

Detection of Perforin in suspension of leukemia lymphocyte culture

الكشف عن البيرفورين (الثاقب) في عالق المزراعة ابيضاض الدم اللمفاوية

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Abstract

This work aimed to study the concentrations and hemolytic assay of perforin in serum and culture suspension of lymphocyte for 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 done then proliferation of leukemia lymphocyte in culture for the detection of perforin concentration by ELISA kit and hemolytic activity of perforin in suspension lymphocyte culture and serum were impacted. The results, showed significant increase in the level of concentrations of perforin in both CLL and ALL as compared with the control, however, showed significant lower in level of hemolytic activity of perforin in these groups as compared to the control. The conclusion is that expression of perforin is strongly associated with leukemia patient in Iraqi population.

Key words: Perforin, leukemia lymphocyte

المستخلص

تهدف الدراسة قياس تركيز وفعالية بروتين البيرفورين الثاقب في مصل الدم ومزرعة الخلايا التانية اللمفاوية للمصابين بابيضاض الدم الحاد والمزمن والسيطرة ، ولتحقيق هذا الهدف جمعت 20 عينة من الدم 10 عينة من ابيضاض الدم الحاد و 10 عينة من ابيضاض الدم المزمن وعزل الخلايا اللمفاوية وتم تحديد تركيز الثاقب والفعالية التحليلية له في المزراعة والمصل اذ اظهرت ارتفاع تركيز الثاقب لمرضى ابيضاض الدم اللمفي الحاد والمزمن بالمقارنة مع السيطرة بينما انخفضت الفعالية للثاقب للمجموعتين بالمقارنة مع السيطرة . الاستنتاجات ، تعبير الثاقب له علاقة قوية مع مرض ابيضاض الدم للمجموعتين العراقي .

الكلمات المفتاحية : البيرفورين ، ابيضاض الدم اللمفاوي

Introduction

Perforin is a cytolytic, channel-forming protein expressed in cytolytic T cells and natural killer cells. It plays a crucial role in the killing cell-mediated machining of virally infected host cells, tumor cells, and allotransplant and endocytic parasitic manifestation [1]. The protein is encoded by the *PRF1* gene which is mapped to 10q22 in human [2]. Its locus is not linked to any genes of the terminal complement system. Two-thirds of perforin sequence is homologous to the lytic, channel-forming complement proteins C6, C7, C8, and C9. The protein attack complex of complement produces pores of up to 20 nm in diameter on target membranes [3]. When the CTLs binds to its target, the contents of the granules are discharged by exocytose and perforin molecules insert themselves into the plasma of target cells forming a pore that enables granzymes to enter the cell [4]. Perforin is a 555 amino acid protein that has a 21 amino terminal signal sequence, an approximately 300 amino acid region that shares homology to the C9 complement protein, a 36 amino acid epidermal growth factor-like domain (EGF), and a 132 amino acid domain homologous to the C2 domain of protein kinase C [5, 2]. Patients with mutations in the perforin gene (*PRF1*) have been found to express low level or even absence of perforin in NK cells and diminished lymphocyte cytotoxicity [6]. Missense mutations in *PRF1* have also been described in an adult with chronic active Epstein barr virus EBV infection [7], in children and adults with bone marrow malignancies [8], and in healthy individuals. The phenotypic expression of *PRF1* mutations is variable, and the spectrum of perforin-related disease may include fatal immune dysregulation in early childhood,

nonfatal, inflammatory reactions at any age, and impaired tumor surveillance in children and adults [8]. The present study aimed to investigate the apoptosis profile of ALL and CLL through the following parameters: Perforin in terms of level and activity in serum and supernatant of cultured T-cytotoxic lymphocyte.

Materials and Methods

Sample Collection

Five ml of blood was collected by vein puncture from 20 cases ALL and CLL who were admitted to the National Center of Haematology/ Al Mustansyria University from May 2010 till March 2011. The disease was 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 [9; 10]. The lymphocytes were isolated from the peripheral heparinized whole blood as follows: three ml of blood was centrifuged at 1000rpm for 15min. The plasma was collected for perforin estimation, Buffy coat was collected in a 10 ml centrifuge tubes and diluted with 5ml RPMI 1640 (cell suspension), five ml of the diluted cell suspension was layered on 3ml of ficoll-isopaque separation fluid, the tubes were centrifuged at 2000rpm for 30min in a cooled centrifuge at 4°C. After centrifugation, the mononuclear cells were visible as cloudy band between the RPMI1640 and lymphoprep layers. The 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, the supernatant was discarded. The precipitated cells were resuspended in 1ml RPMI solution and used in the planned experiments [11]. Counting the cells was performed before experiment [12], the numbers of lymphocytes were counted by improved neuber chamber and the cells concentration was adjusted to 1×10^6 cell/ml. The isolated cells were grown in a flask containing 10ml RPMI 1640 medium supplemented with 10% BSA (Bovine serum albumin) and incubated at 37°C for 48h in CO2 incubator [11].

