Short-term culture for acute myeloid leukemia blast cells
تنمية خلايا سرطان ابيضاض الدم الحاد خارج الجسم الحي

Majeed A. Sabbah **Khulood Al-Samarrae** **Muhammad Rafeeq*** **Ali Muslim****

Biotechnology Research Center/ University of Al-Nahrin
*Biotechnology Department/College of Science/ Al-Nahrain University
**The National Center of Hematology/ Al-Mustansirya University

مجيد ارشيد سباح **خلود السامرائي** **محمد رفيق عبد المجيد *** **علي مسلم****

مركز بحوث التقنيا ت الاحيائية/ جامعة النهرين
*قسم التقنيات الاحيائية/ كليه العلوم/ جامعة النهرين
**المركز الوطني لبحوث وعلاج امراض الدم/ الجامعة المستنصريه

Abstract

This study was carried out to separate acute myeloid leukemia (AML) blast cells and studies their proliferation in short-term culture. The separation procedure include three steps; Ficoll gradient separation, depletion of macrophages and depletion of (lymphocytes and of monocytes) for preparation of highly pure native AML blast cells from blood samples collected from patients with moderate blast percentage. Results showed that this procedure is an inefficient due to a decrease in total cell number and contamination with other cells after each separation step. Proliferation of native AML blast cells in short term-culture by cultivating isolated AML blast cells in RPMI medium supplemented with 20% human plasma at a concentration 1×10^6/ml in the presence and absence of colony -stimulating factor which was provided by conditioned media (PHA-leucocytes-, plasmacytoma cell line- and Hep-2 conditioned medium. The effect of each conditioned medium on proliferation of AML blast cells was studied separately. Results showed that plasmacytoma cell line conditioned medium didnot stimulate the proliferation of native AML blast cells, while cells seeded on media containing 10%PHA-LCM showed an increase in cell number and growth of the cells was observed for approximately 3 days and then decreased.

المستخلص

تهدف هذه الدراسة الى تهيئة خلايا دم سرطانية من نوع ابيضاض الدم الحاد AML مناسبة للدراسات الخلوية والجزيئية . تضمنت الدراسة استخدام عدة خطوات لفصل الخلايا السرطانية من نموذج الدم المعزول من المرضى لغرض الحصول على خلايا سرطانية نقية . النتائج اثبتت أن هذه الخطوات غير كفؤة لهذا الغرض حيث لوحظ نقصان في اعداد ألخلايا المعزولة بالاضافة لتلوثها بخلايا اخرى بعد كل خطوة فصل . تم دراسة نمو الخلايا السرطانية خلال فترة سبعة ايام ، ولوحظ ان هناك عدة عوامل لنمو الخلايا السرطانية منها : ان اعداد الخلايا يجب ان لايقل عن 10^6 ، و وجود عوامل النمو ، وغياب خلايا Neutrophils . عوامل النمو توفرت بوجود وسط نمو خلايا الدم البيضاء المحفزة بـ PHA او وسط نمو خلايا الخط الخلوي Hep-2 . اما وسط نمو خلايا الخط الخلوي Plasmacytoma cell line فلم يساعد على نمو الخلايا السرطانية . اما عند تنمية هذه الخلايا في وسط يحتوي على 10% PHA-LCM استمرت الزيادة في اعداد الخلايا الى ان توقفت عند اليوم الثالث وبقيت على نفس المستوى ليومين ثم بدأت بالانخفاض ابتدأ من اليوم الخامس الى اليوم السابع .

Introduction

Acute myeloid leukemia (AML) is characterized by neoplastic proliferation of myeloid cells. The malignant cells have differentiation block that results in an accumulation of immature cells [1].

In order to separate AML blast cells from blood or bone marrow, combination of several separating techniques had been used. Gradient separation (specific density 1.077 g/ml) is widely used for the preparation of AML cells [2], this method results in populations of >95% purity when samples were denuded from selective patients with a high number of AML blasts relative to other low density cells [3]. Additional positive and /or negative selection is commonly used when gradient–separated blasts contain a relatively high percentage of contaminating cells.

Enriched AML blast population could then be prepared by the depletion of lymphocytes and monocytes, for example by using CD2- (T lymphocytes) and CD19-specific (B-lymphocyte) immunomagnatic negative selection in combination with removal of adherent cells [4].

