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Purification of perforin and study expression of perforin in leukemia patient

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ABSTRACT

This work aimed to study compare of extraction, purification and study gene expression of perforin from ALL, CLL and healthy, to chive this goal, blood samples were collected from 10 Leukemia cases (5 ALL and 5 CLL) and 5 healthy. Comparison of the molecular weight of purified perforin from these groups shows no detectable differences as compared with that of standard perforin which specified as 70KDa using SDS-PAGE. However, gene expression by using western blotting, it was found that the level of perforin in these groups were different due to differential gene expression between patients, and healthy control. Hence high levels were found in healthy individuals and CLL patient as compared with ALL patients as expressed by the presence of intense band.

Keywords: perforin - leukemia patient

NTRODUCTION

A 65-kDa protein with sequence homology to complement components C6 to C9, is stored in cytoplasmic granules of CTL and plays a major role in the secretary pathway (1). Upon binding of CTL to the target cell and appropriate engagement of the T-cell receptor (TCR), the cytoplasmic granules containing perforin and granzymes are released vectorially onto the target cell (2). Perforin monomers assemble into polymeric pore structures that insert into target cell plasma membranes, making the membrane permeable to water and small ions. When perforin is released from the granules of activated CTLs or NK cells, it forms a complex with granzymes and proteoglycans that binds to the target cell plasma membrane and promotes entry of the granzymes into target cell and serves as a channel for the influx of enzyme derived from the CTL granules (3). Target cell lysis, is partially caused by the assembly of tubular transmembrane complexes on target membranes, designated poly perforin 1(poly-P1) and 2(poly-P2) (4). Poly perforin are derived from cytoplasmic granules present in large granular lymphocytes, NK cells or CTL cells, since isolated granules are cytotoxic in the presence of Ca+ and form poly perforin (5). A solubilized partially purified cytolytic fraction of these granules induces marker release from vesicles (6). Moreover, antibodies raised against poly perforin recognize granules and SDS PAGE analysis of isolated granules revealed the presence of three major proteins with approximate molecular weights of 27, 29, and 66 KDa (7).

MATERIAL AND METHODS

Patients Selection

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 Mustanisyria University from May 2010 till March 2011. The disease was clinically diagnosed by the consultant medical staff at the centre. In addition, 5 apparently

healthy controls (blood donors) were also included.

Isolation of Lymphocytes

Preparation of solutions and media were done according to the methods described by {8}{9} unless mentioned. The lymphocytes were isolated from the heparinized whole blood using the method described by {10} 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 2000 rpm for 30min in a cooled centrifuge at 4ºC. 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. Counting the cells were performed before experiment according to {11}, the numbers of lymphocytes were counted by light microscope and the cells concentration was adjusted to $1X10^6$ cell/ml. The isolated cells were grown in a flask containing 10 ml RPMI 1640 medium supplemented with BSA and incubated at 37ºC for 48h.

Purification of Perforin

Preparation of Reagents

Ice Cold Relaxtion Buffer

it was prepared as follow:

Chemical	Amount
KCl	100mM
$MgCl_2$	3.5 mM
ATP	1mM
Hepes (piperazin-N-N-bis (2thane-sulfonic acid))	10mM
EGTA, ethylene glycolbis (B- aminoethylather) N,N-tetra acetic acid	1.25 mM

The mixture were stirred until all components were dissolved. The pH was adjusted to 6.8 and the volume was completed with distilled water to 1000ml and kept at 4ºC until used.

1M $NaKHPO_4$ Buffer

The mixture (Benzamidine HCl 10mM, EDTA 1mM, NaN_3 3mM, and $NaKHPO_4$ 1M were stirred until all component were dissolved. The pH was adjusted to 7.4 and the volume was completed with distilled water to 1000ml and kept at 4ºC until used.

Purification of Granules

The preparation was done according to (12; 13). The cells were harvested by centrifugation at 1500 rpm at 30ºC and washed twice with 50ml Hanks balanced salt solution (Ca+ free) by cooling centrifuge at 1500 rpm at 4ºC. The pellet was resuspended in 20 ml ice cold relaxtion buffer, the mixture was sonicated for 2 min to disrupt the plasma membrane, then centrifuged at 1500 rpm at 4ºC to remove nuclei. The pellet was taken and resuspended in 4 ml relaxation buffer and centrifuged at 1500rpm at 4ºC. The supernatant combined with previous then the hemolytic activity assay using the method described by (14; 15) and concentration of perforin using the method described by (16). Detection of perforin by using SDS-polyacrylamide gel and native polyacrylamide gel.

