Differentiation of Serum-Free Mouse Embryo Cells into an Astrocytic Lineage is Associated with the Asymmetric Production of Early Neural, Neuronal and Glial Markers

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Serum-free mouse embryo (SFME) cells, the astrocyte progenitor cells in the central nervous system (CNS), were exposed to 10 ng/ml leukemia inhibitory factor (LIF) and 10 ng/ml bone morphogenic protein 2 (BMP2) to induce differentiation, and expression of cell-type specific markers. Nestin, a marker of early neural lineage, βIII-tubulin, a marker of neuronal lineage, oligodendrocyte marker O4 (O4), a marker of oligodendrocytic lineage and glial fibrillary acidic protein (GFAP), a marker of astrocytic lineage, were analyzed. Characteristics of SFME cells, as a CNS progenitor, were identified and a possible mechanism, underlying SFME cell specification into an astrocytic lineage upon differentiation, was investigated. These markers were present, both at the initial proliferative phase and after induction of differentiation. GFAP expression increased strongly upon differentiation, while expression of the other markers changed very little. These results indicate that astrocytic differentiation is associated with the asymmetric production of these markers, rather than through induction of astrocytic markers.

Key words serum-free mouse embryo; nestin; βIII-tubulin; oligodendrocyte marker O4; glial fibrillary acidic protein

Strategies using stem cells for cellular therapies are promising approaches for the treatment of several chronic or acute neurological diseases, such as Parkinson’s or Huntington’s diseases or spinal cord injuries. However, some difficulties exist that relate to the origin and to the nature of the cells to be used for such procedures. For instance, clinical use of fetal tissue is limited by ethical issues and by technical problems, as high numbers of the cells are required and immunosuppression of the patient is usually required. Alternatively, somatic stem cells, derived from adult tissues, are possible candidates for cell replacement therapy. These studies raise hopes of successful cell replacement strategies, based on an autograft approach. Further, recent in vivo observations demonstrated that mesenchymal stem cells, a somatic stem cell type, fuse with host neuronal cells. However, the exact mechanism by which some somatic stem cells adopt a neural fate is not completely understood. A better knowledge of the mechanisms underlying the phenotypic plasticity of somatic stem cells and the characterization of their daughter differentiated states is a prerequisite before considering their use in the treatment of human patients.

During development of the central nervous system (CNS), all types of neuronal and macroglial cells derive from neuroepithelial neural stem cells (NSCs), and NSCs have been isolated from immature and from adult mammalian brains. In the adult brain, NSCs that express glial fibrillary acidic protein (GFAP) are found in two regions of the periventricular germinal matrix, the subventricular or subependymal zone of the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus. In addition to the neurogenic regions of the brain, multi-potential NSCs were recently isolated from postnatal cerebellum. These cells express markers associated with neural progenitor and stem cells, and also express GFAP, a defining marker of astrocytes. Recently, GFAP-expressing cells, with characteristics of NSCs, were found in non-neurogenic regions of the developing rodent brain, which indicates that, in addition to the germinal matrix, neural progenitor cells are scattered within the mammalian brain.

A number of recent studies revealed roles of NSCs in brain development and in possible repair of the CNS. Fate determination of NSCs is regulated by cell-intrinsic programs, as well as by extrinsic cues from the surrounding environment. NSCs self-renew and give rise to progenitors that can differentiate into neurons and glial cells. Recent studies have demonstrated that some radial glia, the first cells to differentiate within the embryonic neuroepithelium, have stem cell characteristics and produce neurons and astrocytes. Serum-free mouse embryo (SFME) cells, established by Loo et al., were originally derived from a 16-d-old whole mouse embryo and are maintained in a serum-free culture medium. These cells do not undergo growth crisis, maintain their diploid karyotype for extended passages and are non-tumorigenic in vivo. Consequently, they are non-transformed, behave as primary cultures, have a finite lifespan and display the characteristics of CNS progenitor cells. Although a few molecular markers have been defined for SFME cells upon differentiation into astrocytes, the mechanisms underlying their specification into the astrocytic lineage are not understood. Further, the possibility of SFME cells being able to differentiate into cell lineages other than the astrocytic lineage has never been explored.

In the present study, SFME cells were exposed to leukemia inhibitory factor (LIF) and bone morphogenic protein 2 (BMP2) to induce differentiation, and expression of nestin, a marker of early neural lineage, βIII-tubulin, a marker of neuronal lineage, oligodendrocyte marker O4 (O4), a marker of oligodendrocytic lineage and GFAP, a marker of astrocytic lineage. These data were analyzed to identify characteristics of SFME cells as a CNS progenitor and to elucidate a possible
ble mechanism underlying their specification into an astrocytic lineage upon differentiation.

