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A STUDY ON THE PRESENCE, ROLE AND SIGNIFICANCE OF PERCHLORIC-ACID SOLUBLE PROTEIN (PSP) IN BLAST CELLS OF ACUTE LEUKEMIC PATIENTS.

Abstract

The purpose of this study was to detect the presence of perchloric-acid-soluble protein (PSP) in blast cells of acute leukemic patients and to conduct *in-vitro* study on the possibility of blast cells be proliferated into matured cells when treated with PSP. Samples from acute myeloblastic leukemic (AML) patients and monocytes from healthy donor were used as a test subject. The small amount of perchloric-acid-soluble 14.5 kDa protein (PSP) was isolated from monocytes of healthy donor by a combination of trichloroacetic acid extraction, preparative electrophoresis and CM-Sephadex chromatography. However, this protein was not found in blast cells of AML patients. The 14.5 kDa protein showed a strong cross-reactivity when tested with PSP antibody suggesting a close similarity to p14.5 PSP found in mononuclear phagocytes of human. However in our *in-vitro* study on the proliferation of blast cells of AML samples after treated with PSP showed some reaction, but no significant results. Thus, we believed that the amount of PSP used in this study was too small which was not enough to play a significant role in cell development and maturity.

Keywords: Acute leukemia; Perchloric-acid-soluble protein; blast cells; monocytes

1. INTRODUCTION.

Perchloric-acid soluble protein (PSP) was first isolated and characterized by Oka et al., (1995) from the rat liver (L-PSP1) followed by Ceciliani et al., (1996) from the goat liver, Samuel *et al.* (1997) from the mouse liver, Asagi et al., (1998) from the rat kidney (K-PSP1) and recently by Nordin et al., (2000) from the avian tissues and designated as C-PSP. The PSP isolated from the avian tissues was mainly expressed in the liver, kidney and also significantly expressed in intestine, gizzard, glandular stomach, heart, brain and spleen (Nordin et al., 2000). This protein exhibited a very high degree of identity with a group of protein belonging to the so-called YABJ and Y5GF isolated from *Bacillus subtilis* and *E. coli,* Ceciliani et al., (1996).

The PSP is a homodimer consisting of two identical subunits with a molecular mass of 14 kDa. The cDNA of L-PSP1 contained a 411 bp, encoding a 137 amino-acid protein with a molecular mass of 14149 kDa. The deduced amino acid sequence of L-PSP1 was completely identical with that of K-PSP. L- and K-PSP also inhibited a cell-free protein synthesis in lysate of rabbit reticulocytes in a different manner from RNAse A (Oka et al., 1995; Asagi et al., 1998). These inhibition processes was mainly due to an endoribonucleolytic activity of L-PSP by direct effect on mRNA template and induced disaggregation of the reticulocyte polysomes into 80s ribosomes, even in the presence of cycloheximide (Morishita et al., 1999; Nordin et el., 2000).

In 1996, Schmiedeknecht et al., discovered the presence of the translation inhibition protein (14.5 kDa) designated as p14.5 from the mononuclear phagocytes of human. The

protein was found to show a remarkable similarity with PSP protein as described by Oka et al., (1995). This protein was also present in liver, kidney and vessel wall section when tested by immunohistochemical technique. The expression of the mRNA of the translational inhibitor p14.5 (human homologue of L-PSP1) was significantly up-regulated with the induction of differentiation to macrophages (Schmiedeknecht et al., 1996). A similar phenomenon was observed in the synthesis of K-PSP1 from rat kidney which increases from the 17^{th} day to the 4^{th} postnatal week, and then enters a steady-state level (Asagi et al., 1998). In contrast, the expression of K-PSP1 in renal tumor cells was down-regulated (Asagi et al., 1998). Thus the PSP and PSP-like proteins appear to be expressed in a growth and differentiation-dependent manner.

