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RESEARCH ARTICLE

Ensheathing cell-conditioned medium directs the differentiation of human umbilical cord blood cells into aldynoglial phenotype cells

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Abstract Despite their similarities to bone marrow precursor cells (PC), human umbilical cord blood (HUCB) PCs are more immature and, thus, they exhibit greater plasticity. This plasticity is evident by their ability to proliferate and spontaneously differentiate into almost any cell type, depending on their environment. Moreover, HUCB-PCs yield an accessible cell population that can be grown in culture and differentiated into glial, neuronal and other cell phenotypes. HUCB-PCs offer many potential therapeutic benefits, particularly in the area of neural replacement. We sought to induce the differentiation of HUCB-PCs into glial cells, known as aldynoglia. These cells can promote neuronal regeneration after lesion and they can be transplanted into areas affected by several pathologies, which represents an important therapeutic strategy to treat central nervous system damage. To induce differentiation to the aldynoglia phenotype, HUCB-PCs were exposed to different culture media. Mononuclear cells from HUCB were isolated and purified by identification of CD34 and CD133 antigens, and after 12 days in culture, differentiation of CD34+ HUCB-PCs to an aldynoglia phenotypic, but not that of CD133+ cells, was induced in ensheathing cell (EC)-conditioned medium. Thus, we demonstrate that the differentiation of HUCB-PCs into aldynoglia cells in EC-conditioned medium can provide a new source of aldynoglial cells for use in transplants to treat injuries or neurodegenerative diseases.

Keywords Ensheathing cells · Cell differentiation · Conditioned media · Conditioned medium · Aldynoglia · Umbilical cord blood

Introduction

Human umbilical cord blood (HUCB) precursor cells (PCs) represent an important source of cells capable of proliferating and differentiating into almost any cell type, including neural cells, depending on their environment [1]. While HUCB-PCs share some characteristics with bone marrow PCs, the former are more immature and they exhibit a greater degree of plasticity [2–4].

HUCB-PCs have many advantages over adult PCs as they exhibit a primitive ontogeny and have not been exposed to immunological challenge. This rather naïve immune profile of CB cells may play a significant role in reducing rejection after transplantation into a mismatched host [5]. A major advantage of umbilical cord blood-derived cells is the ease with which they can be obtained in a non-invasive manner following newborn delivery. Moreover, these cells can be cryo-preserved and, thus, made available for autologous transplantation even years after harvesting.

Therapeutically, the use of PCs, and in particular HUCB-PCs, offers many potential benefits [6] for the treatment of neurological disorders [7] and neurologic deficits [8]. These cells have a significant capacity to develop and differentiate into multiple tissues following damage or lesion. Certain cells in HUCB can differentiate into neural cells, which may account for the restorative effects observed in animals injected with HUCB [9]. Although the amount of HUCB-PCs

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available is relatively low, the availability of neural precursor cells from human sources is scarcer still, particularly due to concerns regarding the use of fetal tissue. However, while HUCB represent an accessible cell population that can differentiate into glial cells, neurons or other cell phenotypes [10], the molecular signals that drive their differentiation remain unknown. As such, further understanding of the mechanisms mediating proliferation and differentiation of HUCB-PCs in vitro is essential to develop new therapeutic strategies. Despite growing knowledge in recent years regarding direct stem cell differentiation, methods of differentiating stem cells into specialized cell types remain in their infancy.

Glial cells constitute the main source of neurotrophic factors in the brain, where they participate in neurotransmitter activity and metabolism, and where they regulate the ionic and metabolic environment during degenerative, regenerative, and repair processes following CNS lesion [11]. Some studies have demonstrated that the presence of particular glial cells elicits neuronal growth in the adult CNS. These regenerative properties have been best documented in olfactory bulb ensheathing cells [12]. Together with other glial cell phenotypes, this cell population consists of a central macroglia known as aldynoglia, which promotes neuronal growth and regeneration [13, 14] and constitutes a cell phenotype rather than a particular lineage [15, 16].

Alternative strategies for neural replacement are of particular interest given the problems associated with donated tissue, which include ethical issues (the use of human embryo donors or patients after brain death) and immunological considerations (allogenic transplants) [17]. To overcome these difficulties, we sought to induce the differentiation of HUCB-PCs to generate aldynoglia cells for transplantation into zones affected by CNS pathologies. We have previously demonstrated that neural precursor cells from E14 rats selectively differentiate into the aldynoglia phenotype by induction using olfactory ensheathing cell-conditioned medium [15, 16]. In the present study, we investigated the capacity of two distinct subpopulations of HUCB-PCs to differentiate in EC-conditioned medium.

Experimental procedures

HUCB cells and HUCB-PC primary cultures

A total of 30 HUCB samples were obtained from full-term normal deliveries, with informed consent of the mothers and the approval of the Ethics Committee for Clinical Research of the Hospital "Fray Antonio Alcalde". Samples were collected in sterile bags (Terumo PB-1CD456L) containing sodium citrate, sodium phosphate, dextrose and adenine (CPDA). The samples were stored at room temperature and processed within 24 h.

