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Biochemical and Biophysical Research Communications 323 (2004) 315–322
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Pancreas developing markers expressed on human mononucleated umbilical cord blood cells

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Received 28 July 2004

Abstract

Haematopoietic system represents the main source of haematopoietic stem cells and probably of multipotential adult progenitor cells and mesenchimal stem cells at first described as colony forming unit-fibroblast. Whereas there are many studies on the gene expression profile of the different precursors along their haematopoietic differentiation, few data (sometimes conflicting) have been reported about the phenotype of the cells (present in bone marrow and possibly in cord blood) able to differentiate into non-haematopoietic cells. As both postnatal bone marrow and umbilical cord blood contain nestin positive cells able to proliferate and differentiate into the main neural phenotype (neuron, astroglia and oligodendroglia) many authors considered nestin a neuroepithelial precursor marker that seems to be essential also in multipotential progenitor cells of pancreas present both in rat and in human pancreatic islets (called nestin positive islet derived progenitors). Although the importance of nestin in these cells appears to be evident, it remains yet to clarify the number and the sequential expression of the genes coding all the transcription factors essential for beta cells differentiation and therefore the conditions able to induce the expression of many important transcription factors genes such as isl-1, pax-4, pdx-1 and ngn-3. Among them pdx-1 is a gene essential for pancreas development which is able to control ngn-3 in activating the expression of other differentiation factors for endocrine cells. Here, we describe for the first time in human umbilical cord blood cells (UCB) the pattern of expression of a panel of markers (nestin, CK-8, CK-18) and transcription factors (Isl-1, Pdx-1, Pax-4, Ngn-3) considered important for beta cells differentiation. Our data demonstrate that UCB contains a cell population having a phenotype very similar to endocrine cell precursors in transition to beta cells.

Keywords: Nestin; Umbilical cord blood; Mesenchymal stem cells; Pancreatic markers

Haematopoietic system represents the main source of haematopoietic stem cells (HSC) and probably that of multipotential adult progenitor cells (MAPCs) [1] as mesenchymal stem cells (MSC) previously described as colony forming unit-fibroblast (CFU-F) by Friedenstein et al. [2]. Whereas there are many studies on the gene expression profile of the different precursors along their haematopoietic differentiations, few data (sometimes conflicting) have been reported about the phenotype of the cells (present in bone marrow and possibly in cord blood) able to differentiate into non-haematopoietic cells [3]. According to some authors, HSC express neural markers such as NeuN, MAP2, and GFAP [4,5], and can differentiate into astrocytes when cultured in specific conditions [6]; on the contrary, Wagers et al. [7] think that HSC do not contribute to non-haematopoietic tissue (brain, kidney, liver, gut, and muscle).

Both postnatal bone marrow and umbilical cord blood contain nestin positive cells [8] able to proliferate and differentiate into the main neural phenotype (neuron, astroglia, and oligodendroglia) [9]. On the

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doi:10.1016/j.bbrc.2004.08.088

other hand, a nestin positive stem cell multipotential for neuron, astrocytes, and oligodendrocytes has been described in human brain; this stem cell is also able to reconstitute haematopoiesis if injected in lethally X-irradiated mice [10]. According to many authors, nestin is considered a neuroepithelial precursor marker expressed in many cell types [11,12] but it seems to be essential in multipotential progenitor cells of pancreas, present both in rat and in human pancreatic islets. These cells, called nestin positive islet derived progenitors (NIPs) [13,14], are probably distinct from ductal epithelial cells (because they do not express ductal markers such as CK-19) but express functional receptors for GLP-1 (glucagon-like peptide 1) which is known to induce NIPs to express PDX-1 and to differentiate them into insulin producing cells [15–17].

Studies on pancreas embryogenesis in mouse [11] have found that in embryonic stem cell cultures, serum deprivation increases the portion of nestin positive cells in embryoid bodies. Based on this strategy, Lumelsky et al. [14] used serum deprivation to select nestin positive cells and stimulated them to express insulin, using FGF-2. Although the importance of nestin in these cells appears to be evident, it still remains to clarify the number and the sequential expression of the genes coding all the transcription factors essential for beta cell differentiation. According to Soria [18], strategies to increase islet precursor cells from embryonic stem cells should include conditions able to induce the expression of many important transcription factor genes such as isl-1, pax-4, pdx-1, and ngn-3. Pdx-1 is a gene essential for pancreas development and able to control ngn-3 (which codifies for a neuron-specific basic-helix–loop–helix (bHLH) transcription factor) in activating the expression of other differentiation factors for endocrine cells. Ngn-3 switches off prior to final differentiation into beta cell [19–21].

