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Supportive Effects of Human Embryonic Fibroblast Cell Lines on Growth and Proliferation of EBV-Transformed Lymphoblastoid Cells

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ABSTRACT

Human diploid fibroblast cells produce a spectrum of necessary growth factors and extracellular matrix (ECM) components essential for growth and proliferation of a variety of other cell types. In this study, the effect of five human embryonic fibroblast cell lines, isolated from liver, lung, skin and foreskin tissues, was investigated. A coculture system analyse was employed to cloning efficiency (CE) and DNA synthesis of a human Epstein-Barr virus (EBV)-transformed lymphoblastoid cell line (LCL) in long- and short-term cultures. The fibroblast cells were used as feeder layer after treatment with mitomycin C. Optimal density of the feeder cells induced 10 to 43 times higher CE than cultures supplemented with conditioned media (CM) or cultures without a feeder layer. The stimulatory effect of the feeder cells was partly associated to their tissue origin, with the lung and liver fibroblasts being the most and least effective feeder cells, respectively. Short-term cultures of LCL cells with feeder cells or their CM resulted in a marginal increase in DNA synthesis and proliferation as evidenced by the index of ^{3}H-thymidine incorporation. Our results demonstrated supportive effects of feeder cells on the LCL growth, which can not be replaced by their CM. These supportive effects were partly associated with cell density and tissue origin of the feeder cells. *Iran. Biomed. J. 7 (4): 147-153, 2003*

Keywords: Embryo, Fibroblast, Feeder cells, EBV-transformed B-cells, Cloning efficiency

INTRODUCTION

The use of feeder cells in cell culture was first reported by Puck and *et al.* [1]. Feeder cells are especially effective for the support of growth of cells that are difficult to culture [2]. Furthermore, an integral part of a successful cell cloning is the use of feeder cell layers [3, 4]. Control of cellular proliferation involves interplay between the cell and its environment. Feeder cells provide a suitable environment in the coculture with a variety of cell types through different mechanisms, including cell to cell and cell to extracellular matrix (ECM) interactions [5-7], production of soluble growth factors [8-10] and removal of toxicants from the culture medium [11].

Primary mouse macrophages or thymocytes [12, 13] and human fibroblasts [14-18] are commonly used as feeder layers for cloning of lymphoid cells. Primary cell cultures, however, have several disadvantages. These cultures have a relatively short lifespan in vitro and they must be prepared within a limited time period before experiments. In addition, primary cell cultures are prone to mycoplasma contamination [19]. In contrast, human diploid fibroblast lines are well-characterized cultures [20]. These cultures are easy to propagate and maintain. Fibroblast culture produces a variety of necessary growth factors and ECM components [21-23].

In the present study, four human fibroblast lines were first established from lung, liver, skin and foreskin of a human fetus. These cell lines, together with a previously characterized fetal lung fibroblast (MRC-5) were then used as feeder cells to assess their supportive effects on cloning efficiency (CE) and proliferation of EBV-transformed lympho-blastoid cell line (LCL).

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MATERIALS AND METHODS

Cell lines and cell culture. Four fibroblast cell lines were established in the National Cell Bank of Iran (NCBI) from human fetal tissue, including foreskin (HFFF, NCBI C 170), skin (HFSF, NCBI C176), liver (HFLF, NCBI C168) and lung (HFLGF, NCBI C125), as basically described by Freshney [24]. Briefly, the tissue specimen was placed in a sterile 100-mm Petri dish (Griner, Germany), cleaned of blood clots and finely cut into small pieces at approximately 1 mm^3 dimension. The small pieces were transferred and explanted into a 25-cm^2 flask (Griner, Germany), and cultured in RPMI-1640 medium (Sigma, USA) supplemented with 10% FCS (Seromed, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma) at 37°C with 5% CO_2 and saturated humidity. After the expansion of cultures to approximately 75-80% confluency, cells were trypsinized with 0.25% trypsin/ 0.04% EDTA solution in phosphate-buffered saline (PBS) (all from Sigma) for 1-2 minutes and split in larger flasks. A number of vials from each cell line were cryopreserved at a similar passage numbers (No. 5 to 9) and employed throughout this study. The human fetal lung fibroblast cell line MRC-5 (passage No. 23) was purchased from the European Collection of Animal Cell Culture (ECACC) (Salisbury, Scotland). The EBV-transformed LCL (LCL PI 4, NCBI C173) was established from B-lymphocytes of a healthy adult individual using virus enriched supernatant collected from the B95.8 Marmoset cell line (NCBI C110), as described elsewhere [14, 15].

