Mechanism of erythropoietin action on the erythroid progenitor cells
induced from murine erythroleukemia cells (TSA8)

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Summary

Erythropoietin is a well-known erythroid differentiation and growth factor, but the mechanism of its action is not well understood. In this work, we have examined its mechanism of action on the erythropoietin-responsive murine erythroleukemia cells (TSA8). TSA8 cells become responsive to erythropoietin after induction with DMSO. Stimulatory effects on erythropoietin response are observed with the addition of compounds affecting the cAMP level such as forskolin, phosphodiesterase inhibitor and cholera toxin only in the presence of erythropoietin. cAMP analogues themselves show no stimulatory effect on TSA8 cells, nor does erythropoietin increase cAMP level in the cells. Thus, it is suggested that cAMP does not act as a direct second messenger for signal transduction through erythropoietin receptors, but as a stimulator of the erythropoietin receptor pathway and/or as a second messenger in combination with the receptor pathway. The mechanism for acquisition of responsiveness to growth and differentiation factors of progenitor cells is discussed.

Key words: erythropoietin, erythroid progenitor cells, erythroleukemia cell, mouse, TSA8, forskolin, phosphodiesterase inhibitor, cholera toxin.

Introduction

Growth or differentiation factors have recently been identified in several cell systems and the mechanism of their receptor-mediated signal transductions has been characterized (see review; Nishizuka, 1987).

Erythropoietin is a factor in the differentiation and proliferation of the erythroid progenitor cells such as BFU-E (blast-forming unit-erythroid) and CFU-E (colony-forming unit-erythroid) (Iscove et al. 1974). In contrast to the well-known function in erythropoiesis, its mode of action has not been well documented. Although several approaches to determining the mechanism of action of erythropoietin on these erythroid progenitor cells have been described, no conclusive evidence has yet been obtained because in most of the work a mixed population of bone marrow cells was used, thus limiting the analysis.

We have demonstrated that a murine erythroid cell line TSA8 (Shibuya & Mak, 1983) can be induced to become responsive to erythropoietin; in other words, after the addition of inducers such as dimethyl sulfoxide (DMSO), hexamethylene-bis acetamide (HMBA) and butyric acid the cells can form differentiated colonies in the semisolid medium only in the presence of erythropoietin. These colonies resemble the CFU-E from mouse fetal liver cells or bone marrow cells in several aspects (Mishina & Obinata, 1985; Noguchi et al. 1987, 1988). This in vitro system is applicable to the analysis of action of erythropoietin. We have shown that erythropoietin receptors were present before induction and that their number and affinity did not change following induction (Noguchi et al. 1987). Thus, the change in response to erythropoietin may be due to alterations in the second messenger system rather than the receptor level.

Since the receptor-mediated signal transduction system of erythropoietin has not been well documented, the intracellular mediator for the induction of differentiation response by erythropoietin must be determined. Many glycoprotein hormones elicit a biological response by activation of the adenyl cyclase system (Pierce & Parsons, 1981). Addition of erythropoietin was reported to cause biphasic cAMP levels in rabbit bone marrow cells (Chiuini et al. 1979), but to have no effect on calf or rat fetal liver cells (Graber et al. 1974). It has been reported that erythropoietin activates adenylate cyclase in membrane preparations of progenitor cells (Bonanou-Tzedaki et al. 1986). These contradictory reports on the involvement of the adenylate cyclase system in erythropoiesis may be due to a mixed population of erythroid cells being examined.
Materials and methods

Materials

Forskolin and 3-isobutyl-1-methylxanthine (IBMX, phosphodiesterase inhibitor) were purchased from Calbiochem Co., Ltd, and dibutyl cAMP, 8-bromo-cAMP and 8-bromo cGMP, from Sigma Chemical. Cholera toxin was obtained from the Chemical and Immunological Research Institute. Cyclic AMP assay kit was purchased from Yamasa Co. Purified recombinant erythropoietin was supplied by Kirin-Amgen Co. through Professor F. Takaku of University of Tokyo. IAP (Islet-activating protein) was a gift from Professor M. Ui of University of Tokyo.

