

Induction of glial glutamate transporters in adult mesenchymal stem cells

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Abstract

Adult bone marrow mesenchymal stem cells are multipotent cells that can differentiate into a variety of mesodermal tissues. Recent studies have reported on their ability to also evolve into non-mesodermal cells, especially neural cells. While most of these studies revealed that manipulating these cells triggers the expression of typical neurone markers, less is known about the induction of neuronal- or glial-related physiological properties. The present study focused on the characterisation of glutamate transporters expression and activity in rat mesenchymal stem cells grown in culture conditions favouring their differentiation into astroglial cells. Ten days exposure of the cells to the culture supplement G5 was found to increase the expression of nestin (neuro-epithelial stem cell intermediate filament), an intermediate filament protein expressed by neural stem cells. Simultaneously, a robust induction of the high-affinity glutamate transporter

GLT-1 (and GLAST) expression was detected by RT-PCR and immunocytochemistry. This expression was correlated with a highly significant increase in the Na⁺-dependent [³H]D-aspartate uptake. Finally, while glial fibrillary acidic protein immunoreactivity could not be detected, the induced expression of the astrocytic enzyme glutamine synthetase was demonstrated. These results indicate that *in vitro* differentiation of adult mesenchymal stem cells in neural precursors coincides with the induction of functional glutamate transport systems. Although the astrocytic nature of these cells remains to be confirmed, this observation gives support to the study of mesenchymal stem cells as a promising tool for the treatment of neurological diseases involving glutamate excitotoxicity.

Keywords: glial differentiation, glutamine synthetase, growth factors, nestin, retinoic acid, S100β.

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Bone marrow contains two prototypical stem cell populations, those of the lymphohematopoietic lineage, that continually repopulate the blood circulation, and the stromal cells, which are mesodermal elements that normally give rise to mesenchymal derivatives only. The latter were initially named plastic-adherent cells or colony-forming-unit fibroblasts and subsequently named either marrow stromal cells or mesenchymal stem cells (MSCs). These cells are multipotent as they differentiate in culture or after *in vivo* implantation into osteocytes, adipocytes, and chondrocytes (Pittenger *et al.* 1999). Adult MSCs, however, have the ability to differentiate into a diversity of cell types that may be unrelated to their phenotypical embryonic origin. Indeed, some authors have reported that bone marrow-derived cells can evolve into liver (Petersen *et al.* 1999), heart (Orlic *et al.* 2001), skeletal muscle (Bittner *et al.* 1999), skin (Reyes *et al.* 2002) or vascular endothelium (Jackson *et al.* 2001).

Interestingly, some authors have shown that bone marrow stem cells can differentiate into neurones either *in vitro* (Sanchez-Ramos *et al.* 2000; Woodbury *et al.* 2000; Jiang

et al. 2002) or after transplantation into the brain (Azizi *et al.* 1998; Kopen *et al.* 1999; Brazelton *et al.* 2000; Mezey *et al.* 2000). Indeed, such neuronal differentiation was appreciated after immunological detection of neuronal markers (NeuN and fibronectin) as well as the neuro-epithelial stem cell

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Abbreviations used: CNTF, ciliary neurotrophic factor; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride hydrate; DHK, dihydrokaïnate; EAAT, excitatory amino acid transporters; FACS, fluorescence-activated cell sorter; FGF₂, fibroblast growth factor 2; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; ICC, immunocytochemistry; MAPC, multipotent adult progenitor cell; MSCs, mesenchymal stem cells; nestin, neuro-epithelial stem cell intermediate filament; RA, all-*trans* retinoic acid; t-PDC, L-*trans*-pyrrolidine-2,4-dicarboxylic acid.

intermediate filament (nestin) protein that is expressed in neuro-astroglial precursors. Furthermore, two studies reported on the acquisition, by differentiated MSCs, of electrophysiological properties consistent with a neuronal phenotype (Hung *et al.* 2002; Jiang *et al.* 2003). However, while much efforts have focused on the study of the differentiation of MSCs into neurones, limited data is available regarding the possibility to drive their differentiation into mature and functional astrocytes. Indeed, some authors demonstrated astroglial differentiation by immunological detection of the glial fibrillary acidic protein (GFAP), but no typical functional properties of astrocytes were assessed.

Astrocytes play several critical roles in the CNS, including the dynamic control of the synaptic glutamate transmission (Newman 2003). L-Glutamate is the most common and widespread excitatory neurotransmitter in the CNS of mammals (Fonnum 1984). However, at high concentrations, glutamate may act as a potent endogenous neurotoxin, capable of causing neuronal degeneration. This glutamate excitotoxicity appears to be involved in many neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases, as well as amyotrophic lateral sclerosis, epilepsy and stroke (Meldrum 1998; Trotti *et al.* 1998). Glutamate is removed from the synaptic cleft by a family of membrane proteins known as excitatory amino acid transporters (EAAT). These proteins are expressed by many cell types in the CNS, including neurones and astrocytes (Kanai and Hediger 1992; Rothstein *et al.* 1994). To date, five high-affinity Na⁺-dependent glutamate transporters have been cloned and characterised from rodent and human brain: glutamate/aspartate transporter (GLAST/EAAT1), glutamate transporter-1 (GLT-1/EAAT2), excitatory amino acid carrier (EAAC1/EAAT3), EAAT4 and EAAT5 (Seal and Amara 1999; Danbolt 2001). Immunological studies revealed that GLAST and GLT-1 are primarily localised in astroglial membranes (Chaudhry *et al.* 1995), whereas EAAC1/EAAT3 and EAAT4 are selectively expressed in neurones (Kanai and Hediger 1992). EAAT5 is located in the glia and neurones of the retina (Arriza *et al.* 1997). It is generally accepted that astrocytes, rather than neurones, perform most of the glutamate clearance to maintain low, non-toxic synaptic glutamate concentrations (Rothstein *et al.* 1996; Anderson and Swanson 2000). In addition, astroglial cells are also selectively enriched in glutamine synthetase, an enzyme involved in the breakdown of intracellular glutamate into glutamine (Vogel *et al.* 1975; Norenberg and Martinez-Hernandez 1979).

The aim of the present study is to evaluate whether differentiation of MSCs into astrocytes can be induced *in vitro*. While previous studies reported on the acquisition of typical astrocytic markers by immunological methods, our approach was also focused on the detection of glutamate transporters. Expression of the glial transporters GLAST and GLT-1 was analysed by RT-PCR and immunocytochemistry

(ICC) and their activity was evaluated by measuring the ability of the cells to take up labelled aspartate.

Materials and methods

Animals

Female Lewis rats were obtained from the UCL central animal facility. Animals housed in plastic cages were kept at constant temperature ($23 \pm 1^\circ\text{C}$) and relative humidity (40–60%) on a 12-h light : 12-h dark cycle with *ad libitum* access to both food and water. All animal procedures were conducted in strict adherence to the European Community Council directive of 24 November 1986 (86–609/EEC) and Decree of 20 October 1987 (87–848/EEC).

