Morphological cell typing of osteoid clones derived from human bone marrow

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Abstract

Objective Bone Marrow Stem Cells (BMSC) are a ‘reservoir’ for bone regeneration. BMSC can be studied in vitro by cloning cells which are improperly named colony-forming units of fibroblasts (CFU-f). Thus we decided to study CFU-f organization and morphology to have (A) a parameter by which to compare normal and pathologic conditions and (B) to potentially select the most osteogenic clones.

Methods Two hundred and forty bone samples were collected from 109 patients and primary cultures performed.

Results After two weeks 9 cell types and 6 well organized types of colonies were detectable. Some have alkaline phosphatase (AP) activity.

Conclusion These data could be relevant to estimate the potential regeneration of bone.

Keywords Stem cell · Clone · Morphology · Bone production in vitro system

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Introduction

The processes of structural and functional bone restoration have always drawn attention from both researchers and clinicians. Bone marrow stem cells (BMSC) not only create a microenvironment for the normal reproduction and differentiation of blood cells but also are a ‘reservoir’ for bone regeneration [1]. Indeed, BMSC are a good model for studying the formation, mineralization and maturation of bone tissue [2].

Osteogenic cells – precursors are divided into determined (DOCP) and induced (IOCP) cell types. IOCPs enter within osteogenic differentiation as a result of their induction signals whereas DOCPs do not require induction for bone formation. DOCPs can be studied in vitro by cloning their precursors which are improperly named colony-forming units of fibroblasts (CFU-f). CFU-fs allow the study of in vitro normal and pathologic cells and/or micro-environmental factors [1].

On culturing BMSC in vitro, cells are grouped and each colony is a clone (i.e., it

derives from a single cell) [3]. The high proliferation potential of CFU-f is manifested through its self-supporting ability in vitro. On transplanting in vivo, CFU-f show their ability to proliferate and differentiate into stroma cells required for bone and haemopoietic microenvironment formation [4,5].

In vitro, CFU-fs form collagen and other extracellular matrix components [6]. Fibroblasts of the stroma colonies of human bone marrow synthesize collagen I and collagen II [7]. Collagen I is an adequate support for osteoblast differentiation in vitro and osteogenesis in vivo [8,9].

Since (i) CFU-fs have a key role in osteoblast differentiation in vitro, (ii) they derive from a single cell but differentiate into several cell subtypes and (iii) CFU-f in vitro is potentially a system with which to detect cellular and micro-environmental variables related to normal and pathologic stem cell differentiation, we therefore decided to study CFU-f organization and morphology to have (A) a parameter with which to compare normal and pathologic conditions and (B) which would potentially allow the selection of the most osteogenic clones.

Material and methods

Samples were collected from 109 patients (age ranging from 11 to 80 years) operated at the Department of Maxillofacial Surgery of Kiev and at the Institute of Traumatology and Orthopedics of the Academy of Medical Science of Ukraine after informed consent was obtained. Specimens were cultured in the Laboratory of Immunology of the latter hospital.

Patients were suffering from facial diseases (group 1 and Table 1) or long tubular bone pathologies (group 2 and Table 2) and samples were derived from the splanchnocranium and upper flaring portion of the iliac crest, respectively.

During surgery a bone fragment having a volume of 0.1 ml was collected then immediately placed in a sterile flask. It was cleaned of soft tissues and mechanically fragmented. The cells were immersed in α -MEM culture medium, added with 22.5% FBS, 100 μ M ascorbic acid, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin. The cells were placed in flasks and incubated at 37°C in a 5% CO₂.

Table 1 List of samples derived from patients grouped for pathological condition of the facial region (Group 1)

Diagnosis	Number of patients
Ankylosis of temporo-mandibular joint	16
Fracture of the mandible	14
Fracture of the maxilla	3
Post-traumatic osteomyelitis of the mandible	2
Brachial arc I-II syndrome	4
Facial hemiatrophy	2
Mandibular Class II and III	6
Ameloblastoma of the mandible	2
Dental retention (control)	9
Total	58

Table 2 List of samples derived from patients grouped for pathological condition of long bones (Group 2)

Diagnosis	Number of patients
Chronic post-traumatic osteomyelitis of long tubular bones	28
Chronic hematogenous osteomyelitis of long tubular bones	8
Chronic postoperative osteomyelitis of long tubular bones	13
Hip joint disease	1
Brodie's abscess of long tubular bones	1
Total	51