Here, we describe for the first time the expression in the human umbilical cord blood cells (UCB) of a panel of markers (nestin, CK-8, and CK-18) and transcription factors (Isl-1, Pdx-1, Pax-4, and Ngn-3) considered important for beta cell differentiation. We demonstrate that UCB contains a cell population having a phenotype very similar to endocrine cell precursors in transition to beta cells [18].

Materials and methods

Human mononucleated cord blood cells. The mononucleated cell fraction isolated from human umbilical cord blood (UCB) supplied frozen by Poietic Technologies (Cambrex, NJ, USA) was used as a source of stem cells, according to a protocol approved by the Institutional Review Board (IRB), in cryotubes stored in liquid nitrogen. Immediately before use, the cells were thawed quickly at 37 °C by a technique giving a high percentage of cell recovery and viability (⩾90%). Briefly, warmed IMDM (Cambrex, NJ, USA) containing 10% FBS (Euroclone, UK) with Dnase I (10 U/ml) was added to the cells that were transferred to a conical tube. Gently swirling the tube, enough volume of medium was added to the cell suspension which was centrifuged at 200*g* for 15 min at room temperature.

Most of the wash was removed by pipette leaving 1–2 ml behind so the cell pellet was not disturbed and was then resuspended in the remaining medium. Fresh IMDM with L-glutamine 1% and FBS 10% was added to the cell suspension with gentle swirling and then centrifuged at 200*g* for 15 min at room temperature.

Most of the wash was removed by pipette, again leaving 2 ml medium in which cells were suspended and counted for determining the viability.

Cell suspension was adjusted to achieve the viable cell density required: 5×10^5 cells/ml.

Cell lines used for positive and negative controls in marker assays. To check the expression of Nestin, Pdx-1, Neurogenin-3, Isl-1, CK-8/18, and CK-19 human ductal adenocarcinoma cells (CFPAC-1, ATCC CRL1918) were used as positive control. Mouse myelomonocytic leukaemia cells (WEHI-3B, ATCC TIB 68) and SR4987 stromal cells (ATCC CRL2028) were used as negative control. The cell lines were maintained in our laboratory by serial passages in McCoy's 5A medium (Biochrom Seromed, Germany) + 5% FCS (Euroclone, UK) and from each line slides for immunocytochemistry and cell extracts for Western blotting were prepared, as described below.

Culture conditions. One millilitre of cell suspension (5×10^5 cells/ml) was seeded in Chamber slides (Falcon, BD Biosciences 4102, CA, USA). After 8 days the cultures were observed by microscope to verify the presence of both adherent and floating cells on which were performed immunocytochemistry, Western blotting, and PCR studies.

Antibodies and reagents. The following primary antibodies were used: mouse IgG1 monoclonal antibody anti-human Cytokeratins-8/18 (Santa Cruz Biotechnology, USA) (200 μg/ml); mouse IgG1 monoclonal antibody anti-human Cytokeratin-19 (Santa Cruz Biotechnology, USA) (200 μg/ml); mouse IgG1 monoclonal antibody anti-human Nestin (BD Transduction, USA) (250 μg/ml); and mouse monoclonal IgG1 anti-human Neurogenin-3 (BD, USA) (250 μg/ml). Secondary antibodies used were goat anti-mouse IgG biotin conjugated antibody (Santa Cruz Biotechnology, USA) (400 μg/ml); rabbit anti-goat IgG biotin conjugated antibody (Santa Cruz Biotechnology, USA) (400 μg/ml).

The detection systems were: ECL (SuperSignal West Pico Chemiluminescent Substrate, Pierce Biotechnology, USA); DAB (3,3′ diaminobenzidine tetrahydrochloride, ICN Biomedicals, USA) prepared freshly at a working concentration of 0.5 mg/ml with 0.1% H_2O_2 in distilled water.

In immunocytochemistry, normal goat or rabbit serum was used as blocking serum.

