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TGF-β1-induced IL-6 expression via MEK pathway in mesenchymal stem cells enhances NGF-dependent neurite extension in PC12 cells.

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[Received：December 20 2021：Accepted：December 28 2021]

Abstract: The immunomodulatory ability of mesenchymal stem cells (MSCs) is synergistically regulated through cell contact-dependent mechanisms and secretion of soluble factors. TGF-β1 plays an important role in immunosuppression. In this study, the expression of TGF-β1-induced inflammation-related cytokines, chemokines, and growth factors in the human MSC line, UE7T-13, was investigated. The expression levels of NGF and IL-6 in UE7T-13 cells were significantly enhanced by TGF-β1 stimulation. TGF-β1-induced expression of IL-6 was attenuated by a TGF-β receptor inhibitor and an MEK inhibitor. These results indicate that TGF-β1 increases IL-6 expression via the MEK pathway in human MSCs. Considering that TGF-β1 increased the expression levels of both NGF and IL-6 in the MSCs, we further investigated the effect of the cytokines secreted from UE7T-13 cells on neurite extension of neuronal PC12 cells. We found that the neurite extension in PC12 cells was significantly enhanced in the conditioned medium derived from TGF-β1-pretreated UE7T-13 cells. In addition, it was significantly enhanced by the indirect co-culture of PC12 cells and TGF-β1-stimulated UE7T13 cells under transwell conditions. Interestingly, these enhancements of neurite extension mediated using the conditioned medium or indirect co-culture were negated by the addition of a neutralizing antibody against the soluble IL-6 receptor in the culture media. We also confirmed that the administration of both IL-6 and soluble IL-6 receptors to PC12 cells did not promote these neurite extensions. Overall, these results suggest that NGF secreted from TGF-β1-stimulated MSCs induces neuronal differentiation of PC12 cells, which is further enhanced by IL-6 secreted from MSCs.

Key words: Mesenchymal stem cells, Transforming growth factor-β1, Nerve growth factor, Interleukin-6, Neurite extension

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Introduction

Mesenchymal stem cells (MSCs) are adult stem cells isolated from various tissues such as bone marrow, adipose tissue, and the umbilical cord tissue [1),2)]. MSCs contribute to the repair of damaged tissue owing to their multipotency [3),4)]; they are activated by cytokines released from the immune cells in damaged tissues and have anti-inflammatory effects [5),6)]. The effectiveness of MSCs has been demonstrated in both regenerative medicine and the animal models for inflammatory and autoimmune diseases [7),8),9),10),11)].

The transforming growth factor-β (TGF-β) family, the factors belonging to which have three isoforms (β1, β2, and β3) plays an important role in the regulation of immune responses [12)]. Among all the factors, TGF-β1 downregulates various immune responses as an immunosuppressive molecule and is associated with immune diseases [13)]. TGF-β1 is secreted by immune cells containing macrophages homing to inflammatory tissues, and is known to play an important role in immunosuppression [13),14)]. Macrophages are immune cells that regulate cell proliferation and differentiation by secreting various cytokines. Tumor necrosis factor (TNF) - α, interleukin (IL) -6 and chemokine C-X-C motif ligand (CXCL) family of pro-inflammatory cytokines and chemokines are secreted by M1 macrophages stimulated by lipopolysaccharide (LPS) and interferon (IFN) - γ [15)]. However, growth factors such as TGF-β1, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) are secreted by M2 macrophages differentiated by IL-4 and IL-13 and play an important role in tissue repair [16),17)].

TGF-β1 is known to induce nerve growth factor (NGF) expression in various cell types [18),19)]. While TGF-β1-induced NGF expression is promoted in chondrocytes by Smad2/3-dependent signaling [20)], the activation of the c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) signaling pathways occurs in dental pulp cells [21)]. In addition, our recent study demonstrated TGF-β1-induced NGF expression via the Smad2/3 signaling pathway and p38 MAPK activation in periodontal ligament-derived fibroblasts [22)]. Interestingly, this increased expression of NGF was suppressed by the stimulation of pro-inflammatory cytokines, such as TNF- α and IL-1β.

In this study, the expression of TGF-β1-induced inflammation-related cytokines, chemokines, and growth factors in MSCs was investigated. The expression levels of NGF and IL-6 in MSCs were increased by TGF-β1 stimulation. We also reported that NGF-induced neurite extension is enhanced by the co-stimulation of IL-6 secreted by MSCs along with NGF.

Materials and Methods

Reagents

Recombinant human TGF-β1 was purchased from Pepro Tech., Inc. (Cranbury, NJ, USA). Recombinant human NGF and IL-6 were obtained from R&D systems, Inc. (Minneapolis, MK, USA). Recombinant human soluble IL-6 receptor (sIL-6R) was purchased from Biorbyt LLC. (St Louis, MO, USA). Neutralizing anti-Human IL-6R/CD126 antibody was purchased from Sino Biological Inc. (Wayne, PA, USA). The TGF-β type I receptor inhibitor SB-431542 was supplied by Cell Signaling Technology, Inc. (Beverly, MA, USA). The p38 MAPK

inhibitor SB203580, JNK inhibitor SP600125, MAPK/ERK inhibitor U0126, and Smad3 inhibitor SIS3 were obtained from Merck KGaA (Calbiochem; Darmstabt, Germany).

