Immunophenotypic characterization of rat bone marrow cells

REPORT

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ABSTRACT

Bone marrow cells (BMC) are currently known to be a potential treatment for a number of diseases of hematological or neurological origin. Since BMC are able to differentiate into neural cells, they are regarded as a valuable asset for the treatment of neurological disorders caused by an underlying loss of neural tissue. This work characterizes BMC from rats using immunophenotypic assays. With this goal, 4 monoclonal antibodies directed against different cell surface markers (CD34, CD38, CD45 and CD90) were tittered according to the specifications of the manufacturer, in order to find their optimal working dilution; and were then used to evaluate rat mononuclear BMC isolated through Ficoll gradients (n=20) with an immunocytochemical technique that allows the direct identification of the target molecules on the cellular surface. The results indicate that the study population is positive for all the antibodies under evaluation, and yield quantitative data about the pattern of expression of these markers on BMC. Furthermore, since the optimal working dilutions determined with the methodology used in this work are higher than the published recommendations without resulting in performance degradation, these findings may represent potential economic savings for further studies.

*I*ntroduction

Bone marrow cells (BMC) represent a reliable and important source for cell transplant in diseases where it is necessary to regenerate damaged tissue, such as neurological disorders. Due to the ethical issues surrounding other potential sources for cell transplant, such as fetal tissue, compounded by their limited availability, there has been a need for an alternative non-embryonic cellular supply, enriched with progenitor and stem cells [1], and capable of acquiring a neural phenotype both *in vitro* [2, 3] and *in vivo* after a transplant [4, 5]. BMC transplant has been successfully used in animals with trauma-derived brain damage [6] and to reduce the motor deficit that appears in striatal ischemia after the damage [7]. Recently, autologous BMC transplants have been used to revert the cognitive deficit observed in an animal model for Huntington's disease [8].

The results of this work are centered on the titration of different antibodies used for the detection of several proteins on the surface of mononuclear cells (CD34, CD38, CD45 and CD90). This titration allowed the determination of their optimal working dilution, and the characterization of mononuclear cells obtained from rat bone marrow. The data obtained about the immunophenotypic characteristics of these cells can be used to infer their differentiation status and evaluate their potential for experimental protocols that may include them in treatment design.

*M*aterials and methods

Sprague Dawley (SD) rats weighing from 250 to 300 g (CENPALAB, Havana, Cuba) were used as experimental subjects throughout this work. Five animals were distributed per cage, with water and food provided *ad libitum*, and a light/darkness period of 12 h [9]. Only animals without signs of infection or lesions of any kind were selected, also excluding rats with bald patches or any other alteration suggesting any abnormality. Sample size was n=20.

Obtention of rat bone marrow cells

Male SD rats were intraperitoneally anesthetized with 7% chlorhydrate (0.6 mg/Kg body weight) and a cut on the skin from the hind limbs was performed, separating the tissue parallel to the bone to extract both femurs. The extracted bones were placed for 30 minutes on a Petri dish containing 0.9% physiological saline, after which the bone marrow was obtained by flushing with sterile PBS (NaCl, 8 g/L; KCl, 0.2 g/L; Na_2HPO_4, 1.09 g/L; KH_2PO_4, 0.26 g/L, pH 7.2) through one of the femoral epiphyses, using a syringe. The BMC were collected in sterile containers to be later washed by centrifugation [10].

Isolation of mononuclear cells from rat bone marrow cells

The suspension of bone marrow cells was washed 3 times with 1X PBS by centrifugation for 10 min at 2 000 rpm, 20 °C. An aliquot of 2.5 mL of Ficoll-Hypaque was placed on the bottom of a graduated glass tube, on top of which 5 mL of the cellular suspension in PBS were layered. This was centrifuged for 45 minutes at 2 800 rpm, 20 °C. The mononuclear cell band was extracted with a pipette and washed immediately, discarding the supernatant into a container with hypochlorite and collecting the cellular pellet, which was suspended in PBS 1X [11].