Materials

Culture medium, foetal bovine serum, penicillin, streptomycin, G5 supplement, reverse transcription kit, elongase and PCR primers were obtained from Invitrogen (Merelbeke, Belgium). Fibroblast growth factor 2 (FGF₂) and ciliary neurotrophic factor (CNTF) were from Peprotech (London, UK). Dexamethasone, indomethacine, insulin, 3-isobutyl-1-methyl-xanthine, ascorbic acid, β -glycerophosphate, all-*trans* retinoic acid (RA), poly-L-lysine, 4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) and the polyclonal primary anti-glutamine synthetase Ig (G2781) were from Sigma (Bornem, Belgium). The polyclonal primary anti-GFAP Ig (Z0334) and the Intrastain reagents A and B were from DAKO (Heule, Belgium), the monoclonal primary anti-CD45 Ig was from BD Pharmingen (550566, Erembodegem, Belgium), the polyclonal primary anti-S100 β Ig was a kind gift from Prof. R. Pochet (Université Libre de Bruxelles, Belgium), while the monoclonal primary anti-CD90 Ig (CBL1500), the monoclonal primary anti-nestin Ig (MAB353), the polyclonal primary anti-GLAST Ig (AB1782) and the polyclonal primary anti-GLT-1 Ig (AB1783) were purchased from Chemicon (Chandlers Ford, Hampshire, UK). Secondary antibodies were provided by Jackson ImmunoResearch Laboratory (de Pinte, Belgium). Fluoprep was obtained from Biomerieux (Brussels, Belgium). Tripur isolation reagent was from Roche Diagnostics (Vilvoorde, Belgium). Radioisotope [³H]D-aspartate was purchased from Amersham Pharmacia Biotech (Roosendaal, Netherlands). L-*trans*-pyrrolidine-2,4-dicarboxylic acid (t-PDC) was purchased from Tocris (Bristol, United Kingdom) and dihydrokaïnate (DHK) from Ocean Product International (Nova Scotia, Canada).

Preparation and purification of rat MSCs

Tibias and femurs from 8-week-old Lewis rats were dissected, the ends of the bones were cut, and the marrow was extruded in 5 mL of Dulbecco's modified Eagle's medium (DMEM) using a needle and syringe. The cells were plated on 75 cm² tissue culture flasks in proliferation medium (DMEM supplemented with 10% foetal bovine serum, 85 $\mu\text{g/mL}$ streptomycin and 85 U/mL penicillin) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 24 h, the non-adherent cells were removed as the medium was renewed. The medium was replaced every 3–4 days as the cells were grown to 80% confluence. The cells were lifted with 0.25% trypsin and 1 mM EDTA and replated at a density of 5×10^3 cells/cm². By repeating this protocol, cultures were maintained beyond passage 15. In the present work, cell differentiation

was tested at either low or high passages (passage 5 and passage 15, respectively).

***In vitro* differentiation assays**

To induce adipogenic differentiation, the MSCs were plated at a density of 2×10^4 cells/cm² and incubated in proliferation medium (see above) supplemented with 1 μ M dexamethasone, 0.2 mM indomethacine, 10 μ g/mL insulin and 0.5 mM 3-isobutyl-1-methyl-xanthine. Differentiation was appreciated by morphological examination and Oil Red O staining was performed to detect lipid accumulation (Pittenger *et al.* 1999).

The osteogenic differentiation of MSCs was induced by maintaining cells (plated at a density of 3×10^3 cells/cm²) in the proliferation medium supplemented with 0.1 μ M dexamethasone, 50 μ M ascorbic acid and 10 mM β -glycerophosphate. The medium was replaced every 2–3 days during 3 weeks. Von Kossa's staining was performed in order to demonstrate calcium deposition in differentiated cultures (Pittenger *et al.* 1999).

Neuro-astroglial differentiation of MSCs

To induce neuro-astroglial differentiation, the cells were incubated at a density of 10^4 cells/cm² in a culture medium consisting of DMEM/F12 enriched with the defined growth factors supplement G5 diluted 1 : 100, as suggested by the manufacturer (composition: insulin 500 μ g/mL, human transferrin 5 mg/mL, selenite 0.52 μ g/mL, biotin 1 μ g/mL, hydrocortisone 0.36 μ g/mL, FGF₂ 0.52 μ g/mL and epidermal growth factor 1 μ g/mL). When indicated, FGF₂ 10 ng/mL and CNTF 10 ng/mL, were added sequentially for 5 days each. In all cases, cells were exposed to differentiation media for 10 days. In some cases, RA (0.1 μ M) was added to the culture medium during the entire differentiation step or for a single day (followed by 9 days washout).

Immunological characterisation of MSCs

Cells grown on 12 mm round poly-L-lysine coated glass coverslips were fixed with 4% paraformaldehyde (w/v in water) for 15 min at room temperature (20°C) and permeabilized in TBS (Tris/HCl 50 mM, NaCl 150 mM, pH 7.4)/1% Triton X100 for 15 min. Non-specific binding was blocked by incubating the cells for 1 h in a TBS solution containing non-fat dry milk (15 mg/mL) at 37°C. Cells were then incubated for 1 h with primary antibodies, i.e. a mouse anti-CD90 Ig (1 : 500), a mouse anti-CD45 Ig (1 : 500), a mouse anti-nestin Ig (1 : 500), a rabbit anti-GFAP Ig (1 : 1500), a rabbit anti-S100 β Ig (1 : 200), a guinea pig anti-GLAST Ig (1 : 1000), a guinea pig anti-GLT1 Ig (1 : 1000), a rabbit anti-glutamine synthetase Ig (1 : 1000). Secondary antibodies, applied for 1 h at room temperature (20°C) were fluorescein isothiocyanate

(FITC)-conjugated anti-(mouse IgG) Ig (1 : 500), FITC-conjugated anti-(rabbit IgG) Ig (1 : 500), Cy3-conjugated anti-(guinea pig IgG) Ig (1 : 500). Nuclei were stained for 30 min with the nuclear dye, 4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) (1 : 5000). After three rinses in phosphate-buffered saline (PBS: NaCl 0.137 M, KCl 2.68 mM, KH₂PO₄ 1.5 mM, Na₂HPO₄ 8 mM, pH 7.4) the preparations were mounted in Fluoprep and examined using an Olympus IX70 inverted fluorescent microscope coupled to a CCD camera (T.I.L.L. photonics, Martinsried, Germany). Excitation light (485, 540 and 400 nm wavelength for FITC, Cy3 and DAPI, respectively) was obtained from a Xenon lamp coupled to a monochromator (T.I.L.L. photonics, Martinsried, Germany). Digital images were acquired using appropriate filters and combined using the TILLVISION software (T.I.L.L. photonics, Martinsried, Germany).

