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Proliferation, differentiation, and cytokine secretion of human umbilical cord blood–derived mononuclear cells in vitro

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Objective. **Human umbilical cord blood (hUCB)–derived mononuclear cells were previously shown to exert therapeutic effects in a number of animal models of nervous system impairment. However, the mechanisms underlying the structural and functional improvements are still unclear. As cell replacement seems to be a rare or absent event in vivo, we suggest secondary mechanisms, by which the therapeutic effect of transplanted mononuclear cells might be mediated. We investigated the potential of hUCB-derived mononuclear cells in vitro to proliferate, differentiate, and to secrete factors possibly beneficial for the host brain tissue in vivo.**

Methods. **Using a succession of distinct culture media, mononuclear cells were stimulated by growth factor combinations, e.g., epidermal growth factor (EGF)/fibroblast growth factor-2 (FGF-2) or nerve growth factor (NGF)/retinoic acid (RA). Expression of hematological and neural marker proteins was investigated by immunoblotting, immunocytochemistry, and fluorescence-activated cell analysis. Secretion of proteins was assayed using a human cytokine antibody array, and quantified via enzyme-linked immunosorbent assay.**

Results. **Mononuclear cells were shown to undergo proliferation in the presence of EGF/FGF-2. When cells were cultured in NGF/RA-containing medium, neuronal and glial marker proteins were expressed, indicating differentiation. In the presence of either growth factor combination, cells in vitro secrete interleukins, growth factors, and chemotactic proteins.**

Conclusion. **Although capable of incipient differentiation, cytokine secretion of hUCB-derived mononuclear cells envisages the potential of an indirect effect in vivo. Most factors detected in conditioned medium are renowned for their anti-inflammatory, neuroprotective, angiogenic, or chemotactic actions, thus, providing the means for a therapeutic outcome mediated by secondary effects.** © **2007 International Society for Experimental Hematology. Published by Elsevier Inc.**

During the past few years, stem cell transplantation has become one of the major aspirations for the putative treatment of neurological diseases in humans. Using animal models of various neurological impairments, cell transplantation yielded in the improvement of behavioral and morphological deficits that had resulted from the disease. In some models, there was evidence for functional integration of transplanted stem cells into the diseased neural network with transplanted cells adopting a neural fate [1–5]. Other studies pointed to an indirect mode of action, in that transplanted cells positively influenced the host tissue, resulting in a net improvement [6–8].

In addition to embryonic and neural stem cells, umbilical cord blood was shown to contain multipotent hematopoetic and nonhematopoetic stem cells [9]. The therapeutic potential of umbilical cord blood in the diseased central nervous system have been investigated in a number of animal models, including those of stroke [10], perinatal hypoxic-ischemic brain injury [11], spinal cord injury [12], amyotrophic lateral sclerosis [13,14], and traumatic brain injury [15]. In virtually all of these models, transplantation

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of human umbilical cord blood (hUCB)–derived cells resulted in behavioral improvement of those animals as assessed by sensorimotor testing, determination of neurological scores and, in some studies, cognitive testing.

Although there is evidence for the therapeutic effect of umbilical cells in diseases of the central nervous system, the mechanisms by which these cells exert their beneficial effects still need to be elucidated. Transplanted cells were identified in the brain and investigated for expression of neural marker proteins in vivo, the presence of which would point to the differentiation of umbilical cells to those of neuronal or glial lineages [14,16,17]. Additional in vitro studies have also demonstrated the capability of umbilical cells to adapt neuronal or glial characteristics [18–20]. Thus, some crucial questions remain to be answered: Is the proportion of transplanted umbilical cells expressing markers of neural differentiation sufficient to account for the beneficial effects observed in vivo? Are transplanted hUCB cells capable of conducting indirect actions onto the impaired host tissue, thus exerting secondary therapeutic effects? And if so, how might they contribute to functional recovery?

In this study, the mononuclear fraction of hUCB was taken into culture. Their capability to proliferate and to differentiate into neural-like cells with the simultaneous reduction of the hematopoetic marker CD45 was demonstrated. To investigate the possibility of an indirect mode of action, the ability of umbilical cells to secrete cytokines has been examined in vitro. In response to the growth factor combinations epidermal growth factor (EGF)/fibroblast growth factor-2 (FGF-2) and nerve growth factor (NGF)/retinoic acid (RA), mononuclear cells were shown to secrete substantial amounts of interleukins, growth factors, and chemokines. As most of these secreted factors exert anti-inflammatory, angiogenic, chemotactic, and neuroprotective effects, their release by hUCB cells might explain in part the "therapeutic" effect observed upon transplantation of these cells in vivo.

Materials and methods

hUCB-derived mononuclear cells

Blood from umbilical cord and placenta was obtained from the Departments of Gynecology and Obstetrics (Ruhr-University Bochum and St. Elisabeth Hospital, Bochum, Germany), after receiving the mother's informed consent. The umbilical vein was punctured postpartum, and the blood was collected and stored as described previously [11]. Preparation of the mononuclear cell fraction was performed by the Ficoll gradient technique according to the manufacturer's instructions (Amersham, Freiburg, Germany).

Culture of mononuclear cells

For in vitro analysis, cells were plated at a density of 1×10^7 cells/ml in Dulbecco's minimal essential medium (DMEM) supplemented with 2 mM glutamine, 0.001% β-mercaptoethanol, 1× nonessential amino acids, and 10% fetal calf serum. After 48 hours, i.e., 2 days in vitro (DIV), nonadherent cells were transferred to a new flask in which those cells were cultured for another 2 days (4 DIV) and became adherent. For cell expansion, the medium was replaced with neural proliferation medium (PM), which consisted of 50% DMEM/50% Ham's F12, supplemented with epidermal growth factor (EGF; 20 ng/mL) and fibroblast growth factor-2 (FGF-2; 20 ng/mL) [20], and cells were cultured for 3 days. After 7 DIV, cells were transferred to neural differentiation medium (DM), in which EGF and FGF-2 were substituted by all-trans retinoic acid (RA; 0.5 μM) and nerve growth factor (NGF; 100 ng/mL), and were cultured for another 7 days (14 DIV).

Mononuclear cell–conditioned medium

For preparation of mononuclear cell–conditioned medium 4×10^7 cells were plated in a 12.5-cm^2 plastic flask and cultured in 4 mL medium (as mentioned already). For culture medium (CM), the medium was harvested after 2 days, when cells were transferred to a new culture flask. Conditioning time in PM and DM was identical to the culture protocol described above (3 and 7 days, respectively). Thus, after a total culture period of 2 DIV (2-day–conditioned CM), 7 DIV (3-day–conditioned PM), or 14 DIV (7-day–conditioned DM) the supernatant was collected and either used immediately or stored in bovine serum albumin–coated cryotubes at −20°C.

Fluorescence-activated cell analysis

Expression of cell surface marker proteins was determined with a BD FACS Calibur (BD Biosciences, Heidelberg, Germany), using the following antibodies (BD Biosciences): fluorescein isothiocyanate (FITC)- or peridinin chloraphyll protein-conjugated mouse anti-human CD45, FITC-conjugated mouse anti-human CD3, phycoerythrin-conjugated mouse anti-human CD19, CD34, CD16+CD56, allophycocyanin-conjugated mouse anti-human CD19, and isotype control FITC-conjugated immunoglobulin (Ig) G1 and phycoerythrin-conjugated IgG2a. Erythrocyte lysis was performed using BD FACSTM Lysing Solution (BD Biosciences). Incubation of 50 μL citrate phosphate dextrose-containing umbilical cord blood, of isolated and of cultured hUCB-mononuclear cells was performed with 10 μL of each antibody at 18°C in the dark. After 20 minutes, 2 mL BD FACSTM lysing Solution were added, and incubated for an additional 10 minutes. The cell suspension was then centrifuged at 300*g* at 18°C for 6 minutes, decanted, and washed twice using BD FACSTM Cell Wash (BD Biosciences).

