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Effects of Bone Marrow Conditioned Media on Proliferation of Stromal Clonogenic Cells *In Vitro*

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Fibroblast growth factors aFGF, bFGF, IGF-I, IGF-II, TGF-β, EGF, and PDGF did not stimulate the formation of stromal fibroblast colonies (CFC-F-colonies) in cultured murine adhesive bone marrow cells. It means that colony-stimulating activity of the bone marrow feeder cells with respect to the formation of stromal fibroblast colonies does not depend on the known growth factors. A scheme and conditions of culturing are developed for preparing conditioned media from bone marrow cell cultures which can replace the CFC-F colony-stimulating activity of the bone marrow feeder cells *in vitro*. Primary separation of conditioned media by ultrafiltration reveals that only the fraction with molecular weight of more than 65 kD exhibits CFC-F colony-stimulating activity.

Key Words: *stromal clonogenic cells; growth factors*

Bone tissue stem cells and committed osteogenic bone marrow (BM) precursor cells are normally present in the organism in the G_0 period of the cell cycle. In BM cultures they behave as clonogenic cells with a high proliferative potential and form colonies of stromal fibroblasts (CFC-F-colonies) [1]. The formation of CFC-F colonies in murine and guinea pig BM cultures can be induced in the presence of hemopoietic BM cells as a feeder, besides the serum. Colony-stimulating activity of BM feeder cells is due to the presence of megacaryocyte stem cells releasing unidentified growth factors into the medium [2]. We investigated the effects of conditioned media (CM) of BM cultures on the proliferation of CFC-F *in vitro* and elucidated whether the CFC-F-stimulating activity of BM cells and CM is due to the known fibroblast growth factors.

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MATERIALS AND METHODS

Experiments were carried out on adult CBA mice weighing 18-20 g and guinea pigs weighing 180-250 g. Bone marrow cell suspensions were prepared as described previously [1]. Adhesive BM cell cultures of mice devoid of their own feeder cells were used [3]. To this end, 3×10^5 murine BM cells in 5 ml α-MEM with 5% fetal calf serum (FCS) were incubated in 25-cm^2 culture flasks for 2 h, then the supernatant was discharded, the cultures were twice washed with α-MEM, and complete culture medium containing 80% α-MEM, 20% FCS, 200 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin was added. In some cultures, 1.5×10^7 BM cells from guinea pigs irradiated in a dose of 60 Gy (^{60}Co, 10 Gy/min) were added as a feeder. Feeders for other cultures were the following growth factors: rhPDGF AB (2, 10, and 50 ng/ml), aFGF (1, 10, and 75 ng/ml),IGF-I and IGF-II (5, 10, and 75 ng/ml), TGF-β (0.1, 0.5, and 1 ng/ml, Sigma), purified bovine bFGF (1, 25, and 100 ng/ml), and murine EGF (5, 20, and 100 ng/ml, Sigma), and

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combinations bFGF+IGF-I and bFGF+IGF-II (1+75, 10+10, 75+5, and 1+5 ng/ml). Medium conditioned by guinea pig BM cells was added to some cultures instead of the feeder. For preparing CM, guinea pig BM cells in a concentration of 7×10^6 cells/ml were cultured in 25-cm^2 flasks in a CO_2 incubator at 37°C for 3-7 days in 5 ml medium of the following composition: 1) α-MEM+1-10% FCS; 2) α-MEM+0.5% ITS (CBP) with glutamine and antibiotics in the specified concentrations. After that CM were collected, centrifuged for 15 min at 130*g*, and irradiated (60 Gy). Some CM after irradiation were filtered through a 0.45-μ filter or centrifuged for 30 min at 10,000*g*. CM were added to adhesive murine BM cells (5 ml/flask) and FCS was added to a final concentration of 20%. Some CM were consecutively separated by ultrafiltration through Halipor membranes and the resultant fractions were lyophilized on a Lyolab device. Three CM fractions with molecular weights of >65, 65-10, and <10 kD were added to adhesive murine BM cells in volumes equivalent to 5 and 25 ml whole CM. Murine BM cultures were grown for 12 days in a CO_2 incubator at 37°C, then fixed with ethanol and stained with Azur-eosin. Colonies containing at least 50 fibroblasts were counted and the number of colonies formed by 10^4 explanted cells (ECF-F) was calculated.