Studies of *in vitro* cultured AML cell lines and native blasts have been important for the characterization of proliferation, differentiation, and apoptosis in leukemic hematopoiesis and for the understanding of chemotherapy effects in AML [4] *In vitro* growth characteristics of native AML blasts may be useful as a prognostic parameter in AML. An understanding of these AML models is therefore essential for the interpretation of experimental data and for the understanding of possibilities and limitations of the new therapeutic approaches [3].

In order to establish *in vitro* cell culture system for studying proliferation, differentiation and apoptosis of leukemia blast cells, the aim of this work is separation and short –term cultivation of leukemia blast cells.

Materials and methods

Preparation of solutions

(1): Sheep red blood cells (SRBCs) lyses buffer

Two stock solutions were prepared; solution A,(8.3g of NH_4CL were dissolved in 1L of distilled water), and solution B, (20.6g of Tris were dissolved in 1L of distilled water, adjust to pH7.6). Working solution was prepared by mix (9) volumes of solution A with 1 volume of Solution B, and adjusted to pH 7.2, sterilized by filtration through 0.22μM filter.

(2): Cell culture medium- RPMI-1640

RPMI-1640 culture media was prepared by dissolving 10.4g of RPMI-1640 culture media containing L-glutamine in sterile distilled water up to 1000ml,then 5.98 g/L of Hepes, 2 g/L of Na_2HCO_3, 0.1 g/L of Sodium pyruvate, 0.006 g/L of Penicillin, and 0.013 g/L of Streptomycin were added. The solution was filtered through a 0.22 μM filter then dispensed into 4ml aliquots, and stored at –20 ˚C until used.

(3): Phytohaemaglutinin (PHA)

Crude PHA was obtained as a sterile solution from Iraqi Center for Cancer and Genetic Research, and stored at –20ºC.

(4): Human plasma

Sterile human plasma (AB+ve Blood group) was obtained from the central blood bank, Baghdad, Iraq. Heat inhibition for the complement was done in a water bath at 56 ºC for 30 min. The plasma was dispensed in 20ml aliquots and stored at –20 ºC.

(5): PHA-Leukocyte conditioned medium (PHA-LCM)

Three milliliters of normal heparinized blood were drawn, and stand in a sterile test tube for 80min in order to get leukocyte rich plasma (LRP). LRP was incubated at 37 ºC for 7 days in RPMI-1640 medium containing15% human plasma and 2% PHA. The conditioned medium was separated by centrifugation, sterilized by filtration, and stored at 4 ºC.

(6): Plasmacytoma-conditioned medium (PC-CM):

Plasmacytoma cells was harvested by centrifugation and cultured in RPMI-1640 medium containing 15% human plasma at 37 ºC for 5 days. The conditioned medium was separated by centrifugation, sterilized by filtration, and stored at 4 ºC.

(7): Hep2- conditioned medium (Hep2-CM)

Hep2 cells was harvested by centrifugation and cultured in RPMI-1640 medium containing 15% human plasma at 37 ºC for 5 days. The conditioned medium was separated by centrifugation, sterilized by filtration, and stored at 4 ºC.

Preparation of AML blast cells:

Three milliliters of heparinized blood from AML patient were diluted with RPMI-1640 media up to 10 ml and overlaid on 4 ml of Ficoll-Histopaque (1.077) then centrifuged at 800g for 25 min. The middle layer cells in the interface were removed with a pasture pipette, washed once with phosphate buffer saline (pH 7.2) and suspended in RPMI-1640 medium.

The mononuclear cells separated by ficoll-Histopaque (1.077) were incubated in 5 ml RPMI-1640 medium enriched with 10% of human plasma in a tissue culture flask (25 cm^2) for 30 min at 37ºC. The supernatant were removed and incubated for 2hrs in a second tissue culture flask. The cells in the supernatant after incubation were considered to be non-adherent cells.

The Ficoll separation-mononuclear cells were washed three times with RPMI-1640 medium enriched with 10% of human plasma.

