

Factors controlling induction of commitment of murine erythroleukemia (TSA8) cells to CFU-E (colony forming unit–erythroid)

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Summary

On addition of DMSO, the MEL cell line TSA8 becomes committed into erythroid progenitor cells (CFU-E) which can form differentiated colonies in the presence of erythropoietin. To understand the mechanism of cellular commitment, the number and the affinity of the receptors for erythropoietin were estimated. The affinity of the receptors did not change before or after induction. The number of receptors changed depending on the growth phase, but was not dependent on the addition of the inducer. Thus, the

presence of the receptors for erythropoietin may be required, but are not essential for responsiveness to erythropoietin.

Further examination of the optimum conditions for commitment suggests that the concomitant actions of induced factor(s) with the receptors may control commitment of TSA8 cells to CFU-E.

Key words: CFU-E (colony forming unit–erythroid), commitment, erythroleukemia cells, erythropoietin receptor.

Introduction

Mature haematopoietic cells, such as red blood cells, are derived from several types of progenitor cells, each committed to develop into only one or two cell lineages; but all progenitor cells originate from the same stem cells (Till & McCulloch, 1980). Thus, the maintenance of haematopoiesis requires that a balance be maintained between self-renewal and differentiation (Dexter *et al.* 1984). Progenitor cells, themselves immature progeny of the stem cells, are recognized by their ability to produce colonies including both proliferation and differentiation *in vitro* in the presence of specific growth-regulating molecules. Erythropoietin is a well-characterized molecule and induces *in vitro* the formation of CFU-E colonies which are characterized as an erythropoietin-dependent colony of about 32 terminally differentiated erythroblasts in a semisolid medium (Iscove, Siever & Winterhalter, 1974). However, the mechanism of action of erythropoietin on the proliferation and differentiation of the progenitor cells is not known. Recently, we have demonstrated that a novel MEL cell line, TSA8 cells established by Shibuya & Mak (1983), can be committed to CFU-E by addition of DMSO *in vitro* (Mishina & Obinata, 1985). This *in vitro* system appears suitable for studying how

progenitor cells acquire their ability to respond to erythropoietin. In this work, we examined what factors are required for the commitment of TSA8 cells to CFU-E. The surface receptors for erythropoietin may be one factor. However, our results suggest that the concomitant action of the induced factor(s) and receptors are required for the cells to respond to erythropoietin.

Materials and methods

Cell culture and induction of commitments with several inducers

TSA8 cells were established by Shibuya & Mak (1983) from anaemia-inducing Friend virus complex, FV-A. The cell line was kindly provided by Dr Mak of the University of Toronto. It was maintained for one year in the laboratory of Professor M. Oishi of the Institute for Applied Microbiology and then for one year in our laboratory. The properties of the cells were described previously (Mishina & Obinata, 1985). The cells were grown in Iscove's modified Dulbecco medium supplemented with 15% fetal bovine serum as described (Iscove *et al.* 1974). For induction, the inducer was added to a suspension of the cells at a density of 2×10^5 cells ml⁻¹. For enhanced induction, slightly overgrown cells were passaged and the inducer was added one day after passage, just after the cells had started

to grow. The CFU-E assay was carried out in methylcellulose using techniques by Iscove *et al.* (1974). The cells were transferred to a semisolid medium at various times after the addition of the inducer. 2×10^3 cells were plated in a 24-well multititre dish (Corning) in a mixture containing Iscove's modified Dulbecco medium, 0.8% methylcellulose (Tokyo Chemical Ind., Tokyo), 1% bovine serum albumin (Filtoron, Inc.), 100 mM β -mercaptoethanol and 0.5 i.u. ml⁻¹ of step III sheep plasma erythropoietin (Connaught, Canada). Dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO₂ in air. Colonies were directly stained with benzidine on day 2 and scored with an inverted microscope.

