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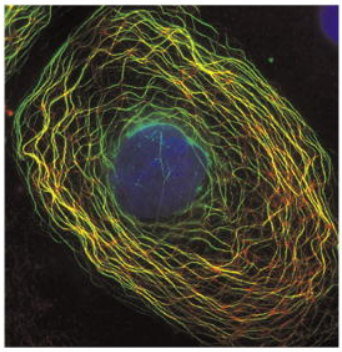

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Induction of podoplanin by transforming growth factor-β in human fibrosarcoma

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Abstract **Podoplanin/aggrus is increased in tumors and its expression was associated with tumor malignancy. Podoplanin on cancer cells serves as a platelet-aggregating factor, which is associated with the metastatic potential. However, regulators of podoplanin remain to be determined. Transforming growth factor-β (TGF-β) regulates many physiological events, including tumorigenesis. Here, we found that TGF-β induced podoplanin in human fibrosarcoma HT1080 cells and enhanced the platelet-aggregating-ability of HT1080. TGF-β type I receptor inhibitor (SB431542) and short hairpin RNAs for Smad4 inhibited the podoplanin induction by TGF-β. These results suggest that TGF-β is a physiological regulator of podoplanin in tumor cells.**

Keywords: Podoplanin; Platelet aggregation; TGF-β; Smad; Fibrosarcoma

1. Introduction

A mucin-type transmembrane sialoglycoprotein, podoplanin (aggrus) is highly expressed in lymphatic endothelial cells (LECs) [1]. Several lines of evidence obtained using podoplanin knockout mice suggest that podoplanin is crucially involved in lymphatic vessel formation [2]. Moreover, podoplanin is reportedly expressed in several tumor cells such as various squamous cell carcinomas, testicular tumors, mesothelioma, and brain tumors, although podoplanin is not expressed in gastrointestinal and pulmonary adenocarcinomas, which frequently undergo metastasis [3]. Recent investigations have reinforced the notion that expression of podoplanin is associated with tumor malignant progression [4].

Podoplanin belongs to the family of type-I transmembrane sialomucin-like glycoproteins and possess the platelet-aggregating activity and metastasis-promoting ability [5,6]. The segment of EDxxVTPG in the extracellular domain, designated as a platelet-aggregation-stimulating (PLAG) domain, is critical for the activity of podoplanin. In particular, this motif, which is highly conserved across species, is triplicated in tandem [7]. In a study of targeted mutagenesis of podoplanin molecules, we obtained evidence that Thr residues in the PLAG domain play an important role in platelet aggregation [5]. Recently, we purified human podoplanin from the glioblastoma cell line LN319 cells using an anti-human PLAG domain monoclonal antibody (NZ-1), and showed that podoplanin possesses a disialyl-core1 structure at Thr52 in the PLAG domain [8]. Furthermore, C-type lectin-like receptor 2 (CLEC-2), which is a non-classical C-type lectin, was identified as an endogenous receptor of podoplanin on platelet [9]. NZ-1 neutralized the association between podoplanin and CLEC-2, and suppressed podoplanin-induced platelet aggregation and metastasis [10].

Transforming growth factor-β (TGF-β) superfamily of extracellular growth factors regulates cell proliferation, differentiation, apoptosis, and morphogenesis. Upon ligand binding to two different types of serine/threonine kinase receptors, type I receptor is activated by type II receptor and transduces intracellular signals by phosphorylation of receptor-regulated Smad (R-Smad). Among R-Smad, Smad1, Smad5, and Smad8 are phosphorylated by the bone morphogenetic protein (BMP) receptors, whereas Smad2 and Smad3 are phosphorylated by the TGF-β/activin receptors [11]. Phosphorylated R-Smad forms a functional signaling complex with Smad4 and translocates into the nucleus to regulate expression of the ligand-responsive genes [12]. TGF-β regulates a number of genes in a cell type and context dependent manner.

Podoplanin has been reported to be expressed in invading front of tumors cells and be involved in tumor invasion [13]; therefore, some stromal factors might be involved in the induction of podoplanin. Previously, Interkeukin-3 (IL-3) was reported to induce podoplanin in human endothelial cells [14]. However, it has not been clarified how the expression of podoplanin is regulated in tumor cells. In this study, we examined the expression of podoplanin by TGF-β using various cell lines.