Immunocytochemical assay

The immunocytochemical assay employed in this study is designed for the specific detection of pro-

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teins on the cellular surface. Briefly, mononuclear cells are bound to 12-well plates, followed by incubation with a monoclonal antibody specific for one of the proteins under analysis (CD34, CD38, CD45 or CD90). After washing off unbound molecules, the amount of bound antibody is detected with antibodies against mouse IgG conjugated to biotin, which are later detected in turn by incubation with a an avidin conjugate, that amplifies the signal, and a chromogenic substrate. The readings of the experiment are carried out on a light microscope [12].

Both the titration of the antibodies and the characterization of BMCs were performed with the methodology described above. Negative controls were included in all cases.

Results and discussion

Table 1 shows the result of the titration of the typing antibodies. Four serial dilutions were assayed per antibody, choosing the highest dilution at which it is still possible to detect an immunophenotypic signal for the cell surface molecule under assay. The CD34 marker was positive at a 1:40 dilution; CD38 at a 1:200 dilution; CD45 at a 1:50 dilution and CD90, at a dilution of 1:20.

Table 2 shows the main characteristics of the antibodies employed in the assay, as well as the working dilution proposed by the manufacturer together with the optimal dilution determined by our methodology. In all cases, the dilution found in this study was higher than that specified by the manufacturer, allowing the processing of a higher number of samples of rat bone marrow mononuclear cells with the same amount of reagent.

In order to characterize the cellular population from rat bone marrow cells, the femurs from SD rats were extracted (n=20), and the percentage of positive cells for the CD34, CD38, CD45 and CD90 markers was determined by our immunocytochemical method. In each case the proper negative control was used (Figure 1 A, B, C, D and E).

Figure 2 shows the average positive cells percentage for each one of the cell surface antigens, and indicates the contribution of each marker to the total percentage of the characterization: CD34=19.33%; CD38=20.80%, CD45=17.27% and CD90=23.52%. The results suggest that the level of expression of these markers in the study population is low.

The cellular surface markers studied in this work have been usually employed for the identification of cells in early stages, but more extensive studies have shown a higher complexity on the immunophenotype of cells from this source, since the range of expressed markers is broader and is also related to the state of cellular differentiation [13, 14]. That result explains the low expression percentages for these markers in this study, since the cell surface antigens appear and disappear from the cellular surface during the evolutionary development of the cell.

Table 1. Working dilution for each monoclonal antibody (mAb).

CD34	1:5	1:10	1:20	1:40*
CD38	1:100	1:200*	1:400	1:800
CD45	1:25	1:50*	1:100	1:200
CD90	1:10	1:20*	1:40	1:80

*Optimal working dilution.

Table 2. Characteristics of the typing antibodies.

AcMo	Subclass	Markers	Working dilution (recommended by the manufacturer)	Working dilution (optimized in this work)
CD34	IgG1	Stromal and hematopoietic cells	1:5	1:40
CD38	IgG1	Hematopoietic cells	1:100	1:200
CD45	IgG2	Hematopoietic cells	1:25	1:50
CD90	IgG1	Stromal cells	1:10	1:20