Preparation of feeder cell layer. Subconfluent feeder cells were treated with 10 µg/ml of mitomycin C (Sigma, USA) for 2 h, rinsed with PBS, trypsinized, and split into 96-well microtitre plates (NUNC, Denmark) at 5×10^3 or 15×10^3 cells/well in a volume of 0.1 ml complete culture medium. In parallel wells, growing fibroblast feeder cells were harvested by trypsinization and re-seeded into 96-well plates at a density of 500 cells/well. The Plates were kept in an incubator at 37°C with 5% CO_2.

Preparation of conditioned medium (CM). Subconfluent cultures were fed with complete culture medium for 3 days and their supernatants were collected, filtered through a 0.22-µm filter (Millipore, USA), aliquoted, and stored at -20°C. The CM was thawed only once before use at dilutions indicated in the text.

LCL cloning efficiency assay. A single-cell suspension was prepared from the bulk culture of LCL cells and then diluted with culture medium and densities adjusted to exactly 100 or 500 cells/ml. The cell suspension was mixed and 0.1 ml (10 or 50 cells/well) transferred and overlaid on the feeder cells, each density in 30 wells. The feeder cells were cultured two days before cloning.

In parallel experiments, LCL suspensions were prepared in culture medium supplemented with 33% CM at the same cell densities. Control cultures without feeder cells were also included in each cloning experiment. Plates were incubated at 37°C and 5% Co_2 in a humidified atmosphere for a maximum of 3 weeks. The cultures were fed twice a week by replacing 2/3 of the medium. Wells with cultures containing colonies with more than 30 cells/colony were counted microscopically 10, 14 and 17 days post seeding.

LCL proliferation assay. The EBV-transformed LCL cells were plated at 2×10^3 cells/well into a 96-well plate in the presence or absence of mitomycin-treated feeder cells (15000 cells/well) or CM (33%) in complete culture medium. Plates were incubated at 37°C in 5% CO_2 for 3 days and then the cells were pulsed with 1 µCi of [^{3}H]-thymidine (Amersham, UK) for 18 h. The cells were harvested and incorporation of radioactive thymidine was counted as previously described [25].

Control cultures without LCL cells were included in each experiment. All cultures were performed in triplicate and repeated at least twice.

Statistical analysis. Chi-square and one-way ANOVA tests were used for comparisons between experimental data. $P<0.05$ was considered significant.

RESULTS

Influence of feeder cell density on cloning efficiency of LCL. The LCL cells were seeded onto various concentrations of feeder cells including MRC-5, HFFF, and HFLGF prepared as either growing or non-growing cultures. The growing feeder cultures attained 70-90% confluency within 7-10 days of incubation, whereas the treated non-growing feeder cultures provided 35%-95% confluency at 5,000 and 15,000 cell densities, respectively (data not presented). The cloning efficiencies were determined at 10, 14 and 17 days after seeding. As illustrated in Figure 1, CE of LCL cells was significantly higher in plates containing

the growth-arrested feeder cells compared with the growing feeder cells at 14 and particularly 17 days intervals. No significant differences were observed between the use of 5,000 and 15,000 non-growing feeder cells on the CE of LCL, although the 5,000 cell density induced better CE in HFFF and HFLGF cultures (Table 1). No growing clamps or colonies of LCL could be observed in the absence of feeder cells (Fig. 1, control culture medium).

Table 1. Statistical analysis of the effect of feeder cell density on cloning efficiency of LCL Feeder Cell Type Feeder Cell No.

Feeder cell type	Feeder cell no.	Culture period (days)	
		14	17
MRC-5	500 vs. 5,000	0.003	0.0005
	500 vs. 15,000	0.020	0.0020
	5,000 vs. 15,000	0.300	0.5000
HFFF	500 vs. 5,000	0.200	0.0050
	500 vs. 15,000	0.300	0.0400
	5,000 vs. 15,000	0.700	0.4000
HFLGF	500 vs. 5,000	0.200	0.0800
	500 vs. 15,000	0.200	0.0300
	5,000 vs. 15,000	0.800	0.700

Ten LCL cells were seeded on MRC-5, HFFF and HFLGF fibroblasts as described in the footnote to Figure 1. Cloning efficiencies were compared on days 14 and 17. The results represent *p* values obtained by comparison of the cloning efficiency in presence of the feeder cells at the specified cell number.