Using a microtiter plate (96 wells) cell culture technique 4×10^5 cells/ml and the complete RPMI 1640 was used as a negative control and complete RPMI1640 with PBS as positive control. The exposure time 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 [11].

Perforin Detection Assay

The perforin concentration was detected using perforin enzyme linked immuno sorbent assay (ELISA) for *in vitro* quantitative determination of perforin in supernatant, buffered solutions, serum and plasma samples. 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 4°C until used then diluents were prepared from 2000 to 62.5 pg/ml and the absorbance 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 25°C. 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. Horseradish peroxidase (HRP) 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. H₂SO₄ 100µl was added into each well as a stop reagent, the absorbance of each well were read on a ELISA micro plate reader (Olympus/ Japan) at 450nm.

Hemolytic Assay for Protein Activity

Perforin activity was determined depending on the hemoglobin released from lysed erythrocytes after treatment with perforin according to the method described by [13,14], 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 37°C, 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:

$$\text{Hemolytic activity} = \left[\frac{\text{Experimental - Spontaneous hemolytic}}{\text{Maximal hemolytic - Spontaneous Hemolytic}} \right] \times 100\%$$

Statistical analysis

The statistical analysis is a very important final step in the research to analyze 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 variables. It is a well used test for the medical statistics. P value <0.05 is considered a significant correlation.

Result and Discussion

Detection of Perforin and Hemolytic Activity in Suspension of Cultured Leukemia Cells

It was found that concentration of perforin was highly significant in the ALL and CLL groups (820.0±193.06 and 1510.4± 263.07) respectively than its controls (243.5±25.59). Perforin concentrations produced from CTLs in both group ALL and CLL have differed significantly from their controls P≤0.001. However, there was significant difference between the CLL and ALL groups, after 24h growth of CTLs P≤0.01, but after 48h perforin concentration highly significantly in ALL and CLL groups (1300.6±274.40 and (2057.2±357.67) respectively than the controls 299.3± 21.94 and have differed significantly from their controls P≤0.001. Hemolytic activity after 24 h for growth of CTLs lowered significantly in the ALL and CLL groups (0.806±0.129 and 0.484±0.006) respectively than its controls (1.065±0.152). Hemolytic activity produced from CTLs in CLL groups has differed significantly from their controls P≤0.001, but there was no significant difference between ALL and control. However, a significant difference between the CLL and ALL group after 24h for growth of CTLs P≤0.01 was found, but after 48h period hemolytic activity lowered significantly in the ALL and CLL groups (0.971±0.14 and 0.704±0.055) respectively than the control group (1.58±0.065). Hemolytic activity of perforin released from CTLs in both group ALL and CLL have differed significantly from their controls P≤0.01 and there was a significant difference between the CLL and ALL group 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.

Groups	No.	Mean ±S.E.			
		Perforin Pg/ 24h.	Hemolytic activity	Perforin Pg/ 48h.	Hemolytic activity
CLL	10	1510.4±263.07 ^A	0.484± 0.006 ^B	2057.2±357.60 ^A	0.704±0.055 ^C
ALL	10	820.0±193.06 ^B	0.806 ±0.129 ^A	1300.6±274.40 ^B	0.971±0.14 ^B
Control	5	243.50±25.58 ^C	1.065± 0.152 ^A	299.3 ± 21.94 ^C	1.58± 0.06 ^A

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

The mean ± standard error was calculated [15] for perforin contents in the natural killer cells and cytotoxic T-cells of controls and found to be 3.561±1.157, but the contents were reduced in individual

with heterozygous and homozygous perforin deficiency family hemophagocytic lymphohistiocytosis and were found to be 2260 and 2120 rMol of anti perforin antibodies per NK cells. [16] refer to higher level expression of perforin from NK cells $94\pm 2.5\%$ of B-CLL patient compared with $75.5\pm 14.7\%$ control and higher expression of perforin from CD8+cells 56.5 ± 17 compared with healthy 27.9 ± 15.6 . On the other hand, [17; 18] 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. [19] refer to loss of perforin protein or production of nonfunctional protein lead to lacks perforin expression in patient with familial hemophagocytic lymphohistiocytosis FHLH and found mutation *A91V* in perforin gene. [20] referred to reduce and absent expression of cytotoxic T-lymphocyte and NK cells activity in FHLH patient from Iran because of two mutations were found at codon 273 (GCC>GCT) and 299 (CAC>CAT). In conclusion, the results appeared significantly elevated levels of concentration of perforin in both CLL and ALL as compared with control. However, the detected hemolytic activity of perforin in these groups at lower levels as compared to control.

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