Erythropoietin binding assay in TSA8 cells

Human erythropoietin purified from the supernatant of COS-7 cells transfected with human erythropoietin cDNA was kindly supplied by Kirin Beer Co. Ltd. Iodination of erythropoietin was done according to the method of Hunter & Greenwood (1962). Usually 1×10^8 cts min⁻¹ μ g⁻¹ of specific radioactivity was obtained. When the labelled products were analysed by SDS-PAGE, 95% of radioactivity was estimated to be incorporated into a single band of $34 \times 10^3 M_r$. The binding activity of a stock solution did not deteriorate for 2 months in a refrigerator. The ¹²⁵I-erythropoietin-binding assay was performed by the modified method of Cartrell & Smith (1984). TSA8 cells were washed three times in medium and then suspended in a buffer containing 1 mg ml⁻¹ BSA (5×10^6 per 100 μ l). The cell suspension was incubated while shaking for 2 h at 15°C in a serial dilution of ¹²⁵I-erythropoietin. The cells were spun through a cushion of *n*-butylphthalate and the pellet was counted in a gamma-counter. Nonspecific binding was measured by incubating the cells with ¹²⁵I-erythropoietin in the presence of 100-fold excess of unlabelled erythropoietin. Usually nonspecific binding was approximately 10% of maximal binding or 0.5% of total input erythropoietin. The average number of erythropoietin binding sites per cell and the affinity of erythropoietin binding were quantified by Scatchard plot analysis (Cartwell & Smith, 1984) of equilibrium binding after subtraction of nonspecific binding.

Results

(A) Effect of known inducers on the induction of commitment of TSA8 cells to CFU-E

We have reported that DMSO can induce TSA8 cells to become committed to CFU-E (Mishina & Obinata, 1985). After exposure to 1% DMSO in the liquid culture, the cells were transferred to a semisolid culture to examine their ability to form erythroid colonies in the presence of erythropoietin. Exposure to DMSO for 2 days was optimum for CFU-E-like colony formation. Without erythropoietin, formation of CFU-E-like colonies was not observed even after induction with DMSO. The CFU-E-like colonies formed in the presence of erythropoietin were very similar to the CFU-E from mouse bone marrows,

Table 1. Effect of inducers on the induction of commitment of TSA8 cells to CFU-E

Inducer	Conc.	Colonies formed in the semisolid medium	
		Partly stained colonies (%)	CFU-E-like colonies (%)
No addition		3.9	1.8
DMSO	1%	10.6	42.1
Sodium butyrate	1 mM	7.6	13.8
	1.25 mM	8.1	19.6
	1.5 mM	6.6	25.2
HMBA	3 mM	5.5	40.0
TPA	10 ng	3.4	2.9
	50 ng	3.3	3.5
	100 ng	6.1	4.7
Hemin	0.05 mM	2.0	1.7
	0.1 mM	1.2	2.0
	0.2 mM	1.9	3.7

TSA8 cells were cultured in liquid medium for 2 days in the presence of inducers and then transferred to semisolid medium containing 0.8% methylcellulose and 0.5 i.u. ml⁻¹ of erythropoietin as described in Materials and methods. Usually 2×10^3 cells were seeded. After 2 days in the semisolid medium, the colonies formed were stained with benzidine and three types of colonies; nondifferentiating (nonstained) colonies, partly stained colonies and CFU-E-like colonies were scored. The percentages were calculated from the numbers of colonies/total number of colonies. In all experiments, the percentage of the CFU-E-like colonies or partly stained colonies in the absence of erythropoietin in the semisolid medium was less than 2%. Total number of colonies formed in the semisolid medium is essentially equal in all drugs used.

although similar colonies were formed in the presence of DMSO in the semisolid medium. We examined whether inducers other than DMSO can induce TSA8 cells to become committed to CFU-E. Many chemicals (Ebert, Wars & Buell, 1976; Friend, Scher, Holland & Sato, 1971; Gusella *et al.* 1976; Leder & Leder, 1975; Lo, Aft, Ross & Mueller, 1978; Reuben *et al.* 1976; Rovera, Santoli & Damsky, 1979; Ross & Sautner, 1976) are known as potent inducers in other MEL cell lines that were established from the spleen cells from polycythemia strain of Friend viruses. Effects of typical inducers on the induction of TSA8 cells to CFU-E are shown in Table 1. In most cases, drugs are not selectively toxic for formation of either the differentiated colonies or the nondifferentiated colonies and thus the total number of colonies formed in the semisolid medium stayed constant. Among the inducers tested, HMBA is efficient for TSA8 cells with the optimum concentration (3 mM) for these cells being lower than that (5 mM) reported for the other MEL cells. Butyrate, though a rather weak inducer, showed a significant effect. Among the drugs tested, Mitomycin C and TPA showed no effect on the induction of commitment. Hemin, which was reported