RESULTS

The ECF-F in cultures of adhesive murine BM cells in the presence of an irradiated feeder was 1.53±0.12, while in the absence of BM feeder cells the CFC-F colonies did not grow. BM cell-conditioned medium stimulated the colony growth. The colony-stimulating activity of CM towards CFC-F depended on the concentration of BM cells used for conditioning (Table 1), attaining the maximum at concentrations 3×10^6-10^7 BM cells/ml. Conditioned media based on α-MEM with both FCS and ITS possessed CFC-F-stimulating activity (Table 2). The colony-stimulating activity of CM depended on the content of FCS and on the duration of conditioning. Increase in the concentration of FCS in culture medium used for conditioning led to an increase in ECF-F in murine BM cultures. With prolongation of conditioning, the colony-stimulating activity of CM increased and reached about 30% of colony-stimulating activity of the BM cell feeder (Table 2).

TABLE 1. ECF-F in Cultures of Murine Adhesive BM Cells in the Presence of Media Conditioned by Different Quantities of Guinea Pig BM Cells for 5 Days (*M*±*m*)

Concentration of guinea pig BM cells, ×10^6 cells/ml	Number of fibroblast colonies	ECF-F
1	2, 2, 2	0.07±0.00
3	10, 10, 14	0.37±0.05
10	12, 13, 14	0.43±0.03
15	3, 3, 6	0.12±0.03

TABLE 2. ECF-F in Cultures of Murine Adhesive BM Cells in the Presence of CM of Different Composition (*M*±*m*)

CM composition		Duration of conditioning, days		
		3	5	7
α-MEM+FCS, %	1	0.02±0.01	0.12±0.03	0.18±0.02
	3	—	0.18±0.03	—
	5	0.42±0.00	0.48±0.06	0.51±0.05
	10	0.26±0.03	0.41±0.02	0.25±0.02
α-MEM+ETS, 0.5%		0.25±0.01		

Note. Dash ("-") means no data.

TABLE 3. ECF-F in Cultures of Murine Adhesive BM Cells in the Presence of Fractionated CM (*M*±*m*)

Molecular weight of fractions, kD	Equivalent of nonfractionated CM (multiplicity)	Number of fibroblast colonies	ECF-F
>65	1	0, 0, 0	0
	5	11, 11, 12	0.37±0.01
65-10	1	0, 0, 0	0
	5	0, 0, 0	0
<10	1	0, 0, 0	0
	5	0, 0, 0	0

The growth of stromal colonies was also seen when BM feeder cells and CFC-F were separated with a Millipore filter [2]. CM filtered through a Millipore filter and ultracentrifuged (10,000*g*) retained its colony-stimulating activity. This suggests that CFC-F-stimulating activity of CM at least partially depends on the presence of a soluble factor released by BM cells. This factor is apparently resistant to low temperature: single freezing at -20°C and subsequent thawing did not decrease the colony-stimulating activity of CM. However, fibroblast growth factors (aFGF, bFGF, IGF-I, IGF-II, TGF-β, EGF, PDGF) added alone or in combinations to cultured murine adhesive BM cells instead of BM feeder did not stimulate proliferation of CFC-F (data not presented). Therefore, the CFC-F-stimulating activity of BM feeder cells does not depend on the known growth factors but is determined by an unidentified growth factor. Primary separation of CM by ultrafiltration showed that only the fraction with molecular weight of more than 65 kD possessed colony-stimulating activity towards stromal clonogenic cells (Table 3).

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