Oligodendrocyte precursor cells (OPCs) can differentiate into oligodendrocytes or astrocytes, depending on cellular microenvironments. OPCs, cultured in medium supplemented with 10% (v/v) fetal bovine serum (FBS), give rise to type II astrocytes that express glial fibrillary acidic protein and a cell surface ganglioside that is recognized by A2B5 monoclonal antibody. However, the factors in FBS that direct the astrocyte differentiation are not determined. Moreover, bone morphogenetic proteins (BMPs) have been reported to be involved in astrocyte differentiation of neural progenitor cells. We therefore examined whether BMPs are responsible for the serum-mediated astrocyte differentiation from OPCs. OPCs were isolated from the spinal cords of Wistar rat embryos (at day 14) using the A2B5 antibody. We measured the concentrations of BMP-2 and BMP-4 in FBS and rat and human sera and the expression of mRNAs for three types of BMP receptors (BMPRIa, Ib and II) in OPCs by RT-PCR. The serum samples of the three species contained BMP-2 and BMP-4, as judged by ELISA with each monoclonal antibody, and the BMP receptor mRNAs are expressed in OPCs. When OPCs were cultured in the medium containing 10% FBS, cells (more than 95%) differentiated into type II astrocytes. However, when OPCs were pretreated with noggin, a soluble antagonist of BMP action, the degree of astrocyte differentiation was markedly decreased from 95.39 to 38.36%. Taken together, these results suggest that BMP signaling may be responsible for the serum-mediated astrocyte differentiation of OPCs. Our findings provide new insights into the molecular basis of differentiation of OPCs.

Keywords: Oligodendrocyte progenitor cells; type II astrocytes; differentiation; bone morphogenetic proteins; serum
OPCs.

To identify whether BMPs are key factor(s) in the astrocyte differentiation of OPCs, we examined content of BMP proteins in FBS and human and rat sera, the expression of mRNAs for BMP receptors in OPCs prepared from embryonic rat spinal cords, and the effect of noggin, a soluble antagonist of BMP action, on the FBS-induced astrocyte differentiation. We provide evidence that BMP signaling may be responsible for the serum-mediated astrocyte differentiation from embryonic OPCs.

Materials and Methods

Isolation and culture of OPCs

OPCs were isolated from the spinal cords of Wistar rat embryos at embryonic day (E) 14 using A2B5 antibody (1:100; Chemicon, Temecula, CA), according to a protocol modified from previous studies (Mayer-Proeschel et al. 1997; Muitaba et al. 1999; Cao et al. 2005; Hu et al. 2009). Immunopanned OPCs were plated onto poly-D-lysine (Sigma, St. Louis, MO) and fibronectin (human plasma; BD Biosciences, Bedford, MA)-coated 10 × 12 cm culture dishes, and growth medium was added and changed every other day. The growth medium contained DMEM/Ham’s F12 (Invitrogen, Carlsbad, CA), 1 × N2 and 1 × B27 supplements (Invitrogen), fibroblast growth factor 2 (FGF-2) (20 ng/ml; Sigma), and platelet-derived growth factor AA (PDGF-AA) (10 ng/ml; Chemicon, Temecula, CA). In all cases, an aliquot of cells was analyzed on the following day to determine the efficiency of immunopanning. Only those preparations in which > 90% of the cells bound to A2B5 were used. After 5–7 d, the cells were propagated. In all experiments, cells at passage 2 (P2) were used.

All embryos were obtained from pregnant Wistar rats bred in the Animal Care Facility at Bengbu Medical College, Bengbu, P.R. China. All animal care was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996) and was approved by the Animal Care Committee of the Use of Laboratory Animals, Bengbu Medical College.

In vitro differentiation of OPCs

To induce differentiation of OPCs, cells of the passage 2 were used. The dissociated OPCs were plated onto PDL/fibronectin-coated coverslips in 24 well plates at a density of 3 × 10⁴ cells/coverslip (12 mm) and allowed to proliferate in OPC growth medium for 24 h prior to differentiation. To identify the effects of serum on OPC differentiation, OPC growth medium was withdrawn and cells were subsequently treated with differentiation medium (consisting of DMEM/Ham’s F12, 1N2, 1B27, 5 μg/ml insulin and 0.1% bovine serum albumin (BSA, Sigma), but without FGF-2 and PDGF-AA) in the absence or presence of 10% FBS. The cells were allowed to differentiate for 5 days before fixation and immunostaining for receptor interaction protein (Rip), GFAP and A2B5 epitope.