Cell count assessment

In order to determine the number of positive cells expressing a particular antigen, data were collected from careful examination of 15 fields from three independent cultures per sample. The total number of MSCs in these samples was determined by counting DAPI-stained cell nuclei and results are expressed as percentages of positive cells for either nestin, glutamine synthetase, GLAST or GLT-1.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells grown on poly-L-lysine coated six-well plates and cDNA was generated by reverse transcription. PCR amplifications targeting nestin, GFAP, S100 β , glutamine synthetase, GLT-1 and GLAST were performed using polymerase elongase in a final volume of 30 μ L. A 300-bp amplicon of GAPDH was amplified using specific primers allowing semi-quantitative analysis of PCR data. Primer sequences and sizes of expected PCR products are summarised in Table 1. After 30 cycles of amplification, consisting of denaturation at 94°C for 30 s, annealing at 54–64°C for 30 s and extension at 72°C for 1 min, samples were electrophoresed on a 1% agarose gel and nucleic acids were visualised by ethidium bromide staining. Densitometric analysis of the PCR signals was performed on digital images using an MCID-M4 imaging system (Imaging Research, Ontario, Canada).

Functional characterisation of MSCs

MSCs were grown on poly-L-lysine coated 24-well plates. Plates were placed at the surface of a 37°C water bath, rinsed twice with preheated Krebs buffer (25 mM HEPES, pH 7.4, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 6 mM Glucose and 140 mM NaCl). In these experiments, L-glutamate was substituted by D-aspartate, its transportable analogue which

Table1 Sequences of DNA primers used in PCR and sizes of expected PCR products.

	Forward primers (5' → 3')	Reverse primers (5' → 3')	Size of product (bp)
Nestin	GAAGCCCTGGAGCAGGAGAAGCA	TCCAGGTGTCTGCAACCGAGAGTTC	159
GLAST	GTGGTGAATTCCTGGAAGATAAAATATGAC	CACATTCTAGAACAGTTTCCAACACCTGGTGC	1535
GLT-1	GAGGAGGGGCGTTCCC	TCCAGGAAGGCATCCAG	531
Glutamine synthetase	TACCCGAGTGGAACCTTTGATG	TAAAGTTGGTGTGGCAGCCTG	598
GAPDH	CGGAGTCAACGGATTGGTCGTAT	AGCCTTCTCCATGGTGGTGAAGAC	300
S100 β	GATGTCTGAGCTGGAGAAG	CTCCTGGAAGTCACACTCC	220

does not interact with glutamate receptors and is not metabolised. [^3H]D-aspartate was used at a final concentration of 20 nM. The uptake was stopped after 6 min by three rinses with ice-cold Na^+ -free Krebs buffer in which NaCl was replaced by choline chloride at the same osmolarity (120 mM). The cells were lysed with 500 μL of 1 M NaOH and the radioactivity of 200 μL of the lysate was determined by liquid scintillation counting. A fraction of the lysate was also used for protein determination. The specific activity of the glutamate transporters (expressed as the uptake velocity per the quantity of protein in mg) was estimated after subtracting the data obtained using Na^+ -free Krebs buffer. When indicated, the inhibitors of glutamate transporters t-PDC (1 mM) and DHK (100 μM), were added 6 min before the addition of the substrate. Results obtained in uptake assays were analysed by one-way ANOVA followed by Newman-Keul's test for multiple comparisons in order to evaluate differences between culture conditions and the influence of transporter blockers.

Fluorescence-activated cell sorter (FACS) analysis

Cells were lifted from culture plates using 0.25% trypsin and 1 mM EDTA and fixed for 15 min with the Intrastain reagent A. After washing with PBS, and permeabilization with the Intrastain reagent B, primary antibodies recognizing either CD45 or CD90 were added for 20 min. Cells were then washed and labelled with a FITC-conjugated goat anti-(mouse IgG) Ig. After 20 min, cells were washed and analyzed using a FACSCalibur flow cytometry system and data collected with CELLQUEST software (Becton Dickinson, Aalst, Belgium).

Results

Characterisation of MSCs

Cells were collected from the bone marrow of femurs and tibias of Lewis rats. From the initial bone marrow cell suspension, ≈ 1 cell out of 10000 (0.01%) was found to adhere to the culture flask and it took more than 2 weeks before enough cells were produced to proceed with the first passage. These cells were then propagated in culture monolayers and maintained for multiple passages. They had a 'healthy' fibroblast-like morphology (Fig. 1a) and their doubling time was estimated at 4 days. When tested by immunocytochemistry (ICC) and FACS for different markers at both low and high passage (passage 5 and 15), these cells were CD45-negative indicating the absence of contamination with cells from the lymphohematopoietic lineage (Figs 1b and c). In contrast, immunofluorescent labelling demonstrated that the cells were CD90 (Thy 1.1) positive, a marker of stromal bone marrow cells (Figs 1d and e) (Wexler *et al.* 2003). In accordance with data from the literature, exposing MSCs to appropriate media, induced their differentiation into adipocytes and osteocytes (Pittenger *et al.* 1999; Wexler *et al.* 2003). When inducing the adipogenic differentiation, the cells adopted a round morphology and accumulated large, cytoplasmic vacuoles in which accumulation of lipid was detected by Oil Red O staining (Figs 1f and h), while in non-

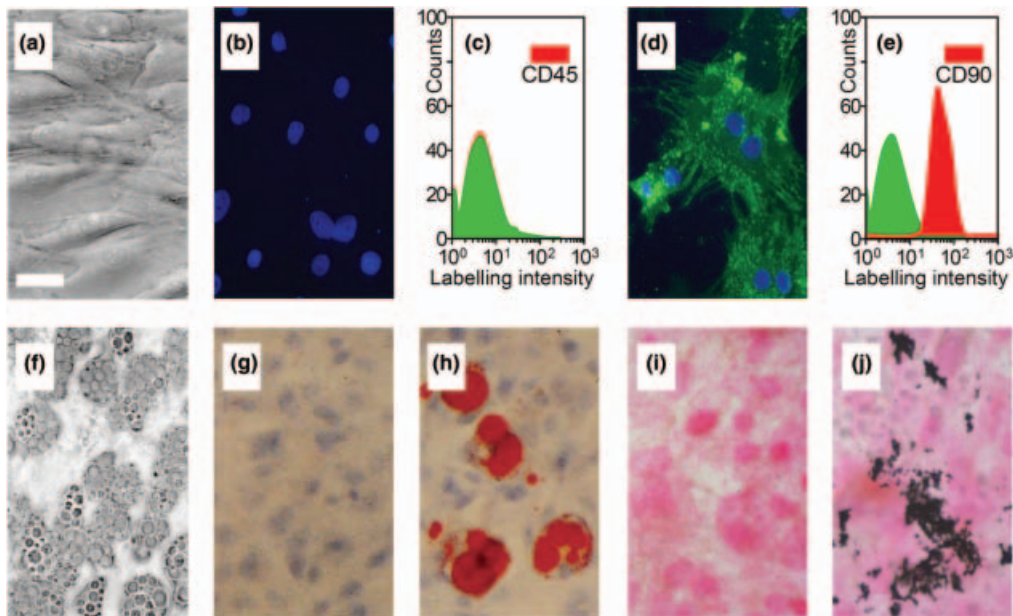


Fig. 1 Characterisation of bone marrow derived MSCs. Isolated cells were propagated in culture and maintained for multiple passages (a). ICC and FACS analysis revealed that proliferating cells at low passage are CD45 negative (b and c), and CD90 positive (d and e). FACS plots show isotype control IgG staining profile (green) vs. specific antibody staining profile (red). The cells at high passage can be differentiated

into adipocytes, as revealed by accumulation of large, cytoplasmic vacuoles (f), that could be histologically stained by Oil Red O (h), or into osteocytes in which calcium deposition is evidenced by Von Kossa's staining (j). Cells maintained in proliferation conditions are negative for these markers (g and i). Scale bar, 20 μm .