Cell culture and induction with DMSO of TSA8 cells

TSA8 cells were grown in Iscove's modified Dulbecco medium supplemented with 15% fetal calf serum as described (Mishina & Obinata, 1985). For induction, DMSO (1%) was added to a suspension of the cells at a density of 2×10^6 cells ml^-1. In this work, the clone shows better inducibility (70–80%) than that in previous work (30–40%) (Mishina & Obinata, 1985; Noguchi et al. 1987). This clone was obtained through several recloning steps after long maintenance (3 years) in our laboratory.

Effect of compounds affecting cAMP level on the erythropoietin responsiveness of TSA8 cells

TSA8 cells were induced as described previously (Mishina & Obinata, 1983). Usually, 1-5–2 days after the addition of DMSO (1-0%) in a liquid culture, the cells were transferred to a semisolid medium and erythropoietin added (0.5 i.u. ml^-1) (Iscove et al. 1974). The single cells formed colonies of 16–32 cells after 2 days of culture. The differentiating colonies producing hemoglobin varied with the amount of erythropoietin added; these were counted after staining with benzidine. In usual induction, on day 1 after the DMSO addition were used. As reported earlier for the effect on erythroid progenitor cells of herbamycin, the specific inhibitor for tyrosine phosphorylation of src-related gene products (Noguchi et al. 1988), both the proliferative and the differentiation responses of the cells induced by erythropoietin can be assayed simply by measuring the colony-forming ability and the differentiated colony formation.

Critical to the characterization of the G proteins that regulate adenylate cyclase was the observation that these proteins serve as substrate for certain bacterial toxins. Cholera toxin, for example, activates the stimulatory G protein of adenylate cyclase (Gs) by catalysing the transfer of ADP-ribose to its 43×10^3 M, α-subunit (Cassell & Pfeuffer, 1978; Gilman, 1984). Pertussis toxin or IAP (islet-activating protein), on the other hand, ADP-ribosylates the 41×10^3 M, α-subunit of the inhibitory G protein Gi (Katada & Ui, 1982; Bokoch et al. 1983). As a consequence of the action of pertussis toxin, Gi is inactivated and hormonal inhibition of adenylate cyclase is blocked. Both toxins can act upon G proteins that regulate processes other than adenylate cyclase.

Thus, we first examined the effect of these toxins on the CFU-E formation. When the drugs were added to the CFU-E assay of the TSA8 cells at 2 days after induction with DMSO, cholera toxin stimulated slightly the formation of the differentiated colonies. No inhibitory or stimulatory effects were detected with the addition of pertussis toxin (data not shown). It is therefore possible that neither the inactivation of Gi nor the inhibition of adenylate cyclase is involved in the erythropoietin action. Neither toxin shows any effect on the colony formation. Thus, neither toxin affects the proliferative response induced by erythropoietin.

The stimulatory effect of cholera toxin was further examined in the TSA8 cells 1 day after induction with DMSO. With the addition of cholera toxin to the induced TSA8 cells in the semisolid medium, an increase in the benzidine-positive colony formation was observed (Fig. 1). The effect of cholera toxin on differentiation and proliferation is shown in Fig. 2. In this experiment, with increase of toxin concentration, the

assayed for cAMP. Radioimmunoassay of cAMP was done with antisera to cAMP using a Yamasa assay kit as described (Pauline & Philip, 1985).

