

Mesenchymal Cells and Fibrocytes in Peripheral Blood. Culture Conditions

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Objectives: The fibrocytes, the mesenchymal cells, and the colony forming units are included in the concept fibroblast-like, peripheral-blood circulating stem cells. The aim of this work was to establish the culture conditions.

Material and method: Peripheral blood mononuclear cells were isolated from 25 samples collected from the cephalic vein. These were cultured in McCoy's 5A enriched with 20% of fetal bovine serum. The culture was read at 7, 14, and 21 days, evaluating the proliferation, confluence, and the round cell/spindle cell ratio. Moreover the 21st day surface markers and phagocytic activity testing was carried out.

Results: Growth was achieved in all the cases but only in 2 dishes were confluence areas retrieved. Spindle cells were 36 (3.71%) (mean [SD]). All the cells showed intense signal against vimentin, CD34 marker, and the NBT test were positives.

Conclusions: Clear CD34 expression, positivity against a connective tissue marker such as vimentin and positivity in a phagocytic assay like the NBT-test, support that stem cells, monocytes, and fibroblasts share common characteristics.

Key words: Fibroblast-like. Mesenchymal stem cell. Fibrocyte. Culture.

Células mesenquimales y fibrocitos en sangre periférica. Condiciones de cultivo

Objetivos: El término *fibroblast-like*, célula madre de sangre periférica, engloba a las unidades formadoras de colonias fibroblásticas, los fibrocitos y las células

mesenquimales. El diseño de este trabajo intenta establecer las condiciones de cultivo.

Material y método: En 25 muestras de sangre venosa se aislaron células mononucleares y se sembraron en medio de cultivo McCoy's 5A con suero bovino fetal al 20%. A los 7, 14 y 21 días se evaluó la proliferación, la confluencia y la relación células redondas/células alargadas. El día 21 se valoró la morfología, los marcadores de superficie y la actividad fagocítica.

Resultados: En todos los cultivos se detectó crecimiento celular positivo para la vimentina, CD34 y NBT. La media \pm desviación estándar del $36 \pm 3,71\%$ eran células alargadas (fibrocitos) y sólo en 2 casos se registraron áreas de confluencia.

Conclusiones: Los métodos de cultivo descritos son adecuados para los seudofibroblastos de sangre periférica. La positividad de CD34, prueba NBT y vimentina demuestra que los seudofibroblastos tienen características comunes con la célula madre, los monocitos-macrófagos y los fibroblastos.

Palabras clave: *Fibroblast-like*. Células mesenquimales. Fibrocito. Cultivo.

Introduction

Fibroblastic morphology cells derived from peripheral blood have both the capacity of self-renewal and to originate specialized cells, characteristics which are typical of progenitor or stem cells (SC)¹ and, in order to integrate all of these morphological variations, the term fibroblast-like² cells is employed, which envelops fibroblastic colony forming units (CFU-F), fibrocytes, and mesenchymal cells (MC). The differences between these cells are still not well defined and the attempts by Castro-Malaspina et al,^{3,4} to demonstrate fibronectin and collagen in the CFU-F, or those of Ucala et al⁵ and le Blanc et al,⁶ using surface markers, were not able to provide a definite answer. A very relevant piece of this puzzle is the one placed by Zhao et al⁷ by confirming the pluripotential stem cell function of monocytes and their derivatives, fibroblast-like cells, and demonstrated that fibroblastoid cells CD14+,

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CD34+, and CD45+ can be induced to differentiate into macrophages, lymphocytes, epithelial cells, neurons, and hepatocytes.

The potential of the clinical use of MC/fibrocytes originating in the bone marrow, is evermore important as their therapeutic usefulness in cartilage⁸ and bone⁹ repair is proven.

The design of this study attempts to establish the culture conditions for MC/fibrocytes from peripheral blood, evaluating cell type identification, and which parameters are the most adequate for their quantification; it is the first step to detect in the future possible variance in the patterns of growth related to inflammatory rheumatic disease.

Material and Methods

Venous blood samples were obtained from 25 healthy donors between 24 and 61 years of age, in sterile tubes with conservative-free heparin. Mononuclear cells (MNC) were isolated after extraction by centrifugation at 400 g for 30 min in a 1077 density gradient. MNC were washed 3 times in McCoy's 5A culture media and were quantified using a Coulter Counter[®].

The culture was done using a modification of the techniques described by Gordon et al¹⁰ and Kaneko et al.¹¹ Using 35 mm diameter Petri dishes, 106 MNC were seeded in 3 mL of McCoy's 5A culture media with antibiotic supplements (100 U/mL of penicillin) and 20% de-complemented fetal bovine serum. They were cultured at 37°C, with a CO₂ pressure at 5% and a humidity at the point of saturation. Half the culture media was removed every week and substituted by fresh media.

