TGF- β receptors in mouse ES-5 cells and their differentiated derivatives¹

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ABSTRACT

By radioreceptor binding studies with iodinated TGF- β 1, it has been shown that an undifferentiated ES-5 cell expresses approximately 3270 receptors with a dissociation constant $K_d=130\text{pM}$, but after the induction of differentiation by retinoic acid and dBcAMP, the receptor number of a differentiated RA-ES-5 cell was increased about 80% and the K_d was also increased to 370 pM. Furthermore, more direct evidence supporting the expression of TGF- β type I and type II receptors in both ES-5 and RA-ES-5 cells has come from dot blot hybridization of cellular mRNA with cDNA probes for type I and type II receptors. Meanwhile, mRNA expression level of types I and II receptors in RA-ES-5 cells were higher than that in ES-5 cells. Down-regulation of TGF- β receptors with a significant decrease in the rate of cell proliferation in both cells, was found by employing a pretreatment with neutralizing antibody to TGF- β 1. The possible role of receptors for TGF- β in cell differentiation is discussed here.

Key words: *mouse embryonic stem (ES) cells, TGF- β receptors, Down-regulation by TGF- β antibody, RA-induced differentiation.*

INTRODUCTION

Transforming growth factor- β s (TGF- β s) are known to be a multifunctional

1. This Paper is dedicated to Professor Zhen YAO, Ph. D, on the occasion of his 80th birthday

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growth regulators. TGF- β s have been proved to play an important role in cell growth and differentiation during embryogenesis[1]. By immunohistochemical procedure, we have also found that abundant TGF- β 1 is present in preimplantation mouse embryos including cleavage, morula and blastocyst stages. In postimplantation embryos, the distribution of TGF- β 1 appears to be related to the differentiation of endoderm and mesoderm, particularly in the histogenetic events in conjunction mainly with mesoderm or mesenchyme cells[2]. It has been reported that the biological effects of TGF- β s are controlled by specific binding of cell surface receptors to TGF- β s. Three types of receptor for TGF- β , named type I, II and III have been identified. From studies on mutated cells and certain tumor cell lines lacking TGF- β responsiveness, types I and II receptors were shown to be essential for TGF- β signalling, while type III (or betaglycan) might play a role in the retention or delivery of TGF- β [3]. TGF- β isoforms bind with different affinities to receptors but each isoform is able to associate with all three receptors[4]. In general, the affinity of the types I and II receptors is higher for TGF- β 1 and TGF- β 3 than TGF- β 2; however, the relative affinities vary with cell types[5]. Only recently, types I, II and III receptors for TGF- β s have been cloned and characterized[6-8]. This provides an immediate strategy for the study of receptor expression during early mouse development as well as in an *in vitro* system of embryonic stem cells (ES cells), as a model for studying growth and differentiation of mammalian embryonic cells[9]. So far, little is known about the characteristics of TGF- β receptors in ES cells and their corresponding differentiated cells. In our earlier experiments, ES-5 cells had been induced to differentiate into more than 90% glial-like cells by retinoic acid (RA) and dBcAMP and these ES-5 cells, and their differentiated glial-like cells, both expressed TGF- β 1, in varying degrees[10]. In this study we further investigated the affinity, number and types of TGF- β receptors in ES-5 cells and their differentiated derivatives by radioreceptor binding assay of 125 I-labeled TGF- β 1 purified from human platelets and dot blot hybridization with cDNA probes for types I and II TGF- β receptors. We also demonstrated the increasing expression of types I and II receptors for TGF- β in differentiated cells treated with RA and dBcAMP. Down-regulation of TGF- β 1-binding sites in the surface of both these cells was found by the pretreatment with neutralizing antibody to TGF- β 1. The possible role of receptors for TGF- β in cell differentiation is discussed.

