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Signaling crosstalk underlying synergistic induction of astrocyte differentiation by BMPs and IL-6 family of cytokines

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Abstract We here show that bone morphogenetic protein (BMP) 7 acted in synergy with the distinct type of cytokines, leukemia inhibitory factor (LIF) and interleukin (IL) 6 that are in the IL-6 family, to induce astrocyte differentiation from neuroepithelial cells as assessed by expression of glial fibrillary acidic protein (GFAP). In this synergistic action, transcription factors, Smads and STAT3 (for signal transducer and activator of transcription 3) activated by respective group of cytokines, as well as a transcriptional coactivator p300 were essential. Taken together with our previous finding that the synergistic astrocyte induction by BMP2 and LIF is attributed to the complex formation of Smads and STAT3 bridged by p300, it is conceivable that this complex formation is a mechanism utilized in common by two different types of cytokines belonging to the BMP and IL-6 families in order to synergistically induce astrocyte differentiation.

Key words: Astrocyte differentiation; Bone morphogenetic protein; Crosstalk; Interleukin-6 family of cytokine; Smad; STAT3

1. Introduction

The central nervous system is organized by neuronal and glial cells generated from common neural precursor cells in the developing brain [1], and fate decision of the precursors is regulated by cell-intrinsic programs as well as extrinsic cues from surrounding environment. Cytokine signaling is one of such extrinsic cues. For instance, it has been indicated that astrocyte differentiaton from neural precursors is induced in a culture for 3–7 days either with BMPs such as BMP2 and BMP4 or with IL-6 family of cytokines like IL-6, IL-11, LIF, ciliary neurotrophic factor and oncostatin M [2–9].

BMPs belong to the transforming growth factor (TGF)-β superfamily and were initially identified based on the osteoinductive property. BMPs are known to signal through heterotetrameric serine/threonine kinase receptors comprising two molecules each of type I and II receptor components. Activated receptors phosphorylate transcription factors Smad1, Smad5 or Smad8, which allows them to associate with a common mediator, Smad4, leading to transactivation of target genes [10].

IL-6 family of cytokines are pluripotent and characterized by the requirement of membrane glycoprotein gp130 in their functional receptor complexes for signal transduction. In response to the binding of IL-6 family of cytokines to their specific receptors, gp130 becomes dimerized either with itself or with another dimer partner like LIF receptor (LIFR) or oncostatin M receptor. This dimer formation triggers the activation of associated Janus kinases and downstream transcription factor STAT3 [11].

Considering that various cytokines exist simultaneously in the physiological condition which may stimulate cells at the same time, it is important to take a possible crosstalk between various types of cytokine signals into account when trying to elucidate bona fide function of cytokines in vivo. We have previously found that BMP2 and LIF synergistically induce astrocyte differentiation of neuroepithelial cells [6]. This synergistic effect has been shown to be explained by cytokine signal-dependent formation of a complex of Smad1 and STAT3 bridged by a transcriptional coactivator p300 which effectively transactivates target genes such as the one for GFAP, an astrocyte marker. In this study, we have observed expression of BMP7, another member of the BMP family, in fetal mouse brain and cultured neuroepithelial cells, implying a role of BMP7 in fate determination of neural precursors. We further demonstrate that BMP7 was capable of cooperating with LIF and IL-6 to induce astrocyte differentiation from neuroepithelial cells through the mechanism analogous to that utilized by BMP2 and LIF.

2. Materials and methods

2.1. Neuroepithelial cell culture

Neuroepithelial cells were isolated from telencephalons of embryonic day (E) 14.5 mice as previously described [5,6]. Dissociated cells were cultured for 4 days in N_2-supplemented DMEM/F12 medium containing 10 ng/ml of basic fibroblast growth factor (R&D Systems) on a 90 mm dish that had been precoated with poly-L-ornithine (Sigma) and fibronectin (Gibco BRL). 4 days later, cells were then detached in HBSS and replated for further experiments. All the cyto-