Table 2. Effect of inhibitors on the induction of commitment of TSA8 cells to CFU-E

Inhibitor	Conc.	Colonies formed in the semisolid medium	
		Partly stained colonies (%)	CFU-E-like colonies (%)
No inhibitor		13.4	34.1
Dexamethasone	9 μM	15.9	31.9
	30 μM	16.5	32.5
TPA	10 ng ml^{-1}	10.8	28.2
	50 ng ml^{-1}	13.6	30.8

Effect of the inhibitors for the induction of commitment was examined as described in Table 1.

to be a potent inducer for the other MEL cells (Ross & Sautner, 1976), as well as to exert a growth-regulating activity on the progenitor cells, however showed no effect on the induction of commitment of TSA8 cells, even if combined with DMSO.

The effects of TPA or glucocorticoids on the induction of commitment of TSA8 cells by DMSO were then examined, since these were reported to inhibit the commitment process in the other MEL cells (Lo *et al.* 1978; Yamasaki *et al.* 1977; Yu & Smith, 1985; Mierendorf & Mueller, 1982) (Table 2). Surprisingly, these inhibitors had no effect on commitment of TSA8 cells to CFU-E by DMSO at the same concentration that inhibits completely the induction of MEL cell with DMSO. These results suggest that the commitment process is not the same in TSA8 cells and other MEL cells.

(B) Level of erythropoietin receptors in the induction of TSA8 cells

The analysis of commitment of TSA8 cells to CFU-E may help to understand the process of commitment of early erythroid progenitor cells. Since the cells can acquire erythropoietin dependency only after the addition of DMSO, the induction of receptors for erythropoietin may be the most likely explanation for the commitment to CFU-E. Thus, we examined whether the commitment of TSA8 cells to CFU-E is primarily due to the induction of erythropoietin receptors. The change in receptors was monitored by the binding of ^{125}I -erythropoietin to the intact TSA8 cells after induction (Fig. 1). The binding of erythropoietin to cell surface receptors was significant, though not high, if specific binding was estimated in the presence of 100-fold excess of unlabelled erythropoietin. The level of the receptors changed dramatically during induction of TSA8 cells with DMSO. To our surprise, however, a similar increase in the receptors was observed in the culture without DMSO and, on DMSO addition, no increase in the number