Purification of Perforin

The supernatants was done according to the method described in section (2-2-8-2) were mixed with equal

volume of 1M $NaKHPO_4$ buffer, after addition of 2mM phenylmethylsulfonyl fluoride, the mixture was placed on ice for 30min. and centrifuged for 30min at 20000 rpm at 4ºC, the clear supernatant was harvested and the pellet was discarded. The hemolytic activity and concentration of perforin was analyzed according to the method described previously. Protein concentration was determined using the method described by (16). Cell free extract (50µl) was mixed with 1.5 ml of Bradford reagent at room temperature about 25ºC and left for 45min, the absorbance was then determined at 595 nm, dilution of standard perforin.

Preparation for Electrophoresis

Stock Solutions Preparation

Solutions were prepared according to the method described by (17). Acrylamide-Bisacrylamide (30:8) was prepared by dissolving 30g of acrylamide and 8g bisacrylamide in 100ml distilled water. The solution was filtered through Whatman No.1 filter paper and stored at 4°C in a dark bottle. N,N,N,N-tetramethylenediamine (TEMED) used as supplied from the company. It is stable in undiluted solution at 4°C in a dark bottle. Ammonium persulphate (1.5%, w/v) was prepared by dissolving 0.15g of ammonium persulphate in 10ml distilled water. This solution is unstable and should be made fresh just before use. SDS (10%, W/V) was prepared by dissolving 10g SDS in 100ml distilled water. The solution is stable at room temperature for several weeks. Stacking gel buffer: Tris-HCl (6g) was dissolved in 40ml distilled water and was titrated to pH 6.8 with 1 M HCl, then the volume was completed to 100ml. Resolving gel buffer: 36.3g Tris -HCl was dissolved in 40ml water and was titrated to pH 8.8 with 1 M HCl. The volume completed to 100ml distilled Water. Reservoir buffer: 3g Tris-Glycine (pH 8.3) and 14.4g Glycine were dissolved in amount of distilled water. The volume completed to 1liter. Reservoir buffer SDS: 0.25 M Tris, 1.92 M Glycine, 1% SDS (pH 8.3) 30.3g Tris, 144g Glycine, and 10g SDS were dissolved in amount of distilled water, then the volume completed to 1 liter, the solution was stored at 4°C.

Polyacrylamide Gel Preparation

The components for preparation of one standard size slab gel using native Polyacrylamide were as shown in table (1).

Table 1: Components of native Polyacrylamide gel.

Stock solution	Stacking gel	Resolving gel 10%	Reservoir buffer
Acrylamide-Bisacrylamide (30:8)	2.5 ml	10 ml	-
Stacking gel buffer	5 ml	-	-
Resolving gel buffer	-	3.7 ml	-
Resolving buffer	-	-	1000 ml undiluted
1.5% ammonium Persulphate	1.5 ml	1.5 ml	-
Water	10 ml	14.7 ml	-
TEMED	0.015 ml	0.015 ml	-

The gel mixture was prepared in a small, thick-walled flask by mixing the components (except TEMED) as shown in table (1). The mixture was degassed for 1 min then the volume of TEMED was corrected, and gently mixed in, the resolving gel mixture was poured into the space between the glass plates leaving sufficient space at the top for a stacking gel to be polymerized later and sample wells formed. After polymerization (10-30 min), the stacking gel was prepared as indicated in table (1). A small volume of the stacking gel mixture was used to rinse the surface of the resolving gel, then the remaining space between the gel plate was filled with stacking gel mixture. The comb was inserted

immediately into the stacking gel mixture. After polymerization, the comb was carefully removed to expose the sample wells which were rinsed with reservoir buffer and then filled with this buffer. Preparation of SDS polyacrylamide slab gel is similar to native polyacralymide gel indicated in table (2).