Immunocytochemical analysis

Cultured cells were fixed in 100% ethanol at −20°C for 10 minutes, or, for nestin and Ki-67 staining, in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 18°C for 20 minutes. After blocking of nonspecific binding sites, incubation with primary antibodies was performed at 18°C overnight; incubation with secondary antibodies at 18°C for 2 hours [11]. Nuclear labeling was performed applying the dye Hoechst 33258 (2 μg/mL in PBS) for 1 minute. Fluorescence was documented using a Zeiss 200M inverted microscope. For quantification, cell numbers were determined by counting at least 200 cells in each of three independent experiments. The amount of immunopositive cells was expressed as percent of the total number of cells.

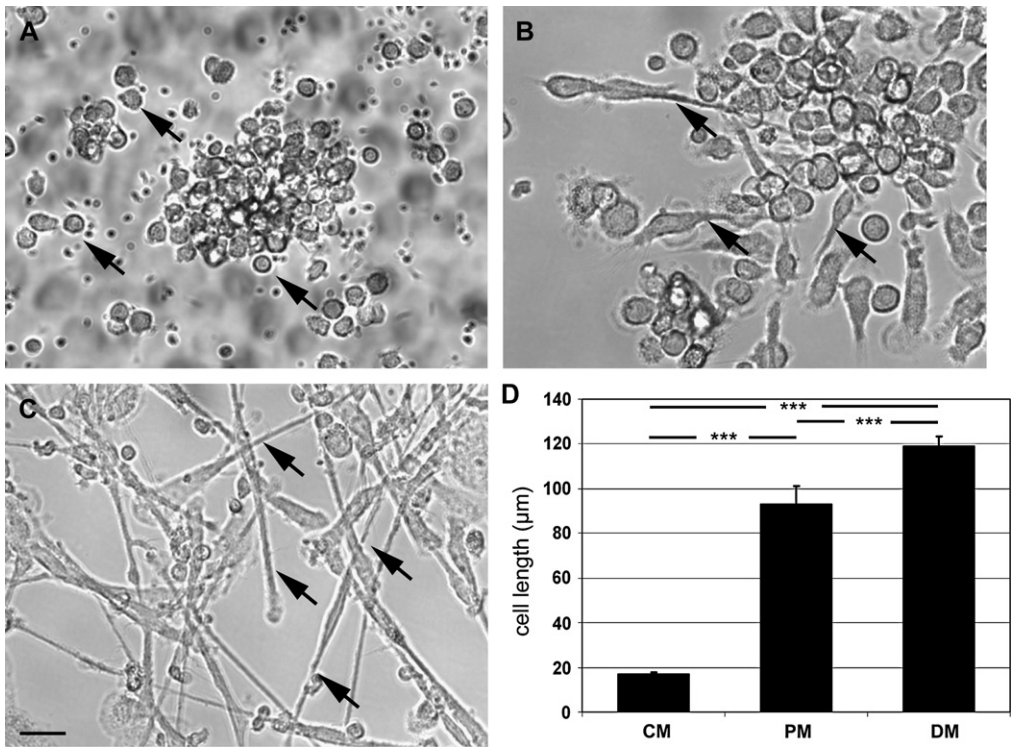


Figure 1. Morphological changes of human umbilical cord blood (hUCB)–derived mononuclear cells in different culture media. (**A**) Phase-contrast photographs of hUCB-derived mononuclear cells cultured in serum-containing culture medium (CM). Cells displayed a round morphology and were mostly nonadherent (arrows). (**B**) hUCB-derived mononuclear cells cultured in proliferation medium (PM) displayed an elongated morphology (arrows). (**C**) When cultured in differentiation medium (DM), cells were even more elongated, with many cells displaying a bipolar morphology (arrows). (**D**) Quantification of cell lengths in the three different media. Compared to the average cell length measured in culture medium, cell length increased significantly (***$p < 0.05$) when cells were cultured in the presence of growth factors. Scale bar: 20 µm (**A**–**C**).

Primary antibodies (dilution and supplier in parentheses) were as follows: monoclonal anti-glial fibrillary acidic protein (GFAP; 1:100), anti–human nuclei antigen (1:50), anti-NeuN (1:100), anti-neurofilament NF68 and NF200 (1:500), anti–musashi-1 (1:500), microtubule-associated protein II (1:100), and anti–Ki-67 (1:75; all from Chemicon, Hampshire, UK); polyclonal anti-GFAP (1:100; Sigma, Taufkirchen, Germany); anti-nestin (1:100; R&D Systems, Wiesbaden, Germany), anti–β3-tubulin (1:500; Covance, Frankfurt, Germany), anti-notch (1:200; Santa Cruz, CA, USA), anti-Cx43 (1:100; Zymed, Berlin, Germany), and anti-CD45 (1:100; BD Biosciences). Secondary antibodies were Alexa Fluor 488 or 546 conjugated goat-anti–rabbit or goat-anti–mouse antibodies (1:3000; Molecular Probes, Leiden, Netherlands).

Western blotting

Protein extracts were obtained by harvesting cells in lysis buffer containing 0.1 M Tris-HCl, 2% sodium dodecyl sulfate, 10% glycerol, 5% β-mercaptoethanol, and 2% protease inhibitor cocktail (Roche, Mannheim, Germany). Electrophoresis was performed using 10% sodium dodecyl sulfate polyacrylamid gels, loading 10 µg protein per lane. After electrophoretic transfer of proteins, nitrocellulose membranes (Amersham Pharmacia Biotech, Freiburg, Germany) were incubated in blocking solution (0.5% blocking reagent [Roche] in PBS) at 18°C for 2 hours, followed by incubation with primary antibodies diluted in 0.2% blocking solution at 4°C overnight. Antibody dilutions were as follows: polyclonal anti-GFAP 1:200; anti–Ki-67 1:500; anti-NeuN 1:1000; anti-NF68 1:250; anti–β3-tubulin 1:500. Equal protein contents per lane were ensured by detection of β-actin (1:8,000; Sigma, Taufkirchen, Germany). Using horseradish peroxidase–conjugated secondary antibodies (1:2000; Dianova, Offenbach, Germany), visualization was performed by enhanced chemiluminescence detection (PicoLuminol; Pierce, Bonn, Germany). Western Blot analyses were performed in three independent experiments.

Human cytokine protein array

A human cytokine antibody array (RayBiotech Inc, Norcross, GA, USA) was used to determine cytokines present in non-conditioned and mononuclear cell-conditioned media. All assays were performed according to manufacturer's instructions. Briefly, after blocking of nonspecific binding sites, membranes were incubated in cell-free–conditioned medium or in the corresponding non-conditioned control medium overnight. After membranes had been washed, a biotin-conjugated antibody was applied at a 1:250 dilution and incubated for 2 hours at 18°C. After repeated washes, membranes were incubated with horseradish peroxidase–conjugated streptavidin at 18°C for 2 hours and, after incubation in detection buffer, exposed to Hyperfilm (Amersham Biosciences). Detectable signals were quantified by densitometric analysis measuring signal intensities (ChemiDocXRS; Biorad, Munich, Germany), with positive controls being normalized to 100, negative controls to 1, and intensity of unknown samples calculated. As with ongoing time in culture, fewer cells were capable of surviving