Cell culture

All cell cultures were maintained at 37° C in a humidified atmosphere with 5% CO_2. Human bone marrow-derived MSC line, UE7T-13, whose life span was prolonged by a retrovirus encoding human papillomavirus E7 and hTERT [23), 24), 25), 26)], were purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB No. 1154, Tokyo, Japan). UE7T-13 cells were cultured on plastic dishes (Thermo Fisher Scientific, Inc) in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich; Merk KGaA) supplemented with 10% fetal bovine serum (FBS), penicillin (50 units/mL) and streptomycin (50 units/mL) (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA). Rat pheochromocytoma PC12 cells obtained from RIKEN BioResource Research Center Cell Bank (Ibaraki, Japan) were cultured on type I collagen tissue culture plastic dishes in DMEM supplemented with 5% FBS, 10% horse serum and penicillin (50 units/mL) -streptomycin (50 units/mL).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using the ISOGEN I reagent (Nippon Gene Co., Ltd., Toyama, Japan). The concentration and quality of the total RNA were measured using a spectrophotometer Bio Drop μLite+ (Biochrom Ltd., Cambridge, United Kingdom). First-strand complementary DNA (cDNA) was synthesized using the PrimeScript RT reagent kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. Fluorescence RT-qPCR was performed through a two-step cycle procedure (denaturation at 95 ° C for 5s and annealing and extension at 60 ° C for 30s) for 40 cycles on a Thermal Cycler Dice Real Time System (Takara Bio) with SYBR Premix Ex Taq II (Takara Bio) and specific oligonucleotide primers (Table 1). Each PCR required cDNA derived from 50ng total RNA as a template and 0.4μM of each primer pair. The mRNA expression level was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the relative amount of mRNA in each sample was calculated via the ΔΔCq method. Relative mRNA expression levels were expressed as the fold increase or decrease relative to those of the control.

Enzyme-linked immunosorbent assay (ELISA)

UE7T-13 cells were stimulated with or without 10ng/mL TGF-β1 for 72h. The amount of secreted IL-6 was measured using sandwich ELISA kits for human IL-6 (R&D Systems Inc.). The IL-6 protein concentration was measured according to the manufacturer's instructions. Absorbance was measured using an MPR-A4i microplate reader (Tosoh Corp., Tokyo, Japan).

Examining neurite extension in PC12 cells

PC12 cells were treated with Calcein-AM solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. The status of neurite extensions emerging from PC12 cells was observed using a DM IL LED fluorescence microscope with an N PLAN L 40×0.55 CORR PH2 objective lens (Leica Microsystems, Allendale, NJ, USA) under phase-contrast or

Table 1. Sequences of the RT-qPCR oligonucleotide primers used in this study.

Reactivity	Target gene	Primer sequence (5' - 3')
Human	GAPDH	F: GCACCGTCAAGGCTGAGAAC
		R: ATGGTGGTGAAGACGCCAGT
	NGF	F: TCAGCGTCCGGACCCAATA
		R: CTGAGTGTGGTTCCGCCTGTA
	IL-6	F: AAGCCAGAGCTGTGCAGATGAGTA
		R: TGTCCTGCAGCCACTGGTTC
	FGF2	F: GTGTGCTAACCGTTACCTGGCTATG
		R: CCAGTTCGTTTCAGTGCCACA
	TGF-β1	F: GCGACTCGCCAGAGTGGTTA
		R: GTTGATGTCCACTTGCAGTGTGTTA
	IL-1β	F: CCAGGGACAGGATATGGAGCA
		R: TTCAACACGCAGGACAGGTACAG
	TNF- α	F: CTGCCTGCTGCACTTTGGAG
		R: ACATGGGCTACAGGCTTGTCACT
	CXCL12	F: GAGCCAACGTCAAGCATCTCAA
		R: TTTAGCTTCGGGTCAATGCACA
	CCL2	F: CTTCTGTGCCTGCTGCTCATA
		R: CTTTGGGACACTTGCTGCTG
	PDGF	F: GGCATCGTGCGTGACAATTA
		R: CTGAGGCTCCCAGAGTGAGA
Rat	GAPDH	F: GGCACAGTCAAGGCTGAGAATG
		R: ATGGTGGTGAAGACGCCAGTA
	NGF	F: TGCCAAGGACGCAGCTTTC
		R: TGAAGTTTAGTCCAGTGGGCTTCAG
	IL-6	F: ATTGTATGAACAGCGATGATGCAC
		R: CCAGGTAGAAACGGAACTCCAGA

fluorescent at 480 nm excitation. In addition, statistical assessment of neurite extension was performed as follows: cells bearing neurites longer than the length of the cell body were counted as cells with neurite extension. Neurite-extended and non-extended PC12 cells were counted in eight different microscopic fields. The ratio of the number of neurite-extended cells to the total number of PC12 cells in the field was statistically analyzed.