2. Materials and methods

2.1. Cells and reagents

HT1080, 293T, HaCaT, and MCF10A cells were obtained from the American Type Culture Collection (ATCC). These cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO)

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Abbreviations: PLAG, platelet-aggregation-stimulating; TGF-β, transforming growth factor-β; R-Smad, receptor-regulated Smad; IL, interleukin; EMT, epithelial–mesenchymal transition

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supplemented with 10% fetal bovine serum (Sigma) and 1% of penicillin–streptomycin solution (Invitrogen Corp., Carlsbad, CA). Puromycin and SB431542 was obtained from Sigma and Calbiochem Novabiochem Corp. (San Diego, CA), respectively. TGF-β was purchased from R&D Systems (Minneapolis, MN).

2.2. DNA constructs

Sequence of oligonucleotides against human Smad4 cDNA was as follows: GGATTTCCTCATGTGATCT. The complementary oligonucleotides were annealed and ligated in a pSUPER. puro vector (OligoEngine, Seattle, WA). Stable HT1080 clones transfected with pSUPER-short hairpin RNAs for Smad4 (shSmad4) were selected and maintained in the presence of puromycin (1 μg/ml).

2.3. Reverse transcription (RT)-PCR

Total RNA was prepared using Isogen (Nippongene Co. Ltd., Tokyo, Japan). Reverse transcription was performed with Superscript III (Invitrogen Corp.), and PCR was performed using the primers listed as follows: podoplanin – 5′-CCAGGAGAGCAACAACTCA A-3′ (forward), 5′-GATGCGAATGCCTGTTACAC-3′ (reverse); Smad7 – 5′-GGAAGTCAAGAGGCTGTGTT-3′ (forward), 5′-GCT TTCTCCTCCCAGTATGC-3′ (reverse); β-actin – 5′-CAAGAGAT GGCCACGGCTGCT-3′ (forward), 5′-TCCTTCTGCATCCTGTC GGCA-3′ (reverse).

2.4. Luciferase assay

Cells were transfected with $(CAGA)_9$ MLP-luc using FuGENE6 (Roche Diagnostics K.K., Tokyo, Japan). Luciferase activities were determined by Luciferase Assay Systems (Promega K.K., Tokyo, Japan) and normalized to β-galactosidase activity of co-transfected CH110 (GE Healthcare UK Ltd., Buckinghamshire, UK).

2.5. Flow cytometry

Expression levels of human podoplanin were compared for confirmation using flow cytometry. HT1080 cells, which were collected by trypsin-EDTA treatment, were incubated with NZ-1 (0.1 μg/ml) for 1 h at 4 °C. Then the cells were incubated with Oregon green-conjugated anti-rat antibodies (Invitrogen Corp.) for 30 min. Flow cytometry was performed using FACS Caliber (BD Biosciences, Barintree, MA).

2.6. Western blot analysis

The cell lines were solubilized with lysis buffer (1% Triton in PBS) and electrophoresed under reducing conditions on 10–20% polyacrylamide gels. The separated proteins were transferred to a PVDF membrane. After blocking with 4% skim milk in PBS, the membrane was incubated with NZ-1 (rat, 0.1 μg/ml), anti-Smad2 (mouse, 0.25 μg/ml; Transduction Laboratories, Lexington, KY), anti-Smad4 (mouse, 0.2 μg/ml; Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-phospho-Smad2 (rabbit) [15], and anti-β-actin antibody (mouse, 0.2 μg/ml; Sigma), and then with peroxidase-conjugated anti-rat, anti-mouse, or anti-rabbit antibodies (1/1000 diluted; GE Healthcare UK Ltd.), and developed for 1 min with ECL reagents (GE Healthcare UK Ltd.) using Kodak X-Omat AR film.

2.7. Platelet aggregation assay by WBA Carna

Heparinized human whole blood (WB) was drawn from healthy drug-free volunteers. Platelet aggregation was measured according to the screen filtration pressure method using WBA Carna (IMI, Saitama, Japan) [3,8,10]. Two hundred microliters each of human whole blood samples in four reaction tubes were stirred at 1000 rpm at 37 °C and pre-incubated for 1 min, followed by addition of 10 μl each of HT1080 cells (1×10^5 cells or 4×10^4). Using a 3.7-mm-diameter syringe containing screen microsieves made of nickel, with 300 openings of $20 \times 20\ \mu m^2$ in a 1 mm-diameter area, WB samples were sucked to detect aggregation pressure at a rate of 200 μl/6.4 s 2 min later. The final platelet aggregation pressure of each reaction tube was determined at the pressure rate (%) of a pressure sensor connected to the syringe.