induced cells, no staining was detected (Fig. 1g). The osteogenic differentiation was demonstrated by calcium deposition in the cell culture by positive Von Kossa's stain in the majority of the cells (Fig. 1j) while non-induced cells were all negative (Fig. 1i). The proportion of cells showing adipogenic and osteogenic differentiation appeared more important at passage 15 in comparison to passage 5 (data not shown). Together, these results demonstrate that the adherent rat bone marrow cultures used in the present study are composed of a cell population that is morphologically and functionally characteristic of multipotent MSCs.

Differentiation of MSCs into cells bearing astrocytic markers

It has been reported previously that the maintenance of undifferentiated MSCs in culture for several passages enhances their differentiation potential (Woodbury *et al.* 2000; Jiang *et al.* 2002). In this respect, MSCs were cultured for up to 30 weeks and biochemical and immunological studies were performed on cells exposed to differentiation media at either low (P5) and high passage (P15).

As detailed under Methods, cultures were exposed to selected media which could favour their differentiation into mature astrocytes. The effect of these media on the acquisition of typical markers of the astro-glial lineage was first studied by examining the expression of nestin, S100 β , GFAP and glutamine synthetase by RT-PCR and ICC. Both approaches were first validated on primary cultures of astrocytes in which these four markers were unambiguously detected.

While the expression of nestin was weakly detected by RT-PCR on total RNA extracted from MSCs maintained in the proliferation medium, a signal was clearly observed in samples from cells exposed for 10 days to the differentiation medium (DMEM/F12 containing the additive G5) (Fig. 2a). Immunocytochemical analysis revealed that induction concerned a low percentage of the cells ($\approx 4\%$) in low passage cultures, whereas up to 20% appeared clearly positive in high passage cultures (Table 2). The addition of FGF₂ and CNTF in the differentiation medium had no influence on the induction of nestin. In contrast, both RT-PCR and immunocytochemical studies revealed a robust increase in the expression of nestin when RA was added to the differentiation medium during 10 days (about 65%) in high passage cultures (Table 2). When RA was only present during the first day of the differentiation step, a marked increase in the expression of nestin was also observed, particularly with high passage cultures (about 40% positive cells). Finally, the effect of RA (a single day) was partially inhibited when the differentiation medium contained the combination of FGF₂ and CNTF (Fig. 2b and Table 2).

The expression of S100 β was examined on proliferating or differentiated cells from high passage cultures. The use of specific PCR primers allowed detection of a weak level of

genetic expression of S100 β in cells maintained for 10 days in the proliferation medium. This expression appeared substantially increased when cultures were exposed for 10 days to the differentiation medium containing G5 and enriched or not with FGF₂ and CNTF. In contrast, S100 β induction appeared strongly prevented when these differentiation media were supplemented with RA for either 1 or 10 consecutive days (Fig. 2a). Contrasting with the detection of S100 β mRNA and its induction in some of the differentiation conditions tested, the expression of the corresponding protein was not revealed by ICC.

In contrast to data obtained with nestin or S100 β , RT-PCR studies failed to demonstrate the expression of the typical marker of mature astrocytes GFAP, neither on cells maintained in proliferation conditions nor in cells submitted to the differentiation protocols (data not shown). Hence, immunocytochemical analysis of GFAP expression did not reveal any positive cells in all cultures tested (data not shown).

Glutamine synthetase constitutes another well established marker of astrocytes. As shown on Fig. 2a, a low, but systematically detectable RT-PCR signal corresponding to glutamine synthetase was obtained using cells maintained in the proliferation medium (both at low or high passage). An increased intensity of the signal was observed on total RNA extracted from cells exposed to all differentiation media tested. The induction of glutamine synthetase in differentiated cells from high passage cultures was confirmed by ICC. Thus, in the proliferation medium, a small proportion of the cells (2.5%) from high passage cultures were glutamine synthetase positive, while in the differentiation medium in absence or presence of the combination of FGF₂ and CNTF, this percentage was two and five times higher, respectively. The addition of RA for a single day was sufficient to raise these levels robustly ($> 50\%$ positive cells). The addition of RA for 10 consecutive days had no additional effect. (Fig. 2c and Table 2).

Expression of glial glutamate transporters in MSCs

The expression of the glial glutamate transporters GLAST and GLT-1 and their induction after exposure to the differentiation media was first investigated by semi-quantitative RT-PCR. The reactions were optimized using total RNA extracted from cultured rat astrocytes, giving the expected amplification products of 531 and 1676 bp for GLT-1 and GLAST, respectively. While we failed to detect *GLT-1* mRNA in MSCs maintained in the proliferation medium, a robust signal was consistently observed in samples from cells exposed to the differentiation medium. As shown on Fig. 3a, induction of the genetic expression of GLT-1 was demonstrated at high passage and was not modified upon combination with FGF₂ and CNTF. In contrast, GLT-1 induction appeared partially prevented when these differentiation media were supplemented with RA for