Evaluation of neurite extension triggered by the conditioned medium of TGF-β1-stimulated UE7T-13 cells

UE7T-13 cells (7.0×10^4 cells/well) were cultured in 12-well tissue culture plates. The cells were stimulated with 20 ng/mL TGF-β1 for 72 h, and the conditioned medium was recovered. The conditioned medium supplemented with or without sIL-6R (50 ng/mL) and/or sIL-6R neutralizing antibody (2 μg/mL) was used as the culture medium

for PC12 cells (1.0×10^4 cells/well) on type I collagen-coated 12-well tissue culture plates. The neurite extension in PC12 cells cultured in the conditioned medium of TGF-β1-stimulated UE7T-13 cells at 72 h was evaluated.

Evaluation of neurite extension triggered by a transwell co-culture system

UE7T-13 and PC12 cells were cultured by indirect co-culture, which was performed using a transwell co-culture system. The transwell co-culture system comprised a polycarbonate transwell chamber (BD Bioscience, Franklin Lakes, NJ, USA), which could be inserted into the wells of the standard 24-well plates. PC12 cells (3.0×10^4 cells/well) were seeded on the bottom of the 24-well culture plates. Subsequently, UE7T-13 cells (5.0×10^3 cells/well) were seeded on the upper membrane (pore size of 0.4 μm) of the transwell chamber. UE7T-13 cells on the upper membrane were stimulated with or without 20 ng/mL of TGF-β1, after which sIL-6R (25 ng/mL) and sIL-6R neutralizing antibody (4 μg/mL) were added to the lower level. After 72 h of culture, the extent of neurite extension in PC12 cells on the lower side was evaluated.

Statistical analysis

The data are presented as the mean ± standard deviation (SD) and were statistically analyzed using Tukey's multiple comparison test. Differences were considered statistically significant at $P < 0.05$. The results are representative of at least three separate experiments.

Results

TGF-β1 increases NGF expression in human MSCs

UE7T-13 cells were stimulated with TGF-β1 and the mRNA expression level of NGF was determined via real-time qRT-PCR analysis. As shown in Fig. 1, the mRNA expression

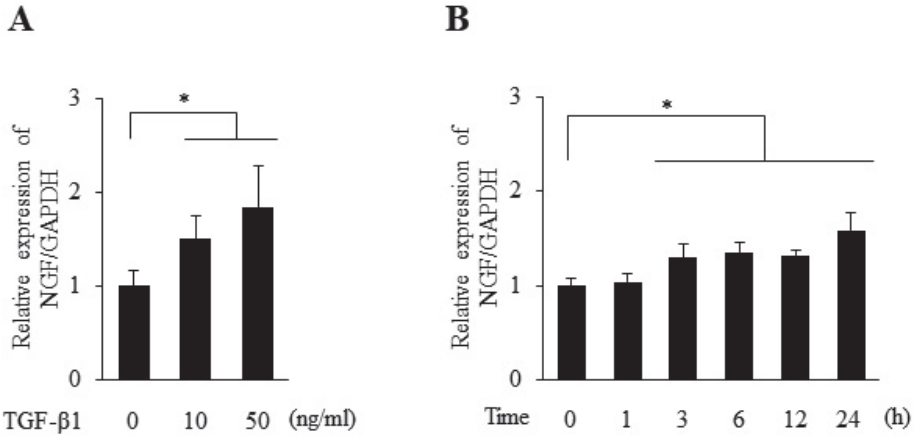

Fig. 1. *Regulation of NGF expression in MSCs stimulated with TGF-β1*
UE7T-13 cells were stimulated with TGF-β1 and then the mRNA expression level of NGF was investigated by RT-qPCR. (A) UE7T-13 cells were stimulated with 10 or 50 ng/mL TGF-β1 for 24 h. (B) UE7T-13 cells were stimulated with 10 ng/mL TGF-β1 in a time period between 1 to 24 h. NGF mRNA expression levels in each sample were normalized to GAPDH, and the results are expressed as the fold change relative to the unstimulated control. Data are presented as the mean ± SD (n=5). The values of $^*P < 0.05$ was considered statistically significant.