2.8. Statistical analyses

Results are expressed as the means ± S.D. Student's *t*-test was used to determine significance among the groups. A value of $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Induction of podoplanin by TGF-β in hunan fibrosarcoma HT1080 cells

To examine the expression of podoplanin by TGF-β, we performed Western blot analysis using anti-podoplanin monoclonal antibody, NZ-1 [3] in various cell lines. As shown in Fig. 1A, TGF-β induced podoplanin in human fibrosarcoma HT1080 cells, but not in breast epithelial MCF-10A cells and human keratinocyte, HaCaT cells, which are known to exhibit the high responsiveness to TGF-β. Although human embryonic kidney 293T cells express low level of podoplanin, the induction of podoplanin by TGF-β was hardly observed. Time course experiment showed that podoplanin mRNA increased at 12 h treatment with TGF-β (Fig. 1B), followed by the podoplanin protein at 24 h (Fig. 1C). Induction of Smad7 which is a target gene of TGF-β signaling, were observed in HT1080 cells, indicating that TGF-β-Smad signaling pathway is intact in HT1080 cells (Fig. 1B). Phosphorylation of Smad2 by TGF-β was also detected (Fig. 1C). Furthermore, we found that SB431542, a selective inhibitor of TGF-β type I receptor, inhibited the induction of podoplanin by TGF-β in a dose dependent manner (Fig. 1D). These results indicated that TGF-β stimulates podoplanin expression in HT1080 cells.

TGF-β is a potent growth inhibitor with tumor-suppressing activity. Cancer cells often show resistance to this growth inhibition either because of genetic loss of TGF-β signaling components or, more commonly, because of downstream perturbation of the signaling pathway, such as by Ras activation [16]. Carcinomas often secrete excess TGF-β and respond to it by enhanced invasion and metastasis [17]. TGF-β is known to induce epithelial–mesenchymal transition (EMT) through the induction of SIP1 and δ EF1 [18], Snail family transcriptional factors [19], and the suppression of inhibitor of DNA binding (Id) proteins [20]. Furthermore, TGF-β promotes bone metastasis through the induction of IL-11 and connective tissue growth factor [21]. Podoplanin has been also reported to induce EMT in MDCK cells linked to the activation of RhoA, and increased cell migration and invasiveness [13]. Although TGF-β induces podoplanin in HT1080 cells, it is not sufficient to induce the invasion and migration of HT1080 cells [22]. Even though, podoplanin might be one of cancer progressive factor, which is regulated by TGF-β.

3.2. Platelet aggregation by HT1080 cells

To confirm the expression of functional podoplanin on cell surface, we performed the flow cytometry using anti-podoplanin (NZ-1). As shown in Fig. 2A, the expression of podoplanin on cell surface was observed in the absence of TGF-β and its expression was further stimulated by TGF-β. We next investigated the platelet aggregation by HT1080 cells in the presence or absence of TGF-β. Platelet aggregation was observed in control HT1080 cells, and was further stimulated by TGF-β-treated HT1080 cells (Fig. 2B). These results suggest that functional podoplanin was induced on cell surface of HT1080 cells.

3.3. Effect of Smad4 knockdown on the induction of podoplanin by TGF-β

We next investigated the involvement of Smad pathway on the expression of podoplanin by TGF-β. We established HT1080 cells which stably expressed short hairpin RNAs for