Another PSP-like protein which belongs to the same family known as UK101 and UK 114 has been shown to be involved in immune control of tumor growth (Bartorelli A et al., 1996). Its role in tumor cell proliferation, however, is still uncertain. Complement-mediated cytotoxic activity has been demonstrated in the sera of UK101 and UK114-treated animals (Bartorelli A et al., 1996), and both cytolysis and tumor inhibition have been observed in the sera of cancer patients (Bussolati et al., 1997). The therapeutic effect of UK101 and UK114 in experimental mammalian tumors has been studied (Bartorelli A et al., 1994; Racca et al., 1997), whereas their role in the prevention of carcinogenesis has not been investigated. Later works by Ghezzo et al., (1998), has shown that the UK101 inhibits carcinogenesis of DMBA-induced Syrian hamster cheek-pouch squamous cell carcinoma.

Similarly this PSP protein's present in tumor cells could have some mediative effect on the cells proliferation and its expression. It is well documented that leukemia is a purposeless, malignant, neoplastic proliferation of abnormal leucocytes in heamatopoietic tissues. Based on the degree of differentiation of the leukemic cell line, leukemias are divided into acute and chronic forms. Undifferentiated heamatopoietic cells are responsible for acute leukemias. Whereas differentiated cells are associated with chronic leukemias. AML is characterized by progressive accumulation of relatively immature, poorly functioning myeloid blasts in the bone marrow (BM) and peripheral blood (PB). It eventually leads to inhibition of the production and proliferation of cells within the normal hematopoietic compartments (Broxmeyer et al., 1985). AML is diagnosed morphologically, using the criteria proposed by the French-American-British (FAB) Co-operative group (Bennet et al., 1976). Based on the previous studies that the PSP play an important role in protein inhibition and regulation of cell proliferation, therefore we undertake this study with the following objectives:

1. To detect the presence of PSP in blast cells of acute leukemias patients
2. To conduct *In-vitro* study on the possibility of blast cells be proliferated into matured cells when treated with PSP.
3. The outcome of this study will be useful to embark further research on the role and significance of PSP in blast cells of acute leukemias patients or perhaps its future treatment.

2. METERILAS AND METHODS

2.1 Normal cells and AML cells

Ten healthy donors of age between 20 to 45 years old and five patients of confirmed cases of AML were used in this study. The experimental protocols were explained to them before they were asked to sign a consent form (Appendix A). Fresh blood samples from healthy donor were collected into EDTA container to make up 1mM concentration and store at 4°C before used. Blood samples from PB and /or BM was drawn at the same time as those for clinical tests before chemotheraphy. The diagnosis of AML was base on May-Grűnwald-Giemsa (MGG), Sudan B black, and esterase staining of bone marrow and blood smear according to FAB classification criteria. The study was carried out in according to the protocol approved by the research and Ethics Committee of the Universiti Sains Malaysia.

2.1.1 Establishment and characterization of AML cells lines

Mononuclear cells (including blast cells) were separated by Opty-Prep density-gradient medium technique as described by Graziani-Bowering et al., 1997). The cells then were cryopreserved at -70°C in the presence of 50% heat-inactivated fetal calf serum (FCS) (Gibco, Grand Island, NY, USA), 10% dimethyl sulfoxide (Aldrich-Chemie, Steinhein, Germany) and α-minimal essential medium (α-MEM, Gibco). For the cultures, the cells were quickly thawed, washed twice with α-MEM and cultured at a high cell density of 1-

U/ml of interleukin-3 and IL-6, 100 U/ml of granulocytes-macrophage colony-stimulating factor and 40 ng/ml of mast cell growth factor. After culturing for 6 to 12 weeks in the above mentioned culture media, the cells were allowed to proliferate in 10% FCS and α-MEM for the next three months. Thereafter the cells were frozen at -70°C. For the experiments the cells were thawed and continuously grown in the presence of 10% FCS and α-MEM in a humid atmosphere at 37°C with 5% CO_2 at a cell density 3-5x10^5/ml. Fresh medium was generally changed every 3-4 days, and every other day for experiments.

