Astrocyte differentiation mediated by LIF in cooperation with BMP2

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Abstract Leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP) 2 signal via different receptor systems. We have recently demonstrated that simultaneous stimulation of fetal mouse neuroepithelial cells with these distinct types of cytokines synergistically induces astrocyte differentiation in a 2-day culture. Here we show that astrocytes spontaneously emerge in vitro without exogenously added LIF and BMP2 in the culture of neuroepithelial cells for a much longer period. This spontaneous astrocyte differentiation is completely impaired when neuroepithelial cells deficient for gp130, a signal transducing receptor component for LIF, are used. We also show that LIF and BMP2 as well as related cytokines and respective receptor molecules are expressed in fetal mouse brain and cultured neuroepithelial cells. Taken together with our previous finding that prenatal mouse brain deficient for gp130 exhibits a severe reduction of astrocytes, it is suggested that LIF acts cooperatively with BMP2 in vivo to induce astrocyte differentiation in mouse developing brain.

Key words: gp130; Leukemia inhibitory factor; Bone morphogenetic protein; Synergy; Neuroepithelial cell; Astrocyte

1. Introduction

It is generally considered that fetal mouse telencephalic neuroepithelial cells contain neural precursors which have a multipotency to differentiate into neurons, astrocytes, and oligodendrocytes [1–3]. The cell fate of the precursors is regulated by various mediators in their environment within the developing brain. We have previously revealed that astrocyte differentiation is synergistically induced in vitro from neuroepithelial cells by leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP) 2, whereas each cytokine alone showed no such activity [4]. The interleukin (IL)-6 family of cytokines includes IL-6, IL-11, LIF, ciliary neurotrophic factor (CNTF), oncostatin (OS) M, and cardiotrophin (CT) 1 [5]. They all share non-kinase membrane protein gp130 as a receptor component critical for signal transduction. All six cytokines trigger dimerization of gp130 with itself or with other dimer partners of structural similarity, activating associated janus kinase (JAK)-type cytoplasmic tyrosine kinases and a downstream transcription factor, signal transducer and activator of transcription (STAT) 3 [5]. BMPs are members of the transforming growth factor (TGF)-β superfamily and signal through heterotetrameric serine/threonine kinase receptors [6,7]. Activated BMP receptors phosphorylate carboxy-terminally located serine residues of downstream transcription factors Smad1, 5 or 8, and this allows them to associate with a common mediator Smad4, leading to up-regulation of the expression of specific genes [6,7]. We have demonstrated that the synergistic effect between LIF and BMP2 to induce astrocyte differentiation is due to the signal-dependent complex formation of STAT3 and Smad1 bridged by transcriptional coactivator p300 to effectively enhance expression of the target genes, such as that for an astrocyte marker, glial fibrillary acidic protein (GFAP) [4].

In contrast to our previous finding as described above, some reports suggested that IL-6 family cytokines (such as LIF and CNTF) [3] or BMPs (for instance BMP2 and BMP4) [8] alone have a comparable potential to induce differentiation of astrocytes from neural progenitor cells. The major difference between the experimental condition in their work and ours is the culture duration in the presence of cytokines. A relatively longer period (3–7 days) of culture was taken in their studies [3,8], compared to ours (only 2 days) [4]. We therefore hypothesized that IL-6 family cytokines and BMPs are endogenously produced and accumulated during the longer culture duration, which may contribute to astrocyte differentiation. In the present study, we show that at least LIF, IL-6, BMP2, BMP4 and their receptor components are indeed expressed in the brain on embryonic day (E) 17, the time when astrocytes are considered to emerge in vivo and in 4-day cultured neuroepithelial cells from E14 brain. We demonstrate that astrocytes spontaneously emerge in the long-term culture of neuroepithelial cells without exogenously given cytokines, which is completely abolished when gp130-deficient cells are used. We further discuss the mechanism whereby IL-6 family cytokines and BMPs cooperatively function in vivo to induce astrocyte differentiation in mouse fetal brain.

