EXPERIMENTAL STUDY

mRNA expression of type I and type II receptors for activin, transforming growth factor- β , and bone morphogenetic protein in the murine erythroleukemic cell line, F5-5.flHikaru Machida¹, Kenji Ogawa^{1,2}, Masayuki Funaba³, Takeo Mizutani¹ and Masafumi Tsujimoto²¹Laboratory Animal Research Center and ²Laboratory of Cellular Biochemistry, RIKEN, 2-1 Hirosawa, Wako, Saitama, 351-0198 Japan and³Department of Nutrition, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Sagami-hara, Kanagawa, 229-8501 Japan

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Email: kkogawa@postman.riken.go.jp)**Abstract**

Objective: Intracellular signaling of activin and transforming growth factor- β (TGF- β) is thought to be mediated by the same molecules (Smad2/3 and Smad4). Although differentiation of murine erythroleukemia F5-5.fl cells is induced by activin, it is not induced by TGF- β , suggesting that at some point TGF- β signaling is defective. The aim of this study was to investigate the unresponsiveness of F5-5.fl cells to TGF- β .

Design: mRNA expression of ligands, receptors, and signal mediators for the TGF- β family was examined in F5-5.fl cells using RT-PCR.

Results: Activin induced erythrodifferentiation of F5-5.fl cells in a dose-dependent manner. Neither TGF- β 1 nor bone morphogenetic protein (BMP)-4 affected the differentiation of F5-5.fl cells in the presence or absence of activin. Although mRNAs of TGF- β s (TGF- β 1, TGF- β 2 and TGF- β 3) were detected, those of inhibin/activin (α -, β A- and β B-subunits) and BMPs (BMP-2, BMP-4 and BMP-7) could not be detected in the cells, suggesting that neither activins nor BMPs are produced in F5-5.fl cells. The expression of both type I (ALK-4/ActRIB) and type II (ActRII) receptors for activin was detected in F5-5.fl cells. In contrast, while the expression of type I receptor for TGF- β (ALK-5/T β RI) was detected, that of type II receptor (T β RII) was not. The mRNA of all Smads examined was detected in F5-5.fl cells.

Conclusions: A defect in the type II receptor might cause unresponsiveness to TGF- β in F5-5.fl cells. An erythrodifferentiation assay using F5-5.fl cells would be useful for measuring net activin activity because it would not be necessary to consider endogenous activins and BMPs.

European Journal of Endocrinology 143 705–710**Introduction**

Activins, transforming growth factor (TGF)- β s and bone morphogenetic proteins (BMPs) are members of the TGF- β superfamily. They are involved in diverse physiological processes, and are believed to act as regulators of cell growth and differentiation (1–4). The TGF- β family members initiate their cellular action by binding to receptor serine kinases consisting of two subfamilies, type I and type II receptors. Each member of the TGF- β family binds to a characteristic combination of type I and type II receptors, both of which are needed for signaling (5, 6). During the past 5 years, Smad proteins have been identified as signal mediators of the TGF- β family members, whereby Smad2 and Smad3 are pathway-restricted Smads for both activin and TGF- β signaling, whereas Smad1, Smad5 and Smad8 are for BMP signaling (5). Upon phosphorylation of the carboxyl-terminal serines in pathway-restricted

Smads, they form complexes with common Smad (Smad4), followed by nuclear translocation and transcriptional regulation of the target gene (5).

Both activin and TGF- β are reported to have roles in the regulation of erythropoiesis (7–10). Activin A and TGF- β 1 induce erythrodifferentiation in the erythroleukemic cell line, K562 cells, with similar efficiencies (11). However, in another erythroleukemic cell line, F5-5.fl cells, although erythrodifferentiation is induced by activin A, it is not induced by TGF- β 1, suggesting defective TGF- β signaling (10).

To explore why F5-5.fl cells do not differentiate into erythroid in response to TGF- β 1, we examined mRNA expression of ligands, receptors and signal mediators for the TGF- β family in F5-5.fl cells. We demonstrate that a deficiency in the type II receptor for TGF- β may be responsible for the lack of TGF- β -induced differentiation in F5-5.fl cells.