Equal volumes of 0.5% SRBCs and mononuclear cell suspension were mixed and incubated at 37 ºC for 5 min. These cells were centrifuged at 200g for 10min at room temperature, and the initial volume was reduced to 5% by gentle aspirating of supernatant. The tubes were placed in the refrigerator at 4 ºC for one hour. At the end of the time the cell pellet was gently resuspended with the complete medium at

concentration ($5X10^5$ cell/ml) and layered on the Ficoll-Histopaque (1.077) and centrifuged as before. The middle layer cells were collected and washed once in RPMI-1640 medium, then treated with a hypotonic solution for 1 min to lyses SRBCs, and the cells were washed twice with RPMI-1640 medium.

Primary culture of AML blast cells

Native AML cells (whole blood, leukocyte-rich plasma, or mononuclear cells) were seeded in RPMI-1640 medium which was enriched with 10% human plasma in the presence and absence of (10% PHA-LCM, PC-CM, and Hep2-CM).

The viable cells count was performed every day, for seven days, by using trypan blue method [10].

Results

Preparation of native AML blast cells

The first step in study is the preparation of highly pure AML blast cells in a suitable number from AML patients with low and intermediate percentages of blast cells. For this aim three separation steps were used:

The first step was gradient separation (specific activity 1.077) by using Ficoll-Histopaque used for preparation of mononuclear cells. Negative selection steps were used in order to exclude monocytes by adherence and T-lymphocytes by E-rosetting with sheep red blood cells, Figure (1). After each separation step viability and differential count were performed with a leishman stain.

In the second step AML-M3 subtype was used; after each separation step the number of cells was decreased significantly, except in the case of adherence (Table 1). Differential count slides, stained with Leishman stain, showed that the separated blast cells were contaminated with lymphocytes. Separation steps showed little effects on the viability of the cells.

In the third step AML-M1 subtype; the same results, as with AML-M3, were obtained except that the number of cells separated by Ficoll was more than in the case of AML-M3 Table (2). Differential count showed that the separated blasts were contaminated with lymphocytes. Separation steps showed little effects on the viability of the cells.

Table (1): The percentages of AML- M3 blast cells after each separation step.

Separation step	Cell number %	Viability %	Granulocytes %	Blasts %	Monocytes %	Lymphocytes %
Initial	**100**	**100**	**100**	**100**	**100**	**100**
Ficoll	**36**	**90**	**8**	**80**	**66**	**88**
Adherence	**35**	**85**	**3**	**77**	**0**	**81**
E-Rosetting	**1.9**	**75**	**0**	**1**	**0**	**0.9**

Proliferation of native AML blast cells in short-term culture:

In order to determine the requirements for the proliferation of native AML blast cells in RPMI-1640 medium supplemented with 20% human plasma, several cultivation experiments were performed.

First: direct cultivation; in order to study the interaction of other blood cells, direct cultivation of whole blood and leucocytes-rich plasma (LRP) from AML-M1 patients with 48% blast cells in RPMI medium conditioned with 10% PHA-LCM were done. By counting the cultivated cells daily with haemocytometer the results showed no growth in a week of cultivation.

The second: the cultivation of separated AML blast cells; in order to study the effect of cell density of separated blast cells, $3x10^3$ cell/ml (which separated by gradient and negative selection) were cultivated. The results showed no growth through one week of cultivation.

The third: the cultivation of mononuclear cells with a conditioned media; in order to determine the effects of the addition of condition media, which contained various types of cytokines, 10% of PHA-LCM, PC-CM, and Hep-2-CM were added separately to the culture medium of native AML blast cells. Results showed that in the case of addition of 10% of PHA-LCM the number of cultivated cells was increased till the third day of incubation, figure (1) and (2), while the number of cells decreased in the case of addition of PC-CM during the period of incubation Figure (1). As shown in Figure (2), the cultivation of AML blast cells without addition of cytokines no growth appeared.

Whereas results of the addition of Hep2-CM showed an increase in the growth of AML blast cells in a manner similar to that of PHA-LCM, but with less activity Figure(2).