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Abbreviations: BMP, bone morphogenetic protein; LIF, leukemia inhibitory factor; IL, interleukin; GFAP, glial fibrillary acidic protein; STAT3, signal transducer and activator of transcription 3; TGF-β, transforming growth factor-β; LIFR, LIF receptor; E, embryonic day; sIL-6R, soluble IL-6 receptor; RT-PCR, reverse transcription-polymerase chain reaction; DN-STAT3, a dominant negative form of STAT3

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kines used in this study (LIF (Gibco), IL-6 and soluble IL-6 receptor (sIL-6R) (kindly provided by Dr. K. Yasukawa, Tosoh Corp), BMP7 (kindly provided by Dr. K. Miyazono, The University of Tokyo), and TGF-β1 (R&D Systems)) were supplemented at a concentration of 80 ng/ml, excepted for sIL-6R (500 ng/ml).

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously described [9]. Used primer sets were as follows: 5′-CGATTTCAGCCTGGACAACG-3′, 5′-CCTGGGTACTGAACACGG-3′ (for BMP7); 5′-CCGGAGAG-CCCTGGATACC-3′, 5′- AGAGGGCAAGGACCTTGCTG-3′ (for TGF-β1); 5′-GGTCTCTGCTTTGTCTCAGTC-3′, 5′-AGACTGG-ACCAGCAATGACAG-3′ (for TβR I); 5′-CGGTCAAGGTTCCA-CAGCTGTGC-3′, 5′-GGTCGTCCTCCAGGATGATGGCA-3′ (for TβR II); 5′-GTCGTCCATCTTGCCATTCAC-3′, 5′-GTTGTGTC-CCACTGATCTACC-3′ (for Smad2); 5′-TGCTGGGTTGGAA-GAAGGGC-3′, 5′-TGTTGAAGGCAAACTCACAGAG-3′ (for Smad3).

2.3. Immunocytochemical staining

Immunocytochemical staining was performed as previously described [5,6]. Briefly, cells cultured for 2 days on chamber slides which had been precoated as described above were fixed in 4% paraformaldehyde in PBS and stained with anti-GFAP antibody (DAKO) and rhodamine-conjugated secondary antibody (CHEMICON) for detection of astrocytes. Bisbenzimide H33258 fluorochrome trihydrochloride (Nacalai Tesque) was used to stain nuclei.

2.4. GFAP promoter assay

GFAP promoter assay was performed as previously described [6]. Briefly, neuroepithelial cells replated on 12-well plates which had been precoated as described above were transfected with a plasmid containing firefly luciferase gene regulated by 2.5 kb GFAP gene promoter (GF1L-pGL3) [6] using TransIt LT-1 (Mirus). Control transfection was made with sea pansy luciferase gene conjugated with human elongation factor 1α promoter pEF-Rluc. On the following day, cells were stimulated with cytokines for 8 h and solubilized. Luciferase activities in the cell lysates were assessed using Pikkagene Dual Luciferase Assay System (Toyo Ink Inc.) with MicroLumat LB96P (WALLAC BERTHOLD) luminometer.

3. Results

3.1. Expression of BMP7 in fetal mouse brain and cultured neuroepithelial cells

BMP family of cytokines are divided into osteogenic protein-1 group, BMP2 group and growth differentiation factor-5 group [12]. We have previously shown that BMP2 is expressed in fetal mouse brain and cultured neuroepithelial cells, and induces astrocyte differentiation in a synergistic manner in cooperation with an IL-6 family of cytokine, LIF [6,7]. However, it remains elusive whether other members of the BMP family contribute to the induction of astrocyte differentiation from neuroepithelial cells. Thus, we first examined the expression of BMP7, a member of the osteogenic protein-1 group in the BMP family. As shown in Fig. 1, transcripts for BMP7 were detectable in mouse brain on E17.5, the time when astrocytes are considered to emerge in vivo, and also in 4 days cultured neuroepithelial cells. Together with our previous data showing the expression of BMP receptors (type I and II) in the fetal brain and neuroepithelial cells [7], it seems likely that BMP7 may play a role in fate determination of neural precursors in the developing brain.