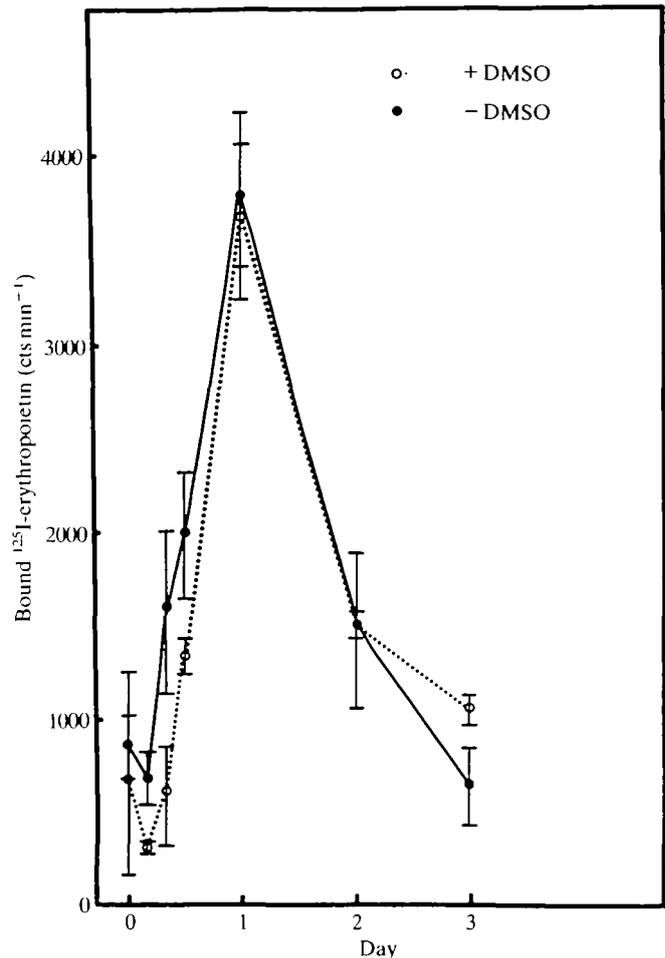


Fig. 1. Change of the erythropoietin receptors in the induction of TSA8 cells with DMSO. TSA8 cells were collected at respective periods in the culture, washed three times with Hanks' balanced solution and resuspended in the binding buffer. 1×10^5 cts min^{-1} (1 ng) of ^{125}I -labelled erythropoietin were added to the suspension of TSA8 cells (5×10^6 cells in 0.1 ml) and left for 2 h at 15°C . The binding assay was performed as described in Materials and methods. Nonspecific binding was 0.4 to 0.5% of the total erythropoietin added to the cell suspension in each point. At a maximum (day 1), approximately 6% of the total erythropoietin was bound. The binding assay was done in a saturation concentration of ligand. Binding of ^{125}I -erythropoietin was expressed as counts per minute per 2×10^6 cells. The values in duplicates in each point are plotted. \circ , culture with DMSO; \bullet , culture without DMSO.

of the receptors was observed. When the level of the erythropoietin receptors was examined in the other MEL cell line B8/3, which was established from a polycythemia strain of the virus and showed no responsiveness to erythropoietin, a similar level of receptors was detected. A change in the level of the receptors similar to that in TSA8 cells was observed in the culture of B8/3 cells with or without DMSO (data not shown). Thus, the drastic change in the

level of receptors may be related to the growth condition of the cells, rather than to inducer addition. One day after addition of DMSO, the number of receptors became maximum. One day later, the level decreased reaching background at 4 to 5 days. The maximum number of receptors (1 day) does not coincide with the formation of CFU-E-like colonies (2 days) or partly stained colonies (1.5 days). The average number of binding sites per cell and the binding affinity were quantified by Scatchard plot analysis of equilibrium binding after subtraction of nonspecific binding (Fig. 2). The highest number of binding sites per cell was estimated to be approximately 500. Scatchard plots of erythropoietin binding to TSA8 cells before and after induction indicated that no gross conversion of a low-affinity component to a high-affinity component occurred after induction. Although the data in Fig. 2 could be interpreted as showing both high- and low-affinity binding sites, the values obtained for dissociation constants of the two components by calculations based on the data in Fig. 2 or on the double reciprocal plots of the data seemed to be the same within the bounds of error. If most binding is expected to be due to a single high-