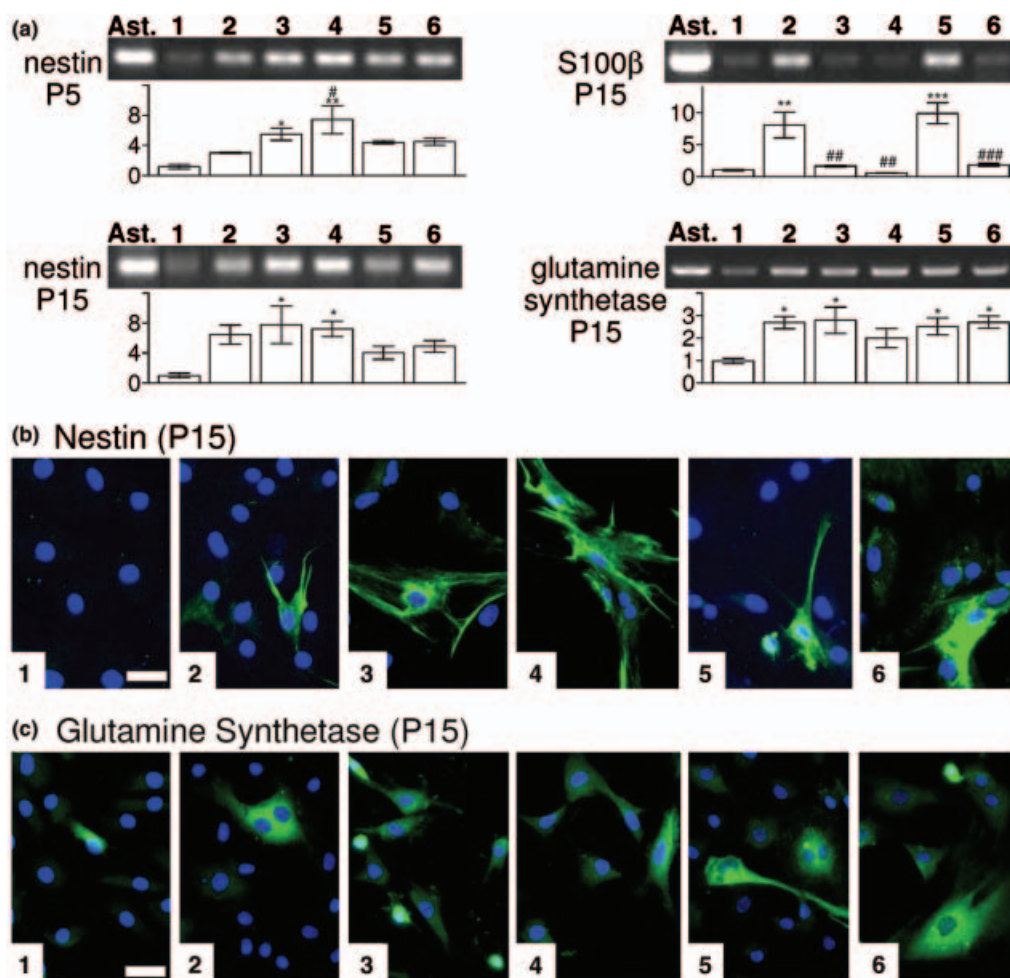


Fig. 2 Expression of astrocytic markers after *in vitro* differentiation of MSCs. (a) The genetic expression of astrocytic markers was evaluated by RT-PCR performed on total RNA extracted from MSCs maintained for 10 days in proliferation medium (1) or in differentiation medium which contains the additive G5 (2–6). In 5 and 6, the combination of FGF₂ and CNTF was added to the differentiation medium. In addition, this medium was also supplemented with RA for a single day (3 and 6) or during the entire differentiation step (4). RT-PCR conditions were validated on total RNA extracted from rat cultured astrocytes (Ast). Data show agarose gel electrophoresis of amplification products (typical experiments) corresponding to nestin (tested both at passage 5 and passage 15), S100β and glutamine synthetase (tested at passage 15). Samples were also used in PCR amplification of the GAPDH allowing semi-quantitative analysis of RT-PCR data and the histograms show relative density of PCR products expressed

in arbitrary units (mean $\pm$ SEM from triplicate measures performed on independent cultures). Statistical analysis was performed by one-way ANOVA followed by the Newman–Keul's test for multiple comparisons. * ($p < 0.05$) and ** ($p < 0.01$) denote significant differences from cells maintained in the proliferation medium while # ($p < 0.05$), ## ($p < 0.01$) and ### ($p < 0.001$) denote a significant influence of added RA when compared to the corresponding medium without RA. The expression of nestin (b) and glutamine synthetase (c) was also detected by ICC using specific antibodies visualised with a FITC-coupled secondary antibody (green). Images shown correspond to high passage cultures maintained in proliferation medium (1) or exposed to the above detailed differentiation media (2–6). Cell nuclei are stained using DAPI (blue). Data are from single experiments performed at least three times on independent cultures and quantitative analysis of specific labellings are summarized in Table 2. Scale bars, 20 μ m.

either 1 or 10 consecutive days. Concerning the GLAST, high passage cultures showed detectable levels of expression when maintained in the proliferation medium. However, a strong induction of the GLAST expression was obtained with all the differentiation protocols tested (Fig. 3a), including those using RA.

The induction of these glial glutamate transporters in differentiated MSCs was further investigated by ICC. Analysis of GLT-1 staining revealed the absence of its expression in both low or high passage cultures maintained in the proliferation medium. In contrast, about 18.7 and 8.6% GLT-1 positive cells were detected when low passage

Table 2 Quantitative analysis of immunocytochemical detection of nestin, GLT-1, GLAST and glutamine synthetase, before and after *in vitro* differentiation of MSCs cells.

Culture conditions	Proliferation medium (DMEM)	Differentiation medium (DMEM/ F_{12} + G5)				
+ FGF ₂ + CNTF	–	–	–	–	+	+
+ RA	–	–	First day only	10 days	–	First day only
Low passage cultures						
Nestin	ND	3.9 ± 1.5	7.8 ± 3.0	12.2 ± 3.0	7.6 ± 2.4	7.6 ± 2.4
GLT-1	ND	18.7 ± 4.8		7.0 ± 2.2	8.6 ± 3.0	
GLAST	8.4 ± 2.6	23.6 ± 7.8		7.3 ± 4.1	27.3 ± 3.8	
High passage cultures						
Nestin	ND	20.0 ± 5.1	39.0 ± 9.5	65.4 ± 8.5	19.7 ± 4.7	23.4 ± 5.6
GLT-1	ND	17.4 ± 4.1		14.4 ± 3.5	31.3 ± 4.8	
Nestin + GLT-1	ND	15.0 ± 4.2		9.7 ± 3.9	17.9 ± 4.6	
GLAST	29.5 ± 5.9	68.0 ± 5.0		40.0 ± 7.9	45.8 ± 5.5	
Nestin + GLAST	ND	12.1 ± 3.5		14.9 ± 5.6	9.5 ± 3.5	
Glutamine synthetase	2.5 ± 0.7	4.8 ± 1.0	54.3 ± 7.4	35.1 ± 5.8	12.8 ± 2.7	58.1 ± 6.7

Cells from low passage (P5) and high passage cultures (P15) were maintained for 10 days in proliferation medium or exposed to the differentiation medium supplemented with or without the combination of FGF₂ and CNTF and RA. At high passage cultures, double-labelling experiments were also performed (Nestin + GLT-1 and Nestin + GLAST). Cells were analysed by immunofluorescence microscopy as shown in Figs 2 and 3 and the number of positive cells expressing each antigen was evaluated by examination of 15 fields from three independent cultures. The total number of cells in these samples was determined by counting DAPI stained cell nuclei and results are expressed as mean ± SEM percentages of positive cells.

cultures were exposed to the differentiation media in absence or presence of the combination of FGF₂ and CNTF. On high passage cultures, GLT-1 positive labelling was not significantly modified in the medium lacking FGF₂ and CNTF, while the addition of these cytokines strongly increased the percentage of GLT-1 positive cells (17.4 and 31.2%, respectively) (Table 2). In accordance with RT-PCR data, the induction of GLT-1 expression by the differentiation medium was sensibly decreased when cells were simultaneously exposed to RA. This negative influence of RA on the induction of this glutamate transporter was more pronounced on low passage cultures (Table 2 and Fig. 3). Confirming these RT-PCR data, the immunocytochemical detection of the GLAST revealed the presence of GLAST-positive MSCs in cultures maintained in the proliferation medium. Exposure of the cultures to the differentiation medium in absence or presence of the combination of FGF₂ and CNTF, resulted in a marked increase in the percentage of GLAST-positive cells in both low and high passage cultures (Table 2). As shown on Fig. 3b, not only the number of positive cells, but also the intensity of the immunostaining was markedly induced after maintaining the cells for 10 days in these selected differentiation media. In order to examine whether the induction of GLT-1 or GLAST expression correlates with the expression of nestin, double-labelling experiments have been performed on high passage cultures. The corresponding data are presented in Table 2. Except for cells exposed to RA in which a robust induction of nestin was observed (see above),

the expression of GLT-1 appeared in the majority of nestin-positive cells. In contrast, no correlation between the expression of nestin and the GLAST induction was observed in all the differentiation conditions tested.