Table 2: Components of SDS Polyacrylamide Gel

Stock solution	Stacking gel	Resolving gel 10%	Reservoir buffer
Acrylamide-Bisacrylamide (30:8)	2.5ml	10ml	-
Stacking gel buffer	5ml	-	-
Resolving gel buffer	-	3.7ml	-
Resolving buffer SDS	-	-	100ml
1.5% ammonium Persulphate	1ml	1.5ml	-
Water	11.3ml	14.45ml	900
TEMED	0.015ml	0.015ml	-
10% SDS	0.2ml	0.3ml	

Sample Preparation (17)

A protein sample was prepared by mixing protein 6 volume with 1volume 0.002% Bromophenol Blue (BPB). All steps involved in the preparation of native protein samples are performed at 4°C. The protein sample for SDS-polyacrelamid were substituted in 0.0625 M Tris-HCl (pH 6.8) is then brought to 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.002% (w/v) bromophenol blue using concentrated stock solution. Samples for SDS-PAGE were heated in a boiling water bath for 3min. This ensures denaturation of the protein, after heating. The sample was allowed to cool to room temperature 25°C and centrifuged at 10000 rpm for 5 min. The method of loading liquid samples was summarized below for both native PAGE and SDS-PAGE, the gels were mounted in place on the electrophoresis set, reservoir buffer was added until the upper line. The sample was carefully loaded onto gel surface using a micro syringe or micropipette. The electrophoresis apparatus was connected to the power pack and the gel subjected to 100 voltage for 2h.

Gel Staining

Coomassie brilliant blue G-250 solution was prepared by dissolving 100mg coomassie blue in 50ml a mixture of Water: Methanol: Glacial acetic acid (5:5:2 by volume) with stirring and the volume was completed to 1L by distilled water and filtered through a whatman No.1 filter paper and stored in a dark bottle at 4°C until used. Staining of perforin in native gel was carried out according to the method described by (18). Protein bands in SDS-PAGE was developed by immersing the gel in to a coomassie blue solution. After three hours, the reaction was stopped by rinsing the stain solution with 5% acetic acid.

Western Blot Analysis for Perforin (19)

5X SDS Running Buffer

The component (Tris-base 15g; Glysine 72g and SDS 5g) were dissolved in 1000 ml distilled water, the mixture was stored at 20°C until used.

Transfer Buffer

Mixture (1X SDS Running buffer 75ml, TBST 5ml and Methanol 20ml) was stored at 20°C until used.

Primary Antibody

A 5µl polyclonal rabbit anti-human perforin antibody (Cat #: GTX102379) was added to 10000µl TBST and 1mg skimmed milk, the mixture was shaked for 2h then stored at 20°C until used.

Secondary Antibody

Horseradish peroxidase labeled rabbit anti–goat (Cat#: 170-6515 /BioRAD) 3µl was added to 10000µl

TBST and 1mg skimmed milk, the mixture was shaking for 2 h then stored at 20°C until used.

Substrate

Chemi-Lumi was used as substrate in the western blot (Cat #: 02230) as supplied by Nacalai Tesque, the kit consists of : solution A (Luminol solution) brown plastic bottle and solution B (Peroxide solution) white plastic bottle. Preparation of Working Solution by mixed solution A was added to solution B in 1:1 ratio.

Western Blot Protocol (19)

SDS–polyacrylamide gel was immersed into transfer buffer by using sandwich electro blot for 10 min at 5 volt. SDS–polyacrylamide gel was soaked into nitrocellulose membrane, then the membrane transferred into 100ml skimmed milk (Skimmed milk 1g was dissolved in 100ml distilled water) with shaking at 60min. The membrane washed by using TBST (TBST was prepared by adding 100µl of 0.05 % Tween 20 to 100ml Tris-buffered saline) with shaking at 5min and the step repeated three time. The membrane was incubated with primary Ab at room temperature at 25°C for 2h. The membrane was washed again by using TBST with shaking for 5min and the step repeated three time. The membrane was incubated with secondary Ab at room temperature for 2h. The membrane was washed by TBST with shaking for 5min and repeated three time then the TBST removed from membrane carefully. The membrane was incubated with working solution for 1min. The working solution was removed and the membrane then placed on X-ray film cassette in dark room. The X-ray film was placed on the wrapped membrane then the cassettes was closed then detects the bands.

RESULT AND DISCUSSION

Polyacrylamide Gel Electrophoresis

Purification of perforin was done using polyacrylamide gel electrophoresis (Native PAGE) without sodium dodecyl sulfate. The results showed that purified perforin from cells of both patient groups and healthy had a similar molecular weight (70 KDa) as compared to standard perforin, this was indicated by the presence of a single band with the presence or absence of 2mM phenylmethyl sulfonylflouride in buffers as shown in (Fig. 1).

