bath at 37ºC for 15 minutes, after the incubation, 1ml of 1.5 M trichloroacetic acid was added to the mixture to stop the reaction, the mixture was centrifuged at 12000 rpm for 10 minutes and the supernatant was collected, then the supernatant was transported to clear test tubes to determine the concentration of ammonia which is liberated from the enzyme action by the method of direct Nesslerization, which was prepared by the concentration of ammonia for each sample by adding 4 ml of distilled water with 0.5 ml of sample to be estimated and 0.5 ml of Nessler′s reagent, the mixture was then shaken well, incubated at 37ºC for 15 minutes and the absorbance was measured at (450nm). The blank was prepared by adding 4.5 ml distilled water with 0.5 ml Nessler's reagent.

Purification of L-aspariginase:

Preparation of ion exchange column (DEAE-Cellulose)

The DEAE-Cellulose column was prepared according to the method according to (11), the resin was packaged gently in glass column, the dimensions of resin was (1 x 24) cm, the equilibration was done by the same potassium phosphate buffer at flow rate approximately 30 ml/hour to next day.

Separation through ion exchange resin (DEAE-Cellulose)

Ten ml of enzyme crude extract was loaded onto ion exchange column, the separated fractions was collected at flow rate 30 ml/ hour approximately, 2 ml for each fraction, the washed using potassium phosphate buffer the same buffer used in equilibration, the elution was done by the same buffer with graduate concentrations of potassium chloride, the flow rate of elution was 30ml/hour too, the protein concentration of the fractions were measured at wave length 280 nm and the enzyme activity was estimated for fractions as in [9], the fractions which give higher activity were collected, lyophilized (freeze dried), stored in the freeze until use.

Gel filtration chromatography

Preparation of Sephadex G-150 column

The preparation of gel was as recommended by supplied company, since 5 grams from gel sephadex G-150 was suspended in 1 liter Tris-HCl buffer 0.1 M with pH 8.6, then the suspension was left in water bath at 90ºC for 5 hours to ensure the swelling of gel beads with gentle agitation from time to time, the gel was transferred to graduated cylinder, left to stagnate for 20 minutes, then the supernatant was removed, the gel was resuspended in 600 ml of Tris-HCl buffer, then the gel was degassed by using vacuum, the gel was packaged gently in glass column with dimensions (1x28) cm, the column was equilibrated using same buffer which used in gel suspension at flow rate 20 ml/ hour approximately to next day.

Separation through Sephadex G-150 column

The lyophilized extract produced from Ion exchange step was suspended in 5 ml Tris-HCl buffer, the suspension was added gently on the surface of gel, the elution was achieved by using the same buffer Tris - HCl at flow rate 20 ml/ hour 2ml for each fraction, the protein concentrations for fractions was measured at 280 nm, the enzyme activity was estimated according to [9], then the fractions with higher enzyme activity were collected, lyophilized, stored at zero ºC for other steps.

Study effect L-asparginase on expression of CD8 and CD95 on lymphocyte leukaemia

The *in vitro* method was used to investigate the effect of pure L- aspariginase on two types of leukemia culture at different concentrations and exposure times.

Preparation of L-aspariginase dilutions

Partially purified L-aspariginase a stock solution was prepared by dissolving 5mg with 1ml PBS, then filtered through millipore 0.22µm filter then was stored at zero ºC until used. Serial dilutions were made starting from the concentration of 100 µg/ml, 50 µg/ml, 25 µg/ml and 12.5 µg/ml. The dilutions were done in a sterile laminar capinate using a sterile BPS and kept in sterile Stoppard tubes.

Sample collection of blood

Five mls of blood was collected by vein puncture from 10 (ALL and CLL) cases for each, who were admitted to the National Center of Haematology/Al-Mustansaria University. The disease were diagnosed by the consultant medical staff at the centre. In addition, 5 healthy looking subjects (controls) were also included.

Isolation of lymphocytes

Preparation of solutions and media were done according to the methods described by [12; 13]. The lymphocytes were isolated from the peripheral heparinized whole blood as follows: three mls of blood were centrifuged at 1000 rpm for 15min, buffy coat was collected in a 10 ml centrifuge tubes and diluted with 5ml RPMI 1640 (cell suspension), five mls of the diluted cell suspension was layered on 3ml of ficoll-isopaque separation fluid, the tubes were centrifuged at 2000 rpm for 30min in a cooled centrifuge at 4ºC. After centrifugation, the mononuclear cells were seen as cloudy band between the RPMI1640 and lymphoprep layers. The sample band was collected in a 10ml test tube and the cells were suspended in 5 ml RPMI 1640. The tube was centrifuged at 2000rpm for 5min (first wash), the supernatant was discarded and the cells were resuspended in 5 ml RPMI 1640 (repeated twice). The suspension was centrifuged at 1000rpm for 10min, then supernatant was discarded. The precipitated cells were resuspended in 1ml RPMI media [14] and counted [15], the numbers of lymphocytes were counted by improved neuberchamber and the cells concentration was adjusted to $1X10^6$ cell/ ml. The isolated cells were grown in a flask containing 10ml RPMI 1640 medium supplemented with BSA (Bovine serum albumin) and incubated at 37ºC for 48h in CO2 incubator [13]. Using a microtiter plate (96 wells) cell culture technique $4X10^5$ cells/ml were exposed to serial dilutions of L-asparagines in the concentrations range mentioned before. The complete RPMI 1640 was used as a negative control and complete RPMI1640 with PBS as positive control, and the exposure times was 48hr. Each plate was designed to contain three replications of each concentration and 12 wells for negative control and 12 wells for positive control [14].