Results

Effect of toxins altering adenylate cyclase on the erythropoietin action on CFU-E induced from TSA8 cells

TSA8 cells were induced with 1% DMSO for 1–2 days and then were transferred to a semisolid medium containing 0.5 i.u. ml^-1 of erythropoietin. Erythroid colony formation was assayed after two days of incubation at 37°C and the differentiated colonies were counted after benzidine staining (Noguchi et al. 1987). On day 1 after induction with DMSO, approximately 20% of the colonies were benzidine-positive and, on day 2, approximately 80% were benzidine-positive. To examine the stimulatory effect of the compounds, cells 1 day after induction were used. As reported earlier for the effect on erythroid progenitor cells of herbamycin, the specific inhibitor for tyrosine phosphorylation of src-related gene products (Noguchi et al. 1988), both the proliferative and the differentiation responses of the cells induced by erythropoietin can be assayed simply by measuring the colony-forming ability and the differentiated colony formation.

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The differentiated colonies of TSA8 cells formed in semisolid medium. TSA8 cells were induced with 1% (v/v) or DMSO in a liquid culture for 1 day and were transferred to a semisolid medium containing 0.5 u. ml⁻¹ of erythropoietin and incubated for 2 days at 37°C. The colonies formed were stained with benzidine. (A) Colonies formed in the presence of cholera toxin (100 ng ml⁻¹). (B) Colonies formed in the absence of toxin. The proportion of the bluish-stained colonies was greater in the presence of cholera toxin.
Erythropoietin action on erythroid progenitor cells

Fig. 2. Effect of cholera toxin on differentiation and proliferation of the erythroid progenitor cells. 4000 TSA8 cells on day 1 after induction were transferred to semisolid medium containing 0.01 i.u. ml⁻¹ of erythropoietin in the presence of different concentrations of cholera toxin. After 2 days culture, the colonies were stained with benzidine as shown in Fig. 1. The stained and unstained colonies were scored and the proportion (%) of the total colonies formed to the seeded cells was expressed as the formed colonies/plated cells (O). The proportion (%) of the stained colonies to the total colonies was expressed as benzidine-stained colonies (%) (●). In the absence of erythropoietin, benzidine-stained colonies (%) were less than 5%.

proportion of differentiated colonies increased from approximately 20 to 70%, while the colony-forming ability did not change. This stimulatory effect was dependent solely on the presence of erythropoietin. Thus, activation of the stimulatory G protein of adenylate cyclase (Gs) may affect the erythropoietin action on the differentiation response.

Effect of drugs affecting the intracellular levels of cAMP

Since cholera toxin is known to increase the intracellular level of cAMP, we examined the effect of the compounds increasing the cAMP intracellular level.

The effect of phosphodiesterase inhibitor (IBMX) was first examined (Fig. 3). In this experiment, with the increasing concentration of IBMX, the proportion of the differentiated colonies increased from approximately 35% to approximately 70% without affecting the colony formation. Again, this stimulatory effect was only observed when erythropoietin was present in the assay. Forskolin, another nonhormonal stimulator of adenylate cyclase, stimulated in a similar fashion (Fig. 3).

The synergistic or additive effect of the two compounds was examined (Fig. 4); the only difference observed was a notable decrease in the colony formation in the presence of both drugs.

Level of the intracellular cAMP after erythropoietin addition

The above results indicate that the increasing level of intracellular cAMP has a stimulatory effect on erythropoietin action on the erythroid progenitor cells. However, these stimulatory effects are only observed in the presence of erythropoietin. Cholera toxin, forskolin and IBMX themselves do not induce the differentiation or proliferation of erythroid progenitor cells in the absence of erythropoietin, nor are they potent inducers for TSA8 cells to commit to CFU-E as shown in Table 1. This result indicates that the cAMP pathway induction is not involved in the commitment event of the cells (Housman et al. 1978). The differentiation response of the progenitor cells to erythropoietin thus cannot be mimicked by simply increasing the intracellular level of cAMP with either forskolin or IBMX. This is confirmed by the fact that the addition of 8-bromo cAMP to the cells showed no differentiation effect on the induced TSA8 cells as shown in Table 1.

