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USE OF A PLASMA CLOT TO ASSESS FIBROBLAST PRECURSORS IN HUMAN BONE MARROW

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UDC 612.419.014.1.086.83

KEY WORDS: bone marrow; culture; fibroblasts; plasma clot

One method of studying the stromal microenvironment of bone marrow is to study the clonal capacity of fibroblasts. As a rule culture of hematopoietic cells from animals, healthy blood donors, or patients with various diseases in liquid media is used for this purpose [3-5]. To increase cloning efficiency, it has been suggested that irradiated hematopoietic bone marrow cells from laboratory animals be added to the cells in culture [2]. The positive effect is probably connected with additional stimulation by platelet-secreted growth factor (PSGF), contained in the platelets and megakaryocytes of the added cells [10, 14], or by other stimulators of hematopoietic cells [12]. Incidentally, comparable used richer culture media without the addition of a feeder [17]. The method of culturing bone marrow cells in methylcellulose medium [22] does not differ likewise from this system in principle. In that system fibroblast colonies are formed from precursor cells which settle on the bottom of the culture vessel and adhere to it.

Methods of culturing fibroblasts in semisolid media are alternatives. Under ordinary conditions fibroblasts do not proliferate in semisolid agar medium. Colony formation in semisolid agar medium by untransformed fibroblasts can be obtained only by the use of transforming growth factors, or with the aid of high concentrations of PSGF and epidermal growth factor [18]. Another possibility, in principle is to use as semisolid medium a collagen gel, in which fibroblasts, proliferating in the semisolid medium, are able to adhere to the substrate [16]. It seems that a semisolid medium containing fibrin threads (plasma clot) may also be used to assess proliferation of connective-tissue cells. An attempt was made to use such a system to culture precursor cells of the bone marrow and hematopoesis [13].

The aim of this investigation was to culture bone marrow fibroblasts and to evaluate their relationship with hematopoietic cells in semisolid medium, using a plasma clot.

EXPERIMENTAL METHOD

Bone marrow was obtained from the sternum of 42 patients with somatic diseases in a stage of remission or compensation. Before culture the bone marrow was carefully treated mechanically and washed to remove plasma factors [4, 5]. The bone marrow from some subjects was treated not only mechanically, but also with collagenase [14]. The bone marrow cells were cultured in medium containing 70% of McCoy' s medium with the addition of 10% placental blood serum from healthy women in labor [16], 20% citrated blood plasma from normal blood donors, and 0.7 ml of 10% calcium chloride solution to 100 ml of medium. Another version of culture was the combined use of plasma and methylcellulose (final concentration 0.8%), made up in double Dulbecco's medium. Culture was carried out in plastic dishes from the "Medpolimer" Factory (diameter 40 mm) under conditions of absolute humidity and with CO_2 concentration of 7%. For morphological study the plasma clot was dried and stained with Wright's stain. For comparison, a bone marrow cell suspension was cultured in Carrel's flasks [3-5]. In some cases, during a change of medium the nonadherent cells were re-explanted into a Carrel's flask with nutrient medium. Marrow from

Immunology Group, Central Research Laboratory, No. 1 Department of Internal Medicine, I. P. Pavlov First Leningrad Medical Institute. (Presented by Academician of the Academy of Medical Sciences of the USSR N. P. Bekhterev.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 110, No. 7, pp. 70-72, July, 1990. Original article submitted December 27, 1989.