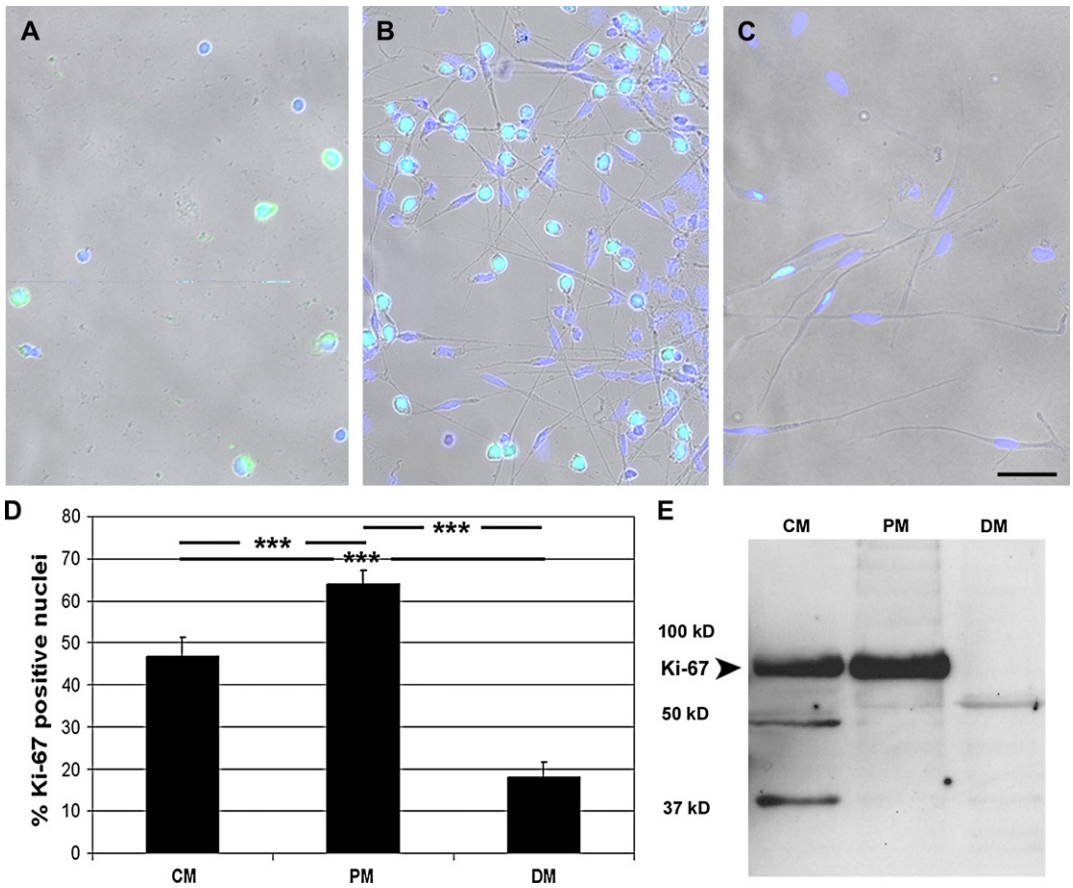


Figure 2. Proliferation of human umbilical cord blood–derived mononuclear cells in the presence of epidermal growth factor (EGF)/fibroblast growth factor-2 (FGF-2). Proliferation was analyzed by immunocytochemical detection of the cell-cycle marker protein Ki-67 (green fluorescence); Hoechst 33258 staining (blue color) labeled all nuclei. (**A**) Few cells expressed Ki-67 when cultured in culture medium (CM). (**B**) The number of Ki-67–immunopositive nuclei increased in proliferation medium (PM). (**C**) In contrast, culture in differentiation medium (DM) resulted in a reduction of Ki-67 expressing cells. (**D**) Quantification of cell proliferation in CM, PM, and DM, expressed as percentage of Ki-67–positive nuclei of the total number of nuclei. ***Significant increase with $p < 0.05$. (**E**) Western blot analysis of Ki-67 expression revealed the presence of a 67-kD–immunoreactive product in samples derived from cells cultured in CM and PM. In contrast, cells cultured in DM did not express Ki-67 protein. Scale bar: 25 μm (**A–C**).

in the respective medium, intensity values were normalized to identical numbers of cells per milliliter medium. Data are derived from three independent experiments and are expressed as mean ± standard error of the mean. Analysis of non-conditioned control media, however, was performed twice and is expressed as mean ± standard deviation. According to the manufacturer, the sensitivity for each cytokine was in the pg/mL range.

Enzyme-linked immunosorbent assay

Quantification of cytokines in conditioned medium was performed by enzyme-linked immunosorbent assays (R&D Systems) following manufacturer's instructions. Angiogenin (Ang), growth-regulated oncogene (GRO), monocyte chemoattractant protein-1 (MCP-1), platelet-derived growth factor-BB (PDGF-BB), and interleukin-8 (IL-8) were analyzed in conditioned CM, PM, and DM. The optical density of the color reaction was detected at 450 nm wavelength using a chemiluminescence reader (Radiometer GmbH, Willich, Germany) and background signal detected at 550 nm was subtracted. Delta values were normalized to the extinction obtained from standard curves, and protein contents were calculated for each sample and cytokine. Amounts were normalized to identical numbers of cells per milliliter medium. Non-conditioned media served as negative controls, and the signal was subtracted from conditioned medium values.

Statistical analysis

Initial one-way analysis of variance was followed by *t*-test. For analysis of microarray data, the logarithm of intensity values was taken prior to statistical analysis. A probability of error less than 0.05 ($p < 0.05$) was considered statistically significant. Percentages are given as cell number in percent ± standard error in percentage points.

Results

In vitro proliferation and differentiation of hUCB-derived mononuclear cells

Upon isolation, cells of the mononuclear fraction were cultured in CM in the presence of fetal calf serum. In this medium, cells displayed a small, rounded appearance (Fig. 1A). In serum-free PM, supplemented with EGF and

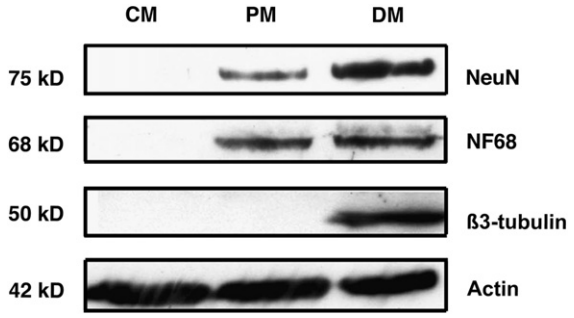


Figure 3. Expression of neural marker proteins in various culture media. Expression of neuronal and glial marker proteins was investigated in human umbilical cord blood–derived mononuclear cells maintained in culture medium (CM), proliferation medium (PM), and differentiation medium (DM). None of the neural proteins investigated was detected in cells in serum-containing CM. In contrast, when cultured in the presence of epidermal growth factor/fibroblast growth factor-2 (PM), mononuclear cells expressed the intermediate filament protein NF68 and the nuclear protein NeuN, both considered as neuronal marker proteins. Nevertheless, expression of most neuronal marker proteins was restricted to cells cultured in the presence of nerve growth factor/retinoic acid (DM), here shown for β3-tubulin. Equal loading was ensured by normalization against actin. Immunoblots presented in this figure show the typical pattern observed in three independent experiments.

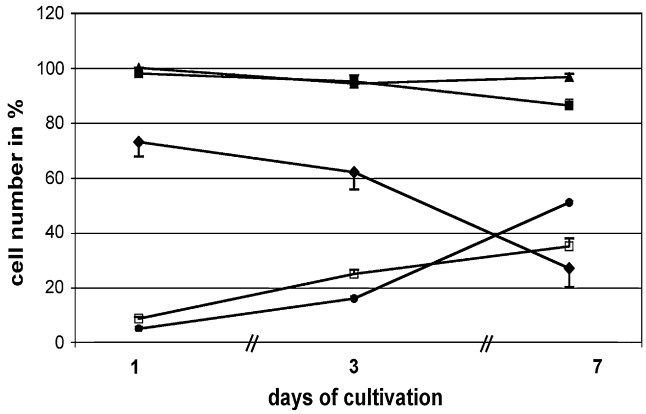


Figure 4. Percentage of cells expressing neural marker proteins after 1, 3, or 7 days in differentiation medium. Whereas the percentage of cells expressing the stem cell marker nestin (◆) significantly ($p < 0.01$) decreased with time in culture, the percentage of cells expressing neuronal marker proteins microtubule-associated protein II (●) or NeuN (□) continuously increased ($p < 0.01$). Interestingly, glial fibrillary acidic protein (■) and NF68 (▲) expression remained nearly unchanged ($p \geq 0.05$) over the 7-day culture period.