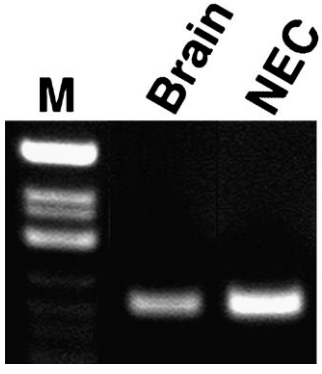

Fig. 1. Expression of BMP7 in fetal mouse brain and neuroepithelial cells. Total RNAs were extracted from fetal whole brain of E17.5 mouse embryo (Brain) or 4 days cultured neuroepithelial cells (NEC) from E14.5 mouse brain, and subjected to RT-PCR using specific primers for BMP7. M indicates molecular marker.

3.2. Synergistic astrocyte differentiation induced by BMP7 and LIF

In light of the above described results and previous findings, we asked whether BMP7 functions in synergy with LIF to induce astrocyte differentiation of fetal brain cells. As shown in Fig. 2H, when neuroepithelial cells were treated with BMP7 and LIF simultaneously for 2 days, cells positive for astrocyte hallmark protein, GFAP, with typical astrocyte morphologies were induced. In contrast, no GFAP-positive cell was observed in the culture with BMP7 or LIF alone (Fig. 2F and G). BMP7 and LIF did not appear to significantly affect the number of neuroepithelial cells as assessed by nuclear staining with H33258 (Fig. 2A–D). These results indicate that BMP7 with LIF can synergistically induce astrocyte differentiation of neuroepithelial cells, as has been reported for a combination of BMP2 and LIF.

3.3. Requirement of Smads, STAT3 and p300 in synergistic astrocyte induction by BMP7 and IL-6 family of cytokines

LIF and IL-6 transduce their signals through either gp130/LIFR heterodimer or gp130/gp130 homodimer, respectively, and transcription factor STAT3. Therefore, we next tried to assess whether BMP7 and IL-6 function in synergy to induce astrocyte differentiation from neuroepithelial cells. In the following experiments, sIL-6R was added to the culture with IL-6 to effectively stimulate gp130 on the cell surface [11]. As in the case of LIF (see Fig. 2), astrocyte differentiation was synergistically induced by a simultaneous addition of BMP7 and IL-6 (Fig. 3H).

Smads and STAT3 have been demonstrated to be indispensable for synergistic activation of the GFAP gene promoter by BMP2 and LIF [6]. We thus examined whether Smads and STAT3 are also prerequisite for the synergistic GFAP expression by BMP7 and IL-6. BMP7 and IL-6 synergistically induced the GFAP promoter-driven luciferase expression in neuroepithelial cells transfected with GF1L-pGL3 ('vehicle' in Fig. 3I), conforming to the results obtained by immunocytochemistry. Smad6 is known as an inhibitory Smad molecule which represses TGF-β and BMP signaling by inhibiting receptor-mediated phosphorylation of signal-specific Smad species or competing with Smad4 for binding to these Smad proteins. Transfection of neuroepithelial cells with Smad6 reduced the promoter activation induced by either BMP7 or a combination of BMP7 and IL-6 (Fig. 3I, hatched and filled columns in the middle four columns). A dominant negative form of STAT3 (DN-STAT3) was further used, which has an amino acid substitution on tyrosine^{705} to phenylalanine and functions as an inhibitory molecule against endogenous STAT3. Overexpression of this DN-STAT3 in neuroepithelial