2. Materials and methods

2.1. Cell culture

Fetuses from intercrossings of mice heterozygous for gp130 mutation on the genetic background of ICR were used [9–11]. Neuroepithelial cells from gp130 null mice and control littermates were prepared and cultured as previously described [2–4]. In brief, telencephalons from mice on E14 were triturated in HBSS (Gibco BRL) by frequent pipetting and dissociated cells were expanded for 4 days in N2-supplemented DMEM/F12 medium (Gibco BRL) containing 10 ng/ml of bFGF (R&D) (N2/DMEM/F12/bFGF) on 6-cm dishes that had been precoated with poly-L-ornithine (Sigma) and fibronectin (Gibco BRL). Cells were then detached in HBSS and replated on chamber slides (Nunc; for immunofluorescent staining) precoated as above at a density of 8×10^4 (for 2-day cultures), 3×10^4 (for 4-day cultures), or 2×10^4 (for 6-day cultures) cells per well. N2/

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Abbreviations: IL-6, interleukin-6; LIF, leukemia inhibitory factor; STAT, signal transducer and activator of transcription; BMP, bone morphogenetic protein; GFAP, glial fibrillary acidic protein; RT-PCR, reverse transcription and polymerase chain reaction

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DMEM/F12/bFGF medium supplemented with LIF (80 ng/ml, Gibco BRL), BMP2 (80 ng/ml) or both cytokines (80 ng/ml each) was used for the cell culture. For a long-term culture experiment, cells that had been prepared and expanded for 4 days as described above were replated and expanded on precoated 90-mm dishes (Nunc) for a further 7 days, replating on day 4. The cells were then replated onto precoated chamber slides at a density of 3×10^4 cells per well and cultured for an additional 5 days without exogenously added cytokines.

2.2. RT-PCR

Total RNAs were isolated from E17 brain or 4-day cultured E14 neuroepithelial cells from normal and gp130-deficient littermates. Reverse transcriptions were performed with the RNAs as templates using Superscript II reverse transcriptase (Gibco BRL). PCRs were carried out using AmpliTaq Gold (Perkin Elmer) with the following settings: 95°C for 9 min; 40 cycles of 94°C 20 s, 60°C 20 s, and 72°C 30 s; 72°C 5 min. Used primer sets were as follows: 5′-GATGCTGGTGACAACCACGG-3′, 5′-CTTGGTTGAAGATATGAATTAGAG-3′ (for IL-6); 5′-AGTGAAGCACGTGGTCCAGG-3′, 5′-CTGTGGTTTACGGTATTGTCAG-3′ (for IL-6 receptor (IL-6R)); 5′-ATGGCAGCGCCAATGCTCGC-3′, 5′-ACAGACGGCAAAGCACATTGC-3′ (for LIF); 5′-TCCTGGAATCTCGGTCGATAG-3′, 5′-GAATCAAGAATTGCCTGGAATTG-3′ (for LIFR); 5′-TGTCAGCACCAAGGATTTGGC-3, 5′-GTAGCTGACCATACATGAAGTG-3′ (for gp130); 5′-CCAGGTTAGTGACTCAGAACAC-3′, 5′-TCATCTTGGTGCAAAGACCTGC-3′ (for BMP2); 5′-TAGCAAGAGTGCCGTCATTCC-3′, 5′-CCAGTCTCGTGTCCAGTAGTCG-3′ (for BMP4); 5′-CAGACTTGGACCAGAAGAAGCC-3′, 5′-ACATTCTATTGTCTGCGTAGC-3′ (for BMP type I receptor (ALK3)); 5′-GCTTCGCAGAATCAAGAACG-3′, 5′-GTGGACTGAGTGGTGTTGTG-3′ (for BMP type II receptor (BMPRII)).

2.3. Immunofluorescent staining

Cells cultured on chamber slides were fixed with 4% paraformaldehyde in PBS and stained with antibody to GFAP (1:2000, Dako) and rhodamine-conjugated second antibody (1:100, Chemicon). Bisbenzimide H33258 fluorochrome trihydrochloride (1 μg/ml, Nakarai) was used to stain nuclei.

2.4. Luciferase assay

Neuroepithelial cells expanded for 4 days were replated on 12-well plates (Nunc) precoated as above at a density of 5×10^5 cells per well and cultured for 16 h. The cells were then transfected with a luciferase reporter construct having the 2.5 kb GFAP promoter (GF1L-pGL3) using Trans-IT LT-1 (Mirus) according to the manufacturer's protocol. As an internal control, a plasmid containing sea pansy luciferase expression construct (R-Luc) was co-transfected. On the following day, cells were stimulated with each cytokine (80 ng/ml) for 8 h and solubilized. Luciferase activity was measured according to the recommended procedures for the Pikkagene Dual Luciferase Assay System (Toyo Ink Inc.). Luminous CT-9000D (Dia-Iatron) was used for quantitation.