Immunocytochemistry. The cells growing in suspension were collected, washed twice in PBS (600*g*, 10 min), and then cytocentrifuged onto slides for cytochemistry at a density of about 30,000 cells/spot. To collect adherent cells, the chamber walls were eliminated, the slides were washed twice in PBS, fixed 10 min in methanol at −20 °C, left drying in air, and then stored at −20 °C. For the immunoassay, the slides were thawed at room temperature and washed in PBS for 5 min; then the cells were permeabilized for 4 min with 0.1% Triton X-100 (Sigma, MS, USA) in PBS. The slides were rinsed with PBS, left drying in air, treated with 1% H_2O_2 in PBS and, after two washings with PBS, the cell spots were treated with 1.5% normal blocking serum in PBS for 40 min at room temperature. The slides were treated with the primary antibody overnight at 4 °C (1:200 in 1.5% blocking serum) and control slides received only serum.

After three washings in PBS, the secondary biotinylated-antibody (1:200 in 1.5% blocking serum/PBS) was added for 1 h at room temperature, the slides were rinsed three times in PBS and then treated with HRP–streptavidin conjugate (Rockland, USA) (1:3000 in PBS) for 30 min in dark at room temperature. After another three washings in PBS, the slides were developed by peroxidase substrate solution (DAB), rinsed in water, and counterstained for 1 min with Mayer's Hematoxylin 0.1% (Sigma, MS, USA).

Cell extracts. Whole cell extracts were prepared as follows. Thawed mononucleated UCB suspended cells and adherent cells (after trypsinization) were washed twice in PBS at 600*g* for 10 min and then pelletted at 1000*g* for 15 min. Cell pellets were suspended in lysis buffer (150 mM NaCl, 0.5 mM EDTA, 0.5 mM $MgCl_2$, 10 mM Tris, 1% Triton X-100, 1 mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 μg/ml trypsin inhibitor), vortexed, incubated on ice for 30 min and after centrifugation at 14,000*g* for 15 min, supernatants containing all cell proteins were collected and stored at −80 °C.

To test transcription factors (like Ngn-3), we used a lysis protocol that could separate cytosolic proteins from nuclear proteins. Cells were collected as described above, then treated with a cytosolic lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, Nonidet-P40 10%, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 μg/ml trypsin inhibitor) for 45 min on ice and pelletted at 14,000*g* for 3 min. Supernatants were collected and discarded, whereas pellets were suspended in nuclear lysis buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 μg/ml trypsin inhibitor) for 5 min on ice and then centrifuged at 14,000*g* for 3 min. Supernatants containing nuclear proteins were stored at −80 °C. Protein concentration of lysates was determined by Bradford assay [22].

Western blotting. Cellular extracts prepared as described above were denatured at 95 °C for 5 min and were then subjected to electrophoresis in 7.5% or 12.5% SDS–polyacrylamide gel by the method of Laemmli [23]. Proteins on the gel were transferred through a semi-dry blotter system (Milliblot-Graphite Electroblotter System, Millipore, USA) to PVDF membranes (Immobilon-P, Millipore, USA) as described by Towbin et al. [24]. After saturation in 1% BSA (Bovine Serum Albumin, AppliChem, Darmstadt, DE) in TBST (Tris buffered solution with 0.2% Tween 20) for 1 h at room temperature, the membrane was incubated overnight at 4 °C with the primary antibody (diluted 1:200–1:1000 in 1% BSA/TBST). After three washings with TBST, the blot was incubated for 45 min at room temperature with anti-mouse antibody diluted 1:10,000 in 1% BSA/TBST. After a further three washings with TBST, membranes were treated for 45 min with HRP-conjugated streptavidin (Biospa, Milano, Italy) 1:8000 in 1% BSA/TBST, and washed three times in TBST and twice in TBS. Finally, the blot was developed by a chemiluminescence technique using Hyperfilm ECL (Amersham, Buckinghamshire, UK).

Image acquisition. WB membranes were analysed by a scanner and the images were elaborated by Adobe Photoshop Elements. Immunocytochemistry slides were read under a microscope and then photographic documentation was obtained by a digital camera (Coolpix 4500, Nikon, Japan). Freshly growing cells were photographed by Nikon Coolpix 4300 (Nikon, Japan).

RNA extraction and purification. Flasks of approximately 2×10^7 cord blood cells (immediately after thawing and after 7 days of culture, both adherent cells and suspended cells), CFPAC-1, and WEHI-3B were selected and total RNA was extracted using the TRIzol kit (Invitrogen, USA); the quality of RNA was verified by 2% agarose gel and stained with ethidium bromide.