Umbilical cord blood was diluted 1:1 in Hanks solution with Ca^{2+} and Mg^{2+}, and subsequently mixed in 4 ml Ficoll. This solution was then centrifuged for 30 min at 300*g* in a Ficoll density gradient (1.077 g/ml; GE Healthcare 171440-03). Mononuclear ring cells were removed and washed in DF10S and centrifuged again for 10 min at 300*g*. Sedimented cells were resuspended in 5 ml DF10S medium. Mononuclear cells were routinely quantified in a Neubauer chamber. Under these conditions, we obtain approximately $4.02 \times 10^6 \pm 2.14 \times 10^5$ mononuclear cells per sample.

Mononuclear cells from HUCB were cultured in different media (described in Table 1) to analyze proliferation: (1) DF20S, (2) umbilical cord blood plasma (UCBP), and (3) B27–EGF + FGF chemically defined medium. Cultures were maintained in a 5 % CO_2 atmosphere and the medium changed twice a week. Cells were maintained in culture for a total of 12 days.

Ensheathing cell cultures and conditioned medium

Primary EC cultures from the olfactory nerve and glomerular layers of adult Wistar rats were prepared, immunopurified and stored at −20°C, as described previously [18]. Conditioned media were obtained as described previously [15]. Briefly, purified OEC were plated at a density of between 1.0 and 2.5×10^4 cells/cm^2, and the medium was collected between 3 and 8 DIV. The conditioned media were maintained at −20°C until it was used, and it was disposed of once it had been used.

Positive magnetic immunopurification and maintenance of CD34+ or CD133+ cells

Ensheathing cells were immunopurified with anti-low-affinity NGF receptor (anti-NGFR, clone 192), as previously described [18]. Before their expansion, mononuclear cells from HUCB were immunopurified in a similar manner and isolated using specific antibodies for CD34 or CD133 antigens. Magnetic beads (Dynabeads, Dynal M-450) were coated with anti-CD34 (Santa Cruz SC7324) or anti-CD133 (MAB 4310) and diluted at approximately 4:1 per cell (4×10^8 dynabeads per ml). This positive purification enabled erythrocytes, platelets and other non-positive cells to be eliminated. The purified cells were then seeded on polylysine-coated culture plates at a density of 1×10^6 cells per cm^2 and maintained in different media (Table 1): UCBP with penicillin/streptomycin (Sigma P4333); 1:100 B27–EGF + FGF media (GIBCO 17504-044); or DF10S (GIBCO 16050-122; Fetal Bovine Serum, Sigma F2442). The cultures (1×10^6 cells/25 cm^2) were expanded

Table 1 Culture medium identification

Medium	ID	Content
DMEM-F12	DF	SIGMA D8900
Bovine fetal serum	BFS	SIGMA F2442
Horse serum	HS	GIBCO 16050-122
DMEM-F12 with one serum	DF10S	DF + BFS (10 %)
Medium with two serums	DF20S	DF + HS (10 %) + BFS (10 %)
B-27 supplement	B27	GIBCO 17504-044
Chemically defined medium	DF-B27	DF:B27 (1:100)
Chemically defined medium plus growth factors	B27–EGF + FGF	DF + B27 + EGF + FGF (10 ng/ml)
Umbilical cord blood autologous plasma	UCBP	DF + plasma (10 %)
Ensheathing cell-conditioned medium	ECcm	B27 conditioned by ensheathing cells

in flasks (Nunc F23-641) at 37°C and 5 % CO_2 in a fully humidified atmosphere for 12 days. Once optimal conditions for the maintenance of the PCs were established, differentiation was induced over 48 h with ECcm or B27 media (control).

Induction of differentiation with EC-conditioned medium

Prior to differentiation, the medium was removed and mononuclear CD34+ or CD133+ cells were recovered by centrifugation. The cell pellet was resuspended in DF-B27 in order to wash the cells and completely change the medium. The cells were then resuspended in ECcm without serum, and plated at a density of 1×10^5 cells/cm^2 on Lab-tech plates coated with poly-L-lysine (Sigma) and laminin (Invitrogen). The cells were left to differentiate for 48 h before they were analyzed by immunohistochemistry.

Differentiation of control cultures was induced with serum-free B27–EGF + FGF, together and separately. Cells were fixed in 4 % paraformaldehyde for 8 min and washed with 1 % PBS prior to analyzing the expression of p75, GFAP, NG2, vimentin, Dlx2, and A2B5 by immunocytochemistry.

Immunocytochemistry

The following primary antibodies were used: polyclonal rabbit IgG anti-GFAP antiserum (DAKO Z0334); monoclonal mouse IgG anti-NGFR (Calbiochem mAb192); monoclonal mouse IgG anti-vimentin (DAKO M0725); polyclonal rabbit IgG anti-NG2 antiserum (Chemicon AB5320); monoclonal mouse IgG anti-A2B5 antiserum (Millipore MAB312); and polyclonal rabbit IgG anti-Dlx2 antiserum (Millipore AB5726). All antibodies were diluted 1:1,000 in PBS containing 0.1 % bovine serum albumin. For intracellular labeling, fixed cells were treated with PBS containing 0.1 % Triton X-100 and 1 % normal goat serum. Alexa Fluor 594 goat anti-rabbit IgG's and Alexa Fluor 488 anti-mouse IgG's (Molecular Probes) were used as secondary antibodies at a dilution of 1:1,000. Immunostaining of cytoskeletal or membrane antigens in cultured cells grown on cover slips was performed as described previously [14]. Preparations were mounted on glycerol:PBS (1:1) and examined on a phase-contrast and fluorescence microscope (Olympus, BX51) fitted with a Penguin 600CL photographic system (Pixera).