3. Results

3.1. Neuroepithelial cells express both LIF and BMP2

We have previously demonstrated that LIF and BMP2 act in dramatic synergy on cultured neuroepithelial cells to induce astrocyte differentiation [4]. In order to determine the role of these two cytokines in astrocyte differentiation during brain development in vivo, we first examined LIF, BMP2, and their receptor components in mouse brain at E17, the time when astrocytes are considered to emerge in vivo (Fig. 1A). Amongst the IL-6 family cytokines, at least LIF, IL-6 and their receptor components were expressed. In the gp130-deficient brain, similar results were obtained except for gp130 expression. BMP2, BMP4, BMP type I (ALK3) and type II (BMPRII) receptors were expressed at comparable levels in both normal and gp130-deficient brains. All the transcripts were detected in neuroepithelial cells from E14 brain cultured

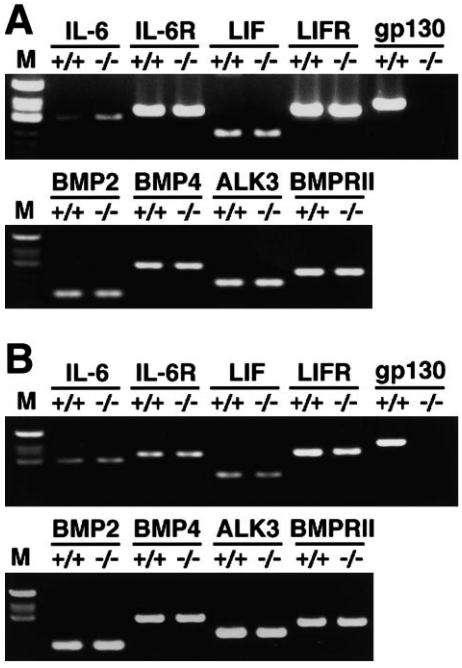

Fig. 1. Expression of LIF, IL-6, BMP2, BMP4 and their cognate receptor components in fetal brain and cultured neuroepithelial cells. Total RNAs were extracted from E17 brain (A) or 4-day cultured E14 neuroepithelial cells (B) from normal (+/+) and gp130 null mutant (−/−) mice, then subjected to RT-PCR using specific primers for each indicated molecule. M indicates molecular markers.

for 4 days (Fig. 1B). Although we have not performed any quantitative experiment such as ELISA, these results provide a possibility that the endogenously expressed LIF, BMP2 and related cytokines may cooperatively act on neural precursor cells to direct differentiation into astrocytes.

3.2. Spontaneous emergence of astrocytes in a long-term culture

In light of the results described above, we expected that astrocytes would emerge spontaneously without exogenously added cytokines in a long-term culture of neuroepithelial cells, owing to the synergistic action of endogenously produced IL-6 family cytokines and BMPs. As shown in Fig. 2, GFAP-brightly-positive cells with astrocytic morphologies actually appeared with an incidence of approximately 55% among

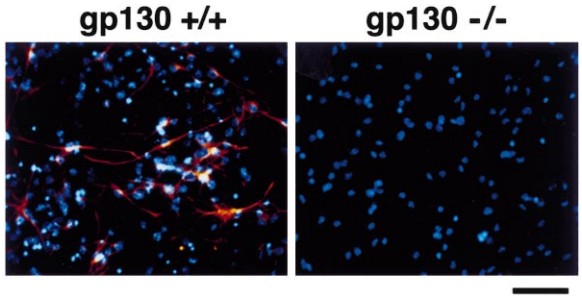

Fig. 2. Spontaneous differentiation of astrocytes in a long-term culture. Neuroepithelial cells prepared from normal and gp130-deficient telencephalons were cultured for 16 days without exogenously added cytokines and subjected to immunofluorescent staining for GFAP. Scale bar, 50 μm.