FGF-2, cell morphology changed to a spindle-shaped phenotype (Fig. 1B). To induce neural differentiation, cells were subsequently switched to DM, containing NGF and RA. Cell morphology appeared elongated, and this effect became more pronounced with time in culture (Fig. 1C). Quantification of the cell length revealed that the average length of cells significantly increased from 17.9 ± 0.5 μm in CM to 93.15 ± 8.0 μm in PM, and to 118.8 ± 4.4 μm in DM (Fig. 1D).

Proliferation of cells was determined by immunocytochemical analysis of Ki-67 (Fig. 2), a nuclear protein expressed during late G1-, S-, and M-phase of the cell cycle, and therefore indicative of cell proliferation [21]. Ki-67 was expressed by 48.6% ± 4.6% of mononuclear cells cultured in CM for 2 days (3.7×10^7 cells per flask). In the presence of EGF/FGF-2, 64.0% ± 3.1% of cells expressed Ki-67. Nevertheless, the absolute number of cells per flask was reduced to 2.0×10^5 in this medium, pointing to the simultaneous occurrence of cell death and proliferation. Stimulation with NGF/RA led to a reduction of Ki-67–expressing cells to 17.9% ± 3.5% with a concurrent further decrease in cell number (1×10^5 cells in DM). Ki-67 protein expression was also investigated by Western blot analysis, confirming hUCB cell proliferation in both CM and PM. In contrast, induction of cell differentiation in DM resulted in the near absence of Ki-67 protein expression, indicating ceased cell proliferation.

One indication of beginning neuronal or glial cell differentiation is the expression of neural marker proteins. Expression of neuron- and glia-specific proteins was therefore investigated in cultured hUCB cells by Western blot analysis, comparing cells maintained in CM, PM, or DM (Fig. 3). Expression of neural marker proteins was absent from cells cultured in CM, which was in line with our immunocytochemical observations (data not shown). In PM, NF68 and NeuN proteins were expressed at low levels. When cultured in the presence of NGF/RA for 7 days, high expression levels of the neuron-specific proteins NeuN, NF68, and β3-tubulin were observed. In addition, GFAP and Cx43, which are markers of astroglial cells as well as neuronal precursors, were also detected in cells cultured in DM (data not shown).

To understand time-dependent changes during differentiation, the expression profile of neural marker proteins was dissected within the 7-day culture period in DM (Fig. 4). The transition of the cell phenotype in DM (compare Fig. 1) was accompanied by changes in the expression of precursor-, neuron-, and glia-specific proteins. Quantification of immunocytochemical staining revealed that, after 1 day in DM, 73% of cells were immunopositive for the progenitor marker nestin. The percentage of nestin-positive cells decreased with time, being reduced to 62% after 3 days and 27% after 7 days in DM. For GFAP and NF68, the number of immunopositive cells remained relatively constant over the culture period of 7 days. In contrast, microtubule-associated protein II was present in 5% of cells after 1 day in DM, and the nuclear antigen NeuN in 9% of cells. With continuing culture time in DM this percentage increased, reaching 51% and 35%, respectively, after 7 days.

Immunocytochemical staining for nestin as well as for GFAP and Cx43 is presented after 3 days in DM (Fig. 5 B–D), whereas immunolabeling for neuronal marker proteins NF68, and NF200 was obtained from cells cultured in DM for 7 days (Fig. 5 E–F). In addition, neuronal transcription factors musashi-1 and notch were also expressed after a culture period of 7 days in DM (data not shown).

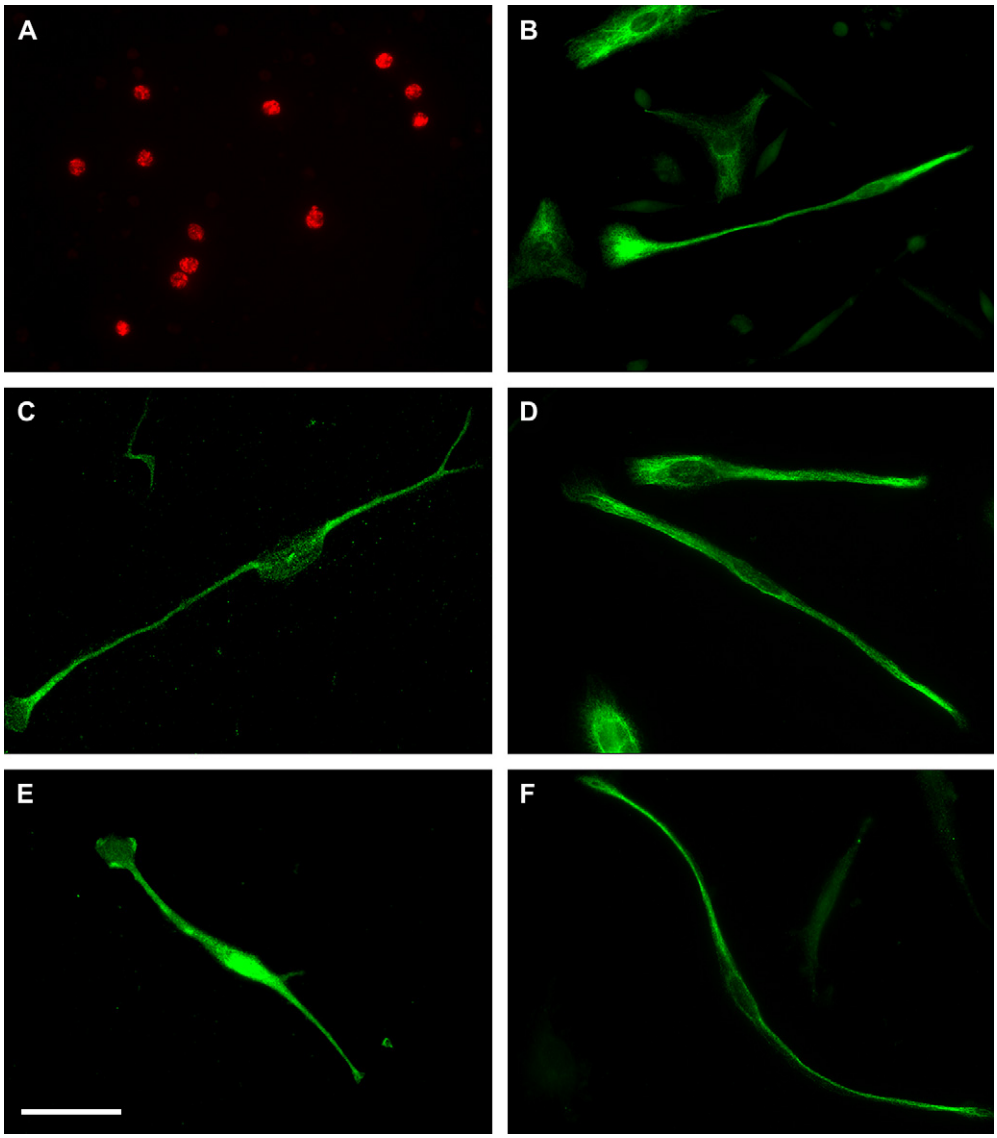


Figure 5. Expression of neural marker proteins in the presence of nerve growth factor/retinoic acid. (**A**) Mononuclear cells from human umbilical cord blood display a round morphology after isolation, as evident from immunolabeling using antibodies against the human nuclei antigen. Immunostaining for nestin (**B**), glial fibrillary acidic protein (**C**), and Cx43 (**D**) was performed after culturing mononuclear cells for 3 days in differentiation medium. Expression of neuronal marker proteins NF68 (**E**) and NF200 (**F**) was investigated immunocytochemically after a culture period of 7 days in differentiation medium. Scale bar: 20 μm (**A**–**F**).

Taken together, consecutive application of growth factors led to morphological changes and proliferation of cultured hUCB-derived mononuclear cells, followed by differentiation as assessed by expression of neural marker proteins.