Immunocytochemistry

The cells cultured on coverslips were rinsed in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA, Sigma) in PBS for 10 min at room temperature (RT). After three rinses in PBS (10 min each), the cells were incubated with 10% normal goat serum (NGS) in PBS in the presence (for intracellular antigens) or absence (for surface markers) of 0.3% Triton X-100 for 1 hour at RT and then with one of the monoclonal primary antibodies against A2B5 epitope and PDGFrα (1:100; all from Chemicon; the markers for OPCs), Rip (1:100; Chemicon; the marker for oligodendrocytes) or both A2B5 epitope and GFAP (1:200; Sigma; the marker for type II astrocytes) overnight at 4°C. On the second day, the cultures were incubated with rhodamine- or fluorescein isothiocyanate (FITC)-conjugated secondary antibody (All from Jackson ImmunoResearch Lab, West Grove, PA) for 1 hour at 37°C. After staining, the coverslips were rinsed and mounted with Gel/Mount aqueous mounting medium (Biomedica Corp., Foster City, CA) containing Hoechst 33342, a fluorescent nuclear dye (1 μg/ml; Sigma). The coverslips were examined using an Olympus BX60 microscope. For cell counts, at least five randomly selected fields with a total of more than 500 cells were counted. In all experiments, primary antibody omission controls were used to confirm the specificity of the immunofluorescence labeling.

Measurement of BMP in serum by enzyme-linked immunosorbent assay (ELISA)

The levels of BMP-2 and BMP-4 in FBS and human sera were quantified using commercially available ELISA kits (R&D Systems, Minneapolis, MN). ELISA was performed according to the manufacturer’s instructions. Briefly, assay diluents (100 μl) were added to each well that had been pre-coated with a monoclonal antibody specific for BMP-2 or BMP-4. Each antibody recognizes rat and human BMP-2 or BMP-4 (R&D Systems). This step was followed by the addition of 50 μl of control standard or sample per well and incubation for two hours at room temperature on a shaker. Substrate solution (200 μl) was added to each well and the plates were incubated for two hours at room temperature on a horizontal orbital microplate shaker at 500 rpm. Each well was aspirated and subsequently washed with wash buffer by use of an autowasher; the preceding step was repeated three times for a total of four washes. After removing any remaining wash buffer, 200 μl of BMP-2 or BMP-4 conjugate was added to each well and plates were incubated for two hours at room temperature on a shaker. Aspiration and washes were repeated as described above. Substrate solution (200 μl) was added to each well and the plates were incubated for 30 minutes at room temperature and were protected from light. Stop Solution (50 μl) was added to each well. The optical density of each well was determined using a microplate reader at 450 nm. The BMP-2 and BMP-4 concentrations were determined from the optical densities in relation to standard experimental curves. No interference and no cross reactivity was expected based on the manufacturer’s instructions. All of the serum samples were measured twice and the mean level of each measurement was used for analysis.

Reverse transcription–polymerase chain reaction (RT–PCR)

RT-PCR was used to detect the expression of BMPR mRNA in OPCs. Briefly, total RNA from OPCs, were extracted with the TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Total RNA from OPCs was extracted with the TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Two micrograms of total RNA were reverse transcribed to cDNA, and PCR was performed by a routine method (Tokumoto et al. 1999). A negative control containing RNA instead of cDNA (without RT) was performed to rule out DNA contamination. PCR products were analyzed on 1% agarose gel, β-actin was used as an internal control. The sequences of specific primers for semi-quantitative RT-PCR were given in Table 1.
BMPs in Serum Induce Astrocyte Differentiation of OPCs

Blocking experiment of noggin, a specific BMP inhibitor

For identifying the role of BMP signal pathway in serum-induced astrocyte differentiation, OPCs were pretreated with noggin (500 ng/ml; R&D Systems Minneapolis, MN), a specific inhibitor of BMPs for 45 min before the addition of serum. Cells were allowed to differentiate for 5 days for assessing their differentiation potential under the treatment of noggin.