Glutamate transporters-mediated [³H]D-aspartate uptake in MSCs

The functional properties of the glial glutamate transporters expressed in MSCs were evaluated by measuring the ability of these cells to take up [³H]D-aspartate. Both low or high passage cultures exerted a very low uptake activity when maintained in the proliferation medium. Thus, velocity of uptake measured with 20 nM radiolabelled substrate was less than 0.1 pmol/min/mg of protein. When low passage cultures were exposed to the differentiation medium with or without the combination of FGF₂ and CNTF and/or RA, a highly significant increase was systematically observed, about 0.5 pmol/min/mg of protein in the sole presence of the supplement G5. The positive effect of these differentiation conditions on aspartate uptake was more pronounced when tested on high passage cultures, reaching values close to 1 pmol/min/mg of protein. While the presence of FGF₂ and CNTF slightly increased this uptake, RA was found to dramatically limit the induction of this functional response. When the assays were performed in a Na⁺-free buffer, a strong inhibition of substrate uptake was observed in all the conditions examined (Fig. 4a–c). In addition, the uptake of [³H]D-aspartate was almost totally abolished when measured

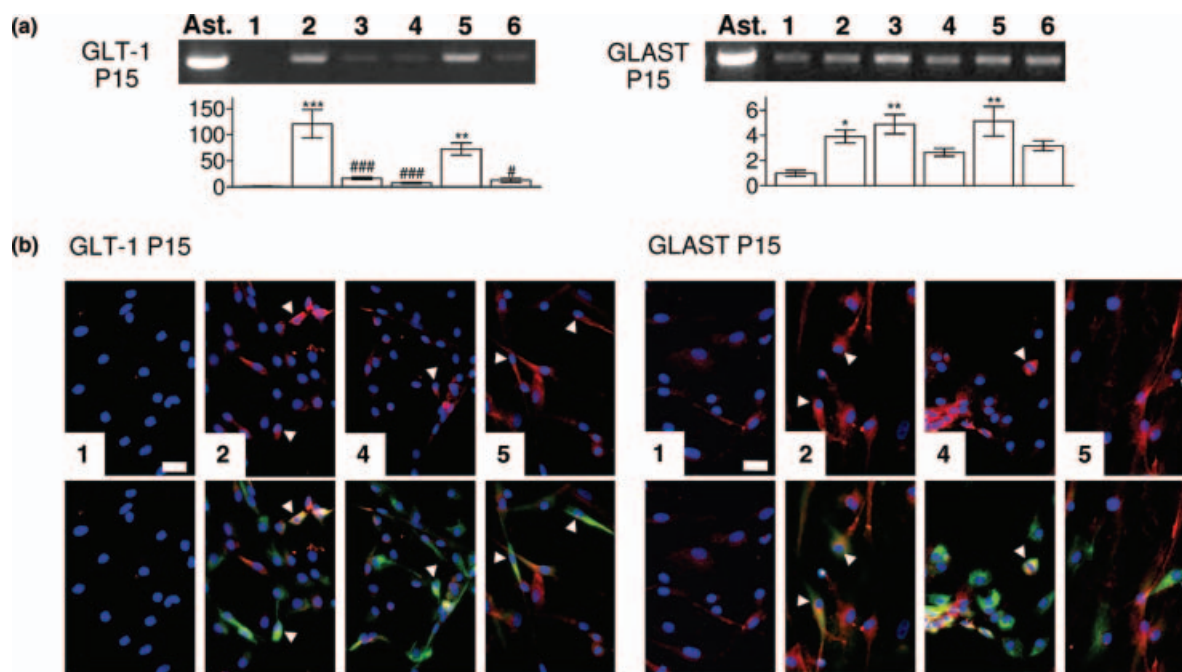


Fig. 3 Expression of the glutamate transporters GLAST and GLT-1 after *in vitro* differentiation of MSCs. (a) The genetic expression of these transporters was evaluated by RT-PCR performed on total RNA extracted from MSCs maintained for 10 days in proliferation medium (1) or in differentiation medium which contains the additive G5 (2–6). In 5 and 6, the combination of FGF₂ and CNTF was added to the differentiation medium. In addition, this medium was also supplemented with RA for a single day (3 and 6) or during the entire differentiation step (4). RT-PCR conditions were validated on total RNA extracted from rat cultured astrocytes (Ast). Data show agarose gel electrophoresis of amplification products (typical experiments) corresponding to GLAST and GLT-1 on samples from high passage cultures. Samples were also used in PCR amplification of the GAPDH allowing semi-quantitative analysis of RT-PCR data and histograms show relative density of PCR products expressed in arbitrary units (mean $\pm$ SEM from triplicate measures performed on independent cultures). Statistical analysis was performed by one-way ANOVA fol-

lowed by the Newman–Keul's test for multiple comparisons. * ($p < 0.05$) and ** ($p < 0.01$) denote significant differences from cells maintained in the proliferation medium; # ($p < 0.05$) and ### ($p < 0.001$) denote a significant influence of added RA as compared to the corresponding medium without RA. The expression of GLT-1 and GLAST (b), shown on the upper panels, was also detected by ICC using specific antibodies visualised with a Cy3-coupled secondary antibody (red). Images shown correspond to high passage cultures maintained in proliferation medium (1) or exposed to the above detailed differentiation media (2, (4) and (5). Cell nuclei were stained using DAPI (blue). Co-expression with nestin was examined using a specific antibody and was visualised with a FITC-coupled secondary antibody (green). This is shown on the corresponding lower panels, where the co-labelled cells are pointed with arrows. Data are from single experiments performed at least three times on independent cultures and quantitative analysis of specific labelling are summarized in Table 2. Scale bars, 20 μ m.

in the presence of t-PDC. Together, these results demonstrate the involvement of functional Na⁺-dependent transporters (Figs 4c and d).