Cell analysis of hUCB-derived mononuclear cells

To understand differentiation and cell-selection events during culture, we performed fluorescence-activated cell (FACS) analysis on cord blood as well as on native and cultured cells of the mononuclear fraction (Table 1). In freshly isolated hUCB-derived mononuclear cells, 59.97% of cells were CD45-positive lymphocytes, with time in culture, however, this proportion was reduced to 45.87% ± 9.88% in CM, 21.56% ± 1.17% in PM, and 2.29% ± 0.42% in DM. The distribution of T cells (CD3-positive), B-cells (CD19-positive), and natural killer cells (CD16+CD56-positive) within the lymphocyte population remained relatively constant (Table 1). The proportion of CD34-positive hematopoetic progenitor cells and of CD14-positive monocytes was also reduced after 2 days in culture when compared to the percentages present in the mononuclear fraction immediately after isolation.

As CD45 is expressed on the surface of all leukocytes, we chose this protein to follow lineage segregation. Thus, immunocytochemistry for CD45 was performed on

Table 1. Incidence of hematopoetic antigens of hUCB cells

Antigen	Expression on	Cord blood	Mononuclear cell fraction	CM	PM	DM
%						
CD45	Leukocytes	32.63 ± 2.50	59.97 ± 9.90	45.87 ± 9.88	21.56 ± 1.17	2.29 ± 0.42
% of Lymphocytes						
CD3	T cells	64.17 ± 8.21	ND	80.09 ± 3.25	63.94 ± 6.83	50.75 ± 10.63
CD16+CD56	B cells	13.54 ± 6.50	ND	9.18 ± 3.25	8.35 ± 2.67	10.79 ± 3.73
CD19	NK cells	12.69 ± 4.43	ND	7.64 ± 1.51	10.79 ± 1.61	1.88 ± 0.83
% of White blood cells						
CD14	Monocytes	8.16 ± 1.00	17.98 ± 3.70	11.24 ± 2.78	ND	ND
CD34	Hematopoetic progenitor cells	0.57 ± 0.22	1.41 ± 0.40	0.27 ± 0.13	ND	ND

Percentage presented as mean ± standard error. The comparison of antigen expression of hUCB cells is made between whole cord blood, isolated mononuclear fraction, and cultured cells in CM, PM and DM.
CM = culture medium; DM = differentiation medium; hUCB = human umbilical cord blood; ND = not determined; NK = natural killer; PM = proliferation medium.

adherent cells cultured in different media (CM, PM, and DM). After 4 days in culture, 93.2% ± 0.8% of cells were CD45-immunopositive, which relates to 95% determined by FACS analysis, and represents the percentage of leukocytes in CM. Numbers significantly ($p < 0.01$) decreased to 29.8% ± 2.1% in PM and 6.6% ± 0.3% in DM (Fig. 6), reflecting the decline of CD45-expressing cells in culture as observed by FACS analysis. Interestingly, cells expressing neural marker proteins like GFAP or NF68 when cultured in the presence of NGF/RA displayed reduced levels of CD45 or no CD45 at all (Fig. 6). In summary, the CD45 expressing leukocyte population is reduced in culture, and the proportion correlates inversely to that of neural marker protein expressing cells.

Secretion of human cytokines, chemokines, and growth factors by cultured hUCB-derived mononuclear cells

The conditioned medium of cultured mononuclear cells was assayed at three independent time points using a human cytokine antibody array (Fig. 7) that simultaneously detected the presence of 79 cytokines in medium. The amount of cytokines secreted by hUCB-derived mononuclear cells was hardly detectable in cells cultured in serum-containing CM with signal intensities below 1.7. These values were consistently low in all classes of cytokines investigated and therefore not considered for further analysis.

In contrast, cells cultured in PM or DM secreted high levels of cytokines, with 18 proteins displaying a significant increase in signal intensity of conditioned media compared to nonconditioned controls (Table 2). In view of their potential action in vivo, proteins secreted by cells cultured in PM or DM have been classified into the groups of interleukins, growth factors, and chemokines.

Interleukins

In the group of interleukins (IL), IL-2, IL-3, IL-4, IL-5, and IL-15 (data not shown) as well as IL-1α, IL-12, IL-13, and IL-16 (Fig. 8A) were secreted at very low levels. In contrast, intensity values for IL-1β, IL-6, IL-7, IL-8, and IL-10 had significantly increased in either one or both of the media (Fig. 8A). Interestingly, the four interleukins demonstrating the most abundant secretion in this assay all belonged to the family of anti-inflammatory cytokines and included IL-1β, IL-6, IL-8, and IL-10. In contrast, the pro-inflammatory cytokine IL-16 was barely detectable.

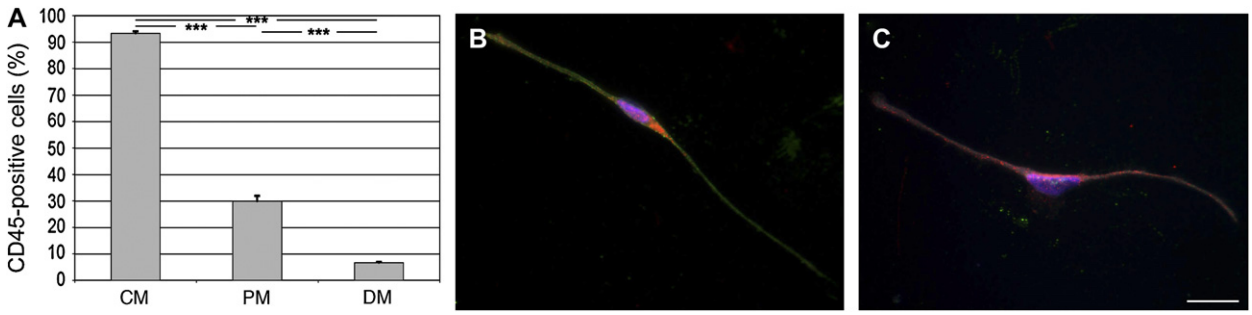
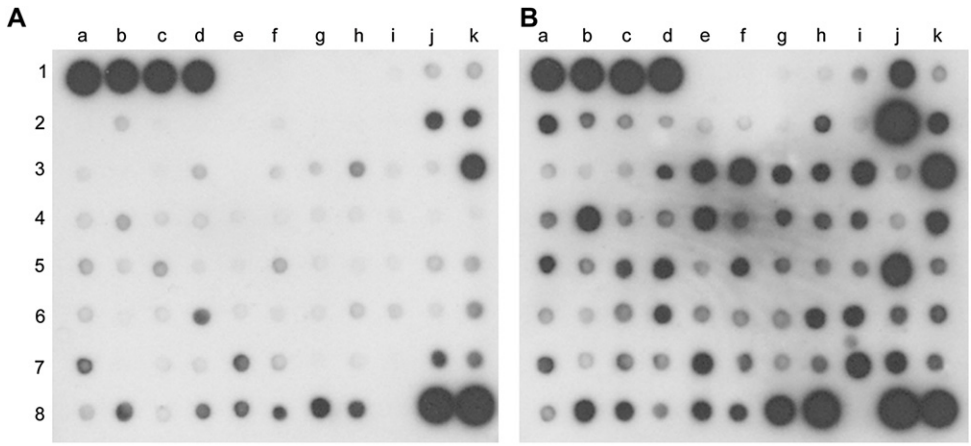


Figure 6. Expression of CD45 in cultured human umbilical cord blood–derived mononuclear cells is reduced in differentiating cells. (**A**) Percentage of CD45 expressing leukocytes cultured in serum containing medium (CM) significantly (***$p < 0.05$) decreased in proliferation medium (PM) and differentiation medium (DM) as quantified from immunocytochemical staining. (**B**,**C**) Immunocytochemistry for CD45 (green fluorescence) and NF68 (red fluorescence), showing reduced (**B**) or absent (**C**) expression of CD45 in elongated cells expressing the neural marker protein NF68. Nuclei are labeled by Hoechst dye (blue). Scale bar: 20 μm.