MATERIALS AND METHODS

1. Culture and induced differentiation of ES-5 Cells

ES-5 cell line was isolated from the inner cell mass of delayed blastocyst of a 129/SV strain mouse[11], and cultured in 80% BRL-CM containing 20% fetal calf serum (FCS) and 10^{-4} M β -mercaptoethanol. Differentiation was induced as described previously[10]. In short, 4 hrs after the plating of ES-5 cells, RA and dBcAMP were added to the BRL-CM at a final concentration of 10^{-6} M and 1 mM respectively. On day 3 the medium was refreshed. ES-5 cells and their differentiated cells (RA-ES-5 cells) induced by RA and dBcAMP were cultured for 6 days in 10 cm dishes. Before ra-

dioreceptor binding assay, both cells were dissociated with 0.25% trypsin and 0.2 mM EDTA in PBS at 37°C for 5 min and the final cell number in each well of 24 well plates was controlled at 4×10^5 /ml.

2. Radioiodation of TGF- β 1

Iodination of TCF- β 1 was performed with Indo-bead shaking procedure established in our institute. Briefly, 2 μ g TGF- β 1 purified from human platelets according to Assoian et al[12] was dissolved in 10 μ l 0.175 M acetic acid, then 1 mCi Na¹²⁵I (Amersham) and 0.05 M, pH7.6 phosphate-buffered solution were added to a final volume of 500 μ l in a plastic tube. Two Indo-beads (N-chlorobenzenesulfonamide) were added and mixed on a shaker at room temperature for 15 min. Free ¹²⁵I was removed from reaction mixture by chromatography on Sephadex G-25. Fraction of ¹²⁵I-TGF- β with highest radioactivity were collected and stored at -30°C.

3. TGF- β receptor binding assay

TGF- β receptor binding assay was carried out according to Enna[13]. Trypsinized ES-5 and RA-ES-5 cells attached separately to 24 well plates for 2-3 hrs were washed once with DMEM without calf serum and then once with binding assay buffer (BAB: MEM containing 0.1% BSA, 25 mM Hepes, freshly prepared before use). A fixed amount of ¹²⁵I-TGF- β 1 (5×10^4 cpm/well) was added to each well and an increasing concentration of unlabeled TGF- β 1 (0-40 ng/ml) to different wells were added and all supplemented with BAB to a final volume of 300 μ l/well. Non-specific binding assay was carried out by using a large quantity of unlabeled TGF- β 1 (20-100 ng/well more than 100 times of the amount of ¹²⁵I-TGF- β 1 used). The reaction was allowed to proceed to equilibrium for 60 min in 5% CO₂ incubator at 37°C. The cells were washed 3 times with precooled Hank's solution containing 0.1 % BSA and the cell membranes were solubilized in 0.5 ml Triton solution (1 % Triton-100, 10 % glycerol, 0.01 % BSA, 20 mM Hepes, pH7.4) with shaking for 20 min at room temperature. Lysates in each well were counted for their radioactivities by a gamma-ray counter (Beckman 5500).

4. Scatchard analysis of receptor binding experiments

The most commonly used approach for data processing of ligand-receptor binding experiments is the preparation of a Scatchard plot of the B/F (amount of bound ¹²⁵I-TGF- β 1/amount of free ¹²⁵I-TGF- β 1) ratio (Y axis) Vs bound ligand concentration (X axis)[14]. The Scatchard equation is $B/F = 1/K_d (R_0 - B)$. The values of B and B/F can be obtained from the transformation of data measured in the receptor binding experiments. The maximum amount of ligand bound to cell surface (Bmax) is X axis intercept from which the receptor number on each cell surface can be obtained. K_d is equilibrium dissociation constant.

5. Treatment of ES-5 and RA-ES-5 cells with antibody to TGF- β 1

When the number of ES-5 and RA-ES-5 cells was set at 4×10^5 /well in 24 well plates respectively, the cells were washed once with BRL-CM/FCS medium without an inducing agent. Then sufficient amount of neutralizing antibodies for TGF- β 1 [15] were added to each well and incubated for 18 hrs at 37°C the before radioreceptor binding assay. The final concentrations of affinity-purified anti-TGF- β 1 IgG used in the well were varied at range of 1-50 μ g/ml. Control IgG was purified from a mouse normal serum. The cell numbers in each well treated by the antibody to TGF- β 1 was estimated by the MTT method[16]. For TGF- β receptor binding assay, both ES-5 and RA-ES-5 cells pretreated with TGF- β antibody were trypsinized and the cell number was adjusted at 4×10^5 /well.