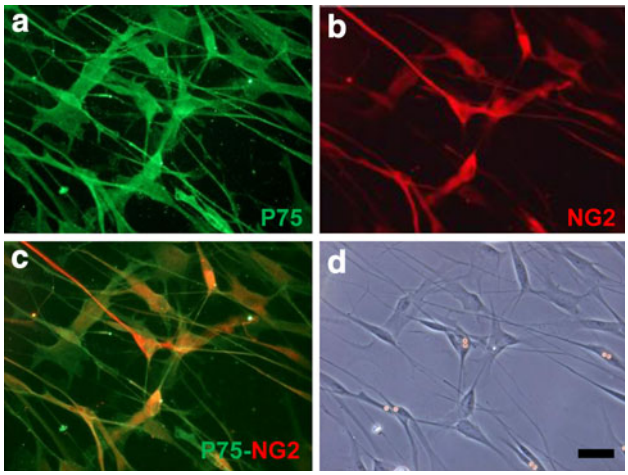

Fig. 1 Ensheathing cells phenotype markers: p75 (**a**), NG2 (**b**), double labeling p75-NG2 (**c**), phase contrast (**d**). *Scale bar* 20 μm

Results

Immunocytochemistry of ensheathing glial cells

After immunopurification and for their identification, EC were co-labeled with either p75 and GFAP (data not shown) or with NG2 and GFAP antibodies (Fig. 1a–c).

Immunopurification and maintenance of CD34+ or CD133+ subpopulations in different culture media

Blood plasma contains a high concentration of growth factors such as PDGF, FGFb, VEGF, IGF-1, and TGF-b [19, 20], which may promote the proliferation and differentiation of mesenchymal stem cells [20–22]. To determine the optimal conditions in which to maintain the cells prior to inducing differentiation, we investigated the survival and proliferation of mononuclear cells from HUCB in 3 different culture media: (1) B27–EGF + FGF, (2) DF20S, and (3) UCBP.

In accordance with previous studies [23, 24], the immunopurified and enriched CD34+ and CD133+ cell populations represented approximately 2 % of the initial cell population. After purification, cells were plated at initial density of 1.0×10^6 cells per cm^2.

When CD34+ cells were cultured in B27–EGF + FGF, the majority of the cell bodies were spherical, although many cells were attached to the substrate and extended bipolar processes. However, a few cells survived up to 72 h in culture. Likewise, in parallel cultures in which the CD34− fraction was maintained under the same conditions, the majority of cells had died after 72 h in culture. Most CD34+ cells cultured in UCBP attached to the substrate and differentiated, adopting a bipolar morphology, with only a few spherical or unattached cells. In parallel cultures of CD34− cells, the majority of cells attached to the substrate and emitted multipolar processes (Fig. 2a, b). CD34+ cells maintained in DF20S remained in suspension and formed colonies or groups. The cell density was preserved over the 72 h in culture but no cells attached to the substrate and extended bipolar processes (Fig. 2c, d). From the initial plated cells almost 90 % survived ($8.9 \times 10^5 \pm 3.0 \times 10^4$) in DF20S. Based on these findings, we concluded that undifferentiated CD34+ cells were best maintained in DF20S prior to differentiation.

The majority of CD133+ cells cultured in B27–EGF + FGF medium differentiated into bipolar cells, in conjunction with a marked decrease in cell density. Most CD133− cells cultured in the same medium died, while spherical cells surviving after 72 h had a smaller soma. When CD133+ cells were cultured in DF20S, the majority of cells attached to the substrate and developed a bipolar soma (Fig. 2e, f). By contrast, cells from the CD133− fraction in the same medium died within 72 h in culture and, due to the low cell density, no immunocytochemical labeling was performed.

The above results demonstrate that the mononuclear cell fraction from HUCB was most effectively maintained in DF20S, in which the CD34+ cell fraction survives better than the CD133+ fraction.