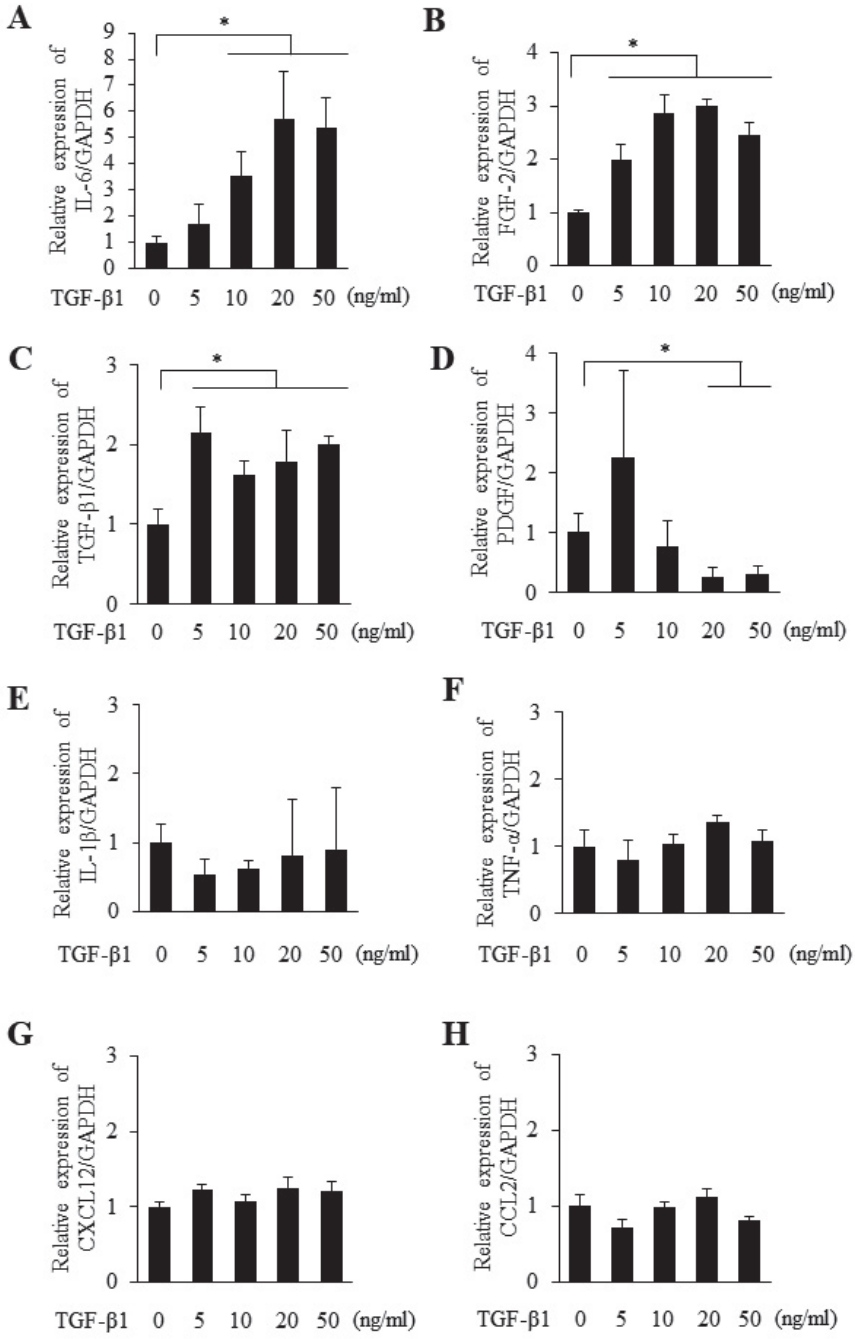

Fig. 2. *Expression of several cytokines, chemokines, and growth factors in MSCs stimulated with TGF- β 1*
UE7T-13 cells were stimulated with various concentration of TGF-β1 for 24 h, and then the mRNA expression levels of (A) IL-6, (B) FGF-2, (C) TGF-β1, (D) PDGF, (E) IL-1β, (F) TNF- α , (G) CXCL12 and (H) CCL2 were investigated by RT-qPCR. The mRNA expression levels in each sample were normalized to GAPDH, and the results are expressed as the fold change relative to the unstimulated control. Data are presented as the mean ± SD (n=5) . The values of *P < 0.05 was considered statistically significant.

level of NGF in the cells significantly increased in a dose- and time-dependent manner following TGF-β1 stimulation. Twenty-four hours after stimulation with TGF-β1, NGF mRNA expression was significantly enhanced at concentrations above 10 ng/mL (Fig. 1A). In addition, when stimulated with 10 ng/mL TGF-β1, its expression level continued to increase from 3 h after stimulation to at least 24 h (Fig. 1B). Therefore, these results