The RNA samples were treated with DNA-free kit (Ambion, USA), according to the manufacturer's protocol, to remove genomic DNA. After DNase treatment, DNA elimination was controlled by PCR, using the DNA random primers according to the manufacturer's protocol, and RNA integrity by 2% agarose gel stained with ethidium bromide.

RT-PCR. Five micrograms of total RNA was reverse-transcribed using Superscript II Rnase H-Reverse Transcriptase kit (Gibco, USA) for 50 min at 42 °C, according to the manufacturer's protocol. For the determination of mRNA levels of nestin, pdx-1, isl-1, CK-8, CK-18, CK-19, neurogenin-3, β-actin, and GAPDH genes, multiplex PCRs were carried out with Hot Star Taq polymerase (Qiagen, USA) and performed in an automated thermal cycle (Peltier Thermal Cycle PTC-200, MJ Research), according to the manufacturer's protocol.

In order to perform PCR analysis 35 amplification cycles were carried out for all genes. For PCR, 1 μl (0.2 μg) of cDNA was included in a total of 20 μl reaction mixture (Qiagen, USA), containing 1× $MgCl_2$ free (Qiagen, USA), 1.5 mM $MgCl_2$ (Qiagen, USA), 0.2 mM each dNTP (Gibco, USA), sense and antisense primers (0.9 μM, for the sequences see Table 1), and 2.5 Units/Reaction HotStartTaq

Table 1
Summary of sense and antisense primer sequences

Gene	bp	Sequence	Reference
Nestin	500	5′-AGAGGGGAATTCCTGGAG-3′ 5′-CTGAGGACCACTCTCTA-3′	Crino et al., PNAS (1996)
PDX-1	200	5′-ACCAAAGCTCACGCGTGGAAA-3′ 5′-TGATGTGTCTCTCGGTCAAGTT-3′	Leicowitz et al., Diabetes (2001)
ISL-1	350	5′-ACACATCTTTGGGGGAAAAG-3′ 5′-AAAAAGCGCAGGAAGAGAG-3′	Steven W. Johnson, PhD, University of Pennsylvania
Neurogenin-3	948	5′-ACGCGTGAATGGGATTATGGGGTG-3′ 5′-CTCGAGGGTAGAAAGGATGACGCCTC-3′	Assady et al., Diabetes 50 (2001) 1691–1697
CK-8	101	5′-CTGGGATGCAGAACATGAGTATTC-3′ 5′-GTAGCTGAGGCCGGCTTGT-3′	Dimmler et al., Lab. Invest. 81 (2001) 1351–1361
CK-18	86	5′-GAGACGTACAGTCCAGTCGTTGG-3′ 5′-CCACCTCCCTCAGGCTGTT-3′	Dimmler et al., Lab. Invest. 81 (2001) 1351–1361
CK-19	103	5′-TGAGTGACATGCGAAGCCAATAT-3′ 5′-GCTACCTCCCGGTTCAAT-3′	Dimmler et al., Lab. Invest. 81 (2001) 1351–1361
PAX-4	125	5′-CTGTTTTGGCTCCAGCTGTC-3′ 5′-CTTTCTCCAGTGCCTCTGCT-3′	Tao et al., Diabetes 47 (1998) 1650–1653
B-actin	460	5′ GGC CCA GAG CAA GAG AGG TAT CC 3′ 5′ AGC CAC GAT TTC CCT CTC AGC 3′	Pesce et al., Mech. Dev. (1998)
GAPDH	500	5′TCTCATGGTTCACACCCATGACGAACATG3′ 5′AAGAAGATGCGGCTGACTGTCGAGCCACAT3′	Clontech

Table 2
Summary of PCR parameters

Nestin	Pdx-1	Isl-1	Neurogenin-3
95 °C for 15 min	95 °C for 15 min	95 °C for 15 min	95 °C for 15 min
94 °C for 1 min	94 °C for 1 min	94 °C for 1 min	94 °C for 1 min
60 °C for 1 min	60 °C for 1 min	57 °C for 1 min	63 °C for 1 min
72 °C for 1 min	72 °C for 1 min	72 °C for 1 min	72 °C for 1 min
×35 cycles	×35 cycles	×35 cycles	×35 cycles
72 °C for 10 min	72 °C for 10 min	72 °C for 10 min	72 °C for 10 min
10 °C forever	10 °C forever	10 °C forever	10 °C forever
Pax-4	**GAPDH**	**β-Actin**	**CK-8/18/19**
95 °C for 15 min	95 °C for 15 min	95 °C for 15 min	95 °C for 15 min
94 °C for 1 min	94 °C for 1 min	94 °C for 1 min	94 °C for 1 min
60 °C for 1 min	60 °C for 1 min	57 °C for 1 min	58 °C for 1 min
72 °C for 1 min	72 °C for 1 min	72 °C for 1 min	72 °C for 1 min
×35 cycles	×35 cycles	×35 cycles	×35 cycles
72 °C for 10 min	72 °C for 10 min	72 °C for 10 min	72 °C for 10 min
10 °C forever	10 °C forever	10 °C forever	10 °C forever