If erythropoietin activates adenylate cyclase and cAMP is used as the second messenger for the intracellular signalling as demonstrated in several hormone systems, the level of adenylate cyclase and therefore the intracellular level of cAMP may increase in the erythroid progenitor cells depending on the addition of erythropoietin. The intracellular cAMP levels were then determined in the TSA8 cells by radioimmunoassay at 2 days after induction with DMSO in the presence of IBMX. As shown in Fig. 5, in the presence
Fig. 4. Additive effect of the compound on the differentiation and proliferation of erythroid progenitor cells. TSA8 cells on day 1 after induction were seeded in semisolid medium in the presence of one or both compounds in the presence or absence of erythropoietin. 500 μM of forskolin and 250 nM of IBMX were used.

Fig. 5. Intracellular levels of cAMP after erythropoietin addition. TSA8 cells on day 1 after induction were incubated in the presence or absence of erythropoietin (0.5 i.u. ml⁻¹) in the presence of IBMX (500 nm). Cells were collected at an appropriate time after erythropoietin addition. Cyclic AMP was extracted by boiling the cells and the amounts were determined immunologically. ●, with erythropoietin; ○, without erythropoietin.

Table 1. Effect of the compounds on the induction of differentiation of TSA8 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Formation of benzidine-positive colony (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td></td>
<td>6 ± 1</td>
</tr>
<tr>
<td>DMSO</td>
<td>1 %</td>
<td>70 ± 5</td>
</tr>
<tr>
<td>cholera toxin</td>
<td>1 ng·ml⁻¹</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>forskolin</td>
<td>500 μM</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>IBMX</td>
<td>200 μM</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>8-bromo cAMP</td>
<td>10 nm</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

Each compound was added to the culture of TSA8 cells in the optimum concentration for stimulation of differentiation. After 2 days culture, the cells were transferred to the semisolid medium containing erythropoietin (0.5 i.u. ml⁻¹) and incubated for 2 days. The colonies formed were stained with benzidine.

Table 2. cAMP level during induction of TSA8 cells with DMSO

<table>
<thead>
<tr>
<th>Days after induction with DMSO</th>
<th>cAMP level* fmole 3×10⁻⁵</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>420 ± 40</td>
</tr>
<tr>
<td>1</td>
<td>240 ± 20</td>
</tr>
<tr>
<td>2</td>
<td>200 ± 20</td>
</tr>
</tbody>
</table>

* TSA8 cells were induced with DMSO and, at an appropriate time after induction, the cells were collected. cAMP was extracted by boiling the cells and the amounts were determined immunologically.

The responsiveness of TSA8 cells to erythropoietin was induced with DMSO and this change is not caused by an increase in the number or affinity of the receptors (Noguchi et al. 1987). Since adenylate cyclase systems did not show a notable change by 2 days after induction with DMSO when the responsiveness to erythropoietin was maximum, the activation or de novo synthesis of adenylate cyclase systems is not involved in acquisition of the responsiveness to erythropoietin. The stimulatory effect of cholera toxin or compounds affecting the
cells to erythropoietin have not been elucidated. Murine erythroleukemia cells (TSA8) are suitable for these analyses, because these cells become responsive to erythropoietin only after induction, and these changes may resemble the differentiation and proliferation processes of the erythroid progenitor cells. (Mishina & Obinata, 1985; Noguchi et al. 1987). The proliferative response and differentiation response of erythroid progenitor cells to erythropoietin are separable (Noguchi et al. 1988). Using TSA8 cells, we were able to measure separately the stimulatory effect of the compounds on the two types of erythropoietin-induced response. In the present studies, we demonstrated that the differentiation response can be mimicked by the compounds affecting the adenylate cyclase system without affecting the proliferation. However, cAMP itself cannot replace the action of erythropoietin. It is likely that the adenylate cyclase systems couple with the ligand-occupied receptor and enhance its function. This is the first report clearly showing the synergism of the adenylate cyclase system in the erythropoietin action on erythroid progenitor cells.