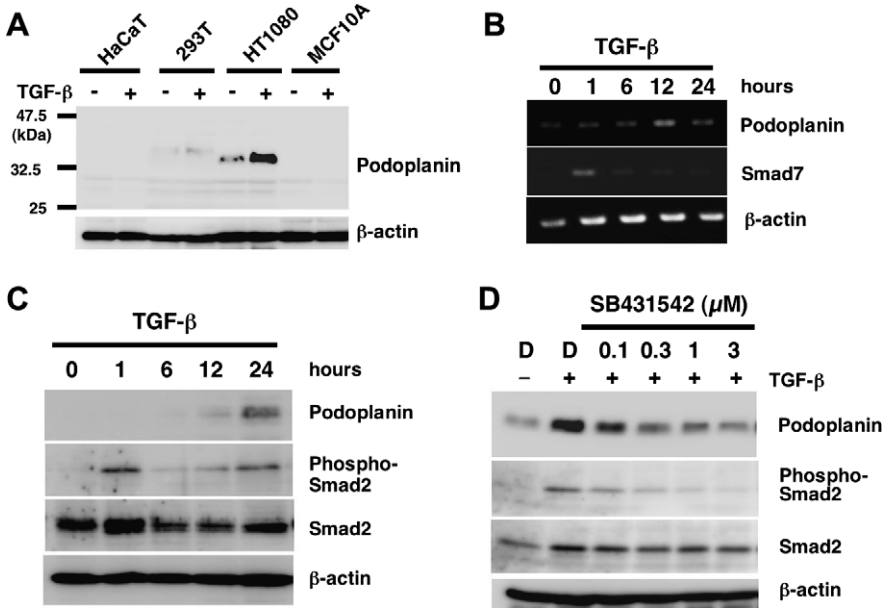

Fig. 1. Induction of podoplanin by TGF-β in human fibrosarcoma HT1080 cells. (A) HaCaT, 293T, HT1080, and MCF10A cells were treated with TGF-β (2.5 ng/ml) for 24 h. Western blot analysis was performed using anti-podoplanin antibody (NZ-1). The β-actin protein is used as a loading control. (B and C) HT1080 cells were treated with TGF-β for indicated time periods. (B) Semi-quantitative RT-PCR analysis was performed to detect podoplanin and Smad7 mRNA. β-actin is used as internal control. (C) Western blot analysis was performed using NZ-1, anti-phospho-Smad2, anti-Smad2, and anti-β-actin antibodies. (D) HT1080 cells were treated with TGF-β in the presence or absence of SB431542 at indicated concentrations for 24 h. Western blot analysis was performed using antibodies as indicated. D indicates dimethyl sulfoxide (solvent).

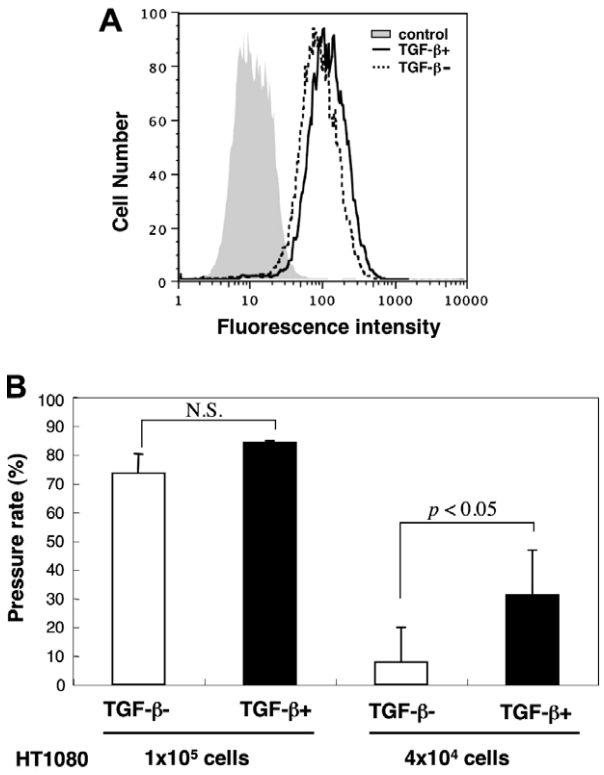

Fig. 2. Cell surface expression of podoplanin and platelet aggregation by HT1080 cells. (A) Flow cytometric analyses of NZ-1 to HT1080 cells which were treated with TGF-β for 24 h. (B) Platelet aggregation was measured using WBA Carna with the screen filtration pressure method. Data are means ± S.D. of three independent experiments.