C

	a	b	c	d	e	f	g	h	i	j	k
1	Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-a
2	I-309	IL-1a	Il-1ß	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
3	IL-12	IL-13	IL-15	IFN-g	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1ß
4	MIP-1d	RANTES	SCF	SDF-1	TARC	TGF-ß1	TNF-a	TNF-ß	EGF	IGF-I	Ang
5	OSM	Tpo	VEGF	PDGF-B	Leptin	BDNF	BLC	Ckß 8-1	Etx	Etx-2	Etx-3
6	FGF-4	FGF-6	FGF-7	FGF-9	Flt-3 Lig.	Fract	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3a	NAP-2	NT-3
8	NT-4	Osteoprot	PARC	PIGF	TGF-ß2	TGF-ß3	TIMP-1	TIMP-2	Neg	Pos	Pos

Figure 7. Protein antibody array detecting cytokines in human umbilical cord blood (hUCB)–cell conditioned media. Representative examples of two antibody array membranes, incubated with either nonconditioned culture medium (**A**) or culture medium conditioned by hUCB-derived mononuclear cells for 2 days (**B**). Increasing intensity reveals increased secretion of the cytokines investigated (for quantification, see Fig. 8 and Table 2). Dots a1 to d1 and j8 to k8 were positive controls (Pos); dots e1 to f1 and i8 were negative controls (Neg). (**C**) Overview of all proteins assayed on the membrane. Boxed factors are color-coded and refer to those proteins secreted at significant levels. Colors designate interleukin proteins (purple), growth factors (blue), chemokines (yellow), as well as tissue inhibitors of metalloproteinase (TIMP)-2 (orange). Ang = angiogenin; BDNF = brain-derived neurotrophic factor; BLC = B-lymphocyte chemoattractant; EGF = epidermal growth factor; ENA-78 = epithelial neutrophil-activating protein-78; Etx = eotaxin; FGF = fibroblast growth factor; Fract = fractalkine; GCP-2 = granulocyte chemotactic protein-2; GCSF = granulocyte colony–stimulating factor; GDNF = glial cell–derived neurotrophic factor; GM-CSF = granulocyte macrophage colony–stimulating factor; GRO = growth-regulated oncogene; HGF = hepatocyte growth factor; IFN-g = interferon-γ; IGF-1 = insulin-like growth factor-1; IGFBP = insulin-like growth factor binding protein; IL = interleukin; IP-10 = interferon-inducible protein-10; LIF = leukemia inhibitory factor; MCP = monocyte chemoattractant protein; MCSF = macrophage colony–stimulating factor; MDC = macrophage derived chemokine; MIF = macrophage inhibitory factor; MIG = monokine induced by γ-interferon; MIP = macrophage inflammatory protein; NAP-2 = neutrophil activating protein-2; NT = neurotrophin; OSM = oncostatin M; Osteoprot = osteoprotegrin; PARC = pulmonary and activation-regulated chemokine; PDGF-B = platelet-derived growth factor-B; PIGF = placenta growth factor; SCF = stem cell factor; SDF-1 = stromal-derived factor-1; TARC = thymus associated and regulated chemokine; TGF-β = transforming growth factor-β; TNF = tumor necrosis factor; TIMP = tissue inhibitor of melloproteinases; Tpo = thrombopoietin; VEGF = vascular endothelial growth factor.

Growth factors

The following growth factors, including neurotrophic factors, were analyzed in the cytokine array: Ang, vascular endothelial growth factor, brain-derived neurotrophic factor, glia-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4, FGF-4, FGF-6, FGF-7, and FGF-9, platelet-derived growth factor-B (PDGF-B), EGF, hepatocyte growth factor (HGF), transforming growth factor (TGF)-β1, TGF-β2, TGF-β3, insulin-like growth factor-1 (IGF-1), leukemia inhibitory factor, stem cell factor, Leptin, and the Flt-4 ligand. Of these, elevated secretion levels (i.e., above 10) were observed for Ang, vascular endothelial growth factor, brain-derived neurotrophic factor, glia-derived neurotrophic factor, PDGF-B, EGF, HGF, TGF-β1, TGF-β2, TGF-β3, IGF-1, leukemia inhibitory factor, stem cell factor, and Leptin, with levels of Ang, PDGF-B, EGF, and HGF being upregulated significantly (Fig. 8B).

Chemokines

A number of chemokines, which are small chemoattractant proteins with molecular weights between 8 kD and 12 kD, have been analyzed: MCP-1 to -4, macrophage inflammatory protein (MIP)-1β/CCL4, MIP-1δ, MIP-3α, stromal-derived factor-1/CXCL12, RANTES (CCL5), macrophage-colony stimulating factor, macrophage-derived chemokine/CCL22, monokine induced by gamma interferon/CCL9, thymus associated and regulated chemokine/CCL17, pulmonary and activation-regulated chemokine (PARC/CCL18),

Table 2. Mean signal intensities of significantly regulated factors

	Conditioned PM (intensity values)	Conditioned DM (intensity values)
Interleukins		
IL-1α	9.38 ± 3.50	10.49 ± 5.51
IL-1β	20.59 ± 8.60	19.90 ± 4.46
IL-6	77.90 ± 21.40	73.15 ± 19.40
IL-7	14.42 ± 7.17	21.21 ± 8.73
IL-8	81.32 ± 26.04	119.68 ± 10.11
IL-10	30.05 ± 11.55	50.02 ± 10.31
IL-12	6.58 ± 3.84	0.67 ± 0.33
IL-13	9.93 ± 4.92	2.04 ± 1.41
IL-16	6.44 ± 5.94	4.77 ± 4.28
Growth factors		
Ang	14.01 ± 5.77	59.76 ± 4.10
VEGF	11.05 ± 5.40	12.27 ± 10.94
BDNF	12.57 ± 6.04	19.97 ± 18.28
GDNF	11.08 ± 5.53	20.23 ± 16.11
NT-3	11.05 ± 5.28	17.83 ± 8.72
NT-4	4.56 ± 4.06	1.30 ± 0.85
PDGF-B	12.91 ± 6.20	22.69 ± 14.55
EGF	—	29.81 ± 6.70
HGF	13.89 ± 7.87	34.12 ± 9.47
Chemokines		
MCP-1	24.88 ± 9.45	57.66 ± 9.61
MCP-4	19.89 ± 7.08	12.62 ± 8.41
MIP-1β	46.96 ± 8.50	70.24 ± 14.86
MIP-3α	23.48 ± 8.47	45.02 ± 14.62
SDF-1	13.66 ± 6.74	15.11 ± 13.79
Etx-2	22.21 ± 10.93	49.75 ± 21.11
PARC	31.95 ± 13.59	40.47 ± 12.94
MIG	14.65 ± 7.09	52.72 ± 11.66
GRO	20.71 ± 9.94	76.42 ± 5.59
Inhibitors of metalloproteinases		
TIMP-1	36.57 ± 8.23	49.49 ± 5.29
TIMP-2	51.99 ± 16.33	106.30 ± 7.42

Signal intensities are presented for those interleukins, growth factors, and chemokines illustrated in Figure 7. In addition, values for TIMP-1 and TIMP-2 proteins are listed.

Values represent the means of three independent experiments ± standard error of the mean. Measurements of secreted EGF levels in conditioned PM have not been considered for analysis, as this medium was supplemented with recombinant EGF.

Ang = angiogenin; BDNF = brain-derived neurotrophic factor; DM = differentiation medium; EGF = epidermal growth factor; Etx = eotaxin-2; GDNF = glia-derived neurotrophic factor; GRO = growth-regulated oncogene; HGF = hepatocyte growth factor; IL = interleukin; MCP = monocyte chemoattractant protein; MIG = monokine induced by γ-interferon; MIP = macrophage inflammatory protein; NT = neurotrophin; PARC = pulmonary and activation-regulated chemokine; PDGF-B = platelet-derived growth factor-B; PM = proliferation medium; SDF-1 = stromal-derived factor-1; TIMP = tissue inhibitors of metalloproteinases; VEGF = vascular endothelial growth factor.

macrophage inhibitory factor, B lymphocyte chemoattractant/CXCL13, GRO, eotaxin-1/CCL11 (Etx), eotaxin-2/CCL24 (Etx-2), and eotaxin-3/CCL26 (Etx-3). For all of these factors, substantial levels of secretion, i.e., intensity values > 10, were detected in either one or both of the conditioned media (PM, DM) investigated. A significant increase was observed for MCP-1, MCP-4, MIP-1β, MIP-3α, Etx-2, PARC, monokine induced by gamma interferon, and GRO (Fig. 8C).