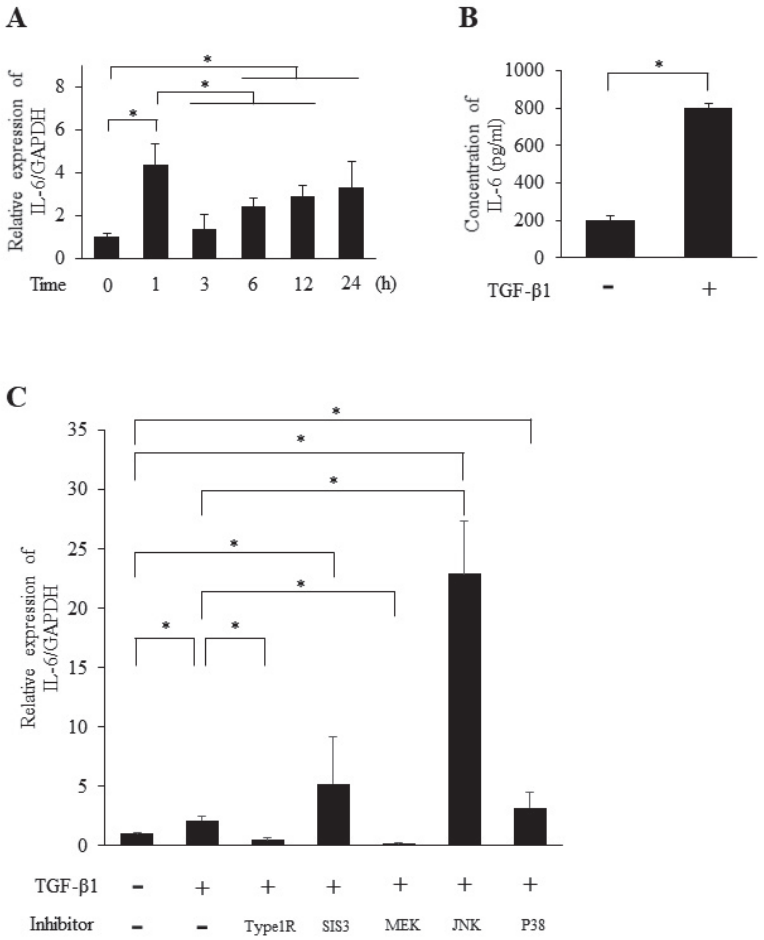

Fig. 3. *Expression and production of IL-6 in MSCs stimulated with TGF-β1*
UE7T-13 cells were stimulated with TGF-β1 and then the mRNA expression levels and protein production of IL-6 were investigated. (A) UE7T-13 cells were stimulated with 10 ng/mL TGF-β1 in a time period between 1 to 24 h. The mRNA expression level of IL-6 was investigated by RT-qPCR. (B) UE7T-13 cells were stimulated with or without 10 ng/mL TGF-β1 for 72 h. The amount of secreted IL-6 was measured using sandwich ELISA kits for human IL-6. The IL-6 protein concentration was measured according to the manufacturer's instructions. (C) UE7T-13 cells were stimulated with 10 ng/mL TGF-β1 supplemented with or without 10 μM each inhibitor described in "Materials and Methods". After 48 h of stimulation, the mRNA expression level of IL-6 was investigated by RT-qPCR. The mRNA expression levels in each sample were normalized to GAPDH, and the results are expressed as the fold change relative to the unstimulated control. Data are presented as the mean ± SD (n=5). The values of $^*P < 0.05$ was considered statistically significant.

suggest that TGF-β1 positively regulates NGF expression in human MSCs.

TGF-β1 increases IL-6 expression in human MSCs

Next, we investigated several cytokines, chemokines, and growth factors by RT-qPCR analysis and revealed that their mRNA expression levels in UE7T-13 cells were increased due to stimulation of the cells with various concentrations of TGF-β1. The mRNA expression levels of IL-6, FGF-2, and TGF-β1 were enhanced due to the dose-dependent stimulation of the cells by TGF-β1 (Fig. 2A-C). In contrast, PDGF mRNA expression was suppressed by stimulation at concentrations of TGF-β1 above 20 ng/mL (Fig. 2D). There was no significant difference with respect to the increase or decrease in the mRNA expression levels of the cytokines IL-1β and TNF-α (Fig. 2E-F) and the chemokines CXCL12 and CC chemokine ligand 2 (CCL2) (Fig. 2G-H).

In our previous study, we investigated the effect of pro-inflammatory cytokines such as IL-1β and TNF-α on TGF-β1-induced NGF expression in fibroblasts [22], thus in our current study we focused on IL-6 as an inflammatory cytokine. The mRNA expression level of IL-6 in UE7T-13 cells continued to increase until 24 h after TGF-β1 stimulation, but a biphasic relationship was observed in which the first peak was observed 1 h after stimulation (Fig. 3A). In addition, we conducted ELISA to measure protein levels and observed that the amount of IL-6 protein secreted by UE7T-13 cells into the cell culture conditioned medium was significantly enhanced by TGF-β1 treatment (Fig. 3B). TGF-β1-induced expression of IL-6 was attenuated by both the TGF-β type I receptor inhibitor SB-431542 and the MEK inhibitor U0126 (Fig. 3C). However, it was not suppressed by the administration of SIS3, a specific inhibitor of Smad3, a major molecule in the TGF-β signaling pathway, and the p38 MAPK inhibitor SB203580. Interestingly, treatment with the JNK inhibitor SP600125 enhanced TGF-β1-induced IL-6 expression in UE7T-13 cells. These results indicate that TGF-β1 increases IL-6 expression via the MEK pathway in human MSCs. In contrast, treatment with recombinant IL-6/sIL-6R was not involved in TGF-β1-induced NGF mRNA expression in UE7T-13 cells (Fig. 4).

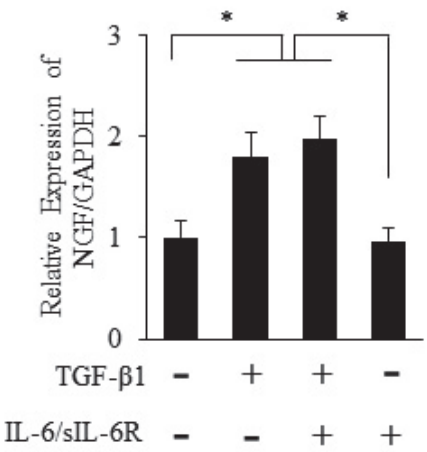

Fig. 4. *Involvement of IL-6 for TGF-β1-induced NGF expression in MSCs*
UE7T-13 cells were stimulated with or without 10 ng/mL TGF-β1, 50 ng/mL recombinant IL-6 and 50 ng/mL recombinant sIL-6R (IL-6/sIL-6R) for 24 h. The mRNA expression level of NGF was investigated by RT-qPCR. The mRNA expression levels in each sample were normalized to GAPDH, and the results are expressed as the fold change relative to the unstimulated control. Data are presented as the mean ± SD (n=5). The values of $^*P < 0.05$ was considered statistically significant.