It has been reported that the adenylate cyclase activity of rabbit marrow immature erythroblasts was activated by erythropoietin and haemin (Bonanou-Tzedaki et al. 1986). However, forskolin failed to produce a mitogenic response in cell culture. Thus, it is not clear that activation of adenylate cyclase by erythropoietin is a key event for the erythropoietin action. On the contrary, in the TSA8 cell system, the intracellular levels of cAMP did not increase with the addition of erythropoietin (Fig. 5). A likely explanation for the discrepancy between the two sets of data may be that the adenylate cyclase system is involved in the proliferation response to the erythropoietin action but not in the differentiation response. It is possible that high levels of adenylate cyclase are required for the proliferation of the normal progenitor cells. Since TSA8 cells are transformed cells and, therefore, possess a high level of adenylate cyclase, further increase in adenylate cyclase is not required to achieve proliferation even after erythropoietin addition.

A tentative model of the process of induction of responsiveness of TSA8 cells to erythropoietin is as follows. Adenylate cyclase systems are active enough to be responsive to erythropoietin before induction, and the increase in the intracellular cAMP by compounds like forskolin, IBMX, and cholera toxin is not enough to account for the differentiation response. The addition of erythropoietin to the cells did not increase the intracellular cAMP level. Thus, the erythropoietin receptor and the stimulatory G protein (Gs) are not linked as observed in other systems. However, during induction, the effect of the compounds that affected the intracellular cAMP level increased transiently. The contribution of cAMP pathway to erythropoietin action may be to facilitate the erythropoietin receptor pathway. It is likely that the cAMP pathway is involved in the activation of protein kinase A and therefore in the phosphorylation of an acceptor protein. The acceptor protein may, in turn, modify some components of the receptor pathway and/or act synergistically with the receptor pathway. The transient contribution of the cAMP pathway during the induction of TSA8 cells as shown in Fig. 6 may reflect the stage of development of erythroid progenitor cells.

Discussion

Erythropoietin stimulates committed erythroid progenitor cells to differentiate, but the mechanism involved is unknown. An erythropoietin preparation obtained using recombinant technology made possible analysis of the surface receptors on erythroid progenitor cells (Mayeux et al. 1987; Tojo et al. 1987) or erythroleukemia cells responsive to erythropoietin (Noguchi et al. 1987; Todokoro et al. 1987). However, the mechanism of erythropoietin action and the developmental response process of the erythroid progenitor cells to erythropoietin have not been elucidated. Murine erythroleukemia cells (TSA8) are suitable for these analyses, because these cells become responsive to erythropoietin only after induction, and these changes...
the erythroid progenitor cells. It is noted that the intracellular cAMP levels decreased depending on the induction of differentiation of TSA8 cells (Table 2). Similarly, the adenylate cyclase levels decreased continuously as the cells developed (Setchenska & Arnstein, 1983). Furthermore, Bonanou-Tzedaki et al. (1986) reported that the magnitude of the response to hormonal stimulation depends on the stage of erythroid cell development and is greater in the more immature cells. The development of the response in erythroid progenitor cells to erythropoietin requires a factor combining both the receptor pathway and the cAMP pathway to produce an intracellular signal. It would be interesting to know whether this type of coupled signalling system is requisite for other blood progenitor cells. In embryonic induction, part of the cells in the embryo are responsive only to the inducer substances; this phenomenon is called competence (see review; Gurdon, 1987). The process in the TSA8 cells of becoming responsive to erythropoietin may be similar to the acquisition of competence. It is hoped that by using these new murine erythroleukemia cells responsive to erythropoietin, the molecular events of the erythropoietin action on erythroid progenitor cells can be elucidated.

Most of the work was performed in the laboratory of Professor S. Natori of University of Tokyo. We thank him for his kind support and helpful discussions. We also thank Drs M. Ui and H. Kurose for their helpful suggestions on signal transduction studies. This work was partly supported by a Grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan.

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(accepted 6 October 1988)