Cultures were evaluated at 7, 14, and 21 days after the date of seeding using an inverted Zeiss Axiovert 25 microscope. On the 21st day, the slides were removed to evaluate the morphology (May-Grünwald-Giemsa stain), surface markers (CD34 and vimentin), and phagocytic activity (NBT test) (Figures 1 and 2). The immunocytochemistry study was done using an alkaline phosphatase-antiphosphatase conjugated with streptavidine technique (BioGenex HK330-59K; BioGenex, San Ramón, California, United States), and a primary rat antibody was used as an anti-CD34 (PeliCluster CD34;CLB; The Sauquin, The Netherlands), and mouse antivimentin for the expression of vimentin (BioGenex, San Ramón, California, United States). For the NBT test a Park et al¹² was followed, incubating cells in a solution with tetrazolium nitroblue, allowing to visualize the formazan (reduced NBT) in the cytoplasm of the cells with a capacity for bacteriolysis.

Quantification was based on 3 parameters: proliferation, confluence, and round cell/elongated cell ratio. Proliferation defined the number of cells on the plate in every 1 of the lectures and was evaluated on a scale of

Figure 1. Mesenchymal cells.

Figure 2. Confluence area at 21 days (May-Grünwald-Giemsa stain).

0 to 3 in which level 1 represented the presence of some cells that did not allow to consider the absence of growth, and in level 2 indicated a very important growth but without filling the whole plate and therefore excluded level 3. Confluence implied the adherence of elongated cells among themselves that can form authentic cell networks covering the plate. It was evaluated with the same scale as that for proliferation. Round cells and elongated cells represent 2 morphological types that are easily identifiable in culture, involving all of the proliferative elements, although each 1 of them probably represents a very heterogeneous cell system with a different identity and functional meaning. To quantify these 2 cell types, they were considered as parts of a population of 100% and were assigned each a percentage.

Results

– Proliferation: in all of the seeded samples a growth was detected upon lecture on days 7, 14, and 21 with mean values 1.6, 1.6, and 1.4, respectively.

The analysis of this data shows that there are no significant differences in the growth among these 3 culture intervals, nor among the different samples (P for growth=.55).

– Confluence: areas of confluence between elongated cells were registered only in 2 cases.

– Relationship between elongated/round cells: the separation of cell types in round cells and elongated cells remained constant throughout the culture. Elongated cells represented 31%, 35%, and 26% of the total growth on days 7, 14, and 21, respectively. There were no significant differences between the 3 phases (P for round cells=.31).

– Immunocytochemistry: vimentin and CD34. All of the cells in the culture, round cells and elongated cells expressed intense positivity for vimentin. CD34 was positive in 53% of elongated cells.

–NBT test: granules of formazan (NBT positive cells) were detected in 80% of round cells and 35% of elongated cells.

Discussion

We still cannot say with certainty if the fibroblastoid cells (fibroblast-like) that can be obtained through liquid culture of peripheral blood are the same cells as fibrocytes or circulating mesenchymal cells, because the surface markers, concretely CD34 –which is an indicator of the status as a hemopoietic stem cell–, are expressed in fibrocytes⁵ and in the cells described by Zhao et al⁷ and is negative in mesenchymal cells⁶. Our results show evidence of a clear expression of CD34+ in fibroblast-like cells that, in addition to the positivity to vimentin (a connective tissue marker) and the NBT-test (an indicator of phagocytic activity), points to a cell with common characteristics of the stem cells, monocytes-macrophages, and fibroblasts.

The lack of uniformity in the expression of surface markers, as documented in the scientific literature, certainly is related to the variability of the culture conditions, including the number of seeded cells, the culture media, the concentration of bovine fetal serum, the culture periods, stimulation factors for differentiation, and the methods for detecting surface markers (immunocytochemistry or flux activated cytometry).¹³

Quantification of cultured cells is conditioned by their characteristic adherence to plastic, and unfolding with trypsin produces distortions in the identification of markers; another relevant fact is the number of cells that are obtained, usually small, that is commonly insufficient for their evaluation using flow activated cytometers. The semi quantitative recount method that we employed allows for

a sufficient evaluation of the degrees of proliferation in order to compare results and is easily reproducible; nonetheless, the search for procedures that allow us to individualize the adherent cells is a primary objective in methodology.

Implications of fibroblast-like cells in rheumatic disease is very significant because of their mesenchymal origin, and some effects of anti-inflammatory drugs such as sodium diclofenac, glucocorticoids, or local anesthetics such as mepivacaine have been described on the proliferation of fibroblast-like cells in liquid medium cultures.¹⁴ Alterations in the pattern of growth have also been registered (unpublished observations) as well as the reduction in the chondrogenic and osteogenic activity of the MC in advanced osteoarthritis.¹⁵ The discovery in these past few years of the pluripotential characteristics of these cells (mesenchymal cells/fibrocytes) and their capacity for tissue differentiation, such as bone, muscle, cartilage, and fat, has introduced them fully into clinical practice and regenerative medicine. Liquid medium culture of fibroblast-like cells, originating from peripheral blood cells, and their expansion using growth factors, open up new diagnostic and therapeutic possibilities.

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ERRATUM

In the “In memoriam” published in *Reumatol Clin.* 2007;3(4):I, an omission has been detected. The author signing this text is Dr Josep Blanch (President of the Spanish Society for Rheumatology).