In addition, a strong chemoattractant activity has recently been described for HGF, which was also secreted at significant levels. Of all factors investigated in this assay, IL-6 and IL-8 (see Interleukins), Ang, MIP-1β, and GRO were secreted at highest levels. In addition, the tissue inhibitors of metalloproteinases-1 and -2 displayed signals of high intensity.

To gain information on the putative biological relevance of secreted proteins, enzyme-linked immunosorbent assay analysis was performed on non-conditioned and conditioned media (CM, PM, DM) for IL-8, Ang, MIP-1β, GRO, and PDGF-BB (Table 3). In conditioned PM and DM, levels of cytokines ranged from 7.11 pg/mL (for PDGF-BB) to 56.84 ng/mL (for IL-8). In contrast, non-conditioned media did not yield any detectable levels of secreted proteins. The clear increase in the protein contents of conditioned media as compared to non-conditioned control media allows speculation on the biological relevance of these factors.

In summary, hUCB-derived mononuclear cells secreted a high number of proteins with anti-inflammatory, angiogenic, neurotrophic, or chemotactic activity.

Discussion

As known from other studies, the mononuclear fraction of hUCB contains multipotent stem cells, which were shown to give rise to either the hematopoetic lineage or to neural cells [9]. In addition, a certain cell population within cord blood, named unrestricted somatic stem cell, was also shown to be capable of differentiation into osteoblasts, chondroblasts, adipocytes, neural and hematopoetic cells [5]. On the other hand, transplantation of the complete mononuclear fraction from umbilical cord blood, including all blood cells and stem cells within, has been demonstrated to ameliorate neurological and sensorimotor deficits in a variety of animal lesion models [10–12,14,15,22]. Thus, we have chosen to investigate the potential of the complete mononuclear fraction in this study. Using a succession of three different media, each of which was supplemented with either serum or certain growth factor combinations, we were able to 1) cultivate an adherent subpopulation of cells, 2) stimulate cell proliferation, 3) induce the expression of neural marker proteins, and 4) activate secretion of a number of chemokines, cytokines, and growth factors in vitro.

In umbilical cord blood, the distribution of lymphocytes and monocytes was determined and values were similar to those obtained in a different study [23]. In culture, the CD45 expressing lymphocyte population decreased substantially in cells stimulated with EGF/FGF-2 and further in NGF/RA-containing medium. As these factors were also shown to induce the induction of marker proteins

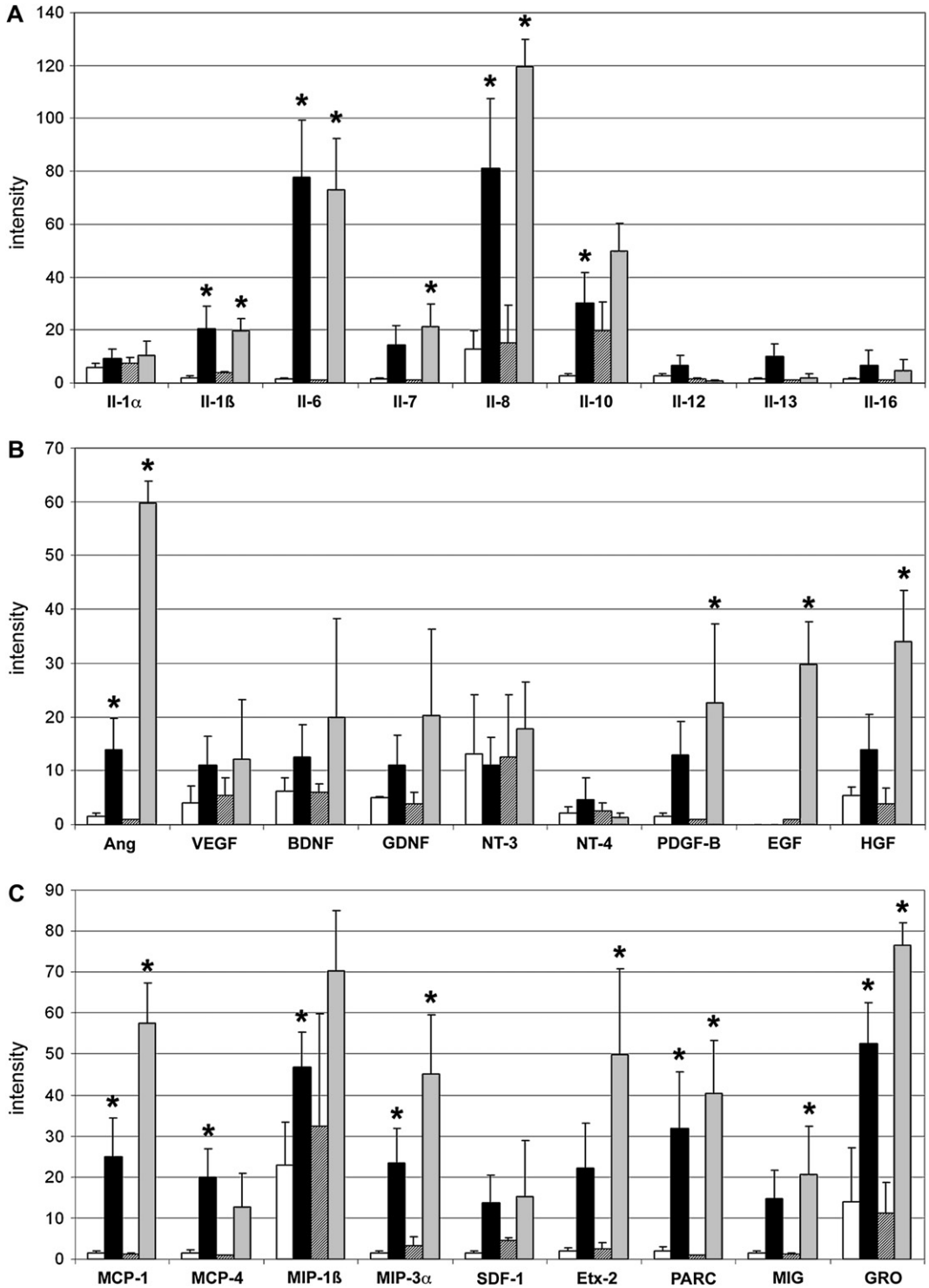


Figure 8. Secretion of interleukins, growth factors, and chemokines by human umbilical cord blood (hUCB)–derived mononuclear cells in vitro. Detection of interleukin secretion in conditioned medium of hUCB-derived mononuclear cells cultured in serum-containing culture medium (CM), serum-free proliferation medium containing epidermal growth factor/fibroblast growth factor-2 (EFG/FGF-2) (PM), or serum-free differentiation medium containing nerve growth factor (NGF)/retinoic acid (RA) (DM). In all data from the cytokine array analysis, intensity refers to the density of the electrochemiluminescence reaction product on microarray films, given in random values. In the conditioned medium of cells cultured in CM, levels of secreted interleukins were hardly detectable (not shown). Non-conditioned media (PM: white columns; DM: hatched columns) served as negative controls. Stimulation by either EGF/FGF-2 (conditioned PM; black columns) or by NGF/RA (conditioned DM; gray columns) resulted in an increase of several factors detected in conditioned medium. (**A**) Growth factor stimulation resulted in the secretion of several interleukins, predominantly IL-6 and IL-8. (**B**) In both PM and DM, angiogenic growth factors (Ang) and neurotrophic factors (PDGF-B, HGF, EGF) were secreted into the culture medium at significant levels. Neurotrophin levels were also