Polymerase (Qiagen, USA). The PCR cycling parameters for cDNA amplification for the genes are shown in Table 2.

PCR products were run on 2% agarose gels and stained with ethidium bromide. The running gels were captured using the FOTO/analyst Image Analysis System (Fotodyn, USA). The NIH Image 1.61 package was then used to estimate the signal of the PCR products. Each RT-PCR was performed twice and three independent experiments were performed. In all PCRs the β-actin and GAPDH genes were used as internal control for mouse and human cells, respectively.

Results

Cultures of umbilical cord blood cells

The mononuclear cord blood cells cultured in chamber slides were observed daily in order to check the growth and morphological changes over the course of 7 days. After 48–72 h a significant number of cells adhered to the plastic/glass substrate. These cells have a fibroblast-like morphology and after 7–8 days 50% of them were spread on the substrate, having very irregular shapes such as stellate or spindle (see Fig. 1). The other cells remained in suspension by floating in the medium supernatant but if they were transferred into a new chamber slide a remarkable number of cells were able to adhere so that the serial subculturing gave a continuous production of adherent cells (at least for the five passages tested). On the contrary, if the adherent cells were trypsinized and passed into another chamber most of them lost the capacity to adhere and to proliferate and after three passages more than 80% of the cells were lost.

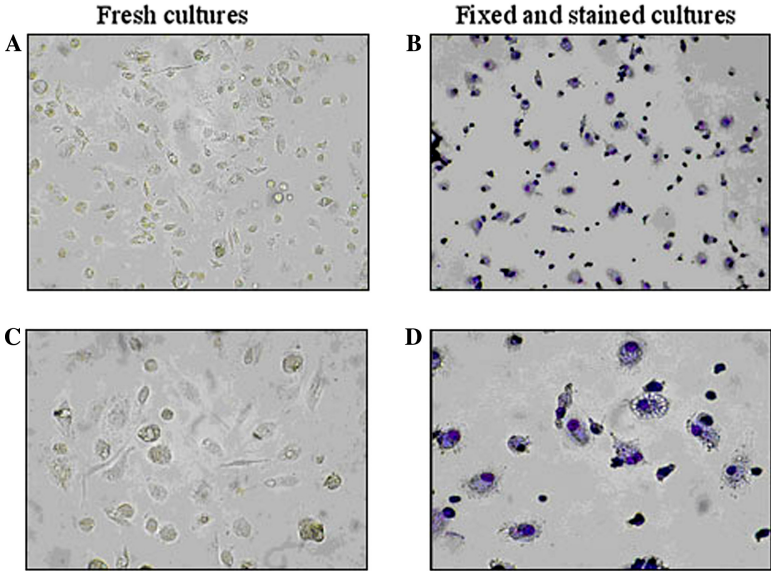

Fig. 1. Cultures of umbilical cord blood cells (UCB). The photos show the morphology of adherent cells after 8 days of UCB culture in complete medium. Cells were observed by using an inverted microscope at 100× magnification (A and C) and 250× magnification (B and D). (A,B) Cells in fresh cultures. (C,D) Cells after fixing and staining with May-Grünwald and Giemsa (Sigma Diagnostics, USA).