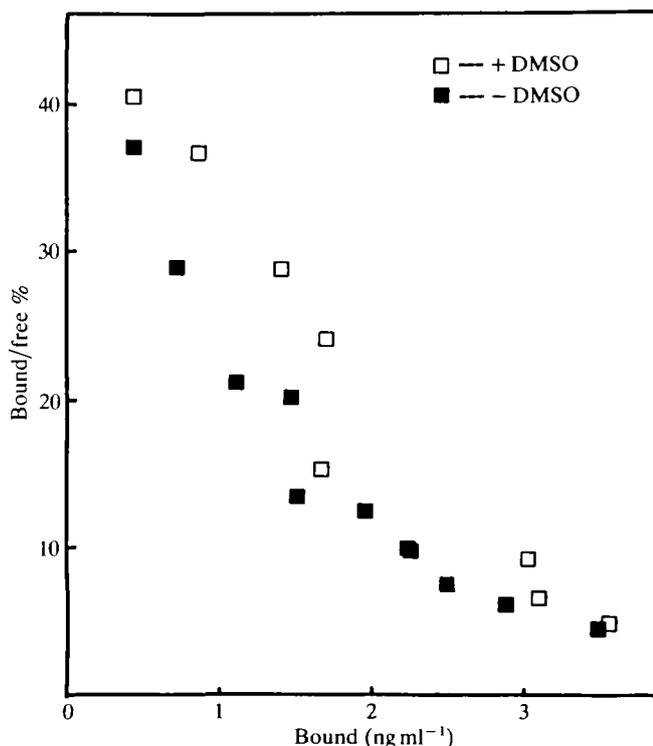


Fig. 2. Scatchard plot of erythropoietin binding. The ¹²⁵I-erythropoietin bound to TSA8 cells was counted and compared with free ¹²⁵I-erythropoietin. Scatchard plot of erythropoietin binding on TSA8 cells was done according to the equation of Scatchard. The cells with DMSO (□) or without DMSO (■) show the essentially similar affinities (0.3–0.7 nM).

affinity component, the dissociation constant is estimated to be 0.3–0.7 nM. Values essentially similar to these were obtained in B8/3 cells. It is demonstrated that the number and the affinity of the receptors do not change with the addition of DMSO. Since, without erythropoietin, formation of CFU-E-like colonies was not observed even after induction with DMSO, a certain level of receptors is likely to be required, but it is not sufficient for CFU-E-like colony formation. Increase of the receptor level is not sufficient to ensure responsiveness since this increase occurs in the absence of DMSO. Some factor(s) induced by DMSO must be required in combination with erythropoietin receptors.

(C) Optimum conditions for formation of the partly stained colonies

Since the appearance of erythropoietin receptors was not dependent on the addition of DMSO, factor(s) other than these receptors had to be investigated. Previously, we observed a fair number of partly stained colonies in addition to CFU-E-like colonies after induction. Appearance of the partly stained colonies preceded formation of the CFU-E-like colonies. Cells forming the partly stained colonies may be a stage midway between the noninduced stage of TSA8 cells and the CFU-E equivalent stage. The shape of the partly stained colonies suggested that they were formed as a result of asymmetric cell division (Mishina & Obinata, 1985). It is likely that generation of the partly stained colonies may be due to the limiting amount of factor(s) after induction. Thus, formation of the partly stained colony may be most sensitive to the amount of such factor(s). If this is true then it is possible to follow the appearance of the factor(s) in the commitment process by examining the optimum conditions for the formation of partly stained colonies. To determine these optimum conditions, the minimum exposure time required was examined. After an interval following the addition of DMSO to the culture, the culture was diluted 10-fold with fresh medium to decrease the concentration of DMSO and allowed to incubate for a total incubation time of 2 days. 0.1% of DMSO did not induce commitment. After 2 days, the cells were seeded on the semisolid medium to score the colonies. The results are shown in Fig. 3. A 6 h exposure gave maximal induction of partly stained colonies. After 6 h, the formation of the partly stained colonies decreased. In contrast, the formation of CFU-E-like colonies increased by 2 days. Thus, during prolonged exposure to DMSO, cells may accumulate sufficient factor(s) to form CFU-E-like colonies.