IL-6 enhances NGF-dependent neurite extension in PC12 cells

As described above, TGF-β1 increased the expression levels of both NGF and IL-6 in MSCs, and we investigated its effect on neurite extension in PC12 cells. NGF-responsive PC12 cells differentiate into sympathetic-like neurons and extend long neurites, providing a useful model for the investigation of neuronal differentiation [27]. Neurite extension in PC12 cells was promoted by stimulation with recombinant NGF, which was further enhanced by co-stimulation with recombinant IL-6 and sIL-6R (Fig. 5A-B). In addition,

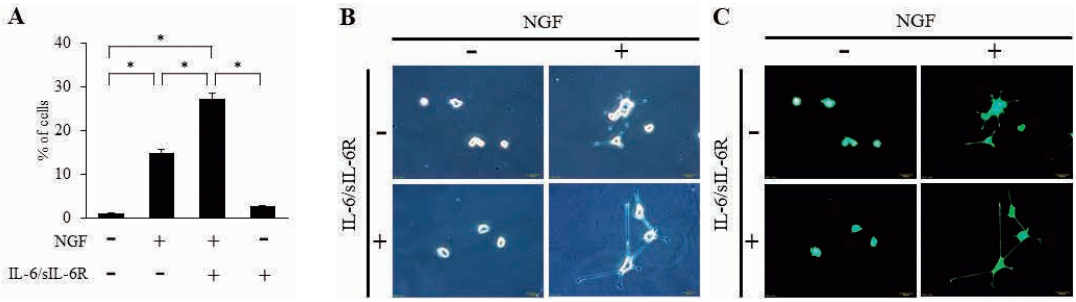

Fig. 5. *Effect of IL-6 on NGF-induced neurite extension in PC12 cells*
PC12 cells were stimulated with or without 20ng/mL recombinant NGF, 50ng/mL recombinant IL-6 and 50ng/mL recombinant sIL-6R (IL-6/sIL-6R) for 24 h. The status of neurite extensions emerging from PC12 cells was observed according to described in "Materials and Methods". (A) The ratio of the number of neurite extension cells to the total number of PC12 cells in the field of microscopy was statistically analyzed. Data are presented as the mean ± SD (n=5). The values of $^*P < 0.05$ was considered statistically significant. (B, C) The neurite extensions emerging from PC12 cells were observed under the phase-contrast (B) and fluorescent (C) field of the microscope.

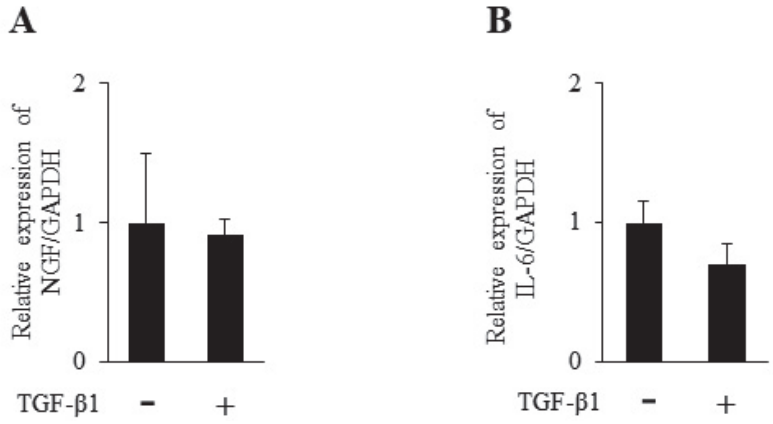

Fig. 6. *Effect of TGF-β1 for NGF and IL-6 expression levels in PC12 cells*
PC12 cells were stimulated with 10ng/mL TGF-β1 for 48 h, and then the mRNA expression levels of (A) NGF and (B) IL-6 were investigated by RT-qPCR using rat-specific oligonucleotide primers. The mRNA expression levels in each sample were normalized to GAPDH, and the results are expressed as the fold change relative to the unstimulated control. Data are presented as the mean ± SD (n=5). The values of $^*P < 0.05$ was considered statistically significant.

treatment with IL-6/sIL-6R alone did not promote neurite extension in PC12 cells. Furthermore, NGF and IL-6 mRNA expression levels in PC12 cells were not significantly affected by TGF-β1 stimulation (Fig. 6A-B).