Statistical Analysis

Data are presented as mean ± standard deviation of the mean (SD). One-way analysis of variance (ANOVA) with post hoc Tukey t-test was used to determine statistical significance. A p value of < 0.05 was considered statistically significant.

Results

Identification and differentiation of OPCs in the absence or presence of serum

OPCs isolated from rat embryonic spinal cord were cultured in OPC-medium supplemented with PDGF-AA and bFGF. Almost all of the cells displayed bipolar or tri-polar morphology, the typical morphology of OPC (Fig. 1A). Immunofluorescent staining showed that more than 95% of cells expressed both A2B5 epitope (Fig. 1B) and PDGFRα (Fig. 1C) and thus were highly pure. When cultured in the differentiation medium (without PDGF-AA and bFGF) for 5 days, these cells displayed multipolar morphology. More than 85% of them expressed an oligodendrocyte-specific marker Rip (Fig. 1D, H), and few cells expressed both GFAP and A2B5 epitope (Fig. 1E, H). However, when OPCs were cultured in the differentiation medium containing 10% FBS, nearly all of them displayed the typical process-bearing morphology of astrocytes and expressed both GFAP and A2B5 epitope (Fig. 1G, H) and few of them expressed Rip (Fig. 1F, H).

Level of BMP-2 and BMP-4 in sera

It has been reported that BMPs contributed to astrocyte differentiation from NPCs (Mabie et al. 1997; Grinspan et al. 2000; Cheng et al. 2007). To explore the possibility whether the serum also contains BMPs that promote astrocyte differentiation of OPCs, we first examined content of BMP-2 and BMP-4 proteins in FBS, rat serum and human serum by ELISA. The results showed that high level of BMP-2 and BMP-4 proteins was detected in the sera of all three species. The concentration of BMP-2 in FBS, rat serum and human serum was 617.24 pg/ml, 269.32 pg/ml and 125.51 pg/ml, respectively, and the concentration of BMP-4 in FBS, rat serum and human serum was 205.85 pg/ml, 463.22 pg/ml and 140.69 pg/ml, respectively (Fig. 2). Thus, both BMP-2 and BMP-4 are present in sera of three different species.

Expression of BMP receptor (BMPR) mRNAs in OPCs

The functions of BMPs rely on binding to their receptors (BMPR). If BMPs induce OPCs to differentiate into astrocytes, the OPCs should express BMPR. Some previous reports indicated that OPCs derived from different sources all expressed BMPR (Mabie et al. 1997; Kondo and Raff 2004; Cheng et al. 2007). To verify whether the OPCs cultured in our system express BMP receptors, we collected the OPCs cultured in OPC-medium and then analyzed the mRNA expression of BMP receptors (BMPRIa, Ib and II) in OPCs using RT-PCR. The results showed that all three BMP receptors could be detected in OPCs (Fig. 3). This finding, together with the result that high level of BMP-2 and BMP-4 proteins in serum, implicates the possibility of BMPs contained in serum are key mediators of the serum-promoted astrocyte differentiation of OPCs.

Effect of noggin, a specific inhibitor of BMPs, on differentiation of OPCs

To further clarify whether BMPs are key factors for the serum-promoted astrocyte differentiation, we next used noggin, a specific inhibitor of BMPs, to observe whether astrocyte differentiation can be blocked or decreased after addition of noggin. When noggin was added before addition of serum, the GFAP/A2B5 epitope-expressing cells were markedly decreased (from 95.39 to 38.36%, Fig.4C, D, I), and in contrast, the Rip-positive cells were increased significantly (from 3.48 to 56.87, Fig. 4G, H, I). The percentages of GFAP/A2B5-positive cells and Rip-positive cells between the two groups (with/without noggin) were significantly different (p < 0.01, Fig. 4I). These results suggest that the BMPs in serum may be the factors that induce OPCs to differentiate into astrocytes.