6. Probes for TGF- β type I and type II receptors and dot blot analysis.

The individual plasmids containing TGF- β RI and TGF- β RII cDNA were generously provided by Dr. Xiao-Fan Wang (Department of Pharmacology, Duke University Medical Center, Durham, North Carolina). 1.7 Kb EcoRI-HindIII fragment of TGF- β RI and 1.2 Kb EcoRI-SacI

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fragment of TGF- β RII were used as hybridization probes and labeled respectively with [32 P]dATP (3000ci/mmol, Amersham) by random priming (GIBCO/BRL). The probes were labeled to a specific activity of 1×10^8 cpm/ μ g for TGF- β RI and 9.3×10^7 cpm/ μ g for TGF- β RII using 32 PdATP, respectively.

According to the methods described in "Molecular Cloning-A Laboratory Manual" edited by J. Sambrook et al[17], extraction of total RNA from ES-5 and RA-ES-5 cells grown in culture was carried out with guanidine isothiocyanate and organic solvent, and poly(A)⁺RNA was purified from the individual preparation of total cellular RNA by chromatography on Oligo(dT)-cellulose. Poly(A)⁺RNA samples were applied onto the nylon membrane (Qiabrane) with the Manifold II (Schleicher and Schuell). After all of the samples were passed through the membrane, was each spot rinsed twice with $10 \times$ SSC. The membranes were then removed from the manifold and allowed to dry completely at room temperature, they were then baked for 2 h at 80°C in a vacuum oven and stored in a plastic bag. Dot hybridization were performed respectively with the 32 P- labeled DNA probes for TGF- β RI and TGF- β RII at 42°C in hybridization buffer containing 50 % formamide overnight and blots were washed in $0.2 \times$ SSC, 0.1 % SDS. The membranes were exposed to X-ray film at -70°C for 4-7 days with an intensifying screen. Later quantification of dot blot was carried out by UltraScan XL laser densitometer (Pharmacia LKB).

RESULTS

1. Demonstration of TGF- β 1 binding to receptors in ES-5 and RA-ES-5 cells

Incubation of ES-5 and RA-ES-5 cells at 4×10^5 /well respectively with increasing concentration of unlabeled TGF- β 1 (0-40 ng/ml) and a fixed amount of 125 I-TGF- β 1 (5×10^4 cpm/well) showed a competitive binding curve (Fig 1). Taking the binding rate of 125 I-TGF- β 1 when concentration of unlabeled TGF- β 1 was at 0 ng/ml as 100%, and the binding of 125 I-TGF- β 1 was decreased in wells with increasing amounts of unlabeled TGF- β 1. Nonspecific binding was determined using an excess of unlabeled TGF- β 1 in one well for each incubation. More than 90% of the bound 125 I-TGF- β 1 was displaced by the highest dose of unlabeled TGF- β 1 tested (100ng/ml). The specific binding was calculated by subtracting nonspecific binding from total binding. The results demonstrated the presence of specific receptors for TGF- β 1 in both ES-5 and RA-ES-5 cells.

Fig 1. Competitive binding curve of ES-5 ($\blacktriangle$) and RA-ES-5 ($\square$) cells: a fixed amount of 125 I-TGF- β 1 (5×10^4 cpm/well) and increasing concentration of unlabeled TGF- β 1 bound competitively to TGF- β receptors on surface of ES-5 and RA-ES-5 cells (4×10^5), (when unlabeled TGF- β 1 concentration at 0, the binding rate of 125 I-TGF- β 1 was 100 %).