NGF and IL-6 secreted by MSCs as soluble molecules accumulate in the culture medium. Therefore, we cultured PC12 cells in the conditioned medium of TGF-β1-treated UE7T-13 cells and then investigated neurite extension. As a result, neurite extension in PC12 cells cultured in the conditioned medium of TGF-β1-pretreated UE7T-13 cells was significantly enhanced by the addition of sIL-6R, and this enhancement was abrogated by the addition of the sIL-6R neutralizing antibody (Fig. 7A). For the same purpose, UE7T-13 cells and PC12 cells were co-cultured under indirect transwell conditions. The neurite extension in PC12 cells was significantly enhanced by the co-culture of UE7T-13 cells pretreated with TGF-β1 and the addition of sIL-6R, and this enhancement was abrogated by the addition of the sIL-6R neutralizing

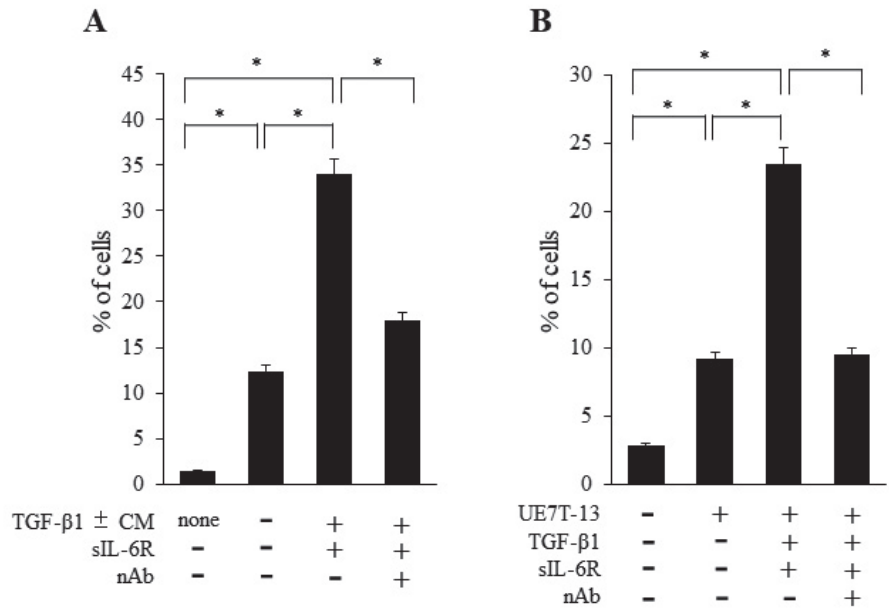

Fig. 7. *Enhancement of neurite extension in PC12 cells by IL-6 secreted from MSCs*
The status of neurite extensions emerging from PC12 cells was observed according to described in "Materials and Methods". (A) UE7T-13 cells were stimulated with 20 ng/mL TGF-β1 for 72 h and the conditioned medium (CM) was recovered. The CM supplemented with or without 50 ng/mL recombinant sIL-6R and/or 2 μg/mL of sIL-6R neutralizing antibody (nAb) was replaced as a culture medium for PC12 cells. The neurite extension of PC12 cells cultured in the conditioned medium for TGF-β1-stimulated UE7T-13 cells at 72 h was evaluated. (B) UE7T-13 cells and PC12 cells were cultured in an indirect co-culture system. UE7T-13 cells were seeded on the upper membrane of the transwell chamber. The UE7T-13 cells in upper side were stimulated with or without 20 ng/mL of TGF-β1, and then 25 ng/mL of sIL-6R and/or 4 μg/mL of sIL-6R neutralizing antibody (nAb) were added to the lower side. After 72 h of culture period, neurite extension of PC12 cells on the lower side was evaluated. The ratio of the number of neurite extension cells to the total number of PC12 cells in the field of microscopy was statistically analyzed. Data are presented as the mean ± SD (n=5). The values of $^*P < 0.05$ was considered statistically significant.

antibody (Fig. 7B). Taken together, these results strongly suggest that NGF secreted by TGF-β1-stimulated MSCs induces neuronal differentiation in PC12 cells, which is further enhanced by the IL-6 secreted from the MSCs.

Discussion

The immunomodulatory effects of MSCs are synergistically mediated by cell contact-dependent mechanisms and the secretion of soluble factors. The functional effects of monocytes/macrophages, dendritic cells, T cells, B cells, and natural killer cells determine the immunomodulatory potential of MSCs. In this study, it was shown that the expression of both IL-6, an immunomodulatory cytokine, and NGF was enhanced in TGF-β1-stimulated MSCs. In addition, it was strongly suggested that the NGF secreted by TGF-β1-stimulated MSCs induces neuronal differentiation in PC12 cells, which is further enhanced by IL-6 also secreted by the MSCs.

TGF-β1 downregulates various immune responses as an immunosuppressive molecule and is associated with immune diseases [13]. It is also known to induce NGF expression in various cell types [18, 19]. TGF-β1-induced NGF expression is promoted in chondrocytes by Smad2/3-dependent signaling [20], and in dental pulp cells by the activation of the JNK and p38 MAPK signaling pathways [21]. In addition, our recent study demonstrated that TGF-β1 induced NGF expression in periodontal ligament-derived fibroblasts via the Smad2/3 signaling pathway and p38 MAPK activation [22]. In the present study, we demonstrated that TGF-β1 induces NGF expression in human MSCs, although the signaling pathway is unknown (Fig. 1). Moreover, stimulation of the MSCs by TGF-β1 enhanced NGF and IL-6 expression in them (Fig. 1, Fig. 2A, Fig. 3A-B). This enhancement of IL-6 expression was shown to be mediated by the TGF-β type I receptor/MEK/ERK signaling pathway (Fig. 3C), and was not suppressed by Smad2/3 inhibitors. Binding of TGF-β1 to receptors on the cell surface triggers the formation of a tetrameric complex of type I and II receptors. Type II receptor kinase activates type I receptor kinase, which transduces the signal through the phosphorylation of receptor-activated Smads (R-Smads) [28, 29, 30, 31]. Smad proteins are central mediators of the TGF-β superfamily signaling. R-Smads, which include Smad1, Smad5 and Smad8 and are primarily activated by bone morphogenetic protein (BMP) -specific type I receptors, whereas Smad2 and Smad3 are activated by TGF-β -specific type I receptors. Activated R-Smads form complexes with the common mediator Smads (Co-Smads e.g., Smad4), which translocate into the nucleus, where they regulate the transcription of specific target genes along with their partner proteins. Abnormal intensity of Smad-mediated TGF-β/BMP signaling is associated with various diseases, including bone and immune disorders, fibrosis, and tumor progression or metastasis [32]. TGF-β also activates intracellular effectors such as MAPKs [33, 34]. There are at least three distinct groups of MAPKs: MEK/ERKs, JNKs and p38 MAPKs. Notably, TGF-β1-induced intracellular signals affect the differentiation of MSCs [26, 35, 36, 37, 38].