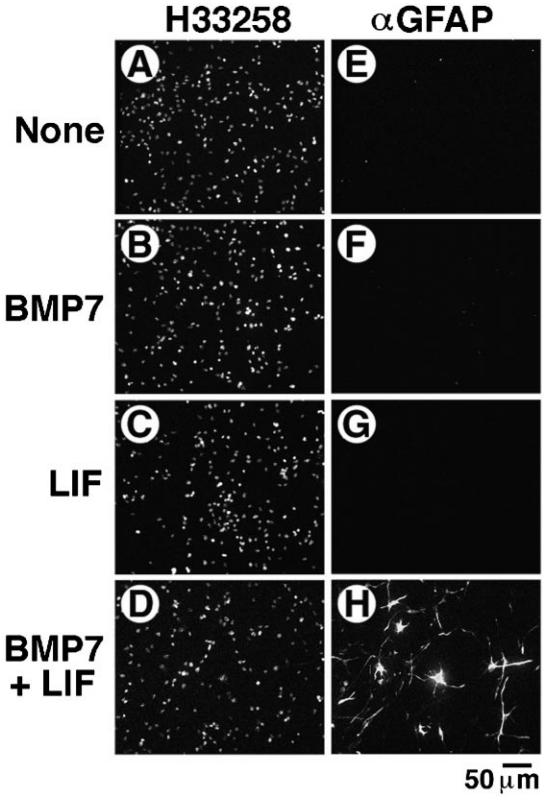

Fig. 2. Synergistic astrocyte differentiation induced by BMP7 and LIF. Neuroepithelial cells from E14.5 mice were cultured with either medium alone (A, E), BMP7 (B, F), LIF (C, G), or a combination of BMP7 and LIF (D, H) for 2 days. Cells were stained for DNA (A–D) and GFAP (E–H), and the same field under each culture condition was photographed.

cells inhibited the GFAP promoter activation induced by BMP7 and IL-6 either alone or in combination (Fig. 3I, the right three columns). These data suggest the requirement of transcription factors, STAT3 and Smads, for synergistic astrocyte differentiation induced by BMP7 and IL-6, implying the existence of crosstalk between BMP7 and IL-6 signaling via Smads and STAT3 transcription factors.

We have previously reported that a complex formation of Smad1 and STAT3 bridged by a transcriptional coactivator p300 is necessary for the synergistic induction of astrocyte differentiation by BMP2 and LIF [6]. Thus, we examined the involvement of the Smad1/p300/STAT3 complex in the synergistic astrocyte differentiation induced by BMP7 and IL-6 as well. p300 is known to interact with various kinds of transcription factors and to function as a molecular glue to form this complex. Recently we reported that p300 preferentially binds to Smad1 and STAT3 at its COOH-terminal and NH_2-terminal regions, respectively. For this reason, two p300 fragments, p300 (1737–2414) and p300 (1–682), have been shown to compete with endogenous p300 for its binding to Smad1 and STAT3, respectively, and to inhibit the GFAP promoter activation induced by BMP2 and IL-6 when overexpressed in neuroepithelial cells [6]. As shown in Fig. 3J, in neuroepithelial cells co-transfected with GF1L-pGL3 and p300 (1–682) or p300 (1737–2414), synergistic GFAP promoter activation induced by BMP7 and IL-6 was severely inhibited. This implies that p300-mediated complex formation of Smad proteins and STAT3 is also involved in astrocyte differentiaton synergistically induced by BMP7 and IL-6. Moreover, synergistic GFAP promoter activation by BMP7 and LIF was also inhibited by overexpression of DN-STAT3, Smad6 or truncated form of p300 (data not shown), suggesting that the Smads/p300/STAT3 complex plays a critical role in common when BMP and IL-6 families of cytokines act in synergy.