Effect of different embryonic fibroblasts on CE of LCL. The differential effects of five human embryonic fibroblast cell lines isolated from lung, liver, skin and foreskin on the CE of LCL cells were compared. There was a significant increase in the CE of LCL cells on one of the feeder layers prepared from the lung fibroblasts (MRC-5) compared to the other four fibroblast cell lines (Fig. 2). The feeder layer prepared from the liver fibroblasts induced the least CE. No significant differences could be detected between the effects of feeder cells from the lung, skin and foreskin tissues on the cloning efficiency of LCL cells, though the ssecond lung fibroblast line (HFLGF) and HFFF induced better CE than the other two feeder cell lines (Table 2).

Comparison between the feeder cells and their conditioned medium (CM). To compare the growth stimulatory effect of the feeder cells and their CM, the CE of LCL was determined after 17 days of culture. While the CM isolated from all feeder cells

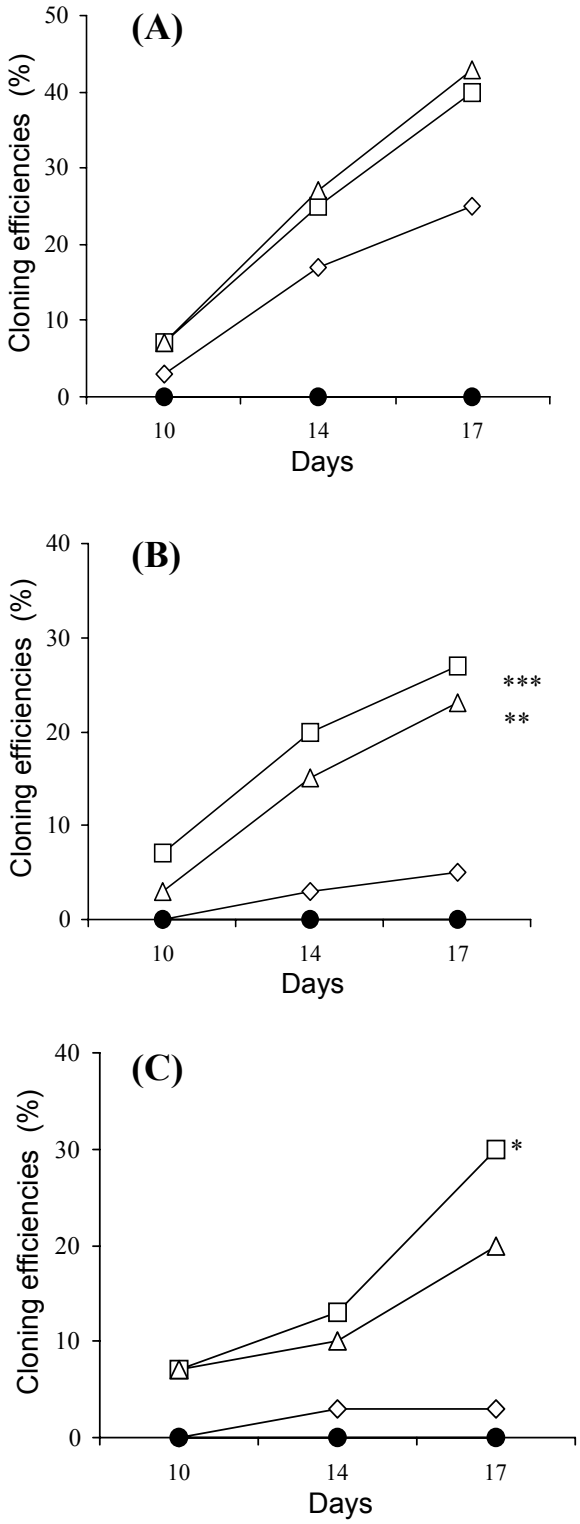

Fig. 1. Effect of different cell densities of human embryonic fibroblasts on cloning efficiency of LCL cells. Ten LCL cells were seeded on proliferating, (◊) 500 cells/well, or non-proliferating (□) 5,000 and (Δ) 15,000 cells/well, **(A)**, MRC-5; **(B)**, foreskin and **(C)**, lung fibroblasts. Cloning efficiencies were determined on days 10, 14 and 17. (•) Control culture medium. Cloning efficiencies were significantly higher in plates containing the non-proliferating feeder cells compared with the proliferating feeder cells, at 14 and particularly 17 days intervals (**P*<0.05, ***P*<0.01, ****P*<0.001, Chi-square test).

Table 2. Statistical analysis of the effect of different embryonic fibroblasts on cloning efficiency of LCL.

