Study the effect of partially purified L-Aspariginase on production perforin concentration of perforin and hemolytic activity from leukemia lymphocyte culture

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Abstract

This work aimed to study the effect of L Aspariginase isolated from local *Withania somnifera* plant on the concentration and hemolytic activity of perforin from leukemia lymphocyte cells of patients with (acute lymphocyte leukemia ALL; chronic lymphocyte leukemia CLL, in addition to healthy subjects). Blood samples were collected from 20 leukemic cases (10 ALL and 10 CLL), and the isolation of lymphocyte than proliferation of leukemia lymphocyte culture for detection of the concentration of perforin by ELISA technique and the activity of perforin by hemolytic erythrocyte assay after and before adding of L-Aspariginase. The results, showed an elevation of concentration and hemolytic activity of perforin before adding L-Aspariginase, then decreasing levels after adding L-Aspariginase compared to control.

دراسة تأثير أنزيم الاسبرجينيزالمنقى جزيئيا على تركيز الثاقب والفعالية الحيوية لمزرعة الخلايا اللمفاوية السرطانية

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الخلاصة

تضمنت الدراسة تأثير أنزيم الاسبرجنيز المعزول من النبات المحلي (سم الفراخ) *Withania somnifera* على تركيز وفعالية للثاقب لمرضى ابيضاض الدم الحاد والمزمن. جمعت 20 عينه من الدم وتوزعت 10 عينه من ابيضاض الدم الحاد و10 عينه من ابيضاض الدم المزمن بدون علاج وعزل الخلايا للمفاويه وتم تحديد تركيز الثاقب باستخدام تقنية اليزا ,وقياس الفعالية بواسطة الفعالية التحليلية للكريات الدم الحمر قبل وبعد أضافه الأنزيم اذ أظهرت ارتفاع تركيز الثاقب وفعاليته قبل إضافة الأنزيم للمرضى ابيضاض الدم الحاد والمزمن قبل أضافه الأنزيم وانخفاضهما بعد أضافه الأنزيم بالمقارنة مع عينات السيطرة .

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. Lymphocytic leukemia are derived from B or T cell precursors. Myelogenous leukemia is derived from granulocyte or monocyte precursors and erythroid leukemia is 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). Acute forms of leukemia are the most common forms of leukemia in children (4). Chronic leukemia is distinguished by the excessive build up of relatively mature, but still abnormal, white blood cells (5). Whereas acute leukemia must be treated immediately, chronic forms are sometimes monitored for some time before treatment to ensure maximum effectiveness of therapy (6; 7). The development of a malignant cell clone is due to the dysregulation of the balance between cell proliferation and the programmed cell death–apoptosis (8) Most missense *PRF1* mutations in FHLH2 patients result in loss of function of perforin, most commonly due to unfolding and faulty trafficking of the protein (9), the mutation identified in perforin result in loss of a functional mRNA and complete loss of perforin protein or nonfunctional protein (10). 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 (11). 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-Aspariginase are known as chemotherapeutic agent against cancer, such as acute lymphoblastic leukemia and lymphosarcoma, which are used mainly in the treatment of children (12).The present study aimed to investigate the molecular immunological profile of ALL and CLL through the following parameters: effect of L-Aspariginase on level of perforin and activity in cultured leukemia lymphocyte.

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-asparaginase 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-Asparaginase activity and protein concentration as explained in (13; 14).

Extraction of L-Asparaginase 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-asparaginase activity (13)

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 (15), 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 graduat 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 (13), 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 (1 x 28) 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 (13), then the fractions with higher enzyme activity were collected, lyophilized, stored at zero ºC for other steps.

Study effect L-asparginase on expression of perforin on lymphocyte leukemia

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

Preparation of L-asparginase dilutions

Partially purified L-asparaginase 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 Mustanisyria University from May 2010 till March 2011. 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 [16; 17]. 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 [18] and counted [19], 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 [16] . 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 [18] .

Perforin Detection Assay

The perforin concentration was detected using perforin kit ELISA (enzyme linked immuno sorbent assay) for *in vitro* quantitative determination of perforin in supernatant, and the effect of L-Aspariginase on perforin concentration and hemolytic activity on culture cell leukemia lymphocyte. The perforin kit is a solid phase sandwich ELISA, a monoclonal antibody specific for perforin has been coated onto the wells of the microtiter plate. Standard Perforin (Cell science company/ USA), two nanogram powder of perforin was dissolved in 1ml of standard diluents to give 2000pg/ml and stored at 4ºC until used. Diluents were then prepared from 2000 to 62.5 pg/ml and the absorbance was read on a spectrophotometer at 450nm.

Perforin Detection Protocol

Standard perforin 200µl was added into wells A1 and A2. Standard diluents 100µl was added to standard wells B1, B2, C1, C2, D1, D2, E1, E2, F1, F2. Serial transfers of 100µl solutions were made in accordance to alphabet and numbers orders, 100µl of standard

diluents was added to the blank well G1, G2 and 100µl of sample was added to sample well. The Covered plate was incubated for 1h at room temperature 25ºC. The plate was washed as follow: The liquid was aspirated from each well, 0.3ml of washing solution was dispensed into well then the content of well was aspirated (washing was repeated twice). Diluted biotinylated anti-perforin 50µl was added to all wells, the wells were covered and incubated for 1h at room temperature then washed as in up step. HRP (Horseradish peroxidase) solution 100µl was added into all wells including the blank wells, incubated at room temperature for 20min then wells were emptied and washed as in up step. TMB (tetra methyl benzidine) substrate solution 100µl was added into all wells, the plate was wrapped with aluminum foil and incubated in the dark for 10-15min at room temperature. H_2SO_4 100µl was added into each well as a stop reagent, the absorbance of each well were read on a ELISA microplate reader (Olympus/ Japan) at 450nm.