Table 1 Sequences of oligonucleotides used for PCR.

Transcript	Primer	Location	Sequence	Product	Ref.
α -subunit	5' primer	341–360	ATGTATTCCGGCCATCCCAA	544 bp	(12)
	3' primer	865–884	CTACCATGGCAGTAGTGGAA		
β A-subunit	5' primer	598–617	GAGAGGAGTGAAGTGTGCT	605 bp	(12)
	3' primer	1183–1202	TACAGCATGGACATGGGTCT		
β B-subunit	5' primer	66–83	GTTCTTCATCGACTTTTCG	281 bp	(12)
	3' primer	331–346	CGATCATGTTGGGCAC		
TGF- β 1	5' primer	462–482	GCTGCGCTTGCAGAGATTAAA	552 bp	(13)
	3' primer	994–1013	TTGCTGTACTGTGTGCCAG		
TGF- β 2	5' primer	521–543	CCAAAGACTTAACATCTCCCACC	684 bp	(14)
	3' primer	1184–1204	GTTTCGATCTTGGGCGTATTTTC		
TGF- β 3	5' primer	635–654	GCTCTTCCAGTACTTCGAC	440 bp	(15)
	3' primer	1055–1074	AGCAGTTCTCTCCAGGTTG		
BMP-2	5' primer	363–382	AGAGATGAGTGGGAAAACGG	547 bp	(16)
BMP-4	3' primer	890–909	GAAGTCCACATACAAAGGGT	526 bp	(17)
	5' primer	698–717	AGGGATTCTGCCCCGTTTGT		
BMP-7	3' primer	1204–1223	GGGAGGAAATTACTGTGCCT	787 bp	(18)
	5' primer	465–484	CCGGTTTGATCTTTCCAAGA		
	3' primer	1232–1251	CTTCAGGATGACATTAGAGC		
	5' primer	855–874	TGACTTTCTGCAGAGGCAGA		
ALK-1	3' primer	1154–1173	CGACTCAAAGCAGTCTGTGC	319 bp	(19)
	5' primer	875–894	ATGACTACCTTCAGTCACT		
ALK-2 (ActRI)	3' primer	1400–1419	CTTCGCCAGAGAAGTTAATG	545 bp	(20)
	5' primer	408–427	TACACTGCCCCCTGTTGTTA		
ALK-3 (BMPRI-A)	3' primer	969–988	CTCTGGTGTCTAGTGTGGCA	581 bp	(14)
	5' primer	884–903	ACCGCTACACAGTGACCATT		
ALK-4 (ActRII-B)	3' primer	1494–1513	TCTTCACATCTTCTGCACG	630 bp	(21)
	5' primer	488–507	ATCCATCACTAGATCGCCCT		
ALK-5 (T β RI)	3' primer	1290–1311	CGATGGATCAGAAGGTACAAGA	824 bp	(22)
	5' primer	882–901	CACCTTAGACGCAAAAGTCCA		
ALK-6 (BMPRI-B)	3' primer	1353–1371	CCATCGATTGGGGAATGAA	490 bp	(23)
	5' primer	835–854	CTTAAGGCTAATGTGGTCTC		
ActRII	3' primer	1518–1537	GACTAGATTCTTTGGGAGGA	703 bp	(24)
	5' primer	479–498	ATCGTCATCGGAAACCTCCC		
ActRII-B	3' primer	1389–1407	CAGCCAGTGATCCTTAATC	929 bp	(25)
	5' primer	258–277	CGTGTGGAGGAAGAACAACA		
T β RII	3' primer	798–817	TCTCAAAGTCTCTGAGGTG	560 bp	(26)
	5' primer	2222–2241	AGATCTATCTCTCCCTAAG		
BMPRII	3' primer	3016–3035	TTAGAATGGACTGCCCTGTC	814 bp	—*
	5' primer	306–325	ACTGAAGCCTCTGGAATGCT		
Smad1	3' primer	905–924	GCGGTTCTTATTGTTGGACG	619 bp	(27)
	5' primer	551–570	CGGAGATTCTAACAGAACTG		
Smad2	3' primer	1377–1396	TGCTTGAGCATCGCACTGAA	846 bp	(28)
	5' primer	642–661	AGCACACAATAAAGTGGACC		
Smad3	3' primer	1254–1277	TAAGACACACTGGAACAGCGGATG	636 bp	(29)
	5' primer	645–664	CATTCTGTGGCTTCCACAA		
Smad4	3' primer	1428–1447	GACTGATGGCTGGAGCTATT	803 bp	(30)
	5' primer	754–773	ATGCCCAGCATATCCAGCAG		
Smad5	3' primer	1371–1390	CAGAAAGAAATGGGGTTTCAGC	637 bp	(31)
	5' primer	532–554	TGTTGCTGTGAATCTTACGGGAAG		
Smad7	3' primer	1167–1187	TGCACGGTGAAACCCGTCCAT	656 bp	—**
	5' primer				