Diluent antibody: Primary Ab diluent buffer 50 µl was added to 1 µl dual monoclonal antibody cluster of differentiation CD marker (anti-CD8 and anti-CD95), then stored at 20ºC until used.

Protein-blocking reagent: Bovine serum albumin (activation) 10µl was added to 200 µl of PBS, and then stored at 20ºC until used.

Washing buffer: Tween20 (100µl) was added to 100ml PBS, and then stored in dark bottle at 4ºC until used.

Monting medium: Glycerol 20 ml was added to 80 ml 50 mM Tris-HCl, the pH of the mixture was adjusted to 8.4 then stored at 4ºC until used.

Detection of expression of CD8 and CD95 Immunocytochemistry

Immunocytochemistry test was used to detect expression of CD8 and CD95. The test was done using kits Dako, LSAB+ System–HRP (Cat# K0679) following the procedure described by [4; 16].

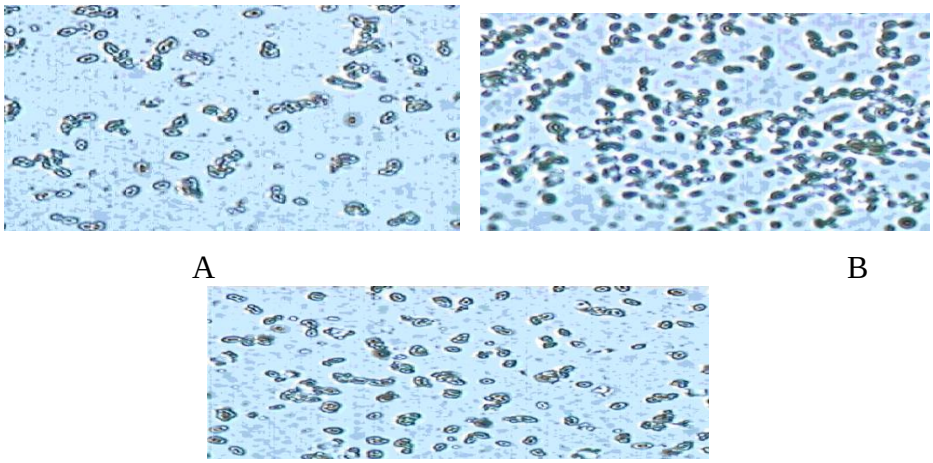
Immunocytochemistry protocol

Slide was floated with 100µl of 3% hydrogen peroxide for 5 min at room temperature about 25ºC then washed by distilled water. Primary antibody (CD8 and CD95) 100µl was added to the slide and incubated for 30min at room temperature about 25ºC then washed by adding washing solution (the step was repeated once more). Biotinylated link 100µl was added to slide and incubated 15 min at room temperature about 25ºC then washed by washing solution (the step was repeated once more). Streptavidin–HRP 100µl was added to slide and incubated for 30min at room temperature about 25ºC then washed by adding washing solution (the step was repeated once more). Substrate–chromogen solution 100µl was added to the slide and incubated for 30 min at room temperature 25ºC, the slide was washed by adding washing solution (the step was repeated once more), the slides were examined under 40X magnification using light microscope.