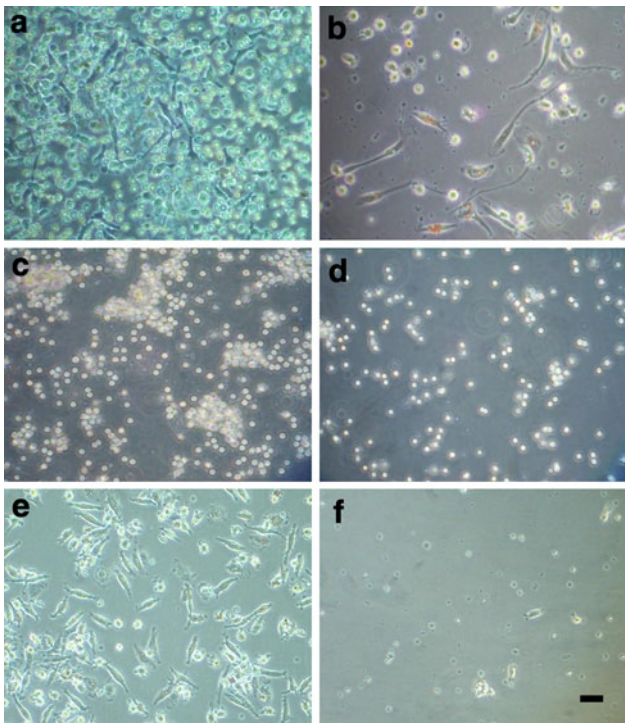

Fig. 2 HUCB-PC subpopulations cultured in different conditions. CD34+ cells cultured in UCBP at 24 h (**a**) and 72 h (**b**). CD34+ (**c**) and CD34− (**d**) cells cultured in DF20S. CD133+ (**e**) and CD133− (**f**) cells cultured in DF20S. *Scale bar* 20 μm

Differentiation of the CD34+ and CD133+ subpopulations in ensheathing cell-conditioned media

HUCB-PCs can differentiate into different cells lineages and express several neuronal markers in vitro, including vimentin, nestin, musashi 1, Oct-4, TuJ1, NCAM, A2B5, GFAP, S100, GalC, and MAP2 [25, 26]. Aldynoglia express some characteristic markers, including p75 (low affinity neurotrophin receptor), estrogen receptor, O_4 (sulphatide seminolipid antigen), vimentin, GFAP, and markers of migration such as PSA-NCAM (reviewed in [27]). Following purification, we characterized the phenotype of the CD34+ or CD133+ HUCB-PCs under differentiation conditions using antibodies against the following markers: GFAP, NGFr (p75), vimentin, NG2, A2B5 and Dlx2.

After culturing both HBC-PCs subpopulations (CD34+ or CD133+) in different media (B27–EGF + FGF, DF20S or UCBP), the differentiation of the cells in EC-conditioned media was analyzed. After 96 h in EC-conditioned media, many CD34+ cells previously cultured in DF20S adhered to the substrate, presenting a bipolar morphology with cytoplasmic prolongations similar to (although shorter than) those of ensheathing cells. Most differentiated cells exhibited intense labeling for p75 but not for NG2, and these two markers were not co-expressed

(Fig. 3a–d), suggesting that the differentiated cells had an aldynoglia phenotype. In contrast, when CD34+ cells were induced to differentiate using B27–EGF + FGF, they developed short prolongations and oval somas, and while most of these cells expressed GFAP, very few expressed p75 (Fig. 3e–h). The co-expression of these markers is characteristic of aldynoglia and, hence, even though these cells appeared to differentiate into a glial phenotype (GFAP+), they did not adopt an aldynoglia phenotype. Furthermore, CD34+ cells cultured in B27–EGF + FGF and DF10S presented a heterogeneous morphology, with some bipolar cells among the mainly spherical cells. Most of these cells were expressed NG2 and only rarely vimentin (Fig. 3i–l). Therefore, the majority of these cells would appear to have differentiated into a neural rather than a glial phenotype.

When CD34+ cells were maintained in UCBP and then differentiated in EC-conditioned media, some cells developed fine but short prolongations, although many cells remained spherical. Few cells expressed the p75 receptor but most were contained vimentin (Fig. 4a–d), suggesting that their differentiation may have been delayed in these conditions. In comparison, only a few cells differentiated with B27–EGF + FGF having been maintained in UCBP developed cytoplasmic prolongations, with almost all cells remaining spherical. Since most of these cells co-expressed vimentin and p75 (Fig. 4e–h), they appeared to be poorly differentiated. Finally, when CD34+ cells were maintained in UCBP, a range of different morphologies was observed, which included rounded, spherical, oval and bipolar somas. Almost all cells expressed p75 and virtually none contained vimentin (Fig. 4i–l).

When CD133+ cells were exposed to EC-conditioned media, the cells differentiated into a phenotype characterized by a large soma, some of which were star-shaped. Most cells expressed vimentin, while some expressed Dlx2 (Fig. 5a–d). CD133+ cells were differentiated in B27–EGF + FGF and DF10S as a control, producing a heterogeneous population of cells, with spherical, bipolar and star-shaped somas. Most cells expressed vimentin and a few expressed Dlx2 (Fig. 5e–h). Finally, differentiation of CD133+ cells in B27–EGF + FGF produced several morphologically distinct cell types, although no cells expressed any of the markers studied by immunocytochemistry (data not shown).