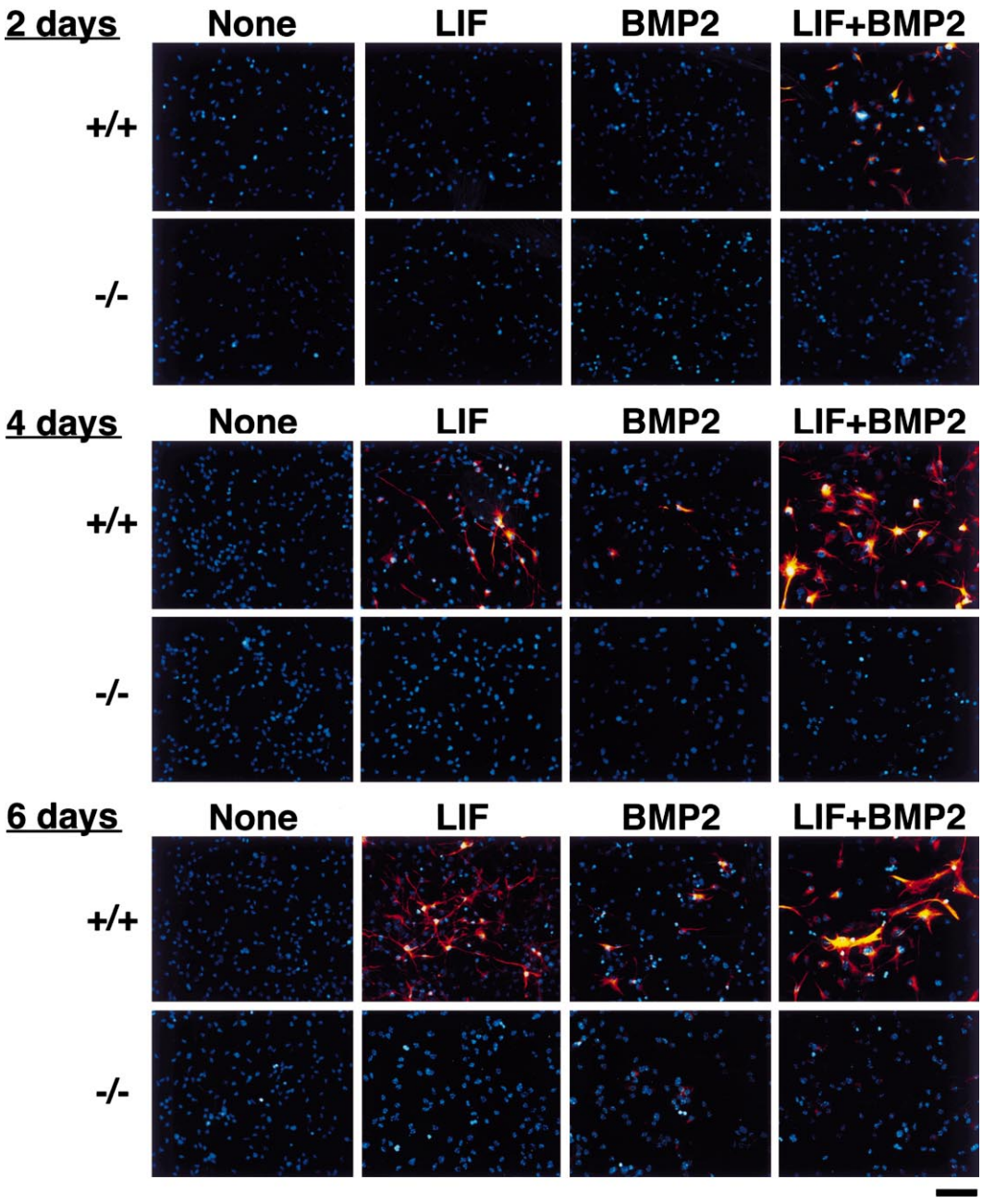

Fig. 3. Astrocyte differentiation induced by LIF and BMP2. Neuroepithelial cells from normal (+/+) and gp130-deficient (−/−) littermates were cultured with medium alone, BMP2 (80 ng/ml), LIF (80 ng/ml), or BMP2 (80 ng/ml) plus LIF (80 ng/ml) for indicated days, and subjected to immunofluorescent staining for GFAP. Scale bar, 50 μm.

the live cells in the 16-day culture of neuroepithelial cells prepared from E14 fetal mice without exogenously given cytokines. No astrocyte was observed in the culture of cells deficient for gp130 under the same condition, confirming that synergistic action of the two distinct types of cytokines is a prerequisite for efficient induction of astrocyte differentiation.