Table 3 Number of specimens derived from the facial region which were successfully cloned

Area of a bone researched	Positive results	Negative results	Total
Body of mandible	17	13	30
Neck of condylar process of mandible	16	10	26
Ramus of mandible	3	5	8
Angle of mandible	7	2	9
Alveolar process of maxillary	1	3	4
Zygomatic bone	0	1	1
Walls of maxillary sinus	0	1	1
Iliac crest	4	1	5
Total	48	36	84

The seeded cellular suspension contains separate suspended cells which were counted by using a Goriaev chamber. The technique for culturing and obtaining a discrete number of colonies required that the density of explanted nuclear cells did not exceed 5×10^5 per 1 cm^2 in the bottom of a culture flask. Bone marrow cells are cloned by using an xenogenous 'feeder' [10,11]. Such a 'feeder' is a suspension of lethally irradiated rabbit bone marrow cells which are added to the culture medium in order to keep the optimal explanting density of human bone marrow cells. The cloning of CFU-f is carried out over 14 days without changing the nutrient environment. The grown fibroblast colonies

were fixed and stained according to the Romanovsky-Gymza method. The obtained colonies were studied by using a light microscope. A cellular aggregation of not less than 50 fibroblasts was considered a colony. Colonies were differentiated as monolayer and multilayer colonies [12]. On studying multilayer colonies, their structure and cellular composition were characterized. To verify the osteogenic differentiation of the cells, alkaline phosphatase (AP) activity was estimated by Kaplow's method [13].

The regenerative potential of a patient was estimated by using (a) the total of nuclear cells 1 cm^3 of a bone, (b) the quantity of the CFU-f in 1 cm^3 , (c) the efficiency of

Fig. 1 General view of culture dishes containing colonies. Macropreparation. Romanovsky-Gymza staining: (A, B) satisfactory growth of CFU-f colonies; (C, D) good growth of CFU-f colonies; (1) CFU-f colony **Fig. 2** Scheme of a type I colony of CFU-f **Fig. 3** A segment of type I colony of CFU-f. Magnification $\times 70$: (1) Parallel arrangement of cells

cloning, (d) the quantity of evolved colonies, and (e) the degree of a differentiation of cells. Eighty four and 156 cultures were obtained from either 58 group 1 patients and 51 group 2 patients, respectively.

Statistical analysis of the obtained data was carried out using acknowledged

methods (Microsoft Excel software). Descriptive statistics (a conventional set of basic statistical indices of an empirical sample) were used [14].

Results

Tables 3 and 4 summarized the results of cloning the osteogenic cells - precursors in group 1. In group 2 there were 111 specimens which were successfully cloned whereas 44 were unsuccessful. In group 2 all samples derived from the iliac crest and the average efficiency of cloning CFU-f was $12.258 \pm 0.297 \times 10^5$ nuclear cells.

The general view of the culture flasks is shown in Fig.1.

A. Type of colonies

Six types of clones are characterized which have different structures and osteogenic potential.

Type I CFU-f has star-shaped structure (Fig. 2). Colonies contain parallel arranged cells, but peripherally cells are arranged more chaotically. Calcium salts are frequently deposited in the centre of such structures where there is a higher density of cells (Fig. 3).

Type I colony contains 7 main kinds of cells (Fig. 4): fusiform cells (which are the main cells at the centre of a colony and dominate over the others - 70.5%); fusiforms with large nuclei (can be found all over a colony and constitute 11.75%); process-containing cells (which spread over the whole area of a colony and constitute 8.75%); triangular cells (located at the periphery of a colony - 4.25%); round cells (2%); long fusiform cells (0.5%); cells with multiple processes (2.25%).

Type II CFU-f are a star-shaped structure CFU-f which have lower structure-forming potential and high osteogenic potential (Fig. 5). In type II colonies, cells are located less regularly as compared with type I colonies. Cells in type 2 colonies are arranged more

Fig. 4 Types of cells forming colonies: (1) round cell; (2) fusiform cell; (3) fusiform with large nucleus cell; (4) long fusiform cell; (5) process-containing cell; (6) multiprocess cell; (7) triangular cell; (8) quadrangular with short processes and large cytoplasm cell; (9) mitotic form

Fig. 5 Scheme of a type II colony of CFU-f

Fig. 6 A segment of type II colony of CFU-f. Magnification x 70: (1) Sites of depositing of calcium salts; (2) Cells are located at different angles to each other