Discussion

OPCs play an important role not only as progenitor
cells that give rise to myelinating cells in the CNS, but also as an active participant in the neural network (Lin and Bergles 2004). However, OPCs have two different differentiation fates which give rise to oligodendrocytes in the absence of serum or type II astrocytes in the presence of serum in vitro (Raff et al. 1983; Louis et al. 1992). This phenomenon suggests that serum induces astrocyte fate of OPCs and certain key factor(s) contained in serum mediate this role. Thus, in this study, we investigated the possible factor(s) involved in serum-induced astrocyte differentiation.

Firstly, we identified the characteristic of OPCs and confirmed the OPCs isolated and used in our experiment were highly pure. When cultured in the medium without PDGF-AA, bFGF and serum, 90% of them differentiated into oligodendrocytes. However, when cultured in the medium containing 10% FBS without PDGF-AA and bFGF, nearly all of them differentiated into both GFAP and A2B5-positive type II astrocytes (G, H) and few of them expressed RIP (F, H). Cells in B~F were counterstained with Hoechst 33342 (blue), a nuclear dye. Bar = 25 μm.

Fig. 1. Identification and differentiation of OPCs prepared from the rat embryonic spinal cord in the presence or absence of serum. A: Phase contrast photomicrograph of OPCs isolated and cultured in growth medium supplemented with PDGF-AA and bFGF. B, C: Almost all OPC cultures were immunopositive with A2B5 (B) and for PDGFRα (C), two specific markers of OPCs. D, E, H: The majority of OPCs differentiated into Rip-positive oligodendrocytes when cultured in the differentiation medium (without PDGF-AA or bFGF) in the absence of serum. F, G, H: when OPCs were cultured in the differentiation medium containing 10% FBS, almost all of them differentiated into both GFAP and A2B5-positive type II astrocytes (G, H) and few of them expressed RIP (F, H). Cells in B~F were counterstained with Hoechst 33342 (blue), a nuclear dye. Bar = 25 μm.

Fig. 2. The concentrations of BMP-2 and BMP-4 in sera of different species. BMP-2 and BMP-4 in three serum samples were measured by ELISA. The graph shows the concentrations of BMP-2 and BMP-4 in FBS and rat and human sera.
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It has been reported that several members of the BMP family, such as BMP-2 and 4, have been implicated as repressors of oligodendrocyte development in vitro by shifting oligodendrocyte precursors into the astrocyte lineage (Mabie et al. 1997; Grinspan et al. 2000; Cheng et al. 2007). Thus, we speculated that BMPs in serum may be the key factors to induce astroglial differentiation of OPCs. To clarify this possibility, we confirmed the high levels of BMP-2 and BMP-4 in serum samples of humans and rats as well as in FBS. It is therefore conceivable that bovine BMPs may be functional in rat cells. The functions of BMPs rely on binding to their receptors (BMPR) (Chen et al. 2004b). It is of interest that both BMP-2 and BMP-4 are closely related by their amino acid sequence and act on the same receptor, implying that these two BMPs might have similar biological functions (Rengachary 2002). The biological functions of BMPs are mediated through the Smad signal transduction pathway via three types of BMP receptors (BMPRIa, Ib and II) (Chen et al. 2004a; Miyazono et al. 2005), and the sensitivity and response of target cells to the BMPs depend on the expression of BMPR. Our results demonstrate that BMPRIa, Ib and II are expressed in OPCs. Thus, these results further support the possibility that BMPs might be the key factors for the serum-induced astrocyte differentiation of OPCs.

Noggin, a soluble antagonist of BMPs, is known to play an important role in the induction of the nervous sys-
In conclusion, the present study has provided convincing evidence to suggest that BMPs contained in serum serve as the regulating molecules that induce astrocyte differentiation from embryonic OPCs. Our findings thus provide new insights into the molecular basis of differentiation of OPCs.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (NO. 30700439, 81071268), the Key Project of Chinese Ministry of Education (NO. 210103), the Science and Technological Fund of Anhui Province for Outstanding Youth (NO. 10040606Y13) and the Natural Science Research Program of Education Bureau of Anhui Province (NO. 2006KJ098A, KJ2010B109).

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