Smad4 (shSmad4). We obtained two transfectants (clones 5 and 10), and these Smad4 were significantly reduced compared with control (pSUPER) transfected cells (Fig. 3A). However, the expression of Smad2 and phosphorylation of Smad2 by TGF-β were similar between control and shSmad4 transfected cells. The transcriptional activation of $(CAGA)_9$ MLP-luc by TGF-β was significantly reduced in shSmad4 transfected cells (Fig. 3B). Using these transfectants, we investigated the induction of podoplanin by TGF-β. As shown in Fig. 3C, the induction of podoplanin by TGF-β was blocked in shSmad4 transfected cells. These results suggest that TGF-β-Smad pathway is important for the induction of podoplanin by TGF-β in HT1080 cells.

TGF-β regulates various target genes via Smad pathway [23]. However, podoplanin was not induced by TGF-β in HaCaT and MCF10A cells (Fig. 1A), which suggested that TGF-β-Smad signaling alone is not sufficient to induce podoplanin. Therefore, Smad would cooperatively regulate podoplanin with other transcriptional factors, which maintain the basal expression of podoplanin in HT1080 cells. Recent report revealed that transcriptional factor Sp1 and Sp3 stimulate basal podoplanin expression, and methylation status of its promoter confers cell-type specific podoplanin expression [24]. Smads have been shown to interact with Sp1, and regulates various target genes including p21 [25]. Further study is needed to identify the transcriptional factors which regulate podoplanin with Smads.

In conclusion, we found that TGF-β induced podoplanin in human fibrosarcoma HT1080 cells. The platelet aggregation by HT1080 cells was stimulated by treatment of cells with TGF-β, which may lead to acquirement of high metastatic ability of cancer cells. Furthermore, we are now investigating whether podoplanin is induced by TGF-β in other cancer cells.

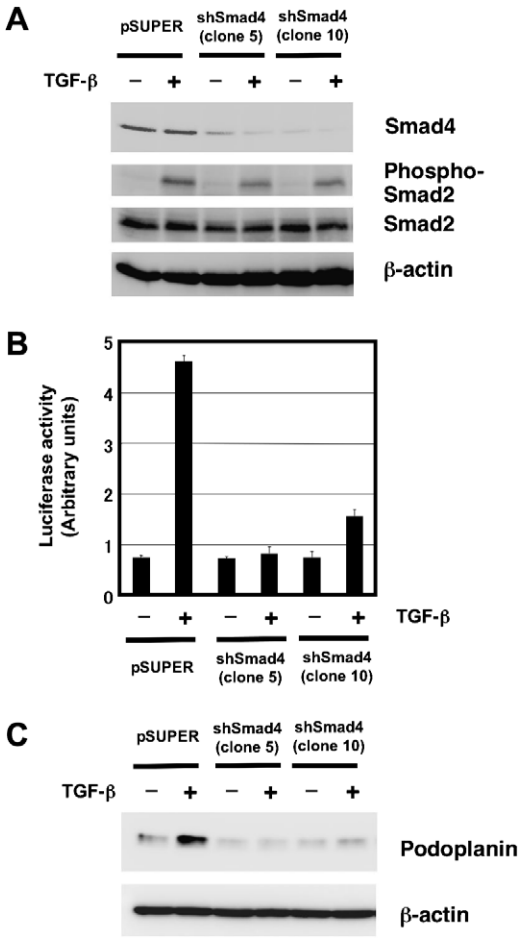

Fig. 3. Effect of Smad4 knockdown on the TGF-β-induced podoplanin expression. (A) Western blot analysis of Smad4 in shSmad4-transfected HT1080 cells (clones 5 and 10). Cells were treated with TGF-β for 1 h. Western blot analysis was performed using anti-Smad4, anti-phospho-Smad2, anti-Smad2, and anti-β-actin antibodies. (B) $(CAGA)_9$ MLP-luc activation by TGF-β was inhibited in shSmad4-transfected cells. Cells were transfected with $(CAGA)_9$ MLP-luc for 24 h and treated with TGF-β for another 24 h. Luciferase activity was determined as described in Section 2. Error bars represent means ± S.D. (C) Effect of Smad4 knockdown on the TGF-β-induced podoplanin expression. Cells were treated with TGF-β for 24 h. Western blot analysis was performed using NZ-1 and anti-β-actin antibodies.

In the future, podoplanin and TGF-β might represent a promising therapeutic target in cancer invasion and metastasis.

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