	Culture period (days)							
Cell lines	14	17	14	17	14	17	14	17
HFFF	0.1*	0.8	0.8	0.8	0.001	0.02	0.0000	0.0000
	HFSF		0.3	1.0	0.100	0.04	0.0000	0.0003
			HFLGF		0.010	0.04	0.0001	0.0003
					HFLF		0.0000	0.0000
							MRC-5	

Fifty LCL cells were seeded on lung (MRC-5 and HFLGF), foreskin (HFFF), liver (HFLF) and skin (HFSF) fibroblasts as described in the footnote to Figure 2. Cloning efficiencies were compared on days 14 and 17. *The results represent *p* values obtained by Chi-square analysis.

induced insignificant growth stimulatory effect comparable to that of the control culture medium, the CE induced by all feeder cells, particularly MRC-5, were several folds higher than those induced by their CM ($p<0.001$) (Fig. 3).

Effect of feeder cells on LCL proliferation. LCL cells were plated at 2×10^3 cells/well in presence of MRC-5 and HFFF cells or their CM. DNA synthesis at day 3 was assessed by pulsing wells with [^{3}H] TdR for the final 18 h. The results depicted in Figure 4 demonstrate that MRC-5 and HFFF cells, and to a lesser extent their CM provided similar growth support to LCL. No significant differences were detected between the two feeder cells or between the feeder cells and their CM.

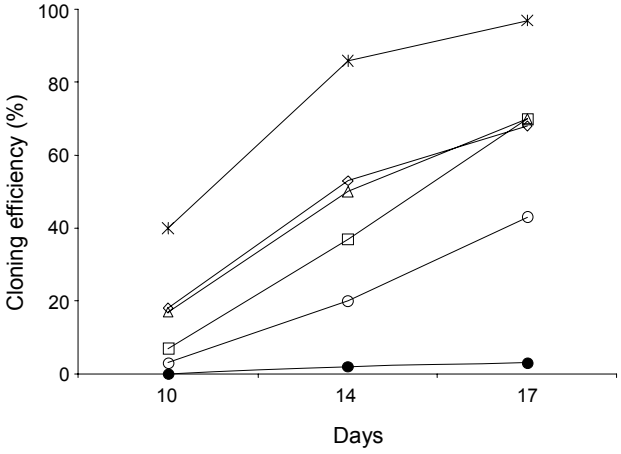

Fig. 2. Effects of different feeder fibroblasts on cloning efficiency of LCL cells. Fifty LCL cells were seeded on non-proliferating fibroblasts (5,000 cells/well); (□) HFSF, (◊) HFFF, ( ) HFLGF, (○) HFLF and (∗) MRC-5 fibroblasts. Cloning efficiencies were determined on days 10, 14 and 17. (•) Control culture medium. Cloning efficiencies were significantly higher in plates containing the MRC-5 cells compared with the other feeder cells ($P<0.001$, Chi-square test), and were significantly lower in the HFLF cells as compared with the HFLGF, HFSF and HFFF feeder cells ($P<0.05$, Chi-square test).

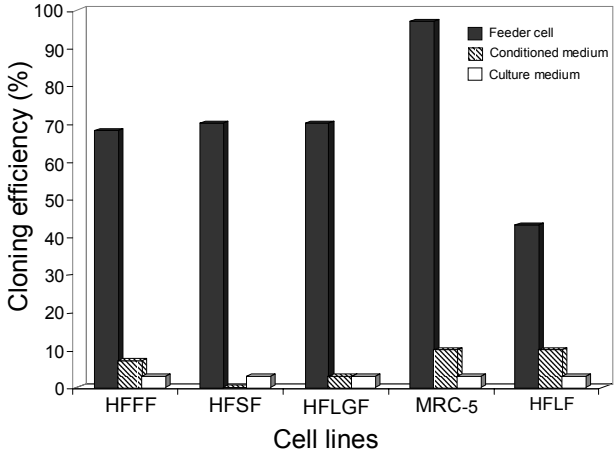

Fig. 3. Comparison between the effect of feeder cells and their conditioned media on the cloning efficiency of LCL cells. Fifty LCL cells were seeded in the presence of non-proliferating feeder cells or their conditioned media as described in the materials and methods. Cloning efficiencies were determined on day 17.

DISCUSSION

Application of feeder cells in co-culture systems is considered as an integral part of cell cloning and replication. In this study, we investigated the effects of human embryonic fibroblast feeder cells originated from different tissues on CE and proliferation of human EBV-transformed LCL cells. The feeder cells were employed as either growing or non-growing cultures. The non-growing cultures were initiated using mitomycin C that could inhibit cell replication without interfering with metabolism, as an alternative to irradiated feeder cells [26]. Our findings clearly demonstrated the positive effects of the feeder cells on cloning and growth of LCL cells. The beneficial effect of the feeder layer, however, was dependent upon three factors, including growing potential of feeder cells, source of feeder cells and direct contact between feeder cells and LCL cells.