* Mouse BMPRII: GB Whitaker, BB Koenig, J Ting, JP Tiesman, AL Limberg, RA Grant, KB Begley & JS Rosenbaum, unpublished (NID: g6680803).

** Mouse Smad7: K Kitamura & K Okazaki, unpublished (NID: g3378187).

Materials and methods

Reagents

RPMI 1640 medium supplemented with 2 mmol/l glutamine was purchased from Iwaki Glass (Chiba, Japan). Fetal bovine serum (FBS) was purchased from

Cansera International (Ontario, Canada). Recombinant human activin A was provided by the National Hormone and Pituitary Program (Rockville, MD, USA). Recombinant human TGF- β 1 was obtained from Pepro Tech EC (London, UK). Recombinant human BMP-4 was purchased from R & D Systems (Minneapolis, MN, USA).

Figure 1 Effect of activin A, TGF- β 1 and BMP-4 on the differentiation of F5-5.fl cells. (A) Activin A dose-dependent stimulation of erythrodifferentiation of F5-5.fl cells. F5-5.fl cells were cultured with different concentrations of recombinant activin A. After 6 days of incubation, the differentiated (hemoglobin-positive) cells were stained with 1% *o*-dianisidine, and the absorbance at 405 nm was determined on a microplate reader as described in Materials and methods. The points represent means of six wells within one representative experiment (means $\pm$ S.D.). (B, C) Effect of TGF- β 1 and BMP-4 on the differentiation of F5-5.fl cells in the absence (B) or presence (C) of activin A. F5-5.fl cells were cultured with recombinant human TGF- β 1 (20 ng/ml) and recombinant human BMP-4 (40 ng/ml) in the absence or presence of 20 ng/ml activin A. After 6 days of incubation, erythrodifferentiation was measured. This is one representative experiment of three individual cultures (means $\pm$ S.D.).

Cell culture

Mouse erythroleukemia F5-5.fl cells (RCB No. 0560, RIKEN Cell Bank, Tsukuba, Japan) were cultured in RPMI 1640 media supplemented with 2 mol/l glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated (56 °C, 30 min) FBS. Activin A, TGF- β 1 or BMP-4 was added to F5-5.fl at a cell density of 1000 cells/well in 200 μ l (96-well plate). After incubation for 6 days at 37 °C, 20 μ l 1% *o*-dianisidine (Sigma, St Louis, MO, USA) were added to stain the differentiated (hemoglobin-positive) cells. After a 15 min incubation,

the supernatant was removed, and cells were incubated in 20% SDS for an additional 6 h at 37 °C. Absorbance was measured at a wavelength of 405 nm on a model 3550 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Figure 2 Expression of inhibin/activin, TGF- β s, and BMPs in F5-5.fl cells detected by RT-PCR. The mRNA expression of inhibin/activin subunits (A), TGF- β s (B), and BMPs (C) in F5-5.fl cells was compared with that in PMSG-primed immature mouse ovary. Lane 1, mouse ovary; lane 2, F5-5.fl; lane 3, no RT control of F5-5.fl cells.