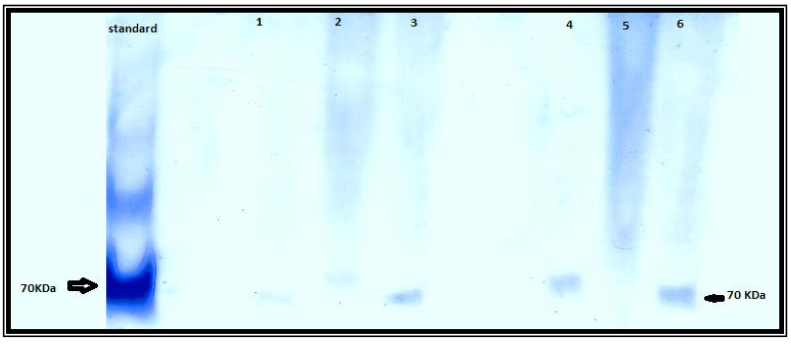

Fig. 1: PAGE gel electrophoresis (native PAGE), bands were fractionated by electrophoresis on a 30/8% acrylaminde-bisacrylamide (2h, 10 V/cm, 1X Resolving gel buffer) and visualized by Coomassie blue. Lane1: Healthy, lane2: ALL, lane3: CLL in 2mM phenylmethyl sulfonylflouride free buffer, lane 4:Healthy, lane 5:ALL, lane 6:CLL in buffer contain 2mM phenylmethyl sulfonylflouride, Standard molecular size perforin 70 KDa.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was also used to detect the presence of purified perforin with the presence of 2mM phenylmethyl sulfonylflouride and high concentration of phosphate 1M NaKHPO4 at pH 7.4 in order to precipitate other protein. The results

showed that only a single band with M.W of 70KDa was detected for purified perforin from both patient groups and healthy as shown in (Fig. 2), however, the presence of purified perforin in buffer frees 2mM phenylmethyl sulfonyl-flouride the results showed many bands as shown in (Fig. 3).

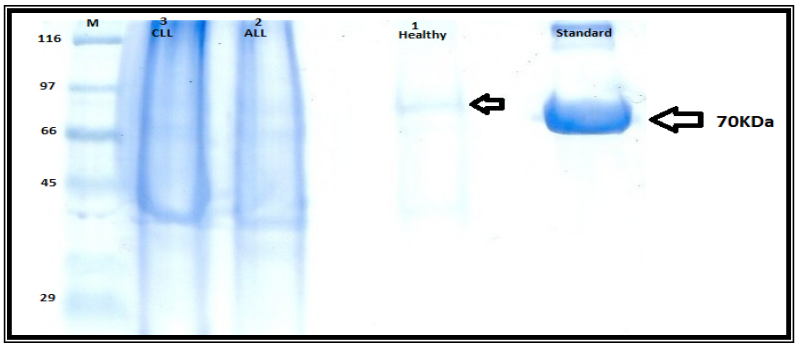

Fig. 2: SDS- PAGE electrophoresis of perforin with the presence of 2mM phenylmethylsulfonylflouride. bands were fractionated by electrophoresis on a 30/8% acrylaminde-bisacrylamide (2h, 10 V/cm, 1X Resolving gel buffer) and visualized by Coomassie blue. Standard perforin have 70 KDa was included. Lane 1: healthy, lane 2:ALL, lanes: CLL, lane M: Marker proteins (corresponds to ovalbumin 116 KDa, BSA 97 KDa, Phosphorylase b 66 KDa, ß – galactosidase 45 KDa, and myosin 29 KDa).

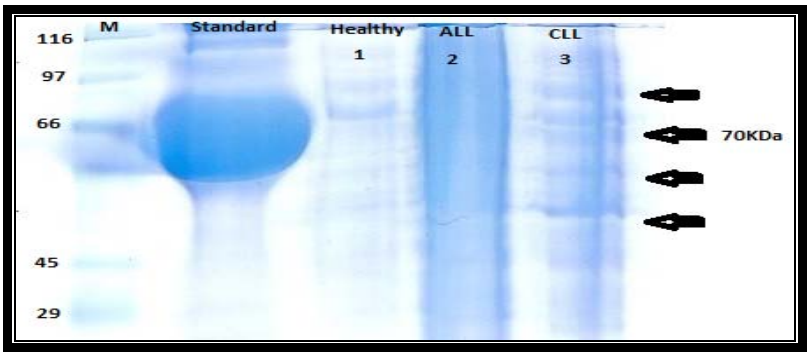

Fig. 3: SDS-PAGE electrophoresis of perforin. Standard perforin (70KDa), bands were fractionated by electrophoresis on a 30/8% acrylaminde-bisacrylamide (2h, 10 V/cm, 1X Resolving gel buffer) and visualized by Coomassie blue. Lane 1: healthy, lane 2: ALL, lane 3: CLL, in buffer free 2mM phenylmethylsulfonylflouride, lane M: Marker proteins corresponding to ovalbumin 116 KDa, BSA 97 KDa, phosphorylase b 66 KDa, ß-galactosidase 45 KDa, and myosin 29 KDa.