Table 2.1.1. *Characteristics of the patients from whom the AML cell lines originated.*

Cell lines	Age/Sex [a]	Blast cell sources [b]	Phase of the disease
AML-1	12/M	BP	Diagnosis
AML-2	9/F	PB	Diagnosis
AML-3	10/M	PB	Diagnosis
AML-4	12/M	PB	Diagnosis
AML-5	8/F	PB	Diagnosis

[a] F, female; M, male; [b] PB, peripheral blood.

PART 1.

1. Preparation of cells.

Isolation of monocytes and blast cells
by OptiPret Method

↓

Centrifuged

↓

Cells were homogenized in 50 mM Tris/HCL pH 7.4

↓

Dialyzed

2. Separation and purification of proteins

Separation and purification of PSP by CM-Sephadex
C-25 column chromatography

↓

Purified PSP were analyzed on SDS-PAGE electrophoresis, blotted onto nitrocellulose membranes and immunoblotted with antisera against rat PSP

PART 2.

3. *In-vitro* study on the proliferation of blasts.

Purified blast cells were cultured as described by Buick et al., (1977)
With or without PSP and incubate at 37°C in 5% CO_2

↓

The cells were blocked, sectioned and stained with immunohistochemical procedures.

↓

Data and results were analyzed.

Figure2.1. Experimental design of the study

2.2 TEST PROCEDURES

2.2.1. Isolation and separation of PSP from normal monocytes and blats cells of AML by OptiPrep method.

Human monocytes from healthy donors and blast cells from AML patients were isolated by the OptiPrep density-gradient medium technique as described by Graziani-Bowering et al., 1997. 10 ml of fresh blood were collected from healthy donor and from patient into EDTA container to make up 1mM concentration. The samples were then brought together with the reagents and equipments to 4°C before use. The OptiPret reagent was gently shaken before removing for aliquot. 4.24 ml of 40% (w/v) iodixanol working solution (WS) was added into 10 ml of blood sample and mixed well. In a 15 ml centrifuge tube, 5 ml of density barrier solution (1.072 g/ml) was layered over 5 ml of the blood and then layered with approx. 0.5 ml of solution B (RPMI containing 10% fetal cuff serum). Centrifuge at 700g in a swinging-bucket rotor for 30 min at 4°C. The cells that float on top was collected and diluted with 2 vol. of solution B and harvested by centrifugation. The cells then were homogenized in 50 mM Tris/HCL pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride ($PhMeS_2F$), 1 mM benzamidine, 1 μM leupeptin, 1 μM pepstatin A at 4°C using a loose-fitting moto-driven glass/Teflon homogenizer. After centrifugation at 10 000 X g for 30 min, the post-mitochondrial supernatant (PMS) was obtained and treated with 60% perchloric acid making a 5% solution of PMS and then again centrifuged at 10 00 X g for 15 min. The supernatant was made up to 25% with trichloroacetic acid, and the precipitate collected

by centrifugation at a similar speed as the later procedure. The precipitate was washed with cold acetone and dried under vacuum. The dried material was then dialyzed against distilled water for 4 h and then dialyzed extensively against 0.1 M sodium phosphate buffer (pH 7.5) overnight.

2.2.2 CM-Sephadex C-25 column chromatography

After clarification by a 10 min centrifugation at 10 000 X g, the proteins in the dialysate were fractionated with saturated ammonium sulfate. The precipitate formed between 0 and 40% saturation was collected by centrifugation at 10 000 X g for 10 min. The precipitate was then suspended in 0.1 M sodium phosphate buffer (pH 7.5) and dialyzed against the same buffer. The dialyzed product was applied through a 2 X 30-cm column of CM-Sephadex C-25. Absorbance readings for protein were taken at 280 nm using Ultraspec 3000 spectrophotometer (Pharmacia Biotech) and the flow-through fractions were collected and electrophoresed by SDS-PAGE. Finally, protein fractions that cross-reacted with PSP antibody were pooled and again subjected to electrophoresis. The corresponding protein fractions obtained were used as the purified protein in the subsequent study.