Figure 3 Expression of type I and type II receptors for TGF- β family proteins in F5-5.fl cells detected by RT-PCR. The mRNA expression of type I (A) and type II (B) receptors for activins, TGF- β s and BMPs in F5-5.fl cells was compared with that in PMSG-primed immature mouse ovary. Lane 1, mouse ovary; lane 2, F5-5.fl; lane 3, no RT control of F5-5.fl cells.

Figure 4 Expression of Smads by RT-PCR of F5-5.fl cells. mRNA expression of Smads in mouse erythroleukemia cell line, F5-5.fl cells was compared with that in PMSG-primed immature mouse ovaries by RT-PCR. Lane 1, mouse ovary; lane 2, F5-5.fl; lane 3, no RT control of F5-5.fl cells.

RNA isolation and cDNA synthesis

Total RNA from F5-5.fl cells was isolated using the RNeasy Total RNA Isolation System (Promega, Madison, WI, USA). Mouse ovary was excised from immature females (4 weeks of age) 48 h after i.p. injection of 7.5 IU of pregnant mare serum gonadotropin (PMSG) (Serotropin; Teikoku Zoki, Tokyo, Japan). One microgram of the recovered RNA was treated with RNase-free DNase I (GIBCO BRL, Gaithersburg, MD, USA) to remove the residual DNA, and reverse transcribed in a 21 µl volume reaction with oligo (dT) primer using the Superscript Preamplification System (GIBCO BRL) to generate the first-strand cDNA. The cDNA products were diluted to a final volume of 200 µl and stored at -80°C until used for PCR.

PCR

The oligonucleotides used for PCR reactions to detect the expression of inhibin/activin subunits (12), TGF- β s (13–15), BMPs (16–18), type I and type II receptors for TGF- β family proteins (19–26), and Smads (27–31) are shown in Table 1. PCR reactions were performed in a total volume of 50 µl containing 10 mmol/l Tris-HCl, 50 mmol/l KCl, 1.5 mmol/l MgCl_2 , 0.2 mmol/l dNTP, 1 µmol/l of each primer, 1.75 U DNA polymerase mixture from the Expand High-Fidelity PCR System (Boehringer Mannheim, Indianapolis, IN, USA), and 5 µl previously diluted RT reaction solution. The thermal cycling parameters consisted of denaturation

Table 2 Type I and type II receptors for TGF- β family.

	Type I receptors	Type II receptors
Activins	ALK-2 (ActRI) ? ALK-4 (ActRIIb)	ActRII ActRIIb
TGF- β s	ALK-5 (T β RI)	T β RII
BMPs	ALK-2 (ActRI) ALK-3 (BMPRI-IA) ALK-6 (BMPRI-IB)	BMPRII ActRII ActRIIb

at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s, for 35 cycles. The PCR products were separated on 1.5% agarose gels in $0.5\times$ TBE and visualized with ethidium bromide.

Results

Effect of activin A, TGF- β 1 and BMP-4 on erythrodifferentiation of F5-5.fl cells

F5-5.fl cells are known to differentiate into erythroid in response to activin A (10). As shown in Fig. 1A, absorbance at 405 nm after staining of hemoglobin with *o*-dianisidine increased with addition of activin within a range of 1.25–40 ng/ml in a dose-dependent manner. A least squares analysis indicated that the EC_{50} on the erythrodifferentiation was 2.94 ± 1.27 (s.d.) ng/ml. We next determined whether TGF- β 1 and BMP-4 affected the differentiation of F5-5.fl cells. Neither TGF- β 1 nor BMP-4 induced the differentiation of F5-5.fl cells (Fig. 1B), and these ligands did not affect activin-stimulated erythrodifferentiation (Fig. 1C). Furthermore, 100 U/ml interleukin (IL)-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-12 or interferon (IFN)- γ did not affect basal and activin-stimulated erythrodifferentiation of F5-5.fl cells (data not shown).

mRNA expression of ligands, receptors and signal transmediators for the TGF- β family members in F5-5.fl cells

The mRNA expression of inhibin/activin subunits, TGF- β s and BMPs in F5-5.fl cells was examined (Fig. 2). RT-PCR of F5-5.fl RNA yielded all examined TGF- β products, although the TGF- β 2 signal was weak (Fig.