3.4. *TGF-β is devoid of activity to induce astrocyte differentiation*

Since we have observed expression of TGF-β1 (another

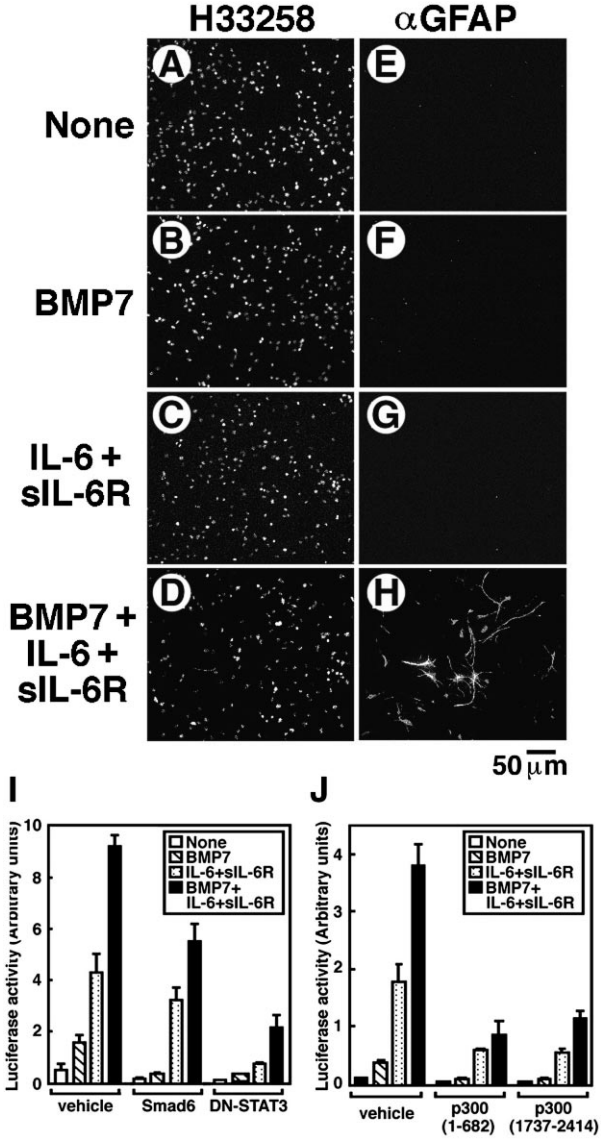

Fig. 3. Synergistic astrocyte differentiation induced by BMP7 and IL-6, and involvement of STAT3, Smads and p300. A–H: Neuroepithelial cells were cultured with either medium alone (A, E), BMP7 (B, F), IL-6/sIL-6R (C, G), or a combination of BMP7 and IL-6/sIL-6R (D, H) for 2 days and stained for DNA (A–D) and GFAP (E–H), and the same field under each culture condition was photographed. I, J: Neuroepithelial cells were co-transfected with GF1L-pGL3, pEF-Rluc and either control vehicle or an expression construct encoding DN-STAT3 or Smad6 (I), or truncated forms of p300 (J). The cells were stimulated on the following day with BMP7, IL-6/sIL-6R or a combination of BMP7 and IL-6/sIL-6R, and luciferase activities in the cell lysates were measured.