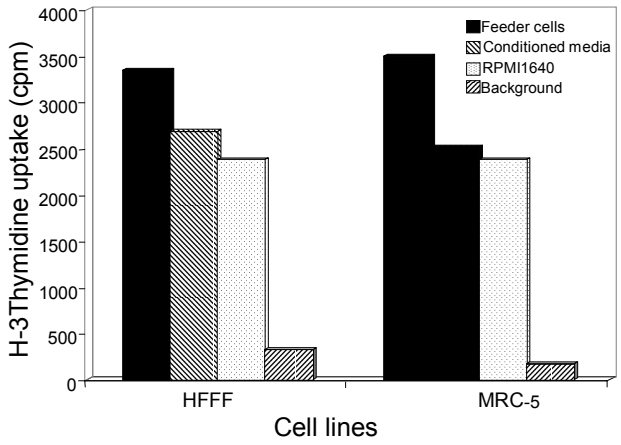

Fig. 4. Effects of fibroblast feeder cells on proliferation of LCL cells. LCL cells were cultured as described in the materials and methods, either in the presence of feeder cells, their conditioned media or fresh culture medium (RPMI 1640). DNA synthesis was assessed by pulsing wells with [^{3}H] TdR for the final 18 h. *Background: C treated non-proliferating feeder cells without LCL cells.

Non-growing feeder fibroblasts provided better support to LCL cells as determined by the significantly higher CE compared to growing feeder cells (Fig. 1).

No significant differences were observed between the 5,000 and 15,000 feeder cell densities, though the 5000 cell density induced better CE. Similar densities (1,500-5,000 cells/com) of mesenchymal and epithelial feeder cells were found to provide optimal growth condition. Increasing the cell density of those feeder cells inhibited the CE of test cells in a density dependent manner [27].

Although, growing fibroblasts are supposed to provide better support through production and secretion of larger quantities of growth factors (28-30), our findings do not support this proposition. It seems that growing feeder cells, particularly in a long- term culture may produce some unidentified toxicants or waste products, which induce negative effect on growth of LCL. These factors, together with the disadvantage of outgrowth of the feeder cells in a coculture system have encouraged the use of senescent or growth-arrested fibroblasts for growth and cloning of lymphoid cells [17, 18, 26, 31].

The growth factors produced by our feeder fibroblasts do not seem to play an important role. Indeed, the CM obtained from our feeder cells did not induce significant supportive effect on CE and replication of the LCL cells as compared with control culture medium (Figs. 3 and 4). Although replacement of feeder cells with CM preparations from fibroblasts and endothelial cells has already been successfully applied [32, 33], controversial results have also been reported. Survival and proliferation of both immature and terminally differentiated B-lymphocytes have recently been shown to be dependent on direct contact with bone marrow fibroblasts, since B cells failed to survive when separated from fibroblasts or stromal cells by microporous membranes [2, 34, 35]. In addition to B-cells, proliferation and differentiation of a variety of hematopoietic and non-hematopoietic cells were linked to direct contact with fibroblasts [36-38].

Fibroblasts seem to regulate proliferation and differentiation of other cell types differentially, based on microenvironment of their tissues of origin. Indeed, coculture of the blood $CD34^+$ progenitors with either dermal or spleen fibroblasts resulted in dendritic cells or natural killer (NK) cells differentiation, respectively [36, 37]. Besides the tissue origin, the developmental stage of the tissue from which the fibroblasts are established could also affect the function of the feeder fibroblasts [unpublished data and 39]. These observations are in accordance with our findings regarding differential effects of the fetal fibroblasts on the CE of LCL cells. Our results indicated a significant increase in the CE of LCL cocultured with the lung fibroblasts (MRC-5), whereas the liver fibroblasts (HFLF) induced the least CE (Fig. 2). Superiority of the fetal lung fibroblasts over fibroblasts of other tissue origins has already been reported in a coculture model investigating the CE of hybridoma cells [31].

Altogether, our findings indicate the importance of cell-to-cell or cell-to-ECM interactions, as has already been demonstrated in a variety of coculture systems [5-7]. The tissue origin of the feeder fibroblasts seems to influence their supportive function, implying differential involvement of membrane-bound molecules [40, 41]. Identification of these molecules and their receptors would certainly further our insights into the mechanisms involved in cell contact-mediated proliferation and differentiation.

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