In comparison with others, (12) purify perforin obtained from CTL L2 cell line, a single band with molecular weight of 75KDa was obtained during their run on SDS-PAGE, while a band of 66 KDa was obtained during the run of purified perforin separated from B6 cell line in the presence of 2mM phenylmethylsulfonylflouride (20).

Western blot of Perforin

Purified perforin was subjected to western blot on nitrocellulose membrane after being blocked with 1% skimmed milk then the membrane was incubated overnight at 4ºC with polyclonal rabbit anti-human perforin diluted at (1:2000) in TBST, 0.1% Tween 20 and 1% skimmed milk, then the membrane was incubated for 1h at room temperature with horseradish peroxidase labeled rabbit anti-goat then substrate Lumiglo chemiluminescence was added and membrane was transferred to X ray film then bands were visualized after autoradiography. Results shown in Fig. (4) indicated the presence of human perforin from all groups ALL, CLL, healthy and standard perforin human. However, in healthy and CLL groups, the levels of gene expression seems to be

higher than that in ALL patients as expressed by the presence of deeper band (Intense band). Other investigators also registered this fact. For instance, low level of human perforin has been expressed in group suppose to have mutation in *T224W* gene as compared with normal group. This result could be attributed to the presence of conformational changes in the protein which lead to loss of its cytolytic function (21). However, (22) characterized two bands which are related to immature and intermediate forms of perforin expressed in hemophagocytic lymphohistiocytosis (HLH) patient, this could be attributed to two mutations in the genes (*A91V* and *W374X*) and no mature active form of perforin was detected as compared with wild type. Later (23) referred to detect the human perforin more abundantly than wild type mouse perforin by western blotting and appear human perforin *A91V* mutant protein was lower levels than human perforin wild type.

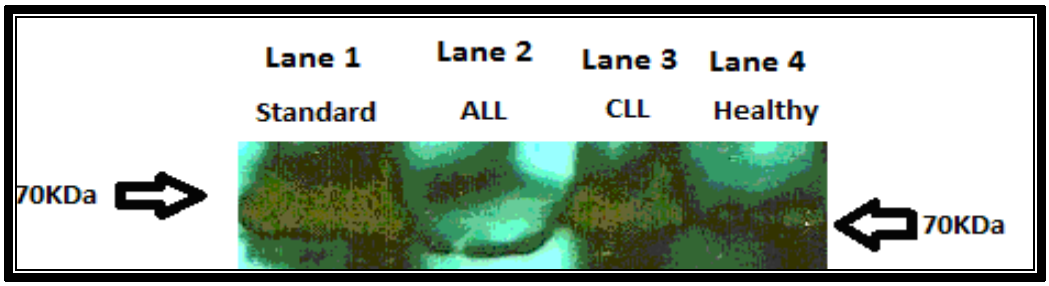

Fig. 4: Western blot X-ray film. Lane: 1. Standard of perforin have 70 KDa, Lane:2. ALL, Lane: 3. CLL, Lane: 4. Healthy perforin.

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ARABIC SUMMERY

تنقية ودراسة التعبير الجيني للبيرفورين فى مرضى ابيضاض الدم الحاد والمزمن

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هدفت الدراسة المقارنة في استخلاص وتنقية ودراسة التعبير الجيني للبيرفورين من عينات الدم مرض ابيضاض الدم الحاد والمزمن وعينات السيطرة وللوصول الهدف جمعت 10 عينات (5 للابيضاض الدم الحاد و 5 للابيضاض الدم المزمن) و5 عينات وبالمقارنة بالوزن الجزيئي للثاقب المنقى من مختلف المجاميع وجد لا يوجد اختلافات بالمقارنة مع الثاقب القياسي والبالغ 70 كيلودالتون باستخدام الهلام SDS-PAGE . بينما التعبير الجيني باستخدام Western blot اظهر اختلافات في مستوى التعبير في مختلف المجاميع بين المصابين والأصحاء وتم العثور على حزمه واضحة جدا لعينة السيطرة وعينة ابيضاض الدم المزمن بالمقارنة مع عينة ابيضاض الدم الحاد حيث ظهرت حزمة باهته .