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.fl

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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-β1 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-βs (TGF-β1, TGF-β2 and TGF-β3) were detected, those of inhibin/activin (α-, βA- and βB-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/TbRI) was detected, that of type II receptor (TBRII) 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.

Introduction

Activins, transforming growth factor (TGF)-βs 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-β1 induce erythroiddifferentiation in the erythroblast cell line, K562 cells, with similar efficiencies (11). However, in another erythroblast cell line, F5-5.fl cells, although erythroiddifferentiation is induced by activin A, it is not induced by TGF-β1, suggesting defective TGF-β signaling (10).

To explore why F5-5.fl cells do not differentiate into erythroid in response to TGF-β1, 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.
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-β1 was obtained from Pepro Tech EC (London, UK). Recombinant human BMP-4 was purchased from R & D Systems (Minneapolis, MN, USA).

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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-β1 or BMP-4 was added to F5-5.fl at a cell density of 1000 cells/well in 200 µl (96-well plate). After incubation for 6 days at 37 °C, 20 µl 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 1 Effect of activin A, TGF-β1 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±s.d.). (B, C) Effect of TGF-β1 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-β1 (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±s.d.).

Figure 2 Expression of inhibin/activin, TGF-βs, and BMPs in F5-5.fl cells detected by RT-PCR. The mRNA expression of inhibin/activin subunits (A), TGF-βs (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-βs 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.
RNA isolation and cDNA synthesis

Total RNA from F5-5.fl cells was isolated using the RNAgents 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 ml 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 ml 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-βs (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 ml containing 10 mmol/l Tris–HCl, 50 mmol/l KCl, 1.5 mmol/l MgCl2, 0.2 mmol/l dNTP, 1 μmol 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 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× TBE and visualized with ethidium bromide.

Results

Effect of activin A, TGF-β1 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 EC50 on the erythrodifferentiation was 2.94 ± 1.27 (S.D.) ng/ml. We next determined whether TGF-β1 and BMP-4 affected the differentiation of F5-5.fl cells. Neither TGF-β1 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)-1β, 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-βs and BMPs in F5-5.fl cells was examined (Fig. 2). RTPCR of F5-5.fl RNA yielded all examined TGF-β products, although the TGF-β2 signal was weak (Fig.

Table 1: Type I and type II receptors for TGF-β family.

<table>
<thead>
<tr>
<th>Type I receptors</th>
<th>Type II receptors</th>
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<tbody>
<tr>
<td>Activins</td>
<td>ALK-2 (ActRI)</td>
</tr>
<tr>
<td>TGF-βs</td>
<td>ALK-4 (ActRII)</td>
</tr>
<tr>
<td>BMPs</td>
<td>ALK-6 (BMPR-IB)</td>
</tr>
</tbody>
</table>

Table 2: Combinatorial expression of type I and type II receptors for TGF-β family in F5-5.fl cells.

<table>
<thead>
<tr>
<th>mRNA expression in F5-5.fl cells</th>
<th>Combinatorial expression of type I and type II receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activins</td>
<td>ALK-4 (ActRI)</td>
</tr>
<tr>
<td>TGF-βs</td>
<td>Negative</td>
</tr>
<tr>
<td>BMPs</td>
<td>Negative</td>
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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 F5-5.5f cells (Fig. 3). RT-PCR of F5-5.5f RNA yielded two type I receptor PCR products (ALK-4 (ActRIB) and ALK-5 (TβRII)), 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 TβRII cDNAs were not detected by RT-PCR of F5-5.5f cells (Fig. 3B). The mRNA expression of Smads in F5-5.5f cells suggests that all Smad products examined in this study were not detected by RT-PCR of F5-5.5f cells (Fig. 3B). The mRNA expression of Smads in F5-5.5f cells suggests that all Smad products examined in this study were not detected by RT-PCR of F5-5.5f cells (Fig. 3B).

Discussion

In the present study, we first confirmed that activin induced erythroid differentiation of the erythroleukemic cell line, F5-5.5f cells. Activin A, but not TGF-β1, stimulated hemoglobin production in F5-5.5f 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 F5-5.5f cells, and neither TGF-β1 nor BMP-4 affected activin-induced erythrodifferentiation. Furthermore, incubation with 100 U/ml of IL-1β, 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 F5-5.5f 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-β1 (11). Therefore, F5-5.5f cells are more suitable for measuring activin activity than K562 cells.

Analyses of the expression of the TGF-β family ligands in F5-5.5f cells detected mRNA of TGF-β1 (TGF-β1, TGF-β2 and TGF-β3), whereas the expression of inhibin/activin subunits (α, βA and βB) and BMPs (BMP-2, BMP-4 and BMP-7) was not detected. The results suggest that neither activins nor BMPs were produced in F5-5.5f cells. Thus, use of F5-5.5f 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 F5-5.5f 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 F5-5.5f cells. Expression of all the molecules involved in activin signaling may have been responsible for the sensitivity to activin in the differentiation of F5-5.5f cells. In contrast, although expression of the type I receptor for TGF-β (ALK-5/TβRI) was detected, that of the type II receptor (TβRII) was not detected in F5-5.5f cells. Since downstream signaling of activin and TGF-β is thought to be mediated by the same molecules (5), the inability of TGF-β1 to stimulate differentiation of F5-5.5f cells may be attributed to the lack of TβRII expression.

Acknowledgements

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