Based on these findings, we conclude that, once exposed to EC-conditioned media, cells derived from HUCB can acquire the characteristics of the aldynoglia phenotype, although some cells also express these markers when exposed to DF-B27 medium. Furthermore, cells of the CD34+ population express markers of the phenotype of

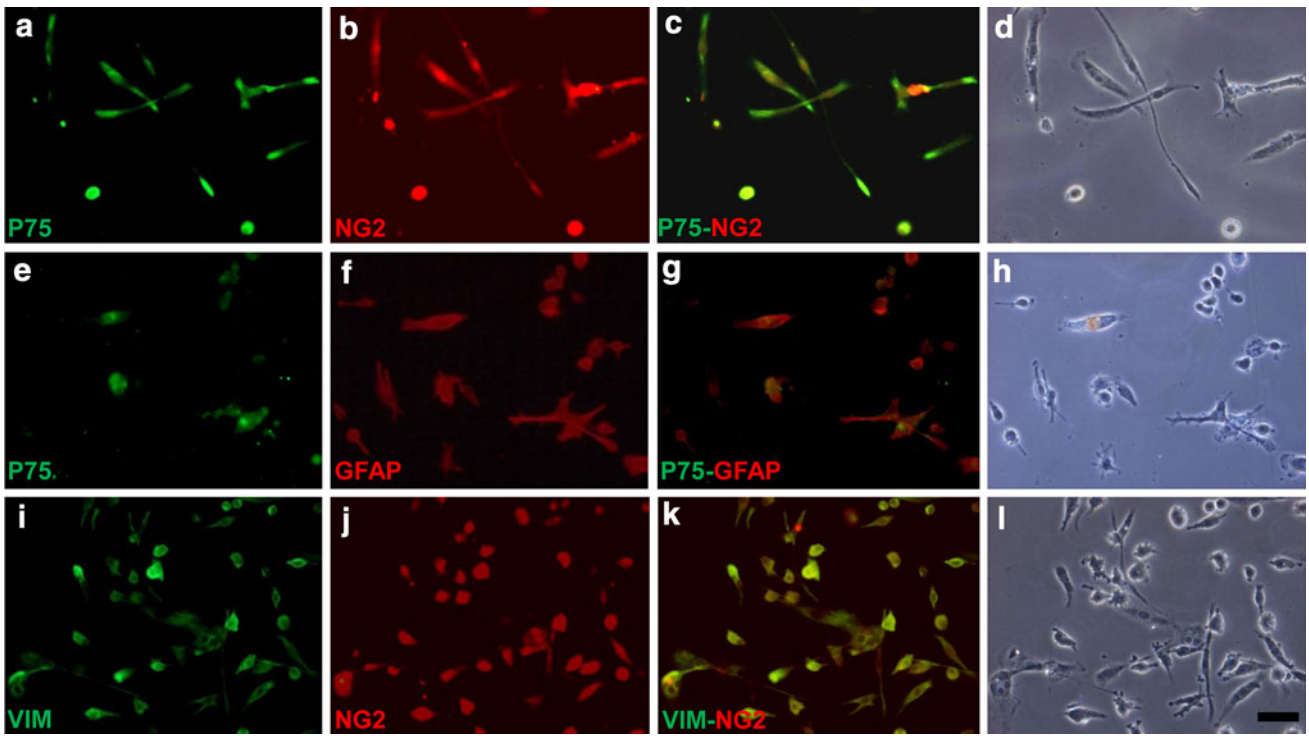

Fig. 3 Immunocytochemistry of CD34+ HUCB-PCs cultured in DF20S and differentiated over 96 h in different media. Differentiation induced with ECcm (**a**–**d**), B27–EGF+FGF (**e**–**h**) and in DF10S (**i**–**l**). Labeling is as follows: **a**, **e** p75; **b**, **j** NG2; **f** GFAP; **i** vimentin; **c** p75/NG2 double-labeling; **g** p75/GFAP double-labeling; **k** vimentin/NG2 double-labeling; and **d**, **h**, **l** phase contrast. *Scale bar* 40 μm