Table 3. Mean concentrations of significantly regulated factors as determined by ELISA

	Conditioned CM	Conditioned PM	Conditioned DM
Interleukin-8 (ng/mL)	1.75 ± 0.23	79.4 ± 14.67	56.84 ± 26.99
Growth factors			
Ang (pg/mL)	2.78 ± 0.72	78.67 ± 53.31	565.89 ± 79.81
PDGF-B (pg/mL)	1.71 ± 1.07	7.11 ± 0.61	24.08 ± 1.83
Chemokines			
MCP-1 (ng/mL)	0.06 ± 0.02	11.39 ± 2.72	7.98 ± 1.55
GRO (pg/mL)	292.62 ± 95.16	744.65 ± 272.40	537.82 ± 158.75

Mean concentrations of selected interleukins, growth factors and chemokines are listed. Values represent the means of a minimum of three independent experiments ± standard error.
Ang = angiogenin; ELISA = enzyme-linked immunosorbent assay; GRO = growth-regulated oncogene; MCP = monocyte chemoattractant protein; PDGF-B = platelet-derived growth factor-B.

associated with neural differentiation, reduction in the number of CD45 expressing cells is likely to be inversely correlated to differentiation events. In addition, CD45 levels seemed to be reduced at the cellular level upon expression of neural proteins, as described previously for the microtubule-associated protein II [24]. The inverse correlation of hematopoetic and neural marker proteins might therefore be indicative of transdifferentiation, as has been suggested by other studies [24–27]. However, alternative or additional mechanisms like lineage selection cannot be excluded.

Incipient differentiation of the adherent mononuclear cell population in vitro was demonstrated by morphological changes as well as by expression of neural marker proteins, which had also been observed previously using different culture protocols [18–20,24,28]. However, there is only one report on successful neuronal differentiation that includes demonstration of the electrophysiological properties of these cells in vitro [29]. Thus, it seems likely that treatment with the combination of NGF/RA is able to induce expression of neural proteins, but might not be sufficient to advance cell differentiation to a mature neuronal phenotype. Expression of neuronal proteins by transplanted hUCB cells has also been reported in vivo [14,16]. Theoretically, additional differentiation factors might be present in lesion areas in vivo and, thus, be capable of inducing full differentiation of these cells. Nevertheless, as the percentage of neuronal differentiated cells was very low (2–4%) and their physiological function remains to be demonstrated, the impact of neuronal cell differentiation in vivo is still uncertain. One further reservation came from transplantation studies in a model of perinatal brain damage [11], in which human leukocyte antigen DR–immunopositive mononuclear cells—despite migrating to a hypoxic-ischemic brain lesion and displaying significant therapeutic effects—demonstrated no obvious signs of differentiation.

It was, therefore, suggested that secondary mechanisms might be responsible for the beneficial outcome upon hUCB mononuclear cell transplantation in this lesion model. One potential mode of interaction might be the release of neuroprotective, anti-inflammatory, or angiogenic factors by transplanted cells, thereby providing the basis for an indirect effect on host tissue.

Analysis of medium conditioned by hUCB-derived mononuclear cells by two independent methods revealed substantial secretion of a number of cytokines, chemokines, and growth factors, induced by EGF/FGF-2 or NGF/RA treatment. Treatment with these two combinations did not result in a differential outcome of cytokine secretion, indicating that the cues for stimulation might be more universal than anticipated. Interestingly, many of the secreted factors are renowned for their beneficial effects under inflammatory or brain-damaging conditions. In the group of interleukins, IL-6, IL-8, and IL-10 were most abundant in the conditioned medium, and are known to exert anti-inflammatory effects [30]. In a study by Newman et al. [31], IL-1α and IL-8 were also described to be consistently expressed by cultured umbilical cord blood cells. Liu and Hwang [32] reported an increased secretion of IL-6 by hUCB-derived mesenchymal stem cells upon stimulation

high, but not significant. In contrast, for several factors investigated, including members of the fibroblast growth factor family (FGFs) and the Flt-4 ligand, there was no significant difference between nonconditioned and conditioned media (data not shown). (**C**) Significant levels of secretion of chemotactic factors were observed in the presence of EGF/FGF-2 (PM) as well as NGF/RA (DM), and these factors included members of the MCP and MIP families, MIG, PARC, and GRO. *Significant increase ($p < 0.05$) of signal intensity in conditioned media as compared to nonconditioned control media. Ang = angiogenin; BDNF = brain-derived neurotrophic factor; EGF = epidermal growth factor; Etx = eotaxin; GDNF = glial–derived neurotrophic factor; GRO = growth-regulated oncogene; HGF = hepatocyte growth factor; Il = interleukin; MCP = monocyte chemoattractant protein; MIG = monokine induced by γ-interferon; MIP = macrophage inflammatory protein; NT = neurotrophin; PARC = pulmonary and activation-regulated chemokine; PDGF-B = platelet-derived growth factor-B; SDF-1 = stromal-derived factor-1; VEGF = vascular endothelial growth factor.

with IL-1β. However, FGF-2 alone failed to stimulate IL-6 secretion in those cells [32], whereas in our study the combination of FGF-2/EGF resulted in a significant increase of IL-6 secretion by hUCB-derived mononuclear cells. Interestingly, IL-8 had also been demonstrated to induce angiogenesis [33,34], and might therefore act in concert with the angiogenic factor Ang, which was secreted by mononuclear cells. Most recently, additional factors, also secreted in our experimental paradigm, were associated with the induction of angiogenesis, including stromal-derived factor-1, MCP-1, and eotaxins [35,36].

Another important aspect of a putative "bystander" effect might be the neuroprotective action of hUCB-secreted factors on host neurons. Receptors for PDGF-B were detected on oligodendrocytes and astroglia cells, and the factor was found to promote proliferation and mobility of astrocytes, two effects of major importance with respect to glial scar formation. In addition, PDGF-B is involved in nervous system development and was shown to exert neurotrophic and neuroregenerative activities as well as the induction of neurite outgrowth [37,38]. Interestingly, the effect of PDGF-B on astrocytes was enhanced by GRO [39], one of the most abundant factors in mononuclear cell conditioned medium. GRO is found predominantly on monocytes after cell activation, and was also described as a strong mitogen for oligodendrocyte precursor cells [39].

Various chemokines were secreted by hUCB-derived mononuclear cells, including MCP-1, MIP-1β, and PARC. In general, chemokines are involved in cellular migration and intercellular communication. The PARC protein, for instance, has been well-characterized in the immune system, where it acts chemotactically and contributes to the physiological homing of lymphocytes and dendritic cells [40]. In the brain, macro glia cells and infiltrating leukocytes are the most abundant source of chemokines [41–43], however, microglia cells and neurons were also shown to secrete chemokine proteins [44–46]. Thus, these factors might exert their effects on host immune cells, including microglia, on astrocytes, and on other mobile cells within the host brain. On the other hand, chemoattractive factors secreted by umbilical cord blood cells might also enhance migration and "homing" of transplanted hUCB-derived mononuclear cells in vivo in an autocrine enhancement loop.

In our assay system, secretion of factors was strongly induced by stimulation via EGF/FGF-2 or by NGF/RA. As these factors are also present in the injured brain, it is conceivable that transplanted umbilical cord blood cells are attracted to the brain lesion, where they might be exposed to these stimulating growth factors in vivo. Thus, hUCB-derived mononuclear cells might be activated in vivo to secrete some if not all of the factors described previously. As hUCB cells display a selective "homing" to lesioned brain areas [11], the release of these factors in vivo would be highly specific and locally limited to the lesioned brain area. Thus, transplanted hUCB-derived mononuclear cells might improve functional outcome through a bystander effect, possibly mediated by a cocktail of factors secreted by umbilical cells exerting anti-inflammatory effects and resulting in improved angiogenesis, neuronal and glial cell survival.

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