2. Number and affinity of TGF- β 1 receptors in ES-5 and RA-ES-5 cells

Scatchard analysis of the binding data presented in Fig 1 was carried out with the "LIGAND" program developed by Munson[14] and transformation of linear regression revealed the presence of some higher affinity binding sites in both cells. Curvilinear Scatchard plots of TGF- β 1 binding in ES-5 and RA-ES-5 cells were presented in Fig 2. The intercept of a oblique line in the X axis was the maximum binding of ligand TGF- β 1 (B max) and the reciprocal of the slope was dissociation constant Kd. By the calculation of Scatchard equations the affinity site had a dissociation constant of 110 to 150 pM with approximately 3270 receptors per ES-5 cell, while the affinity site had a dissociation constant of 300 to 440 pM with approximately 5840 receptors per RA-ES-5 cell (refer to Tab 1). Therefore, these results suggest that a change in the number and affinity of TGF- β receptors on the surface of undifferentiated ES cells occurred during the period of their differentiation into glial-like cells: an increase in the number of TGF- β receptors was approximately 80 and a decrease in affinity was about 3 times less than their parent cells.

Fig 2. Scatchard plot of the binding of TGF β 1 in ES-5 and RA-ES-5 cells.
 □ ES-5 cells, ■ RA-ES-5 cells

Tab 1. Down regulation effect of TB21 McAb on TGF- β 1-binding sites of ES-5 and RA-ES-5 cells

Cell type	Group	Dissociation constant (Kd, nM)	Binding site / cell ($\times 10^3$)
ES-5		0.13 ± 0.02	3.27 ± 0.15
ES-5	NMIgG	0.19 ± 0.02	3.19 ± 0.10
ES-5	TB21IgG	0.14 ± 0.04	1.56 ± 0.04
RA-ES-5		0.37 ± 0.07	5.84 ± 0.15
RA-ES-5	NMIgG	0.34 ± 0.04	5.6 ± 0.10
RA-ES-5	TB21IgG	0.32 ± 0.03	1.9 ± 0.05

3. Expression of TGF- β type I and II receptor RNA in ES-5 and RA-ES-5 cells

Dot blot hybridization of total RNA isolated from ES-5 and RA-ES-5 cells with the probes for TGF- β RI and TGF- β RII showed that both ES-5 cells and RA-ES-5

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cells expressed the two receptor mRNAs, but mRNA level of TGF- β RI was higher than that of TGF- β RII in both cells (Fig 3). However, the differentiated cells, and this expressed more TGF- β RI and TGF- β RII mRNA than undifferentiated parent cells which was particularly evident from the scanning data of low- loaded samples, as shown in Tab 2. The results thus suggested that the expression level of mRNAs for TGF- β RI and TGF- β RII was somewhat increased after induced differentiation and this increase was conformable to the data of TGF- β receptor binding assay mentioned above.

Tab 2. Scanning data of dot blot signals for TGF- β RI and TGF- β RII shown in Fig 3 by UltraScan XL laser densitometry

No*	Component name	Position mm	Height Au	Area Au* mm	Relative area %
A-1	ES/TGF- β RI	61.56	1.199	5.96224	53.0
A-2	RA/ES/TGF- β RI	70.52	1.077	5.27736	47.0
B-1	ES/TGF- β RI	62.68	0.340	1.39488	34.8
B-2	RA/ES/TGF- β RI	70.84	0.575	2.61888	65.2
C-1	ES/TGF- β RI	63.96	0.390	1.71800	49.2
C-2	RA/ES/TGF- β RI	72.76	0.370	1.7712	50.8
D-1	ES/TGF- β RI	64.20	0.137	0.72856	39.0
D-2	RA/ES/TGF- β RI	72.76	0.220	1.13872	61.0

* Refer to Fig 3.

Note that when the amount of total RNA loaded was 10 μ g for each dot, the expression level of mRNA for TGF- β RI and TGF- β RII was increased by about 87% for RI (compare B-2 to B-1) and 56% for RII (compare D-2 to D-1) after ES-5 cells were differentiated into RA-ES cells, although no significant change was found in case 20 μ g of total RNA was loaded (compare A-1 and A-2, C-1 and C-2), possibly due to the high background.