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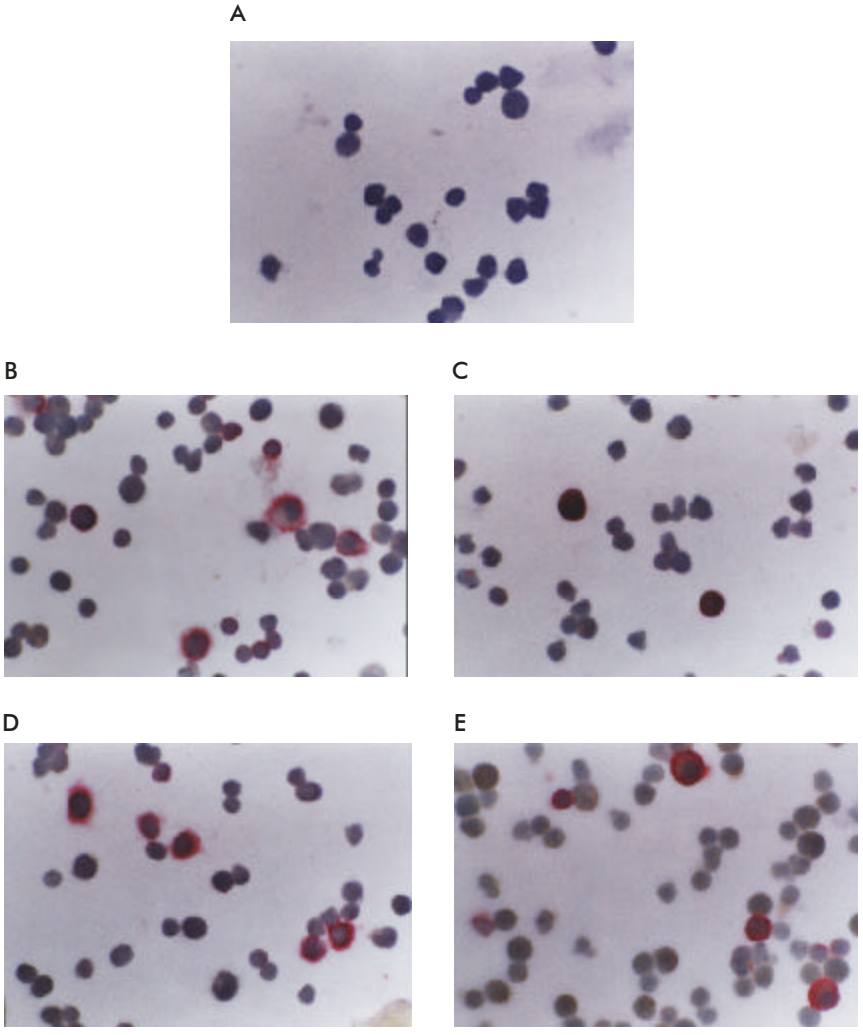

Figure 1. Characterization of mononuclear cells. A) Negative control; B) CD34-positive bone marrow cells, 1:40 dilution; C) CD38-positive bone marrow cells, 1:200 dilution; D) CD45-positive bone marrow cells, 1:50 dilution; E) CD90-positive bone marrow cells, 1:50 dilution.

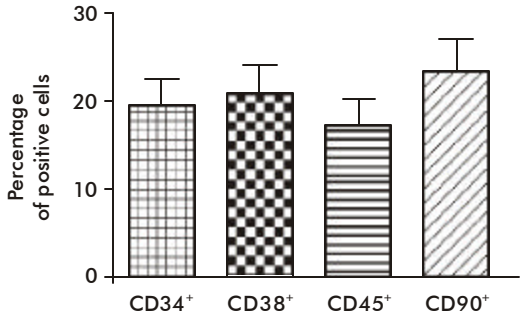

Figure 2. Average positive percentages for the CD34, CD38, CD45 and CD90 markers.

The characteristic immunophenotype for hematopoietic cell subpopulations is defined by the presence of CD34, CD38 and CD45, which constitute cell lineage markers [15, 16]. An additional antigen, CD90, has been identified which is useful for the characterization of the mesenchymal cell subpopulation (also known as stromal stem cells) from the cellular repertoire of the bone marrow [13, 17]. This marker was detected satisfactorily among the cells used in this study and contributed, like the other markers, to the total percentage in their identification.

Several decades ago it was agreed that among the adult stem cells from bone marrow, only one type conserved any kind of regenerative capacity. However, it is currently acknowledged that the cellular composition of bone marrow is far more complex than originally thought, since a heterogeneous group of adult stem cells has been identified that includes, besides the previously mentioned hematopoietic and mesenchymal cells, the so-called lateral population [18] and the adult multipotent progenitor cells [19].

The results from the present study support the current concept of heterogeneity of the population of adult stem cells from bone marrow, since the CD34, CD38, CD45 and CD90 antigens, which represent subclasses of bone marrow cellular subpopulations in different stages of differentiation, were detected in all cases.

Conclusions

1. The population under study was positive for CD34, CD38, CD45 and CD90 at the different working dilutions used in this work.

2. A working dilution was obtained for each antibody which was higher than that recommended by the manufacturer but performed equivalently under our conditions.

3. This study allowed the determination of the differential percentage contributed by each of the cellular markers to the general percentage of the immunophenotypically characterized cells.

4. Our results can represent significant cost savings, taking into account that with the optimization of the working dilution for the antibodies, the amount of reagent used per determination is reduced.

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