0007-4888/90/0007-0935$12.50

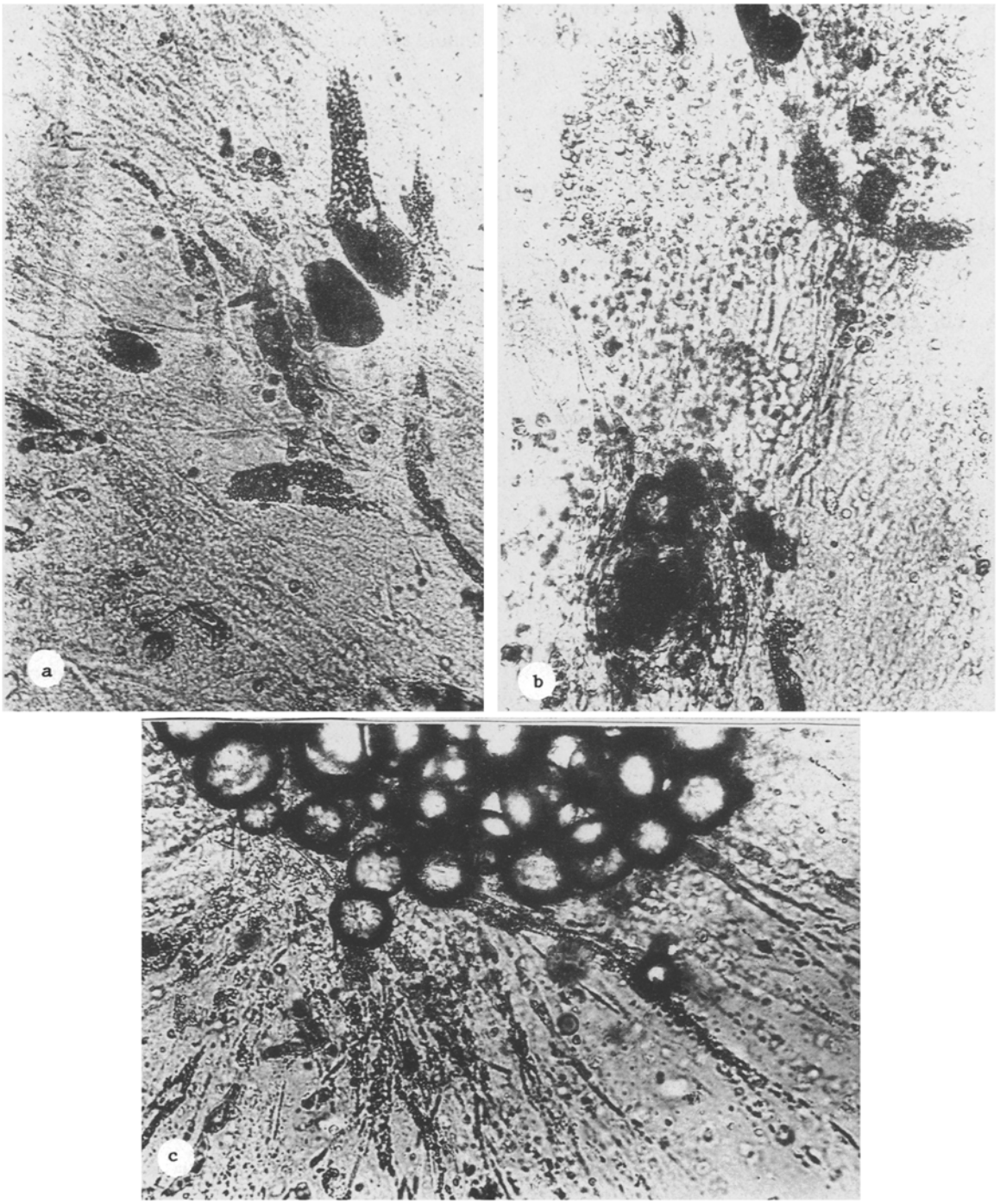

Fig. 1. Fragment of colony containing fibroblasts and adipocytes (a), hematopoietic cells, fibroblasts, and adipocytes (b), and large quantity of fat droplets (c). ×90.

five subjects was cultured in liquid medium with the addition of irradiated rabbit bone mitrow, by a method developed in Fridenshtein' s laboratory [8].