2.2.3. Western blotting

We analyzed the pure proteins in homogenate from normal human monocytes and from blast cells by SDS-PAGE using polyacrylamide gels (15%) according to the method described by Laemmli (1970). After electrophoresis, the proteins on the gels were blotted

onto nitrocellulose membranes (Schleicher and Schuell, Germany) and immunoblotted with antisera against rat PSP (Oka et al., 1995).

2.2.4. Immunohistochemistry

The cells were prepared for immunohistochemistry as described earlier (Soini et al., 1992) by fixing in 10% neutral formalin for 2-3 days at room temperatures, after which the cells were pelleted by centrifugation. The cell pellet was suspended in melted 2% agarose, and the agarose block was further embedded in paraffin. About four µm-thick (4 µm) sections were placed on slides and fixed with 70°C alcohol for 30 min. The slides then were blocked with PBS containing 20% normal goat serum and 1% BSA for 1 h and incubated for 1 h with polyclonal antibodies against PSP in PBS containing 0.1% BSA in a moist chamber. The slides were washed extensively with PBS and then incubated for 1 h with 8 µg/ml horseradish peroxidase-conjugated goat anti-rabbit IgG (Oka). After washing with PBS, the slides were stained by the addition of 0.05% diaminobentizine and 0.1% H_2O_2. After washing with PBS, the smears were counterstained with methyl green for 20 min, dehydrated and mount. Negative controls were prepared by using non-immunized rabbit IgG as a primary antibody.

2.2.5. *In-vitro* study on the proliferation of blast cells.

2.2.5.1. Colony forming assay

A modified semi-solid methylcellulose (mc) method described by Buick and co-workers (Buick et al., 1977) was used to investigate the effect of PSP on the formation of clonogenic blast cell colonies. The cells were plated in 96-microwell plates in 0.1 ml of

basic growth medium [10% heat-inactivated FCS in α-MEM] and 0.9% mc (Aldrich-Chemie, Steinheim, Germany) with or without of PSP and incubated in a humidified atmosphere at 37°C with 5% CO_2 at a cell density of 3 x 10^3 cell/well. All the cultures were performed in triplicate. Colony formation was observed and classified using an inverted microscope. Colonies from three wells/sample containing more than 20 cells were counted and the mean colony number was calculated.

2.2.5.2. Suspension culture assay

In the suspension culture, 1 x 10^6 cells were incubated in the presence or absence of PSP in 1 ml of basic growth medium in 24 multi-well plates (Becton Dickinson & Company, Lincoln Park, New Jersey, USA) as described previously by Nara & McCulloch,1985. The cell number and viability were determined by vital dye exclusion (0.4% trypan blue, Sigma Chemical Co.Ltd., Irvin, UK) using standard hemocytometer.

3. RESULTS

3.1. Isolation and purification of PSP from normal human monocytes and blast cells of AML.

In this study, we described the isolation and purification of PSP-like protein found in the healthy donor monocytes for further investigation. However, this protein was not found in any blast cells of AML samples (Fig. 3.1). The protein obtained by extraction with 5% perchloric acid and 25% trichloroacetic acid from the supernatant was almost pure at this step. The PSP-like protein appeared in the flow through fractions of CM-Sephadex

chromatography and was shown to be homogenous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and contained single protein with a molecular mass of 14.5 kDa (Fig.3.1). The immunoblotting result showed a strong cross-reaction with rat PSP antibody suggesting that the purified protein is similar to a perchloric-acid-soluble protein purified from rat liver (Fig.3.1.1).

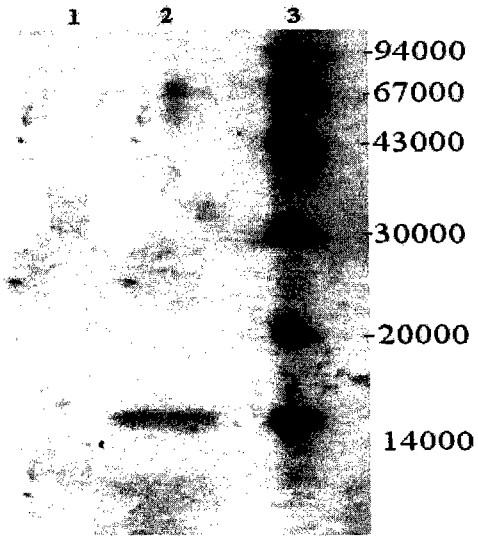

Figure3.1. SDS-PAGE of the purified PSP protein.
3: Molecular weight marker,
2. PMS of normal human monocytes.
1: PMS of blast cells of AML.