Table 4 Efficiency of cloning of specimens derived from the facial region

Area of a bone researched	Efficiency of the cloning (10^5 nuclear cells)
Body of mandible	3.74 ± 0.57
Neck of condylar process of mandible	16.39 ± 1.49
Ramus of mandible	0.92 ± 0.92
Angle of mandible	5.2 ± 1.85
Alveolar process of maxillary	0.105 ± 0.105
Zygomatic bone	0
Walls of maxillary sinus	0
Iliac crest	13.94 ± 1.55

Table 5 Percentage of different types of cell forming clones of CFU-f

Activity of AP	Kinds of cells	Type I colony	Type II colony	Type III colony	Type IV colony	Type V colony	Type VI colony
↑	Triangular	4.25	9	17	33.25	24.75	6.25
	Quadrangular with short processes and large cytoplasm	-	-	3.5	2.25	1.5	70
	Multiprocess	2.25	5	9	7.25	15.75	4
	Process-containing	8.75	10.75	24.5	13.25	10.75	5.25
	Long fusiform	0.5	-	-	-	-	0.25
	Fusiform with large nucleus	11.75	26.25	3.5	2.5	2	2
	Fusiform	70.5	46.75	41.5	40.5	44.25	12.25
	Round	2	2.25	1	1	1	-
(-) Means a lac of the specified kind of cells		Quantity of the cells in colonies having high AP activity					

Table 6 Colony types of CFU-f of the bone marrow of the iliac crest of patients of different age with chronic osteomyelitis of long tubular bones

Year of birth	Age	I type colony	II type colony	III type colony	IV type colony	V type colony	Total
1940	50 – 60 years old	+			+	+	3
1946			+		+	+	3
1949					+		1
1965	20 – 50 years old		+				1
1973		+			+		2
1977					+	+	2
1980	10 – 20 years old			+			1
1981				+	+	+	3
1983				+	+	+	3
1984	Before 10 years			+		+	2
1985			+				1
1990					+	+	2
Total		2	3	4	8	7	

Table 7 Colony types of CFU-f of the bone marrow of the iliac crest of patients of different sex with chronic osteomyelitis of long tubular bones

Sex	I type colony	II type colony	III type colony	IV type colony	V type colony	Total
Man	+			+	+	3
		+				1
				+	+	2
			+			1
			+	+	+	3
			+		+	2
Woman	+	+				1
		+		+	+	3
				+		1
				+		2
			+	+	+	3
				+	+	2
Total	2	3	4	8	7	

Table 8 Colony types of CFU-f of the bone marrow of the iliac crest of patients of different blood group with chronic osteomyelitis of long tubular bones

Blood Group	I type colony	II type colony	III type colony	IV type colony	V type colony	Total
O (I) Rh+			+	+	+	3
				+		1
		+				1
A (II) Rh+				+	+	2
				+	+	2
			+	+	+	3
B (III) Rh+	+		+			1
				+	+	3
		+				1
AB (IV) Rh+		+		+		2
Total	2	2	3	7	5	

chaotically thus forming a more gentle and light structure. Most of the triangular cells and some cells with large nuclei are located at an angle to each other, and such cells form structures resembling spider's webs. In the

centre of such structures, deposits of calcium salts can frequently be found (Fig. 6). The other kinds of cells are evenly spread over the colony and do not produce characteristic forms.

Type II colony contains 6 kinds of cells: fusiform cells (which can be found all over a colony - 46.75%); fusiform with large nucleus (which can be found all over a colony - 26.25%); process-containing cells

Fig. 7 Scheme of a type III colony of CFU-f

Fig. 8 A segment of type III colony of CFU-f. Magnification x 70: (1) Sites of depositing of calcium salts; (2) Islands

Fig. 9 Scheme of a type IV colony of CFU-f

Fig. 10 A segment of type IV colony of CFU-f. magnification x 70: (1) carpet-forming islands

Fig. 11 Scheme of a type V colony of CFU-f.

Fig. 12 A segment of type V colony of CFU-f. magnification x 70: (1) curves resembling Foucault currents

Fig. 13 Scheme of a type VI colony of CFU-f

Fig. 14 A segment of type VI colony of CFU-f. Magnification x 70: (1) Site of depositing of calcium salts (a lump)

Fig. 15 The triangular cells with long processes and highest activity of AP. (magnification x 400)

Fig. 16 The products of reaction occur in extracellular matrix and near triangular and quadrangular cells which have high AP activity in the cytoplasm. (Magnification x 400).