Table 3 Combinatorial expression of type I and type II receptors for TGF- β family in F5-5.fl cells.

	mRNA expression in F5-5.fl cells		Combinatorial expression of type I and type II receptors
	Type I receptors	Type II receptors	
Activins	ALK-4 (ActRI)	ActRII	Positive
TGF- β s	ALK-5 (T β RI)	Negative	Negative
BMPs	Negative	BMPRII/ActRII	Negative

2B). In contrast, inhibin/activin subunits and BMPs cDNA products were not detected (Fig. 2A and C).

We next analyzed the expression of mRNA coding six type I and four type II receptors for the TGF- β family in F5-5.fl cells (Fig. 3). RT-PCR of F5-5.fl RNA yielded two type I receptor PCR products (ALK-4 (ActRIB) and ALK-5 (T β RI)), while the other four cDNAs (ALK-1, ALK-2, ALK-3 and ALK-6) were not detected (Fig. 3A). For the type II receptors, ActRII and BMPRII cDNAs were yielded, whereas ActRIIB and T β RII cDNAs were not detected by RT-PCR of F5-5.fl cells (Fig. 3B). The mRNA expression of Smads in F5-5.fl cells suggests that all Smad products examined in this study were expressed, although the Smad1 signal was slightly weak (Fig. 4).

Discussion

In the present study, we first confirmed that activin induced erythrodifferentiation of the erythroleukemic cell line, F5-5.fl cells. Activin A, but not TGF- β 1, stimulated hemoglobin production in F5-5.fl cells in a dose-dependent manner, which was consistent with a previous study (10). In addition, BMP-4 had no direct effect on the differentiation of F5-5.fl cells, and neither TGF- β 1 nor BMP-4 affected activin-induced erythrodifferentiation. Furthermore, incubation with 100 U/ml of IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-12 or IFN- γ did not change basal or activin A-induced erythrodifferentiation (data not shown). These findings suggest that measuring erythrodifferentiation of F5-5.fl cells is a useful bioassay to quantify activin activity. Another erythroleukemic cell line, K562 cells, also differentiates into hemoglobin-positive cells in response to activin. However, these cells also respond to TGF- β 1 (11). Therefore, F5-5.fl cells are more suitable for measuring activin activity than K562 cells.

Analyses of the expression of the TGF- β family ligands in F5-5.fl cells detected mRNA of TGF- β s (TGF- β 1, TGF- β 2 and TGF- β 3), whereas the expression of inhibin/activin subunits (α , β A and β B) and BMPs (BMP-2, BMP-4 and BMP-7) was not detected. The results suggest that neither activins nor BMPs were produced in F5-5.fl cells. Thus, use of F5-5.fl cells to measure activin activity has another merit in that it would not be necessary to consider endogenous activins and BMPs.

For signaling of the TGF- β family members, an appropriate combination of type I and type II receptors is needed (6). Each member of the TGF- β family binds to a characteristic combination of type I and type II receptors (Table 2) (5). mRNA expression of receptors for the TGF- β family members in F5-5.fl cells is summarized in Table 3. The results revealed the expression of both type I receptor (ALK-4/ActRIB) and type II receptor (ActRII) for activin. Furthermore, mRNAs of the Smads responsible for activin signaling (5), Smad2/3 and Smad4, were also detected in F5-5.fl

cells. Expression of all the molecules involved in activin signaling may have been responsible for the sensitivity to activin in the differentiation of F5-5.fl cells. In contrast, although expression of the type I receptor for TGF- β (ALK-5/T β RI) was detected, that of the type II receptor (T β RII) was not detected in F5-5.fl cells. Since downstream signaling of activin and TGF- β is thought to be mediated by the same molecules (5), the inability of TGF- β 1 to stimulate differentiation of F5-5.fl cells may be attributed to the lack of T β RII expression.

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