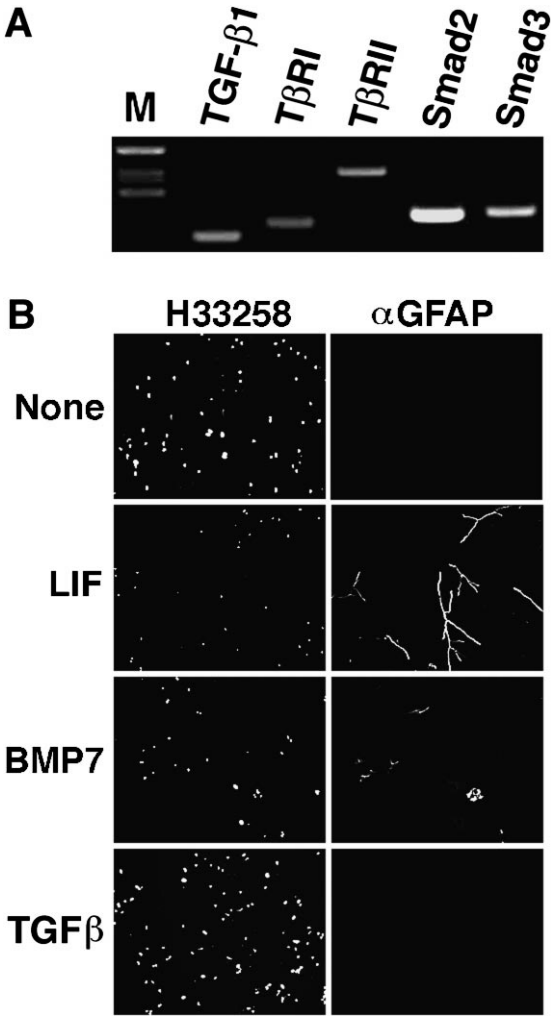

Fig. 4. TGF-β is incapable of inducing astrocytes from neuroepithelial cells. A: Expression of TGF-β1, its receptor components (TβR I and II) and the downstream transcription factors (Smad2 and Smad3) in neuroepithelial cells was examined by RT-PCR using specific primers. B: Neuroepithelial cells were cultured for 6 days with either medium alone, or either of TGF-β, LIF and BMP7 as indicated. Cells were stained for DNA (right panels) and GFAP (left panels), and observed by fluorescent microscopy.

member of the TGF-β superfamily to which the BMP family belong), its cognate receptor components (TβR I and II) and its downstream transcription factors (Smad2 and Smad3) in neuroepithelial cells as in Fig. 4A, we asked whether TGF-β1 is capable of contributing to the induction of astrocyte differentiation. We have previously indicated that exogenously added BMP2 acts in synergy to induce astrocyte differentiation with endogenously expressed and accumulated IL-6 family of cytokines in the culture, so that GFAP-positive astrocytes emerge when the cells were cultured for 6 days with exogenously added BMP2 alone [7]. This is also the case for BMP7 (Fig. 4B). However, TGF-β1 failed to induce astrocyte differentiation in this culture condition (Fig. 4B), even though its receptor components and the downstream transcription factors were detectable in neuroepithelial cells (Fig. 4A). TGF-β1 activates Smad2 and Smad3 as 'pathway-specific Smads' but not Smad1, Smad5 and Smad8 that are BMP pathway-specific [10]. Hence, it is likely that the activation of BMP pathway-specific Smads is required for the synergistic astrocyte induction with IL-6 family cytokines.

4. Discussion

In the present study, we found that BMP7 as well as BMP2 induce astrocyte differentiation in synergy with IL-6 family of cytokine, LIF or IL-6, and tried to elucidate the molecular mechanism underlying this phenomenon. We have demonstrated the critical role of Smads and STAT3 in this synergistic astrocyte induction because synergistic GFAP promoter activation by BMP7 and LIF or IL-6 was inhibited by overexpression of Smad6 or DN-STAT3. LIF and IL-6 are known to signal via different types of gp130-containing dimers, i.e. gp130/LIFR heterodimer and gp130/gp130 homodimer, respectively, yet both of these dimer formations lead to the activation of STAT3 [11]. Accordingly, STAT3 activation is ultimately important, regardless of the type of dimer formation, for the IL-6 family of cytokines to participate in astrocyte differentiation. As for BMPs, BMP pathway-specific Smads are essential to induce astrocyte differentiation, because TGF-β1, which activates TGF-β pathway-specific Smads, Smad2 and Smad3, is incapable of inducing astrocyte differentiation. The synergistic effect between BMP and IL-6 family of cytokines to induce astrocyte differentiation observed in the present in vitro culture system seems to function in vivo as well, since these cytokines and their receptor components are expressed together in developing brain [7–9,13]. In support of this idea, GFAP-positive astrocytes were severely reduced in fetal mouse brain deficient for gp130 even though the expression of BMPs and their receptor component was intact [5,7].

Supposing that various factors which regulate cell fate determination may exist at the same time in the developing brain, it is important to take the signaling crosstalk into consideration for the better understanding of in vivo function of these factors in the brain development. We have reported the molecular mechanism whereby BMPs and the IL-6 family of cytokines function in synergy to promote astrocytogenesis. Smads and STAT3 form a complex in response to this cytokine stimulation with a help of p300, and this complex formation is essential to effectively activate the target gene transcription. Considering that p300 is capable of associating with a variety of transcription factors, other signaling crosstalks in which p300 is involved may exist.

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