MATERIALS AND METHODS

Materials LIF and BMP2 were purchased from CHEMICON International Inc. (Temecula, CA, U.S.A.) and from PeproTech House (London, U.K.), respectively. Anti-nestin polyclonal antibody, anti-βIII-tubulin monoclonal antibody and anti-O4 monoclonal antibody were obtained from R&D Systems Inc. (Minneapolis, MN, U.S.A.). Anti-GFAP monoclonal antibody was obtained from YLEM (Rome, Italy). HilyteFlour™488-conjugated antibody to mouse IgG heavy and light chains (H+L), HilyteFlour™555-conjugated antibody to mouse IgG H+L, and HilyteFlour™555-conjugated antibody to goat IgG H+L were obtained from AnaSpec Inc. (San Jose, CA, U.S.A.).

Cell Culture SFME cells, a gift from Dr. S. Shirahata (Kyushu University, Fukuoka, Japan), were cultured in a humidified 7% CO₂–93% air atmosphere at 37 °C in 60 mm diameter dishes, pre-coated with 10 μg/ml fibronectin. The basal nutrient culture medium was a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12, containing 15 mM HEPES, pH 7.4, 1.2 g/l sodium bicarbonate, 10 mM sodium selenite and 10 μg/ml gentamicin, supplemented with insulin (10 μg/ml), transferrin (25 μg/ml) and epidermal growth factor (50 ng/ml). Cell passages were accomplished by rapid trypsinization with 0.2% crude trypsin and 1 mM ethylenediaminetetraacetate in phosphate-buffered saline (PBS) without calcium or magnesium, followed by dilution in the culture medium at room temperature. The medium containing the collected cells was centrifuged at 250 g at 4 °C for 7 min and the supernatant was removed. The cells were suspended in the culture medium without the supplements, plated at 1×10⁶ cells/dish and cultured again in the medium with the supplements. After 48 h, 10 ng/ml LIF and 10 ng/ml BMP2 were added to induce differentiation of the cells and they were cultured for another 48 h, followed by immunofluorescence staining.

Immunofluorescence Staining and Confocal Laser-Scanning Microscopic Observation As previously reported, the specificity of the anti-GFAP antibody was determined by western blot analysis, in which it reacted with a single peptide. All the other antibodies in the present study showed appropriate specificities (data not shown). SFME cells cultured in the serum-free medium were fixed in 4% p-formaldehyde and then blocked in Tris-buffered saline containing 0.05% Tween-20 and 3% non-fat dried milk. Next, the cells were incubated with combinations of anti-nestin polyclonal antibody, anti-βIII-tubulin monoclonal antibody, anti-O4 monoclonal antibody and anti-GFAP monoclonal antibody at room temperature for 1 h, washed with PBS and then incubated with appropriate combinations of HILightly™488-conjugated goat anti-mouse IgG, HILightly™555-conjugated goat anti-mouse IgG and HILightly™555-conjugated rabbit anti-goat IgG, at room temperature for 30 min. After mounting with ProLong Gold anti-fade reagent (Molecular Probes Inc., Eugene, OR, U.S.A.), confocal imaging was performed using an LSM5 PASCAL confocal laser-scanning microscope equipped with Ar and He–Ne lasers. Images from the microscope were analyzed with Confocal Microscopy Software Release 3.2a (Carl Zeiss Japan, Tokyo, Japan).

Statistical Analysis Data are expressed as means±S.D. The significance of differences among means was determined by ANOVA and the t-test. Differences were considered significant at the level of p<0.05.

RESULTS AND DISCUSSION

Expression of Nestin and of βIII-Tubulin in SFME Cells at the Initial Proliferative Phase and after Induction of Astrocytic Differentiation by LIF and BMP2 Nestin is a class VI intermediate filament protein and was originally identified as a marker for neural progenitor cells during early CNS developmental stages. It is predominantly expressed in neural stem/progenitor cells and in a few other cell types. It is also expressed during the regeneration of injured adult tissue. Although expression of nestin is detected in various cells and tissues, the factors and mechanisms controlling nestin expression remain unknown. Tubulin is a ubiquitous structural protein and is found in cells as microtubules consisting of α- and β-tubulin monomers. Mammalian β-tubulin exists as seven isotypes, namely βI, II, III, IVa, IVb, V and VI, each a separate gene product, synthesized without alternative splicing. Expression of some tubulin isotypes is restricted to specific tissues, whereas other isotypes are constitutively expressed, resulting in a unique pattern of expression for each tissue. The neuronal specificity of βIII-tubulin is conserved in the developing and the postnatal CNS, including expression in putative bi-potential neuronal/glial progenitors and/or glial restricted precursor cells. It is also detected in neuronal and glial tumors. In neuronal tumors, its expression is differentiation-dependent; it is associated with neuronal morphological changes and with decreased cell proliferation. Conversely, in glial tumors, such as astrocytomas and oligodendrogliomas, its expression is aberrant. The functional significance of variations in tubulin expression in both normal and in tumor cells is not fully understood.