(10.75%); triangular cells (most frequently, such cells are located at the periphery of a colony - 9%); round cells (2.25%); cells with multiple processes (5%).

Type III CFU-f is an island-shaped colony structure that has significant structure-forming potential and high osteogenic potential (Fig. 7). It is very difficult to differentiate all kinds of cells in type III colonies; however, cells having one long process are fairly distinguished. The structure of the whole colony consists of islands of cells (Fig. 8). In such islands, cells are located very closely to each other and even overlap. Such islands are linked to each other with long processes or with different kinds of cells. The centers of such

islands contain the focus of the deposits of calcium salts.

Type III colony contains 7 kinds of cells: fusiform cells (41.5%); fusiform with large nucleus (3.5%); process-containing cells (24.5%); triangular cells (17%); round cells (1%); cells with multiple processes (9%) and quadrangular cells with short processes and large cytoplasm (3.5%).

Type IV CFU-f has diffused clusters of cells and calcium deposits along the periphery (Fig. 9). Triangle cells form islands linked to each other with long processes or with cells. In colonies, such islands tend to merge, forming a continuous carpet (Fig. 10). The type IV colonies have predominantly peripheral deposits of calcium salts.

Type IV colony contains 7 kinds of cells: fusiform cells (40.5%); fusiform with large nucleus (2.5%); process-containing cells (13.25%); triangular cells (33.25%); round cells (1%); cells with multiple processes (7.25%) and quadrangular cells with short processes and large cytoplasm (2.25%).

Type V CFU-f is a mixed colony (Fig. 11). The cells of this colony are organized in accordance with type I and type IV (centre and periphery). Islands merge with each other (while preserving their centers) gradually from the periphery to the centre of a colony. Then a transition to an orderly strict growth occurs, and parallel lines are formed. At the sites of maximum accumulation of cells, calcium salt deposits are detected (Fig. 12).

Type V colony contains 7 kinds of cells: fusiform cells (can be found around the whole colony: they are the main cells at the centre of a colony - 44.25%); fusiform with large nucleus (2%); process-containing cells (10.75%); triangular cells (located at the periphery of a colony - 24.75%); round cells (1%); cells with multiple processes (15.75%) and quadrangular cells with short processes and large cytoplasm (1.5%).

All the previous 5 types described are detected in facial bone and iliac crest. In the colonies derived from the mandible, a greater amount of calcium salts is deposited as compared with other colonies. When the deposition of calcium salts increases, it affects the shape of the colonies.

Only mandibular fibroblast produced Type VI CFU-f colony (Fig. 13). The periphery of type VI resembles type I colony. However, close to the centre of type VI, cells are arranged evenly and in parallel with each other. However, unlike the cells of type I, all cells of type VI are directed toward the centre of the colony (Fig. 14) and a large amount of calcium salts (a lump) is deposited in the core of a type VI colony. Since there was a large amount of calcium salts masking the shape and structure of cells, we have failed to identify all kinds of cells.

The periphery of a colony of the type VI contains 7 kinds of cells: fusiform cells (12.25%); fusiform with large nucleus (2%); process-containing cells (5.25%); long fusiform cells (0.25%); triangular cells (6.25%); cells with multiple processes (4%) and quadrangular cells with short processes and large cytoplasm (70%).

B. Cell morphology and AP activity

Colonies of CFU-f are composed of several types of cells in different proportions (Table

5) and cells with morphology have different AP activity.

Not all fusiform cells (being the basic cells of all types of colonies) contain the products of the reactions mediated by AP. Enzyme activity is lowest in such cells as compared with other types of cells. The incidence of mitotic forms is higher in the fusiform cells.

All fusiform cells with large nuclei occurring most frequently in the type II colonies contain AP. Long fusiform cells can rarely be seen only in type I and VI colonies. They show very low AP activity.

Cells having 2 to 3 processes most frequently observed in the type III colonies show AP activity with moderate intensity whereas cells with multiple processes occurring in all of cells (Fig. 15).

Quadrangular with short processes and large cytoplasm at low percentage occur in types III-V colonies and form 2/3 type VI colony. These cells show high AP activity.

Deeply colored reaction products occur in cytoplasm, and outside cells in sites with a high density of triangular and quadrangular cells (Fig. 16).