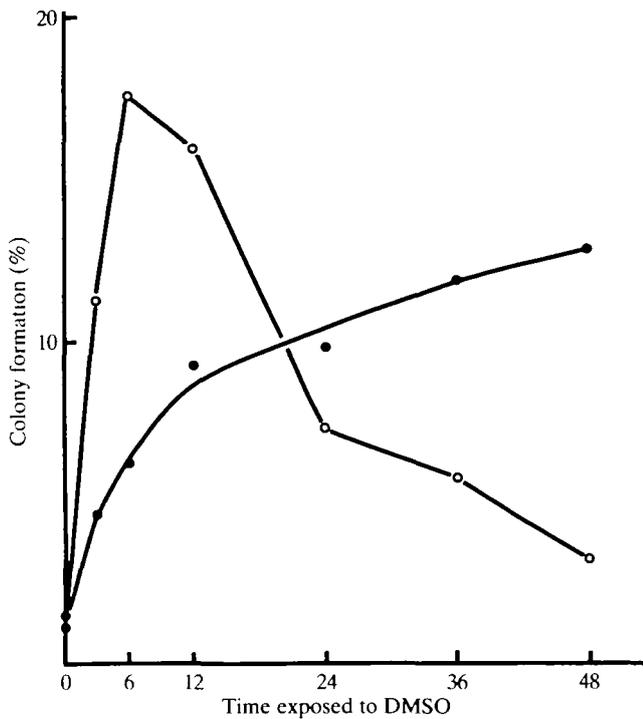


Fig. 3. Minimal requirement of inducer to induce the partly stained colonies. TSA8 cells at the concentration of 5×10^5 cells ml^{-1} were exposed to 1% DMSO in the liquid culture, then at the times indicated, the cells were diluted 10-fold with fresh medium to decrease the concentration of DMSO and were incubated until total incubation time reached 48 h. The cells were then seeded in the semisolid culture for the CFU-E assay. The appearance of the CFU-E-like colonies and the partly stained colonies at the time exposed to 1% DMSO is shown. 6 h exposure was optimum for the formation of partly stained colonies (○), while 24 h exposure was required for the maximum formation of CFU-E-like colonies (●). The percentage of colonies is expressed as the number of colonies/number of seeded cells. The values are the average of duplicates in each point and their errors are within 5%.

Discussion

TSA8 cell, a novel MEL cell line, was established from mouse spleens infected with an anaemic strain of Friend virus by Shibuya & Mak (1983). We have demonstrated that this cell line can be induced to commit to CFU-E, erythropoietin-dependent differentiated colony formation. Examination of many chemicals known as potent inducers or inhibitors for the differentiation of other MEL cells which were established from mouse spleens infected with a polycythemia strain of Friend virus indicated that TSA8 cells show significantly different responses. The differences can be explained by the fact that commitment of TSA8 cells to CFU-E occurs earlier than that of other MEL cells. We examined what factors are required for the commitment process of TSA8 cells.

The receptors for erythropoietin may be one of the most-likely factors. Thus, we estimated the number and affinity of erythropoietin binding to the surface receptors of TSA8 cells before and after induction. The highest number of receptors per cell was estimated to be approximately 500. Scatchard plot analysis showed that the binding affinity was 0.3–0.7 nM, if the receptors are expected to be a single component. The affinity of erythropoietin receptors in these cells was higher than that estimated in Friend-virus-infected spleen cells and the numbers were essentially similar to those previously reported (Krantz & Goldwasser, 1984). This may be due to different preparation of labelled erythropoietin. The essentially similar values were obtained in the other MEL cell line B8/3, which showed no responsiveness to erythropoietin. The number of receptors changed from 100 per cell to 500 per cell during the time course of the induction, but this change was also observed in cells without DMSO. Thus, the change in the number of receptors may not be dependent on induction. The time course of appearance of receptors does not coincide with that of CFU-E activity. A change in the level of receptors, similar to that in TSA8 cells, was observed in the culture of B8/3 cells with or without DMSO. Therefore, the appearance of the receptors in TSA8 cells may be required, but not sufficient, for the responsiveness of TSA8 cells to erythropoietin after induction. It is possible that the receptors acquire the ability to transmit signals into the nucleus by the erythropoietin binding only after induction. This suggests that factor(s) other than receptors may be required for the cells to respond to erythropoietin. To search for the factor(s) controlling the commitment of TSA8 cells, we examined the optimum conditions for generation of partly stained colonies since this is most sensitive to the limiting amount of the factor(s). Partly stained colonies were observed after a shorter exposure time of the cells to the inducers than is required for induction of CFU-E-like colonies. It is likely that during commitment of TSA8 cells to CFU-E, possibly intrinsic factor(s) may be induced, which act synergistically with signals transmitted through erythropoietin receptors present before induction.

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