Several secreted growth factors, including BMP2, BMP4, LIF and ciliary neurotrophic factor, stimulate differentiation of cultured neural precursors into astrocytes. In the present study, SFME cells were treated with 10 ng/ml LIF and 10 ng/ml BMP2, cultured for 48 h and immunolabeled with anti-nestin and anti-βIII-tubulin antibodies. Addition of LIF and BMP2 caused morphological changes and the cells acquired an elongated fibroblast-like character, which indicates differentiation into an astrocytic lineage (Fig. 1). Observation of the immunofluorescence staining with a confocal laser-scanning microscope revealed that both nestin and βIII-tubulin were present in SFME cells. Although no statistical significance was found, nestin expression was weaker and βIII-tubulin expression was stronger, following the addition of LIF plus BMP2 (upon differentiation). These results indicate that SFME cells retain early neural and neuronal characteristics and can possibly differentiate into neuronal lineages.

Expression of O4 in SFME Cells at the Initial Proliferative Phase and after Induction of Astrocytic Differentiation by LIF and BMP2 Oligodendrocytes, which make the myelin sheaths in the CNS, develop from NSCs through a series of developmental stages. The first stage involves
round pre-progenitors that express nestin, and the second stage involves early bipolar progenitors or oligodendrocyte-type-2 astrocytes, expressing A2B5, a marker of early oligodendrocytic lineage, that later become multipolar and positive for chondroitin sulfate proteoglycan. The third stage involves multipolar or arborizing late progenitors expressing O4 sulfatide glycolipids. The forth stage is characterized by arborized premyelinating oligodendrocytes positive for galactocerebroside and O1, and the final stage involves mature oligodendrocytes synthesizing the myelin membrane with its structural components such as myelin basic protein. O4-positive oligodendrocytes can be induced by triiodothyronine treatment.

In the present study, O4 was very weakly detected in SFME cells (Fig. 2). Although no statistical significance was found, a minimal increase in O4 expression was found by the addition of LIF plus BMP2. These results indicate that SFME cells have early oligodendrocytic characteristics and
can possibly differentiate into oligodendrocytic lineages.

Expression of GFAP in SFME Cells at the Initial Proliferative Phase and after Induction of Astrocytic Differentiation by LIF and BMP2  
GFAP is an intermediate filament protein found in astrocytes \(^{51}\) and has been reported to be involved in multiple functions in the CNS, such as long term upkeep of normal CNS myelination \(^{52,53}\) and astrocyte-neuron interactions. \(^{54}\) Nakashima \textit{et al.} \(^{55}\) and Yanagisawa \textit{et al.} \(^{43}\) reported that synergistic astrocyte induction from neuroepithelial cells by LIF and BMP2 could be attributed to formation of a complex between LIF-activated STAT3 and BMP2-activated Smad1, bridged by the transcriptional co-activator p300. This activation could result in increased GFAP levels.

In the present study, GFAP expression was significantly increased by the LIF and BMP2 treatment (Fig. 3). Several culture conditions have been defined under which CNS progenitor cells differentiate into neuronal, astrocytic and oligodendrocytic lineages. Our approach was to culture in serum-free, defined medium, in which SFME cells survive, proliferate and differentiate into an astrocytic lineage when treated with LIF and BMP2. Figure 4 shows relative intensity of immunofluorescence for nestin, βIII-tubulin, O4 and GFAP. These markers were present both at the initial proliferative phase, and after induction of differentiation by the LIF and BMP2 co-treatment. GFAP expression was strongly increased upon differentiation, while expression of the other markers changed very little. 

In conclusion, we found that at the initial proliferative phase, SFME cells express the glial marker GFAP, as well as nestin (a marker of early neural lineage), βIII-tubulin (a marker of neuronal lineage) and O4 (a marker of oligodendrocytic lineage). It was also found that differentiation of these cells into an astrocytic lineage is associated with asymmetric production of these markers, rather than by acquiring an astrocytic marker upon differentiation. SFME cells were originally derived from a 16-d-old whole mouse embryo \(^{29}\) and have been documented to increase GFAP, the astrocyte intermediate filament marker protein, following the addition of serum, TGF-β \(^{56,57}\), LIF \(^{58}\) or BMPs \(^{59}\) or following the addition of LIF plus BMP2. \(^{60}\) These cells offer advantages for studying cell differentiation because they serve as progenitor cells and proliferate without senescence \textit{in vitro}, \(^{34,59}\) and have long been considered to differentiate into only an astrocytic lineage. Our present study showed that the cells express early neural, neuronal and oligodendrocytic markers, as well as the astrocytic marker. This is an indication that these cells, at this embryonic stage (16-d gestation), may play an important role of differentiating into diverse cell types in CNS. Further investigations would be expected to elucidate if these cells have multi-potent characteristics and are capable of dif-
ferentiation into cell lineages other than the astrocitary line-
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REFERENCES