A pharmacological characterisation of the glutamate transporters involved in the uptake of aspartate was obtained using DHK, a specific blocker of GLT-1. By focusing our study on the two conditions favouring a maximal uptake velocity (differentiation medium without RA), we observed a lower uptake velocity when DHK was included in the assay. Thus, in low passage cultures exposed to the differentiation medium in absence or presence of the combination of FGF₂ and CNTF, a 30 and 14% decrease in uptake values were measured, respectively. Under the same conditions, in the high passage cultures, DHK significantly decreased aspartate uptake by 38 and 63%, respectively (Figs 4b–d). Together,

these results suggest that differentiated MSCs express functional glutamate transporters and that the addition of a combination of FGF₂ and CNTF promotes the expression of GLT-1.

Discussion

Challenging the traditional concepts of terminal differentiation, the presence of stem cells in the vast majority of adult tissues is now clearly demonstrated. These adult stem cells have been identified in the bone marrow, the liver, the muscles, the skin and the CNS. A widespread interest in these adult stem cells recently arose, on the basis of several reports showing that these cells have the ability to differentiate into a diversity of cell types that may be unrelated to their

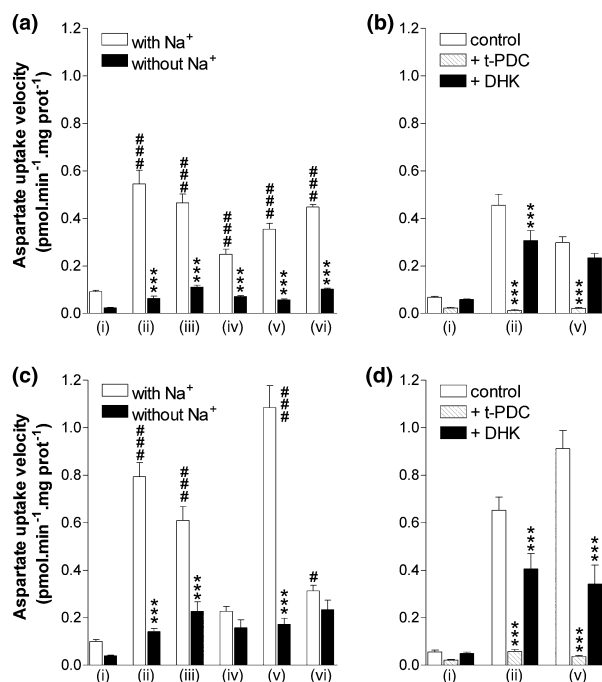


Fig. 4 Functional measures of glutamate transporters activity after *in vitro* differentiation of MSCs. The rapidity of [³H]D-aspartate uptake (20 nM) was measured in MSCs from low (a–b) and high passage (c–d) cultures maintained for 10 days in proliferation medium (i) or in differentiation medium which contains the additive G5 (ii–vi). (v and vi) The combination of FGF₂ and CNTF was added to the differentiation medium. In addition, this medium was also supplemented with RA for a single day (iii and vi) or during the entire differentiation step (iv). Panels (a) and (c) indicate the uptake values determined in the presence (open bars) or in the absence (closed bars) of extracellular Na⁺. Panels (b) and (d) indicates the Na⁺-dependent uptake determined in the absence (open bars) or in the presence of the glutamate transporter inhibitors t-PDC (hatched bars) or DHK (closed bars). Data shown are mean ± SEM from triplicate measures performed on three independent cultures. Statistical analysis was performed by one-way ANOVA followed by the Newman–Keul's test for multiple comparisons (###*p* < 0.001 with cells maintained in the proliferation medium and for each condition tested; ****p* < 0.001 with the corresponding values obtained in Na⁺ containing buffer).

phenotypical origin. In particular, the differentiation of MSCs into neurones, hepatocytes, myocytes and epithelial cells was experimentally reported both *in vitro* and *in vivo* (Bittner *et al.* 1999; Petersen *et al.* 1999; Reyes *et al.* 2002; Jiang *et al.* 2003). Based on this observation, we investigated the ability of MSCs isolated from the bone marrow to differentiate into cells belonging to the astroglial lineage. These cells were obtained after extensive proliferation of an extremely low proportion of plastic-adhering cells. A recent study reported the characterisation and clonal amplification of very rare pluripotent stem cells present in the bone marrow (Jiang *et al.* 2002). While the protocols used to culture the MSCs in our study differ from those which could favour the

isolation of these multipotent adult progenitor cells (MAP-Cs), the presence of these primitive precursors in our cultures is not ruled out. Indeed, data obtained using these cells indicate that the differentiation potential increased as cultures were maintained for several passages. This could suggest that repeated passaging increases this potential or that prolonged proliferation favours the selection of these rare pluripotent stem cells.

In order to induce the *in vitro* astro-glial differentiation by soluble factors, proliferating MSCs cells were exposed to a chemically defined medium classically used for the culture of neural stem cells (DMEM/F12) which was supplemented with G5, a commercially available cell culture additive specifically designated to induce the growth, differentiation and maturation of primary cultured astrocytes (Michler-Stuke *et al.* 1984). In addition, while several differentiation inducers such as leukemia inhibitory factor (Gegelashvili *et al.* 2000), bone morphogenetic proteins (Chang *et al.* 2003) and dimethyl sulfoxide (Dinsmore *et al.* 1996) have been reported to contribute to differentiation of embryonic stem cells or neural stem cells into astrocytes, the present study focused on FGF₂ and CNTF, two polypeptides with potent trophic and protective effects on the brain. Indeed, authors have reported on their potential therapeutic application after brain ischemia, or in neurodegenerative processes such as Huntington's disease and amyotrophic lateral sclerosis (Aebischer *et al.* 1996; Emerich *et al.* 1997; Li and Stephenson 2002; Song *et al.* 2002). These cytokines were applied sequentially, according to Morrow, which stipulates that FGF₂ induces glial fate while CNTF favours terminal glial differentiation of neural stem cells (Morrow *et al.* 2001). The latter was also shown to induce a transient up-regulation of *GFAP* mRNA in astrocytes. (Kahn *et al.* 1997). Another differentiation inducer, RA was also used in some protocols, as it is known to induce differentiation of embryonic stem cells and embryonic carcinoma cells into specific cell types, including neurones and astrocytes (Dinsmore *et al.* 1996; Sanchez-Ramos *et al.* 2000; Jang *et al.* 2004).

Nestin is an intermediate filament protein expressed by proliferating neuro-epithelial cells during the development of the CNS. It is also expressed by neural stem cells in adult mammals and used to identify adult neuronal progenitors in culture. MSCs submitted to differentiation conditions were found to express nestin, revealing that the protocols tested trigger their differentiation into neuro-astroglial progenitors. Higher expression levels were detected on high passage cultures confirming that the differentiation potential of the MSCs is enhanced when cells are maintained in culture for prolonged period of time. According to a previous study by Sanchez-Ramos, the induction of nestin expression was increased considerably when the differentiation medium was supplemented with RA (Sanchez-Ramos *et al.* 2000).