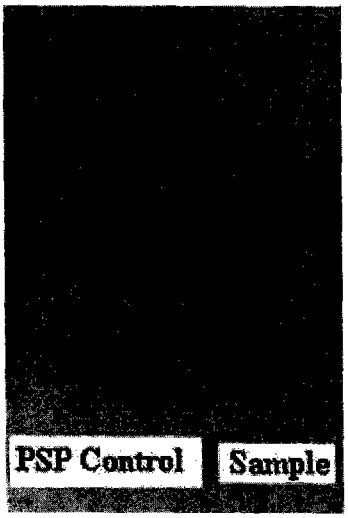

Fig. 3.1.1. Immunoblot analysis of PSP-like protein from normal human monocytes. The Were electrophoresed in 15% SDS-PAGE And immunoblot was carried out as Under section 2.2.5.

3.2. Immunohistochemistry

To further support the data related to the detection of PSP in monocytes and to prove that PSP was not present in blast cells of AML, immunohistochemistry was performed. In this preparations we showed that some immunopositive deposits were seen in smears containing monocytes obtained from the healthy donor but, no immunoreaction were

observed in smears containing blast cells from AML samples (Fig.3.2). No reaction products were found in any control smears prepared using non-immunized rabbit IgG as the primary antibody (Fig.3.2).