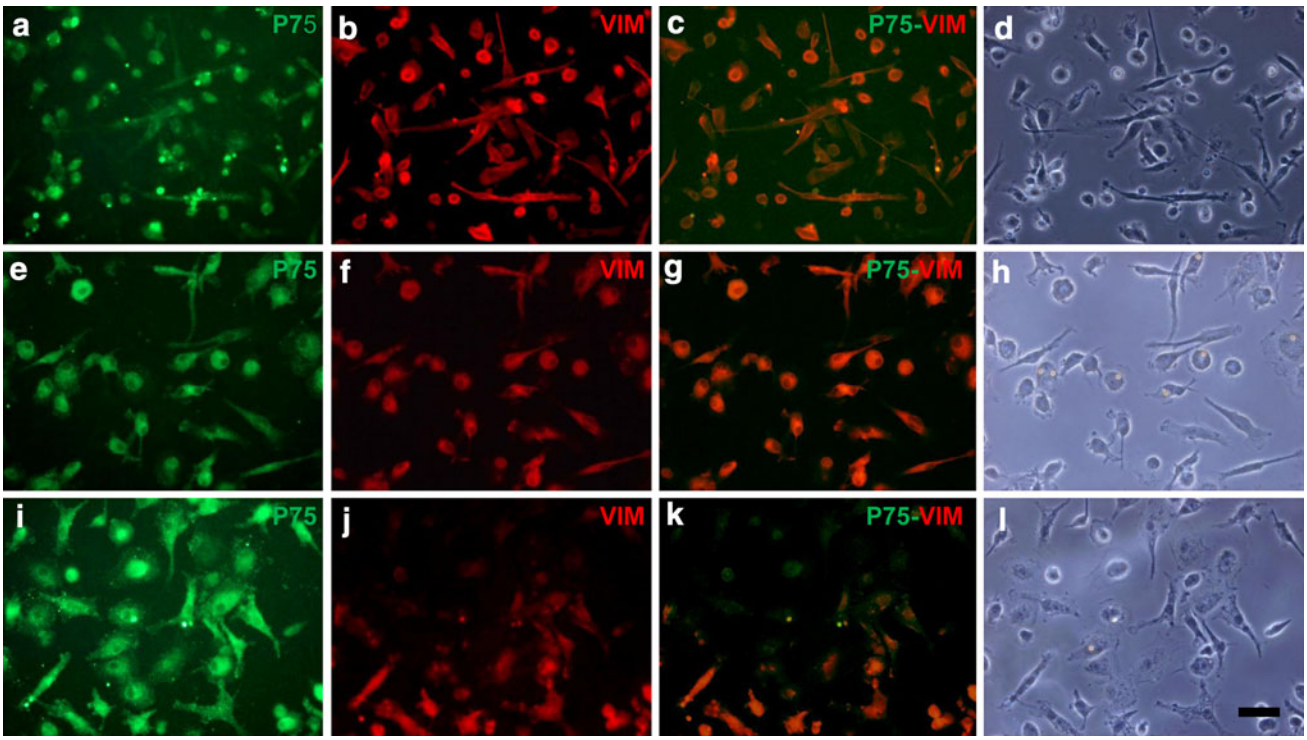

Fig. 4 Immunocytochemistry of CD34+ HUCB-PCs cultured in UCBP and differentiated over 96 h in different media. Differentiation induced with ECcm (**a**–**d**), B27–EGF+FGF (**e**–**h**) and in DF10S (**i**–**l**). Labeling as follows: **a**, **e**, **i** p75; **b**, **f**, **j** vimentin; **c** p75/vimentin double-labeling; **g** p75/vimentin double-labeling; **k** double p75/vimentin double-labeling; and **d**, **h**, **l** phase contrast. *Scale bar* 40 μm

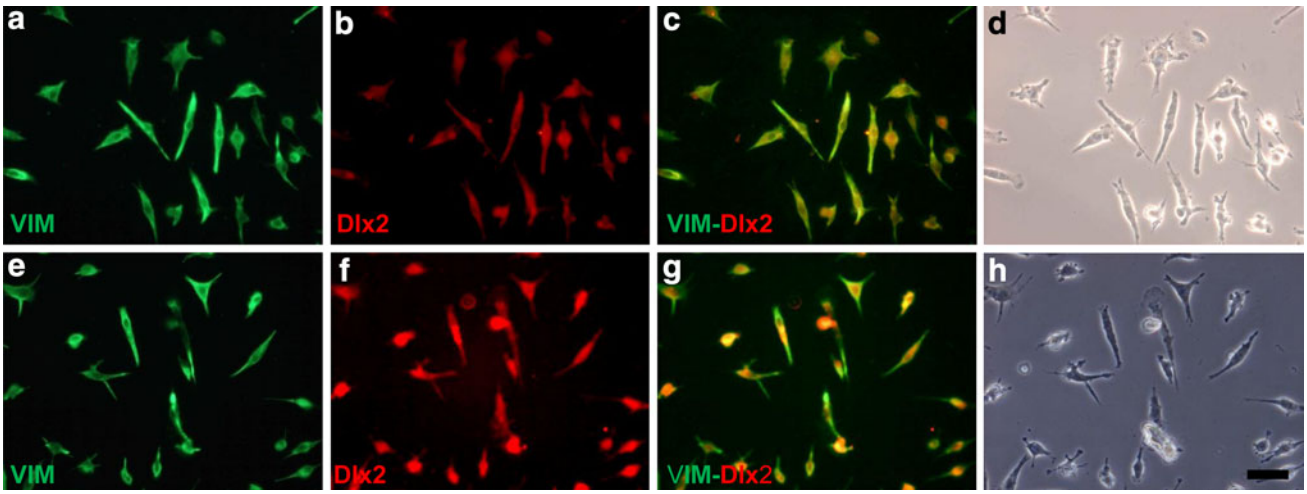

Fig. 5 Immunocytochemistry of CD133+ HUCB-PCs cultured in DF20S and differentiated over 96 h in different media. Differentiation induced by ECcm (**a**–**d**) and DF10S (**e**–**h**). Labeling as follows: **a**, **e** vimentin; **b**, **f** Dlx2; **c**, **g** vimentin/Dlx2 double-labeling; and **d**, **h** phase contrast. *Scale bar* 40 μm

interest, including p75, GFAP, vimentin and NG2 (Figs. 2e–h, 5a–d). Some cells differentiated from the CD133+ population also expressed some of these markers (GFAP and A2B5), although they also expressed neuronal markers such as vimentin and Dlx2 (Figs. 5a–d, 6e–h; see summary in Table 2).