Equally low percentages (1–2.25%) of round cells occur in all colonies. In such cells, AP has either no activity or its activity is negligible. The morphology of such cells is actually identical to that of the cells which adhered on the first day of culturing.

A layer of cells having the morphology of typical osteoblasts occurs at the periphery of large calcium deposits in the type VI colony.

C. Other variables

Gender, age and patient's blood group have no impact on colony subtype formation (see Tables 6, 7 and 8) instead donor site does have an impact. CFU-f derived from iliac crest bone produce only type I-V colonies and type IV and V colonies were more frequent whereas type I and II colonies were less frequent.

Discussion

Morphological heterogeneity of fibroblasts in cultures has been repeatedly emphasized in literature. It is known that cultures contain haemopoietic cells and macrophages during the early two weeks of culturing [15]. However, by the end of the first week, haemopoietic cells undergo dystrophic changes, many of such cells are in a state of cytolysis, and only fibroblast-like cells can survive and proliferate.

Macrophages disappear in two weeks. Fibroblasts are present as large, different-shaped cells: round, diamond, fusiform and quadrangular. In addition, the same culture may contain colonies of different numbers of cells (from 50 to 140) having different shapes and packing [16]. Separate colonies differ from each other in their microscopic structure and size [17].

Studies of stroma precursor cells in vitro have revealed that CFU-f of human bone marrow is a heterogeneous population that manifests different 'feeder' sensitivity and the ability to form two different types of colonies – monolayer and multilayer – in culture [11]. Monolayer colonies are loose, are 3.3 ± 0.05 mm in diameter and contain 50 to 500 cells. Multilayer colonies are dense, are 4.6 ± 0.09 mm in diameter and contain more than 500 cells having high structure-forming potential. A multilayer colony is densely packed with cells which form structures oriented in a helix around the centre of a colony. Calcium deposits are found exclusively in multilayer colonies. The intensity of calcium deposits increases with an increase in the number of layers in a colony. Calcification is rather intense in the centre of a colony, and typical fibroblasts are observed at the periphery of a colony. The ratio of multilayer to monolayer colonies is different in different cultures and depends on the source of bone marrow cells and efficacy of cloning [11,13].

In this study additional data on the heterogeneity of human bone marrow CFU-f are reported. Eight types of CFU-f cells differing in their morphological characteristics (i.e., shape, size, 'nucleus/cytoplasm' ratio) and in their functional characteristics (i.e., capacity to organize monolayer and multilayer colonies, occurrence of 6 types of colonies differing in their architectonics and composition of CFU-f, different rate of depositing of calcium salts by the cells of a colony, peculiarities of type VI colonies which are observed only as derivatives from mandible).

Cytochemical studies make it possible to estimate the AP activity of cells and the trends of differentiation. AP is an osteoblasts marker enzyme and it mediates in the ossification of bone and cartilage [18]. The occurrence of this enzyme in a cell culture testifies to the osteogenic differentiation of such cells [19]. Thus, according our data, the round cells may be considered stem cells in themselves or cell-precursors and have several mitosis. All types of fusiform cells showing mitotic

forms are more mature but still capable of mitosis. Such cells containing no AP are less mature than those containing the enzyme marker. This fact complies with the data from 7-day-old cultures where there is a drop in the activity of AP: almost all cells forming such a colony have no AP. Triangular and quadrangular cells showing high activity of AP may be considered as preosteoblasts.

Type VI colony is peculiar to the mandible; the centre of the colony is a deposit of plenty of calcium salts (under the guise of a block); the periphery of the colony contains 76.25 % of cells with high activity AP. This datum is new to the best of our knowledge.

Type IV and V colonies are more frequent and they contain more cells having the highest AP activity (26.25%–35.5%). Therefore, we can suggest that colony type IV and V testify to the normal function of osteogenic cell-precursors.

Conclusion

In conclusion, nine kinds of cells differing in their morphological, functional and cytochemical characteristics are observed in CFU-f colonies. There are 6 basic types of CFU-f colonies: type VI is produced only by mandibular samples. A stem stroma cell of the human bone marrow undergoes a whole cycle of differentiation from stem cells to osteoblasts during 12–14 days in vitro. All stages of the development of a stem stroma cell can be found within one colony. This data could be relevant to estimate the potential regeneration of bone.

The reported data add new information as regard cell morphology and colonies organization. In addition the efficiency of colony formation can be utilized as a preliminary test of bone regeneration potential.

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