RESULTS AND DISCUSSIONS

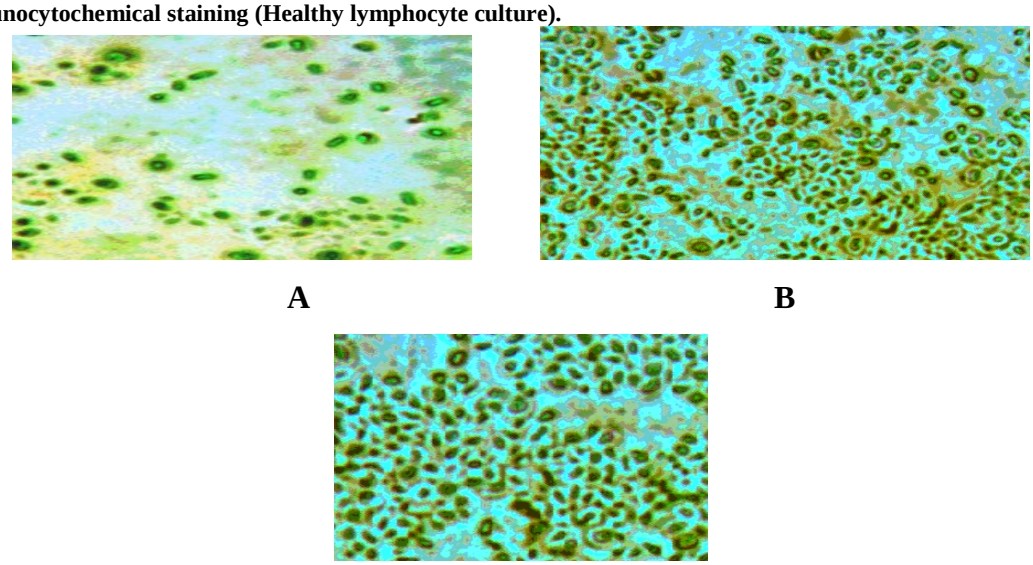
Immunoperoxidase staining using anti-CD8 and anti-CD95 on suspension lymphocyte leukemia type ALL and CLL and healthy lymphocyte and study effect L-Asparagines on cell surface expression of CD8 and CD95 appear this study, immunocytochemistry demonstrated that CD8 and CD95 expression is very low in all healthy suspension lymphocyte, however appear increased cell expression of CD8 and CD95 were observed in most of the lymphocyte leukemia

ALL and CLL culture that indicated the cytotoxic T-lymphocyte are capable to higher expression of CD8 and CD95 on cell surface after add L-Aspariginase camper with healthy and lymphocyte leukemia culture without treatment, shown as figure (1 and 2) and moreover, increased proliferation and effector T cell infiltration (CD8, and CD95) were observed in treated lymphocyte leukemia culture that enhanced immune effector function is an important drug induced mechanism



A B C

Figure 1: Lymphocyte expresses CD95. (A): CD95 immunocytochemical staining (Brown) is shown in a representative leukemia lymphocyte without treatment respectively. (B): CD95 positive immunocytochemical staining is shown leukemia lymphocyte after exposure the culture to L-asparaginase (C): CD95 immunocytochemical staining (Healthy lymphocyte culture).



A B C

Figure 2: Lymphocyte expresses CD8. (A): CD8 positive immunocytochemical staining (Brown) is shown in a representative leukemia lymphocyte without treatment respectively. (B): CD8 positive immunocytochemical staining is shown leukemia lymphocyte after exposure the culture to L-asparaginase (C):CD8immunocytochemical staining (Healthy lymphocyte culture).

In comparison with other result, B-cell chronic lymphocyte leukemia is the most common adult leukemia in the western world and is characterized by the accumulation of circulating sIgM+, CD19+, in addition, higher absolute number of circulating T-cells and increase in CD8 T-cells results than control healthy [17]. However, the experiment of [18] Tinhofer *et al.* showed higher expression of CD4+ and CD8+ (4.2±0.63 and 2.8±0.29) compared with healthy control (2.1±0.5 and 2±0.7) respectively of

B-CLL patient. [18] Tinhofer *et al*. refer to higher expression of CD95+ (mean 53%) in the natural killer cell and (mean 45%) in the cytotoxic T cell compared with healthy (mean 22%), and higher expression of CD95+ on B-CLL cell patients (45.7%) compared with healthy (4.1%) shown as [19] Loffler *et al.*, and Bennett *et al.*, [4] refer to increase cell expression of CD95 on human esophageal carcinomas by using immunohistochemistry staining CD95 specific rabbit polyclonal IgG compare with control negative. Refer by Aue *et al.*, [20] shown that lenalidomide up regulates the antigen presenting function of CLL cells 2 fold increase in CD80 and CD95 expression in agreement with the findings of the studies discussed. L-asparaginase is an important anti-cancer drug and was the focus of intensive investigations during 1970s that made large scale industrial manufacture of this enzyme practical in both Japan and the United States from wild type *E coli* strains [21]. L-asparagines are known as chemotherapeutic agent against cancer, such as acute lymphoblastic leukemia and lymphosarcoma, which are used mainly in the treatment of children [8; 22]. Leukemic cells lacking the mammalian asparagine-synthetase enzyme depend on exogenous sources of asparagine for protein synthesis and survival. Theoretically, the deamination of serum asparagines selectively kills leukemic cells, leaving normal cells, which have the ability to synthesize asparagine intracellularly, and unaffected [23]. Moreover, studies of the action of aspariginase upon neoplastic cells with respect to the nutritional requirements caused by the lack of asparagine, led to the introduction of new drugs as well as the combination of aspariginase with drugs with similar modes of action in the clinical treatment of lymphoblastic leukemia [24]. The conclusion is that there is enough evidence to support the claim that there is L-asparagines are known as chemotherapeutic agent against cancer.

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