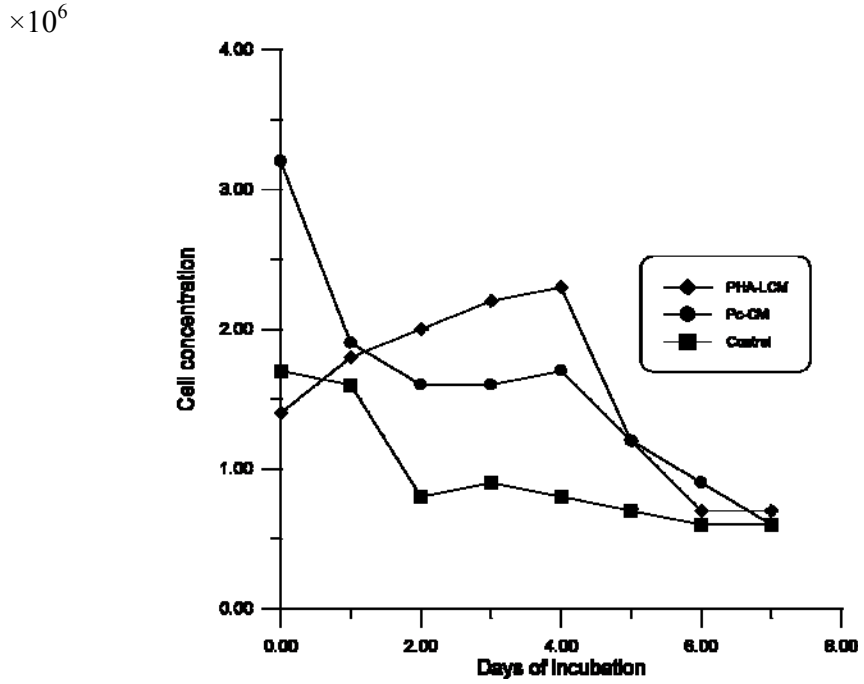

Fig (1): Proliferation of AML blasts in short-term culture with and without conditioned medium (CM, PHA-LCM and PC-CM).

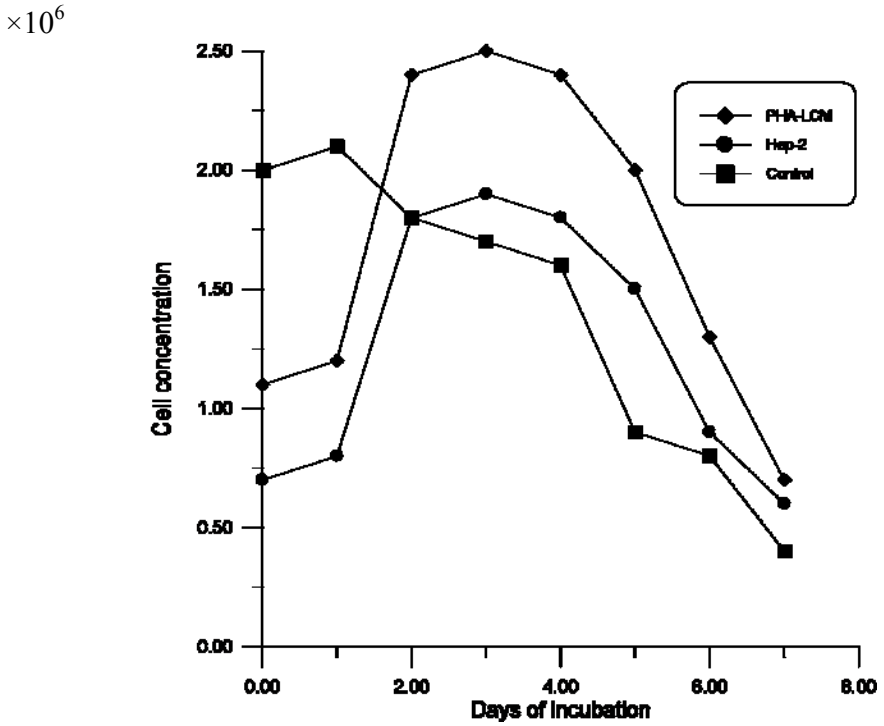

Fig (2): Proliferation of AML blasts in short-term culture with and without addition of conditioned medium (CM, PHA-LCM and Hep2-CM).

Discussion:

Normal human peripheral blood contains a variety of progenitor cells capable of giving rise to colonies under appropriate conditions in culture [5]. These include the myelopietic progenitors [6] and lymphopoietic progenitors [7]. The peripheral blood of patients with AML contains, in addition to these, a population capable of giving rise to colonies of cells with a blast like morphology [8]. The number of these progenitors significantly correlates with blast concentrations [9].