4. Effect of TGF- β 1 antibody on ES-5 and RA-ES-5 cells

a. Growth inhibition of ES-5 and RA-ES-5 cells

The growth inhibition of ES-5 and RA-ES-5 cells followed the treatment of them

with a monoclonal antibody against human TGF- β 1. The percentage of inhibition in both types of cells increased with increasing concentration of the antibody IgG tested. In the same concentration of TGF- β 1 IgG, inhibitory effect on RA-ES-5 cells appeared to be higher than that of ES-5 cells. When the dose of TGF- β 1 antibody IgG was increased up to 50 μ g/ml, the growth inhibition of RA-ES-5 cells reached 61%, but it only reached 46% in the ES-5 cells. In a controlled experiment with mouse IgG, no effect on growth of both cells was found in corresponding concentration (Fig 4).

Fig 4. Inhibitory effect of TB21 antibody IgG on ES-5 and RA-ES-5 cell. See text for details.

- RA-ES-5+ Mc TB21 IgG
- RA-ES-5 + normal mouse IgG
- ▲-▲ ES-5 + McTB21 IgG
- △-△ ES-5 + normal mouse IgG

b. Down-regulation of TGF- β receptors

There was a rapid decrease in the binding of 125 I- TGF- β 1 to both the ES-5 and the RA-ES-5 cells, if these cells were pretreated with TB21 IgG antibody against TGF- β 1. Scatchard analysis of the binding data from radioreceptor assay for these cells, as presented in Figs 5 and 6, suggested that TGF- β 1 binding sites (receptors) of ES-5 and RA-ES-5 cells treated with TB21 IgG were significantly less than those of corresponding untreated cells or cells treated with normal mouse serum IgG. The number of TGF- β receptors in ES-5 cells treated with TB21 IgG was decreased from

Fig 5. Scatchard plot of the binding of TGF- β 1 to ES-5 cells after treatment with TB21 antibody

- ES-5+NIgG
- ES-5+TB21 IgG

Fig 6. Scatchard plot of the binding of TGF- β 1 to RA-ES-5 differentiated cells after treatment with TB21 antibody

- RA-ES-5NIgG
- RA-ES-5+TB21 IgG

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an initial 3270/cell to 1560/cell, and in RA-ES-5 cells from initial an 5840/cell to 1900/cell (Tab 1). The results indicated that down-regulation of TGF- β 1 binding sites was followed by the pretreatment of ES-5 and RA-ES-5 cells with the TGF- β antibody IgG.

DISCUSSION

The interaction of TGF- β with target cells involves a set of membrane proteins with both a distinct structure and function. It has been shown that there exist membrane proteins binding to TGF- β in many cell types using receptor affinity labeling procedure[4-5]. Binding studies with iodinated TGF- β 1 have shown that undifferentiated EC and ES cells have low levels of cell surface receptors for TGF- β . After the induction of differentiation by RA, however, high affinity binding sites for TGF- β 1 become detectable[18-19]. In this study we have carried out TGF- β 1 binding experiments and dot blot hybridizations for TGF- β type I and type II receptors on undifferentiated ES-5 cells and their differentiated derivatives. Preliminary results have shown that an undifferentiated ES-5 cell expressed approximately 3270 receptors with a dissociation constant $K_d=130\text{pM}$. This number was increased by about 80% when ES-5 cells were differentiated into a population of glial-like cells by RA and dBcAMP. Concomitantly, the K_d was increased to 370 pM. Our results which are slightly different from that from other reports[19], may be due to the fact that we have used trypsinized ES-5 cells which are easy to grow *in vitro* in form of aggregates in the presence of LIF/DIA. We have made some trial experiments to test the effect of trypsinization before receptor binding assay, and found that the number of binding sites was variable in non-trypsinized ES-5 cells, often being less than that of trypsinized ES-5 cells. On the other hand, RA-ES-5 cells grown in single cells did not display any significant change in the number of binding sites regardless of whether cells were treated with trypsin or not. Since most of ES-5 cells sampled without trypsinization contained a number of small aggregates, it appears that cell aggregation might cause an apparent decrease in the number of TGF- β binding sites in radioreceptor binding assay.