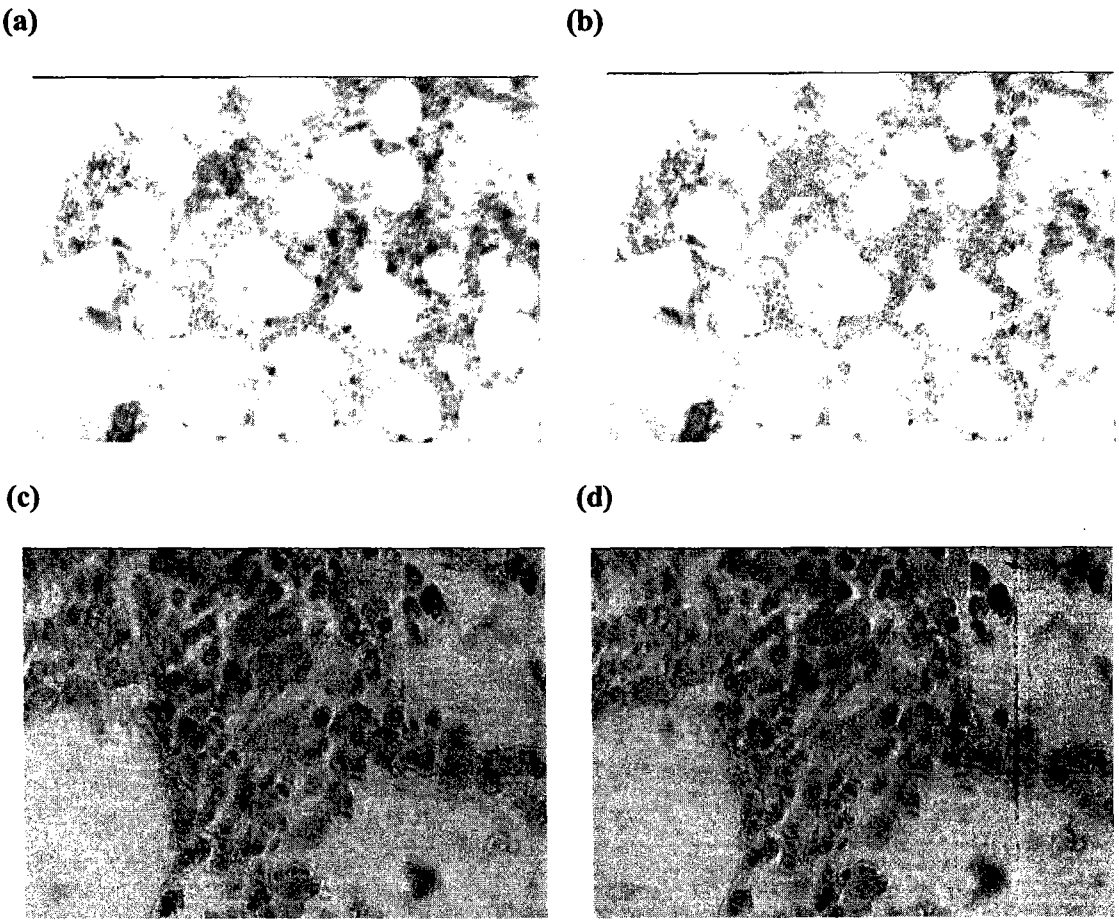

Figure. 3.2. Immunohistochemical staining of monocytes and blast cells of AML for PSP-like protein. Left panels (b,d) show negative control, and right panels (a,c) are shown to be immunostained with anti C-PSP rabbit IgG. Immunoreactive product are shown in the monocytes section (a) but not blast cells of AML (c).

3.3. *In-vitro* study on proliferation of blast cells

3.3.1. Effect of PSP on AML cell growth

3.3.1.1. Colony-forming assay and Suspension culture assay

Due to a small amount of PSP-like protein were used in this study, *in-vitro* study of blast cells proliferation using colony-performing assay and suspension culture assay was not significant.

4. DISCUSSION

In the present study, a small amount of PSP-like protein was isolated in the monocytes of the normal healthy donor (Fig.3.1). However, this protein was not found in blats cells of AML patients (Fig. 3.1). The molecular mass of the PSP-like protein was found to be 14.5 kDa as assessed by SDS-PAGE (Fig.3.1). The cDNA of a 14.5 kDa protein showed a remarkable similarity to the p14.5 PSP found in mononuclear phagocytes (MNP) of human as described by Schmiedeknecht et.al., (1996). Immunohistochemical observations also revealed that the PSP-like protein was cross-reacted with PSP anti sera. Study by Schmiedeknecht et.al. (1996) shown that circulating monocytes have only weak expression of PSP, while the higher differentiated alveolar macropages and the lipid-laden macrophages within the atherosclerosis plague have more intensive

immunolabeling of this protein suggesting, that expression and appearance of PSP-like protein are cell-specific processes.

In our study, although many healthy donor samples (10 donors) has been analyzed and tested several times however, the amount of PSP-like protein isolated was too little. This is probably due to the separation technique we used was not appropriate or could be the samples used was not enough. Because of that the *In-vitro* study on possibility of blast cells of AML proliferation when treated with PSP using colony-forming assay and in suspension culture assay was shown no significant results.

Based on our observation and supported by the previous studies (Schmiedeknecht et.al., 1996) shows that PSP play an important role in protein inhibition and regulation of cell proliferation, therefore we believe this protein possibly could play a significant role in helping blast cell of AML be proliferated into matured cells. To embark further research on the role of this PSP-like protein on blast cell of AML in future we suggest, the leukapheresis technique may be used to isolate monocytes as described by Schmiedeknecht et.al., (1996) or perhaps commercially prepared PSP may be useful.

ACKNOWLEGEMENTS

Our special thank are due to all healthy donor who have participated in this study. We are very grateful to Associate Prof. Mustapha Musa, Head of Immunology Department for granting us permission to use their laboratory and equipments during the study. We are also indebted to the staff of the Immunology laboratory, namely; Ms Malisa Yoong Abdullah, Ms Kamariah Abdul Rahim and Mr.Abdul Razak and Ms Asiah from the cell culture section, PPSG for their technical assistance during the entire course of this study. We are also grateful to all the staff of the Pediatric Wad who helped us collecting blood samples during the experimental processes. The research was financially supported by a short-term gram (304/PPSP/6131243) from Universiti Sains Malaysia.

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