In order to study AML blast cells at cellular and molecular levels, AML blast cells must be separated from other blood cells. Peripheral blast counts may be high, intermediate or low. When blast counts are high one separation method requires, the ficoll method, to get a highly pure preparation of blast cells. When blast counts are intermediate or low, additional separation methods required for getting highly pure preparation of blast cells.

This study has tried to establish a separation procedure for small-scale (about 5 ml) leukemia peripheral blood samples used for cellular and molecular studies of leukemia. This separation system was used by many researchers in the field of AML studies [9, 10, 11, 12] before introducing the developed equipments for cells separation such as Flow cytometery [13] and immunomagnatic beads [3].

A single-density step method using ficoll of 1.077g/ml density was used for the separation of peripheral blood mononuclear cells (lymphocytes, monocytes, and blast cells) from granulocytes and mature red cells. The less dense mononuclear cells were recovered at the plasma / ficoll interface, while granulocytes and mature red cells usually were sediment at the bottom of the tube. The efficiency of ficoll separation seen in M1 was more than in M3, this might be due to the difference in the densities among these types of cells, because the diameter of myeloblast was about (15 μm) while the cell diameter of promyelocyte was about (20-255) μm [14].

The depletion of T-lymphocytes using E-rosetting method resulted in a decrease in cell number and did not give a pure preparation of blast cells this was due to the inefficient removal of T-lymphocytes by E-rosetting (remove about 50% of T-lymphocytes from blood sample [15]. These results agreed with the results of [16], he mentioned that this separation system was inefficacious in removal of all T-lymphocytes.

This separation system can be used in cell culture studies but it should start with large peripheral blood volume (about 20 ml). For molecular studies, pure preparations of AML blast cells can be obtained by using ficoll separation followed by the depletion of lymphocytes and monocytes using CD2- (T-lymphocytes) and CD19-spesefic (B-lymphocytes) immunomagnatic negative selection in combination with the removal of monocytes by adherence [17].

Highly pure AML blast cells may be prepared by starting with large peripheral blood volumes (bout 50ml), which were used by international studies [9].

[17] the first to mentioned that AML blast cells were capable of proliferation in simple suspension cultures are supported only by fetal calf serum, minimal essential medium (MEM), and a stimulator provided by media conditioned by normal leucocytes in the presence of PHA (PHA-LCM). Certain cell lines such as, human bladder carcinoma, could also provide such a stimulation [18]. These conditioned media contained multiple growth factors such as colony stimulating factors and interleukins [19]. By analysis of different recombinant cytokines on AML blast cells culture, it was found that recombinant granulocyte colony-stimulating factor, granulocyte-monocyte colony-stimulating factor, and inteleukin-3 stimulated the growth of leukemic blast cells in over 90% of AML patients [2].

More than 20 human tumor cell lines were reported to produce CSF in the conditioned medium [20].

AML blast cells responded to conditioned media contains CSF with an increased cell proliferation [3]. In agreement with these studies, results of this study showed that Hep-2 cell line, like PHA-LCM but with less efficiency, can induce proliferation of AML blast cells.

The murine cell line plasmacytoma show no effect on the proliferation of AML blast cells *in vitro*. This either due to that this cell line don't produce CSF or produce it but show no effect on human AML blast cells, may be due evolutionary reasons because we don't find

in the literature any studies on effect of murine CSF on AML blast cells or human bone marrow cells.

The analysis of growth kinetics of our samples shows no autonomous proliferation *in vitro*.

[21] Showed that about one-third of AML patients had blast cells which could proliferate autonomously *in vitro*. Other AML patients had blast cells which needed CSF for proliferation *in vitro*. Autonomous proliferation was considered as a determinant of prognosis in acute myeloid leukemia.

By addition of PHA-LCM, the number of cells increased till the third day then began to decrease. This result is in agreement with the results of [12]. Determination of growth kinetics is important for cytotoxicity tests in the next experiments. Hep-2 conditioned medium also induce AML proliferation but with little effect than PHA-LCM. Thus in this study cytotoxicity tests we depend on the addition of PHA-LCM.

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