Recently, it was reported that undifferentiated ES cells and differentiated ES cells all produced a significant amount of biologically active TGF- β [20] and the TGF- β 1 has been detected in the ES-5 cell line[21]. Meanwhile, ES cells and their differentiated cells have been shown to be responsive to TGF- β [22]. It is therefore not surprising to find specific, high affinity receptors for TGF- β in these cells. In general the number and affinity of TGF- β receptors on the cell surface might reflect the difference in cellular function and capacity to grow or differentiate. However, no definite relationship between the number of TGF- β receptor and sensitivity to growth inhibition by TGF- β has been found in several cancer lines[23-24]. Moreover, some results indicated that the difference between TGF- β responsive and nonresponsive cells with regard to their total number of high affinity receptors was small[25].

Further studies, consequently, will be required to define the relationship between the number/affinity of TGF- β receptors and their ability to regulate growth and differentiation in ES cells.

In the present studies, more direct and consistent evidence supporting the expression of TGF- β type I and type II receptors in both undifferentiated ES-5 cells and their differentiated derivatives has come from dot blot hybridization of individual cellular mRNA with cDNA probes for TGF- β RI and TGF- β RII. With respect to general properties of three types of TGF- β receptors, only types I and II receptors are directly involved in TGF- β signaling. In fact, the intracellular signaling events initiated by TGF- β receptor-ligand interaction are not fully understood. It has been shown that the type I and II receptors might interact with each other in TGF- β signaling. The type II receptor could bind TGF- β in the absence of the type I receptor, but type I receptor could not bind TGF- β in the absence of type II receptor[4]. So it appears that TGF- β signals through a heteromeric complex of the type I and II TGF- β receptors. Recently, It has been shown that the type II receptor belongs to a transmembrane receptor of the serine-threonine kinase family, and the type I receptor has an intrinsic serine-threonine kinase activity, which was essential for signal transduction[8]. Such being the case in our experiments that simultaneous expression both of type I and type II TGF- β receptors occurred in ES cells and their differentiated cells, and the molecular events of TGF- β signal transduction by this TGF- β receptor complex must exist in the process of ES cell differentiation.

One of the common properties of growth factor receptors is the ability of the ligand to down-regulate the receptor. Receptor "down-regulation", or the ligand-induced decrease in the cellular content of receptors, is a mechanism by which the cells regulate their responsiveness to TGF- β . The decrease in the TGF- β receptor number that occurs in cells exposed to TGF- β antibody is a characterized example of down-regulation. In the present studies we have found that the rate of cell proliferation of ES-5 and RA-ES-5 cells was dramatically reduced by the pretreatment with the monoclonal TGF- β antibody IgG and that the number of TGF- β receptors was also decreased simultaneously to two-three-fold in both groups of cells during the period of the treatment with the TGF- β 1 antibody. The indirect evidence obtained from these data supports the possibility that the neutralization of TGF- β 1 present in cellular micro environment by the TGF- β 1 antibody would result in a decrease in the local concentration of TGF- β content which might induce relevantly down-regulation of the TGF- β receptors on cell surface. By this reasoning it seems likely that, at least *in vitro*, TGF- β antibody may be an effective down-regulating agent for TGF- β receptors. Taken together, our data and those from other papers[19-22] all suggest that TGF- β which exists in cellular microenvironment might act by an autocrine/paracrine mechanism in ES cells and their differentiated cells. TGF- β may act as a direct intercellular signal by regulating proliferation of competent target cells or as indirect signals by inducing the expression of a cascade of other genes which, in turn, trigger specific differentiation events.

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