Besides nestin, we evaluated the expression and functional properties of glial glutamate transporters. Together, GLAST

and GLT-1 ensure >80% of total glutamate clearance from extracellular space in the CNS and are frequently considered as astrocyte-specific proteins (Suarez *et al.* 2002). The cognate mRNAs, however, are also found in peripheral tissues and the expression of functional GLAST has been reported in the bone (Danbolt 2001). Hence, considering GLAST, both RT-PCR and immunocytochemical studies revealed detectable expression levels in MSCs maintained in proliferation conditions. The corresponding signals appeared noticeably increased in cells exposed to the differentiation medium containing G5 with or without the combination of FGF₂ and CNTF. RA was without effect on the induction of GLAST at the genetic level and the protein level. Considering the above-mentioned effect of this inducer on nestin expression, these data suggest that induction of GLAST expression occurs as an early event during *in vitro* differentiation of MSCs. Alternatively, as GLAST is also expressed in bone, another hypothesis could be that the detection of this transporter reflects the persistence of contaminating precursors committed to the osteogenic pathway. However, undifferentiated cells, even at passage 15, were shown to be negative for Von Kossa staining (calcium deposition), which is characteristic of osteocytes. Similarly, cells exposed to the differentiation media and in which induction of transporters was observed appeared negative in the Von Kossa staining assay. In contrast to GLAST, we failed to detect any expression of the GLT-1 protein and its mRNA in MSCs maintained in proliferation medium whereas a robust induction was observed in the conditions favouring differentiation of cultured astrocytes, and more notably with the medium supplemented with FGF₂ and CNTF. The growth factors present in the differentiation media seem to be at least partially responsible for this differentiation as several authors reported on the ability of EGF and FGF₂ to induce differentiation, and to stimulate GLT-1 expression in astrocytes (Zelenai *et al.* 2000; Figiel *et al.* 2003). Other researchers showed an up-regulation of the glial glutamate transporter GLAST in response to FGF₂ and CNTF in cultured astrocytes. (Gegelashvili *et al.* 2000; Suzuki *et al.* 2001). Interestingly, RT-PCR studies revealed that the addition of RA dramatically reduced the positive influence of the supplement G5 or the growth factors FGF₂ and CNTF on the expression of GLT-1. ICC assays showed that even in differentiation media supplemented with RA for 10 days, there is a significant but reduced expression of GLT-1. This further supports our hypothesis suggesting the positive but restricted influence of the addition of RA on the differentiation of MSCs into astrocytes progenitors. Double-labelling experiments indicated that the induction of GLT-1 expression concerns a high proportion of the nestin positive cells. On the contrary, induction of GLAST expression appeared in both nestin positive and nestin negative cells. In many conditions, the percentage of GLAST positive cells was higher than the percentage of nestin positive cells, indicating that at least a

proportion of the nestin negative cells were GLAST positive. In addition, in the control condition (non-differentiated cells) some cells expressed GLAST but were totally nestin negative. These results are in accordance with the documented expression of GLAST in several tissues while the GLT-1 subtype is generally considered as a typical glial glutamate transporter (Danbolt 2001).

The induction of both GLT-1 and GLAST in the medium supplemented with the G5 additive and in the presence or not of the combination of FGF₂ and CNTF correlated with a highly significant increase in the Na⁺-dependent uptake of aspartate, confirming that differentiation of MSCs favours the expression of functional transporters. Indeed, while the uptake was close to the limit of detection in proliferating cells, the uptake velocity reached up to 1 pmol/min/mg of protein in high passage cultures. Using the same protocol, the corresponding value measured on primary cultured astrocytes was close to 3 pmol/min/mg (Vermeiren *et al.* 2004). Considering immunocytochemical data showing that all the cells do not express GLAST or GLT-1, this result indicates that differentiated MSCs effectively take-up the excitatory amino acid from the extracellular medium. The use of the selective inhibitor of GLT-1, DHK, allowed to further demonstrate that the increased aspartate uptake measured in differentiated cells reflect the involvement of this transporter in MSCs. Furthermore, the inhibition of substrate uptake by DHK was higher when the cells were tested at higher passages, indicating that cells maintained in culture for a prolonged period of time tend to express the GLT-1 transporter at higher levels upon differentiation. In agreement with RT-PCR and immunological data, cells exposed to RA during the differentiation step showed dramatically lower uptake values. Together, these data related to the influence of RA on nestin, GLAST and GLT-1 could indicate that RA triggers neuro-astroglial differentiation, but maintains the cells in a non-fully differentiated state.

In addition to the expression of nestin, and the expression of functional glial glutamate transporters, we further investigated the presence of other traditional markers of astrocyte differentiation. GFAP is the main subunit of intermediate filaments of glial cells and astrocytes. However, in the present study, none of the differentiation conditions tested were found to trigger GFAP expression in MSCs. The total absence of GFAP detection could indicate that the cells did not reach full maturation after the 10 days differentiation protocol. Indeed, immature astrocytes do not readily express GFAP, but express nestin (Privat 2003). The S100 β protein, which belongs to the S100 family of calcium binding proteins is also considered a reliable marker of astrocytes (Pinto *et al.* 2000; Donato 2001). This protein could not be detected in the differentiated MSCs, but the corresponding mRNA was clearly amplified by RT-PCR. While a weak signal was observed in proliferating cells, a robust induction was obtained in cells exposed to the differentiation media

enriched with G5 and growth factors. In contrast, when RA was added to the culture medium containing these additives, a rather modest induction of *S100 β* mRNA was obtained. These observations are in correlation with the results obtained for the glial glutamate transporter, GLT-1. A third marker of astrocytes is the glutamate metabolizing enzyme glutamine synthetase. In cells maintained in the proliferation medium, low levels of *glutamine synthetase* mRNA could be detected while a considerable induction was observed in all differentiation media tested. Accordingly, immunocytochemical analysis revealed that the expression of this enzyme is increased in the differentiation media supplemented or not with the combination of FGF₂ and CNTF. Short-term or prolonged exposures to RA were found to strongly enhance the expression of this enzyme. Accordingly, RA was also found to induce the expression of glutamine synthetase in astrocytes differentiated from mouse embryo cells (Loo *et al.* 1995). In the light of our data concerning the influence of RA on nestin and glutamate transporter expression, its effect on glutamine synthetase was rather unexpected considering the essential role of this enzyme in mature astrocytes, where it specifically ensures the conversion of glutamate into glutamine (Vogel *et al.* 1975; Norenberg and Martinez-Hernandez 1979). Limited data are available concerning the regulation of this enzyme during *in vitro* maturation of cultured astrocytes. However, some authors already reported that the glutamine synthetase activity significantly declined during astrocyte maturation (Stanimirovic *et al.* 1999).

Though it is at present unclear which factors are implicated in the differentiation of adult MSCs, this study provides important evidence that manipulating the composition of the culture medium may be sufficient to trigger their differentiation into cells showing some essential properties of astrocytes. Indeed, by taking up glutamate from the extracellular space, astrocytes play a critical role in protecting the nervous system against potential excitotoxicity. The possibility to achieve this activity in cultured MSCs may allow use of these bone marrow derived cells in the treatment of neurodegenerative disorders.

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