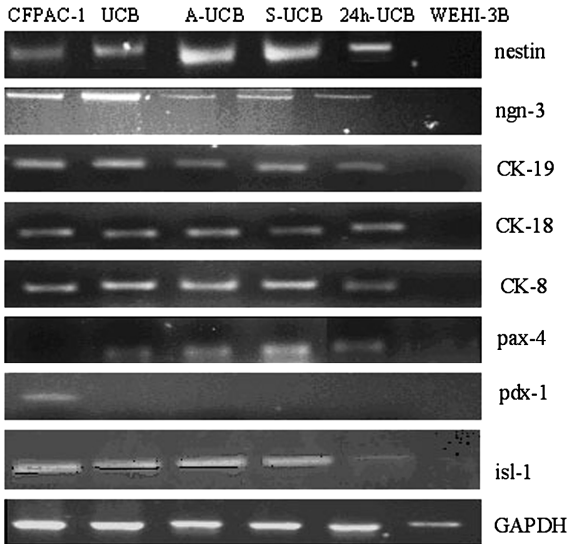

Fig. 2. RT-PCR analysis of specific markers expressed in human umbilical cord blood cells. CFPAC-1, human ductal adenocarcinoma (positive control); UCB, thawed mononucleated umbilical cord blood; A-UBC, adherent fraction of UCB after eight days of culture; S-UCB, suspension fraction of UCB after eight days of culture; 24 h UCB, UCB after 24 h of culture; WEHI-3B, myelomonocytic murine leukemia (negative control). Experiments were performed twice and the same result was obtained.

RT-PCR for mRNA expression

The expression of mRNA for eight different markers was checked on umbilical cord blood cells both before culturing and after 8 days of culture, on the two fractions (adherent and floating cells) obtained. We used CFPAC-1 cells (positive CTRL) and WEHI-3B cells (negative CTRL) as control cells for the different markers checked.

As reported in Fig. 2, UCB cells before culturing were positive for mRNA expression of nestin, CK-18, CK-8, CK-19, isl-1, ngn-3, and pax-4 but were negative for pdx-1. A further control of 24 h cultures of the same cells gave the same pattern of mRNA expression with a weaker signal for Isl-1. After 8 days of culture, both the adherent and the floating cells expressed all the mRNA, with the exception of pdx-1 and with a significant increase of the nestin expression.

Immunocytochemistry

The marker detection by immunocytochemistry is reported in Fig. 3.

As positive and negative controls, respectively, CFPAC-1 and SR-4987 cells were used. Basal

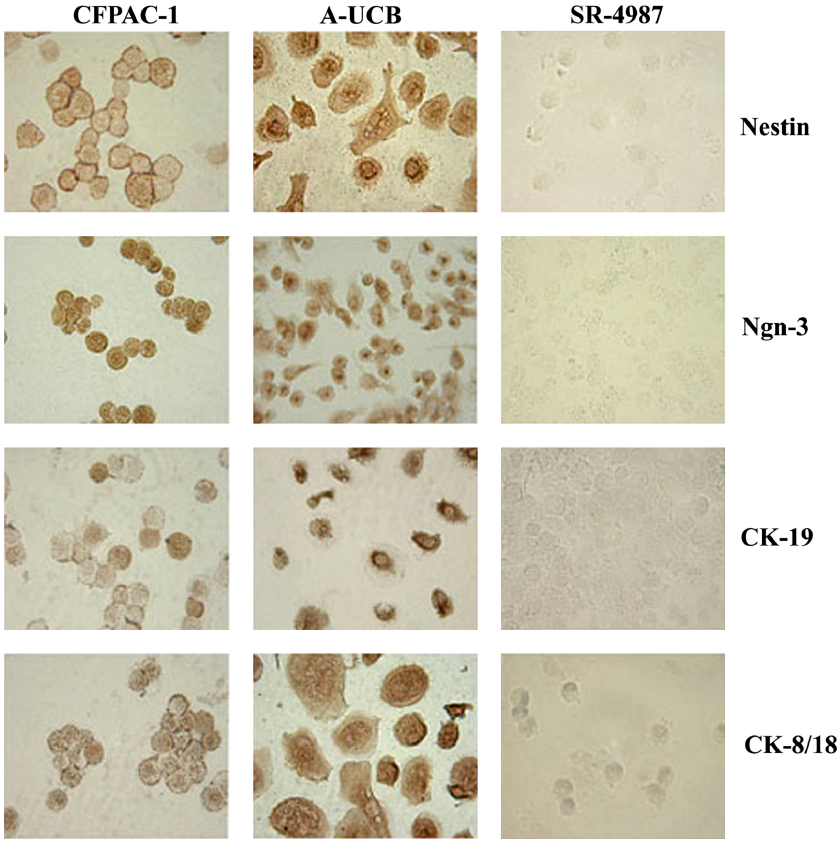

Fig. 3. Immunocytochemistry analysis of specific markers expression in human umbilical cord blood cells. As positive control were used CFPAC-1 cells (human ductal adenocarcinoma) and as negative control a murine stromal cell line SR-4987. A-UCB, adherent cells after 8 days of culture of umbilical cord blood cells. Experiments were performed three times and gave the same result. Photographs were performed at 100× magnification.

expression of markers in UCB before culturing was found to be negative both for Cytokeratin-19 and Cytokeratins-8/18 whereas for Neurogenin-3 and Nestin, some positive cells were identified (1–2 cells for each cytospin spot that means about 1–2/10^4 cells) (photographs not shown).