3.3. *Astrocyte differentiation induced by cooperative action between exogenously added LIF or BMP2 and their endogenously produced counterpart cytokines*

Based on the result described above, we examined whether exogenously given LIF or BMP2 alone acts in cooperation with its endogenously expressed and accumulated counterpart cytokine to induce astrocyte differentiation in a medium-term culture. In the 2-day culture of neuroepithelial cells from normal mice, many GFAP-brightly-positive cells with typical astrocyte morphologies were induced only when they were treated with LIF and BMP2 simultaneously, while no astrocyte was observed in the culture containing each cytokine alone (Fig. 3, top panels), as previously demonstrated [4]. In contrast, LIF or BMP2 alone could induce astrocyte development as the culture duration was extended to 4 days or 6 days, implying that exogenously added LIF or BMP2 cooperatively functions with an endogenously produced and accumulated counterpart cytokine (e.g. BMP2 or LIF, respectively) within

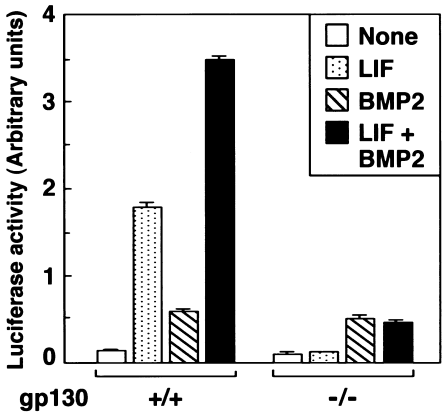

Fig. 4. GFAP promoter activation induced by LIF and BMP2. Normal and gp130-deficient neuroepithelial cells were co-transfected with GF1L-pGL3 and R-Luc, and then stimulated with indicated cytokines (80 ng/ml).

the culture. The phenomenon of single cytokine-induced astrocyte differentiation became more obvious when the culture duration was further extended to 6 days, supporting the idea of accumulation of counterpart cytokines. Astrocytes were not induced from gp130-deficient cells under any conditions taken for the 4-day culture as above. A small number of GFAP-dull-positive cells was observed in the 6-day culture of gp130-deficient cells containing BMP2 (Fig. 3, bottom panels). This is consistent with the result that LIF had no effect on GFAP promoter activation in cells deficient for gp130, while BMP2 induced weak activation of the promoter (Fig. 4). In neuroepithelial cells from normal mice, the synergistic activation of GFAP promoter by LIF and BMP2 was obvious (Fig. 4) as previously demonstrated [4]. These results indicate that exogenously added LIF or BMP2 functions in cooperation with its endogenous counterpart cytokines to induce astrocyte differentiation. While our results have not resolved whether LIF signaling alone is able to induce astrocyte differentiation in the complete absence of BMP signaling, the result provided in this study suggest that both IL-6 family cytokines and BMPs are required for effective induction of astrocyte differentiation from neuroepithelial cells.

4. Discussion

In our present and previous studies [4], we have shown that LIF and BMP2 act in synergy to induce astrocyte differentiation from neuroepithelial cells in the 2-day culture in vitro. GFAP-positive astrocytes were induced in neuroepithelial cells treated with a combination of LIF and BMP2, though not with either LIF or BMP2 alone. Some previous reports suggested, inconsistently with these 2-day culture results, that IL-6 family cytokines and BMPs each alone have a potential to induce astrocyte differentiation from neural progenitor cells [3,8]. However, contrasting to our culture period (2 days), relatively longer periods (3–7 days) were taken in these previous reports [3,8]. We propose here in the present study a model explaining this discrepancy. We show that LIF, IL-6, BMP2, BMP4, and their respective receptor components are expressed in primary cultured neuroepithelial cells, and that exogenously given LIF or BMP2 alone can induce astrocyte differentiation when the culture duration is extended to 4 days or 6 days, possibly in cooperation with an endogenously produced counterpart cytokine, i.e. BMP2 or LIF, respectively. In addition, cooperative function between endogenously produced IL-6 family cytokines and BMPs may lead to the spontaneous emergence of astrocytes in long-term culture (for 16 days) of neuroepithelial cells. Requirement of the signal through gp130 in the effective induction of astrocyte differentiation was demonstrated by the results that GFAP-positive cells were not significantly induced in any in vitro culture of neuroepithelial cells deficient for gp130. A small number of GFAP-dull-positive cells was observed when the gp130-deficient neuroepithelial cells were cultured for 6 days in the presence of BMP2. This is consistent with the observation of a significant but only weak response of the GFAP promoter to stimulation with BMP2 alone in neuroepithelial cells from gp130 null mutant mice. In support of these in vitro findings, the number of astrocytes was dramatically reduced in E18 gp130-deficient mouse brain [12], in which the expression of BMP2, BMP4 and their cognate receptors was unaffected. This in vivo observation also suggested that signals from both IL-6 family cytokines and BMPs are indispensable for the integrity of astrocyte differentiation in fetal mouse brain during development.

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