EXPERIMENTAL RESULTS

The use of a plasma clot with or without the addition of methylcellulose led to the development of three types of colonies in semisolid medium: hematopoietic; fibroblast, and mixed, containing fibroblasts and hematopoietic and fat cells. The hematopoietic colonies consisted of granulocytes, granulocytes and macrophages, macrophages alone and, finally, a combination

TABLE 1. Results of Culture of Bone Marrow Cells in Plasma Clot

Number of explanted cells	Number of colonies formed	Cloning efficiency, $\times 10^5$
$1 \cdot 10^5$	12,2±2,1	12,2±2,1
$3 \cdot 10^5$	28,2±3,0	9,4±1,0
$5 \cdot 10^5$	42,5±3,5	8,5±0,7

TABLE 2. Results of Reculture of Nonadherent Cells after Culture of Bone Marrow Cells in Carrel's Flasks ($\times 10^6$)

Days after primary explantation	Primary culture	Days					
		1	2	3	4	5	6
Number of fibroblast colonies after 14 days	32	28	0	14	12	18	6

of macrophages, neutrophils, and eosinophils. The fibroblast colonies consisted entirely of connective-tissue cells, and sometimes fatty infiltration of the fibroblasts was observed (Fig. 1a). Mixed colonies contained fibroblasts, sometimes with marked fatty infiltration, and hematopoietic cells (Fig. 1b), mainly of granulocytic-macrophagal-eosinophilic branches. The last two types of colonies frequently contained large drops of fat (Fig. 1c).

Quantitative analysis of the fibroblast colonies formed in the plasma clot revealed that they numberred 12.1 ± 1.1 after explantation of 10^5 bone marrow cells. This high cloning efficiency was achieved in myelokaryocytes in liquid medium on the addition of feeder cells [2]. We were unable to reproduce the feeder effect with irradiated bone marrow.

The use of different concentrations of bone marrow cells for culture in this system revealed a linear relationship between the number of colonies formed and the number of cells explanted (Table 1). The cloning efficiency under these circumstances was reduced a little, probably due to the inhibitory effect of monocytes on proliferation of the fibroblasts [1].

The use of methylcellulose did not affect cloning efficiency. Treatment of the bone marrow with collagenase led to a decrease in the number of very large mixed hematopoietic-fibroblast-adipocyte colonies and to an increase in the number of small (20-50 cells) mixed hematopoietic fibroblast colonies.

On the whole, by the plasma clot method it is possible to culture fibroblasts in disposable plastic Petri dishes of USSR manufacture. In this case a semisolid medium not requiring imported collagen is used. Moreover, colony formation in liquid medium depends not only on the number of clonogenic cells, but also on the ability of the cells to adhere to the bottom of the culture vessel. According to our own observations, a week or more after explantation of bone marrow cells many forming fibroblast colonies on re-explantation are found in the suspension medium among nonadherent cells. Representative data of one of four reculture experiments are given in Table 2. These results are in agreement with data obtained by other workers [15, 19]. In a plasma clot, colony formation does not depend on the ability of the cells to settle and to adhere to the surface of the vessel, or on migration of daughter precursor cells.

By the use of this method it was possible to detect associated proliferation of hematopoietic and stromal cells in culture, which was impossible by the use of methylcellulose medium [7]. This is evidently because of pre-existing association of stromai and hematopoietic cells in vivo [9] and also the possibility of establishment of contacts of this kind in vitro [6, 21]. It also follows from the results that by no means all concentrations of stromal cells in culture are colonies, for they originate from association of different cells. This was particularly evident when the bone marrow was not treated mechanically or with proteolytic enzymes. The very large concentrations containing adipocytes and hematopoietic cells frequently observed in agar medium during culture of human bone marrow have the same origin. The diversity of colonies developing in semisolid medium may perhaps reflect the situation in vivo: stromal fibroblasts, not associated with hematopoiesis, form fibroblast colonies, fibroblasts associated with hematopoietic cells, and on proliferation they form mixed colonies.

The reason for the higher level of colony formation in the plasma clot system than in suspension medium is not quite clear. There are evidently two relevant factors. One is independence of the ability of the cells to adhere to the bottom of the culture vessel. The other is connected with the unusual way of stimulating connective-tissue cells with thrombin. This substance has been shown to be capable of stimulatine proliferation independently of other growth factors [8]. It has been found that

thrombin is degraded in blood serum in the course of a ew minutes, whereas in the plasma clot it can preserve its activity for a very long time.

On the whole the results are evidence that culture of human bone marrow can result in a sufficiently high cloning efficiency, and it may also be used to evaluate relations between bone-marrow fibroblasts and hematopoietic cells under plasma clot conditions.

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