Discussion

In the field of regenerative medicine, HUCB-SCs represent promising candidates for cellular and tissue replacement, largely due to their increased immunological compatibility and lower probability of rejection when compared with

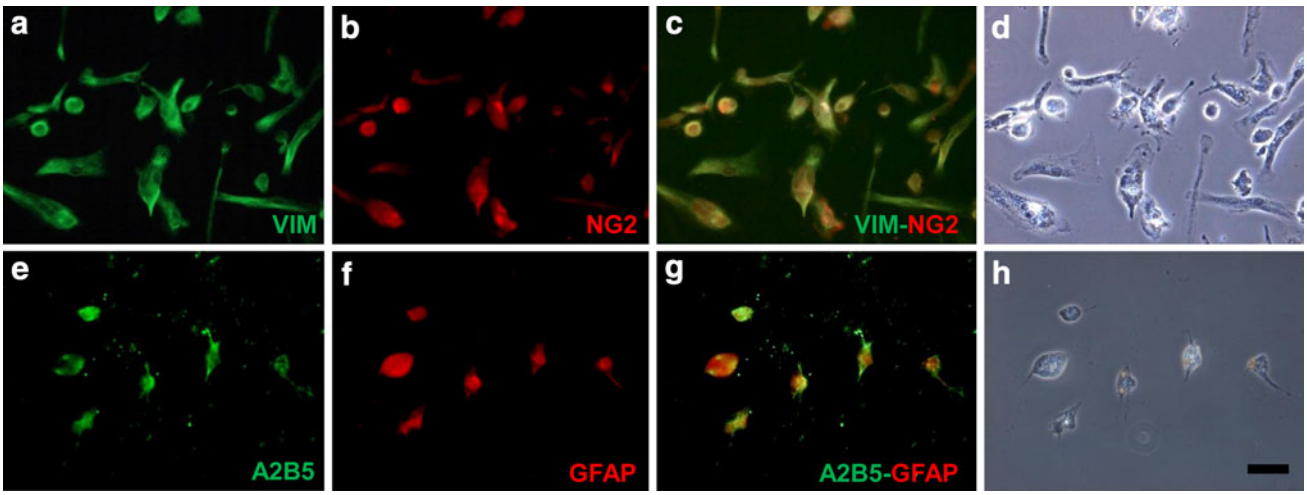

Fig. 6 Immunocytochemistry of CD34+ or CD133+ HUCB-PCs cultured in DF20S and differentiated over 96 h in different media. Differentiation induced with B27–EGF+FGF (**a**–**d**) and ECcm (**e**–**h**). Labeling is as follows: **a** vimentin; **b** NG2; **e** A2B5; **f** GFAP; **c** vimentin/NG2 double-labeling; **g** A2B5/GFAP double-labeling; and **d**, **h** phase contrast. *Scale bar* 40 μm

Table 2 Expression of markers in CD34+ or CD133+ HUCB-PCs after differentiation with EC-conditioned media

Marker	CD34+	CD133+
Vimentin	++	++
P75	++	–
GFAP	++	++
NG2	+	–
A2B5	–	+
Dlx2	–	+

currently available methods [28–30]. The present study demonstrates that an aldynoglia phenotype can be successfully induced in a subpopulation of HUCB cells in vitro using EC-conditioned media. This method provides an important alternative means of obtaining EC for autologous transplants to treat specific lesions or diseases.

Effect of serum on HUCB-PC proliferation

One of the main factors influencing the in vitro differentiation of PCs is the serum used. Chemically defined culture media generally lack the factors required to induce differentiation [31]; however, serum contains a large variety of factors that can influence the fate of PCs [32], although this hampers the identification of individual factors responsible for differentiation into specific cell phenotypes. The substrate on which cells are grown is a second factor that strikingly influences the differentiation process in vitro. Several extracellular matrix molecules can affect the fate of PCs, activating different signaling pathways [33, 34]. Thus, better understanding of the culture conditions and factors that guide PC differentiation is required to design experimental protocols that direct the differentiation of HUCB-PCs into neuronal or glial cells. Here, we demonstrate that PCs can be successfully obtained from HBC-specific subpopulations grown on a poly-L-lysine/laminin substrate in DF20S medium, and then differentiated using EC-conditioned media.

In vitro maintenance of HUCB-PCs under different culture conditions

Varying degrees of success have been obtained when attempting to expand HUCB-PCs by adding different factors to the medium, including: fetal calf serum and FLT3L, thrombopoietin and chemokines, IL-8, MIP1a, VEGF, and glycosaminoglycans [35–37]. Similarly, co-culture with stromal cells or mesenchymal cells has also been attempted [38]. After exposure to mitogens or neuromorphogens, HUCB-PCs can differentiate into neuronal phenotypes that express typical markers of neurons, astrocytes, and oligodendrocytes [31]. However, the differentiation process and the resulting phenotype is significantly influenced by the culture medium [39], supporting the view that HUCB-PCs are not committed to a specific cell fate at this stage. We used three different culture media previously thought to favor stem cell expansion [40]. However, under our experimental conditions, an adequate amount of HUCB CD34+ PCs for subsequent differentiation was only yielded using EC-conditioned media.