Hemolytic Assay for Perforin Activity

Perforin activity was determined depending on the hemoglobin released from lysed erythrocytes after treatment with perforin according to the method described by [14; 20], 2% erythrocyte prepared by mouse blood sample was centrifuged at 1500rpm for 10 min, the serum was removed and the cells were resuspended in 1ml PBS saline then 2% erythrocyte was prepared by adding Tris-buffer saline. 100µl samples including purified perforin, blood serum from patients; healthy and cell culture suspension were added to 100µl of 2% erythrocyte. The mixture was incubated for 30 min at 37ºC, then diluted to 2ml Tris HCl/NaCl, the mixture was centrifuged at 1500rpm for 10min and the supernatant was subjected to hemoglobin assay using spectrophotometer at 420nm, the following formula:

$$\textbf{Hemolytic activity} = \left(\frac{\text{Experimental - Spontaneous hemolytic}}{\text{Maximal hemolytic - Spontaneous hemolytic}} \right) \text{x } 100$$

The statistical analysis is a very important final step in the research to analyse and evaluate the obtained results. Medical statistics of this study was conducted via computer based statistical program which was :

1- SPSS for Windows computer package (Programmer 11.5).
2- Microsoft Excel 2003.

The statistical analysis tests which used in this were as follows:

Duncan test is non-parametric test which used to determine whether there is a significant difference between the expected frequencies with respect to two variable. It is a well used test for the medical statistics. P value <0.05 is considered a significant correlation.

Results and Discussion

It was found that the concentration of perforin was decreased significantly in the ALL and CLL culture (912.00±11.930 and 810.40±23.652) respectively they have 100µg/ml L-Aspariginase compare with culture without L-Aspariginase. Perforin concentration from lymphocyte culture has 100µg/ml of L-Aspariginase in ALL and CLL differed significantly compare to control ($P \leq 0.001$). However , there was highly significantly increase in ALL and CLL culture (50µg/ml; 25µg/ml and 12.5µg/ml) than 100µg/ml and have different significantly($P \leq 0.001$). It was found concentration of perforin was the same in healthy culture 100µg/ml and 50µg/ml compare with healthy culture without L-Aspariginase, as shown in table (1). Hemolytic activity of perforin was higher

significantly in ALL and CLL (0.971±0.14 and 0.704±0.055) without L-Aspariginase compare with added 100µg/ml of L-Aspariginase which decreased significantly in ALL and CLL (0.434±0.018 and 0.383±0.016) respectively (P≤ 0.001) as shown in table (1).

Table (1): Percentage frequency of perforin concentration and hemolytic assay in leukemia patients (CLL and ALL) and controls after effect different concentrations of L-Aspariginase (72 hour).

Treatment	Concentration	Time (72 hours)	
		Perforin concentration	Hemolytic activity
ALL	100µg/ml	912.00±11.930 c	0.434±0.018 d
	50 µg/ml	1121.33±65.498 b	0.608±0.005 b
	25 µg/ml	1115.00±14.571 b	0.708±0.028 b
	12.5 µg/ml	1104.00±63.814 b	0.699±0.007 b
	Without	1300.6±274.40a	0.971±0.14a
CLL	100µg/ml	810.40±23.652 c	0.383±0.016 d
	50µg/ml	919.50±10.583 b	0.539±0.012 c
	25µg/ml	831.56±19.812 c	0.608±0.010 b
	12.5µg/ml	901.30±18.240 b	0.522±0.005 c
	Without	2057.2±357.60a	0.704±0.055a
Healthy	100µg/ml	255.66±57.13 a	1.455±0.099 a
	50µg/ml	285.23±21.48 a	0.974±0.071 a
	25µg/ml	201.60 ±4.33 b	0.814±0.068 b
	12.5µg/ml	232.50±23.08 a	0.970±0.083 a
	Without	299.3 ± 21.94a	1.58± 0.06 a

*Different letters: Significant difference (P ≤ 0.001) between means of columns (Duncan test).

(21) calculated the mean ± standard error for perforin contents in the natural killer cells and cytotoxic T-cells of controls and found to be 3.561±1.157, however, the contents were reduced in individual with heterozygous and homozygous perforin deficiency family hemophagocytic lymphohistocytosis and were found to be 2260 and 2120 rMol of perforin from NK cells and cytotoxic T-cells . (22) refer to higher level expression of perforin from NK cells 94±2.5% of B-CLL patient compared with 75.5± 14.7% control and higher expression of perforin from CD8+cells 56.5±17 compared with healthy 27.9±15.6. On the other hand, (23, 24) referred to lower expression of perforin from hemophagocytic lymphohistiocytosis patients and detected partial or complete perforin deficiency because missense and nonsense mutations in exon 2 (del 207C) and exon 3 (del 1090-91CT) in coding region of perforin gene (25), refer to loss of perforin protein or production of nonfunctional protein lead to lacks perforin expression in patient with FHLH and found mutation *A91V* in perforin gene. (26) referred to reduce and absent expression of cytotoxic T-lymphocyte and NK cells activity in FHL patient from Iran because of two mutations were found at codon 273 (GCC>GCT) and 299(CAC>CAT). This some my result lower hemolytic activity of perforin from Iraqi ALL and CLL lymphocyte culture from population of Iraqi. 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 (27). In this study, the results appeared significantly elevated levels of concentration of perforin and hemolytic activity in both CLL and ALL before add L- Aspariginase as compared with control.

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