After 8 days of culture (Fig. 3), Nestin, Cytokeratins-8/18, Cytokeratin-19, and Neurogenin-3 were found to be expressed on 100% of the adherent cells whereas all the suspended cells were negative (with the exception of nestin which is expressed on 60–70% of the floating cells).

Western blotting

The results of Western blotting analysis indicate that in the cell lysate before culturing (basal expression) none of the markers studied was detectable. As shown in Fig. 4, adherent cells expressed Nestin, Ngn-3, Cytokeratin-19, and Cytokeratins-8/18. Only Nestin and Ngn-3 were also expressed in floating cells (data not shown). A comparison of the results obtained by PCR, immunocytochemistry, and Western blotting is reported in Table 3.

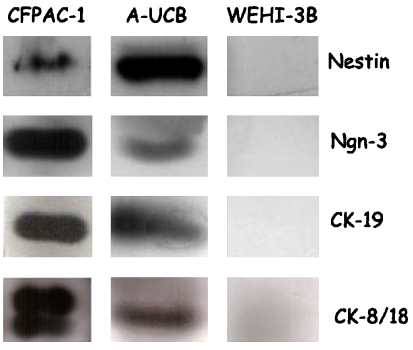

Fig. 4. Western blotting analysis of specific markers in human umbilical cord blood cells. As positive control were used CFPAC-1 cells (human ductal adenocarcinoma) and as negative control the murine WEHI-3B cell line. A-UCB, adherent cells after 8 days of culture of umbilical cord blood cells. For nestin, CK8/18 and CK19 were used 40 μg of whole cell lysate; for Ngn-3 were used 40 μg of nuclear lysate. Experiments were performed three times and gave the same result. Photographs were performed at 100× magnification.

Table 3
Comparison of results from PCR, ICC, and WB

Marker	Test					
	RT-PCR		ICC		WB	
	UCB	A-UCB	UCB	A-UCB	UCB	A-UCB
Nestin	+	+	+/–	+	–	+
Ngn-3	+	+	+/–	+	–	+
CK-8/18	+	+	–	+	–	+
CK-19	+	+	–	+	–	+

(+/–): 1–2/10^4 cells.

Discussion

Although Friedenstein et al. [2] demonstrated that bone marrow in long-term cultures favoured the selection of stromal cells (CFU-F), only very recently the role of multipotential stem cells, called mesenchymal stem cells (MSC), has been attributed to these cells and have demonstrated that they are able to differentiate into mature fat, bone, cartilage, endothelium, and muscle [25]. Furthermore, although umbilical cord blood (UCB) cells have been well characterized for haematopoietic markers, in the literature there are few reports describing markers not related to haematopoietic function as described above for bone marrow. Some authors [26,27] have also suggested that, in contrast to bone marrow, the umbilical cord blood (UCB) does not contain or produce mesenchymal progenitor cells. Our results support the observations of Lee et al. [28] which demonstrate that cryopreserved UCB contains cells having the capacity to strongly adhere to the substrate and showing a morphology very close to stromal cells (MSC o MPCs) as found in bone marrow. Furthermore, the analysis of their phenotype gave a surprising result because these cells share many markers expressed on the precursors of pancreatic cells. In fact, the expression of Nestin, Ngn-3, CK-8, CK-18, CK-19, Isl-1, and Pax-4 is considered important during the development of the islets of Langerhans, both for exocrine and endocrine cells [29] and, as seen in Fig. 1, the PCR study demonstrates the presence of cells expressing mRNA for all the above genes in UCB. Of course, the cells do not express Pdx-1, which is the transcription factor for insulin.