HUCB-PC subpopulations

HUCB contains a mixture of different stem cell types in numbers not found in any other location. These include embryonic-like, hematopoietic, endothelial stem cells,

epithelial, mesenchymal, unrestricted somatic and neuronal stem cells [10, 41]. Subpopulations of HUCB-PC can be isolated according to the expression of hematopoietic stem cells markers such as CD34+ [42], CD133+ [43] and CD45+ [44], and these cells have previously been induced to differentiate into a neuronal-like phenotype in vitro. Given the wide variety of subpopulations, we preselected and immunopurified two HUCB-PC subpopulations, CD34+ and CD133+, to determine their behavior in EC-conditioned media.

HUCB-PCs that characteristically express CD34 and CD133 exhibit a certain degree of plasticity and, thus, they are suitable candidates to induce differentiation for therapeutic applications [45]. CD34 is an integral glycoprotein that regulates cell adhesion in hematopoietic cells. Its expression is downregulated during differentiation, diminishing gradually as differentiation proceeds, and, thus, CD34+ is related with an undifferentiated precursor cell phenotype [46–48]. CD34+ cells constitute are very heterogeneous population and only 30–50 % of these cells are precursors. Moreover, in HUCB, not all precursors express CD34+ [49]. Nevertheless, CD34+ cells constitute an important source of cells for clinical trials of graft survival, and a large number of CD34+ cells are used by blood units as selection criteria for transplantation purposes [48].

CD133+ is a surface protein associated with the PCs of many cells types, including hematopoietic, endothelial and neuronal phenotypes. Moreover, in hematopoietic lineages, CD133+ PCs also express CD34 [50]. Interestingly, the expression of CD133 [51], a marker typically associated with stem cells, is lost following differentiation.

Induced differentiation

HUCB-PCs can differentiate into cells of the neuroectodermic lineage and neural cells under certain culture conditions [26, 30, 31, 39, 40, 52–55], and in the stage prior to differentiation the result of this process can be influenced. Co-culturing cells with astrocytes or hippocampal slices provides adequate signaling to direct differentiation towards neural phenotypes [39], and stimulation with 3-isobutyl-1-methylxanthine, dibutyryl-cAMP and retinoic acid results in cells expressing tyrosine hydroxylase (TH) and Nurr1, markers typically associated with DA neurons [56]. Similarly, epigenetic mechanisms have also been implicated in HUCB-PC differentiation [57]. HUCB-PC differentiation can also be induced by the addition of NGF and retinoic acid, resulting in cells expressing some markers of neural development [58]. Most of these cells differentiated into GFAP-expressing cells, rather than cells expressing neuronal markers such as β-tubulin III, suggesting that it may be easier to generate glial cells than neurons from HUCB-PCs. The possibility to direct PC fate in the initial culture period, which may serve as a reprogramming stage rather than representing a merely proliferative phase, has recently attracted much attention.

Most studies of HUCB-PC differentiation analyze the total mononuclear fraction, without separating these cells into distinct precursor subpopulations. Using this approach, it remains unclear which subpopulations differentiate into cells of a neural phenotype. This information is crucial in order to better understand the biology of HUCB-PC differentiation, and to develop new methods by which to specifically induce and direct differentiation into neural cells for therapeutic use [59]. While some attempts to direct HUCB-PC differentiation into specific neural phenotypes have been reported [39, 56], it is still not possible to control differentiation precisely.

We observed that when CD34+ cells were cultured in EC-conditioned media, cells adhered to the substrate within 4 h and, shortly afterwards, they began to undergo morphological changes. This time course coincides with the changes in gene expression previously reported in neural precursor cell differentiation in EC-conditioned media [16]. Subsequently, after 24 h, characteristic markers of aldynoglial cells are co-expressed in newly differentiated cells, such as p75, GFAP, and vimentin [14]. By contrast, differentiation of the CD133+ cell fraction in EC-conditioned media was less apparent, even though these cells adhered to the substrate within 8 h. Moreover, the cells that differentiated from the CD133+ subpopulation expressed Dlx2, in addition to GFAP and vimentin, but not p75 or NG2. Thus, this subpopulation did not preferentially differentiate into an aldynoglia cell phenotype. Based on these findings, we propose that the CD34+ fraction of HUCB-SCs is the most appropriate cell population to direct differentiation into cells towards the glial phenotype.

Possible therapeutic applications of HUCB-PCs differentiated in vitro

HUCB-PCs constitute an important alternative stem cell source, particularly since they can be obtained by non-invasive methods and they have only weak immunogenicity [60]. Due to the radically different characteristics, properties, functions and responses of these cells at different stages of differentiation, it is important to define whether these cells will be transplanted as partially differentiated precursors or as cells with a specific differentiated phenotype when considering their therapeutic potential. Developing clinical serum-free media to maintain and expand human stem cells is an important area of research in regenerative medicine. Some success has been recently achieved with the use of defined serum-free medium to support the in vitro proliferation of human

mesenchymal stem cells [61, 62]. Several of the factors involved in the differentiation of aldynoglia phenotype cells from PCs have also been identified [15, 16]. Here, we demonstrate that HUCB-PCs respond to EC-conditioned media by differentiating into an aldynoglia phenotype. Further studies will be necessary to identify the specific factors required to direct this differentiation process, which will provide a new source of aldynoglial cells for transplantation to treat CNS injuries or neurodegenerative diseases.

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Conflict of interest None.

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