The rat pheochromocytoma PC12 cell line has been established and used as a model for the growth and differentiation of neural crest cells [27]. NGF-responsive PC12 cells differentiate into sympathetic-like neurons and from long neurites, thereby providing a useful model for the investigation of neuronal differentiation. In this study, neurite extension

in PC12 cells cultured in TGF-β1 pretreated-MSCs conditioned medium was significantly enhanced by the addition of sIL-6R (Fig. 7A). Similarly, neurite extension in PC12 cells grown in a co-culture with MSCs was also significantly enhanced under transwell conditions with TGF-β1 pretreated-MSCs supplemented with sIL-6R (Fig. 7B). The enhancement of neurite extension was abolished by the addition of the sIL-6R neutralizing antibody. IL-6 is a pleiotropic cytokine that regulates the immune and inflammatory responses [39), 40)]. Although IL-6 has been reported to be a principal regulator of acute phase proteins [41)], other cytokines, such as IL-1β and TNF-α also participate in the induction of a broad subset of acute phase proteins [41), 42)]. Previously, we reported that the expression levels of inflammation-related chemokine CCL2 were enhanced by stimulation with IL-6, IL-1β and TNF-α in gingival- or periodontal ligament-derived fibroblasts [43), 44)]. Interestingly, CCL2 specifically induced the migration of MSCs but not that of fibroblasts, suggesting that CCL2 specifically recruits MSCs to the inflammatory site but not normal fibroblasts in the damaged tissue. IL-6 function is dependent on how its signal is transmitted to target cells. It acts via its classical signaling pathway by binding to its membrane-bound IL-6R, which then dimerizes with glycoprotein 130 (gp130) [40)]. This event triggers the activation of Janus kinases (JAK) which phosphorylate distinct intracellular tyrosine residues of gp130. These residues serve as docking sites and activate downstream pathways, at least two distinct signaling, namely the signal transducer and activator of transcription (STAT) -3 and MAPKs [45)]. In addition, IL-6 can act via its sIL-6R such as the soluble form of IL-6R coupled with gp130 protein to act on non-IL-6R expressing target cells [46)]. This is referred to as the trans-signaling pathway. It has been suggested that IL-6 trans-signaling is pro-inflammatory. Besides its physiological and pathophysiological effects on immune system regulation, IL-6 impacts neural development via the activation of the JAK/STAT and MAPK pathways activation. It contributes to adult neurogenesis and induces the differentiation of neural progenitor cells derived from induced pluripotent stem cells [47), 48), 49)]. Mice lacking IL-6 showed decreased neural progenitor maturation in neurogenic brain regions such as the hippocampal dentate gyrus compared to that in their wild-type littermates [50)]. Additionally, IL-6 signaling induced neurite extension in PC12 cells [51), 52), 53), 54), 55)]. The protein is also associated with a various neuropathologies [56)]. Recently, Bongartz et al. showed that IL-6 signaling induced the mRNA expression of proto-oncogene c-Fos and early growth response protein 1 (Egr1) in PC12 cells [57)]. c-Fos and Egr1 are immediate early genes and their expression is essential for physiological brain development and plasticity [58)]. Blocking the expression or function of either of these proteins impairs NGF-induced neurite extension in PC12 cells [59), 60)]. In the present study, our findings strongly suggest that NGF secreted from TGF-β1-stimulated MSCs induces the neuronal differentiation of PC12 cells, which is further enhanced by IL-6 secreted from MSCs. However, the molecular mechanisms underlying IL-6-induced neural differentiation and neurite extension remain unclear.

In conclusion, TGF-β1 has been shown to induce NGF and IL-6 expression in MSCs, and this NGF induces neurite extension in PC12 cells. Importantly, this neurite extension was

enhanced in the presence of IL-6, which is also secreted by the MSCs. Therefore, in a microenvironment where MSCs are exposed to TGF-β1, the induction of neuronal differentiation and subsequent growth are expected.

Acknowledgments

The present study was supported in part by JSPS KAKENHI grant nos. 19K19173 to M, O., 16H05534 and 20K09883 to A, I. and 16K11654 and 20K10014 to N, C. from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Competing interests

The authors declare that there are no competing interests associated with this manuscript.

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