Immunocytochemistry confirmed that Nestin and Neurogenin-3 were naturally expressed by very few cells (about $4–6 \times 10^5$) of the mononucleated fraction of UCB (positive for five markers) whereas all the five markers (Isl-1 and Pax-4 have not been tested) were expressed by 100% of the adherent cells. This explains the apparent discrepancy between the data found in RT-PCR and those found by Western blotting (Table 3): in fact, for the RT-PCR assay, very few positive cells were needed to give a positive result (due to the high mRNA amplification), whereas the same low number of positive cells is probably not enough to isolate such an amount of proteins to make positive the Western blotting analysis. However, in general we can also suppose that all the cells in UCB have mRNA for Nestin and Ngn-3 that need to be regulated by a posttranscriptional mechanism.

After 8 days of culture, the results obtained by RT-PCR assay correlate well with those from western blotting and immunocytochemistry, being 100% of the adherent cells positive for Nestin, Ngn-3, CK-8/18, and CK-19. Of interest is the observation that cytokeratins are maximally expressed in cells showing the capacity to adhere to the substrate, whereas Nestin and

Neurogenin-3 were similarly expressed (100%) in floating and adherent cells.

Our data also underline that the single expression of Nestin cannot (alone) demonstrate the neuronal fate of a cell, as some authors suggest [30] and that Nestin is probably an important master control gene (common for neural, epithelial, and haematopoietic lineages and perhaps of other tissues) which remains active in the control of other genes' expression [9,10,31].

The strong positivity of adherent cells for the abovementioned markers is of interest because it has been reported that in Nestin+, Ngn-3+ ES cells the activation of Pax-4 and Pdx-1 produces differentiation into insulin secreting cells [31–33]. In our study we found that umbilical cord blood cells were negative for Pdx-1 both before and after cell culture. However, UCB were positive for Pax-4, which during embryogenesis is considered a key factor in the differentiation of insulin producing beta cells [34] and also positive for Ngn-3 which acts as a pro-endocrine gene [35]. In fact, in the endocrine islets in cells ngn-3+ contribute to pancreatic islet renewal. By taking into account the different stages of the endocrine cell precursors in relation to their phenotype, as suggested by Soria [18] (Table 4), in the UCB it is possible to identify Pax-4+, Ngn-3+, and Isl-1+ cells which are precursors of endocrine cells. According to other studies, also Cytokeratins-8/18 (the first intermediate filament proteins expressed during embryogenesis) are expressed in exocrine and endocrine differentiated islet cells and Cytokeratin-19 is expressed in ductal cells, considered the progenitors of islets [36].

Our data represent the first evidence that umbilical cord blood cells, after culture in medium supplemented with foetal calf serum (in the absence of specific cytokines or growth factors), show a panel of markers consistent with the characters of epithelial cells expressing genes considered essential in the differentiation steps towards pancreatic cells. According to our observations, it could be possible to work along two sequential culture phases: the first one in complete medium, to select the adherent cell population Nestin+, Ngn-3+, CK-8/18+, and CK-19+; the second one (based on the replacement of complete medium with a definite one containing a specific cocktail of cytokines), to guide these cells into beta cells. Further studies in this direction are in progress in our laboratory as according to our data UCB can be used for basic research in therapeutic strategies as has been the case for bone marrow cells.

Table 4
Gene expression profile along pancreatic development[a]

Early pancreatic development	Endocrine cells precursors	Transition to β-cell	β-Cell
Nestin+	Nestin+	Nestin+	Nestin+
Pdx-1+	*Pdx-1−*	*Pdx-1−*	Pdx-1+
Ngn-3−	Ngn-3+	Ngn-3+	*Ngn-3−*
Isl-1−	Isl-1+	Isl-1+	*Isl-1−*
Pax 6−	Pax 6+	*Pax 6−*	*Pax 6−*
Pax-4−	*Pax-4−*	Pax-4+	*Pax-4−*
β2/*NeuroD−*	β2/*NeuroD−*	β2/NeuroD+	β2/*NeuroD−*
Nkx 2.2−	*Nkx 2.2−*	*Nkx 2.2−*	Nkx 2.2+
Nkx 6.1−	*Nkx 6.1−*	*Nkx 6.1−*	Nkx 6.1+
Glut 2−	*Glut 2−*	*Glut 2−*	Glut 2+

(+) Expressed gene and (−) not expressed gene.
[a] This table is constructed by considering the observations suggested by Soria [18].

Acknowledgments

The authors thank Ms. Loredana Cavicchini for her excellent technical assistance and Dr. Arianna Bonomi for assistance in preparation of the manuscript.

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