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Modulation of Growth Factor Receptors on Acute Myeloblastic Leukemia Cells by Retinoic Acid

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The effects of retinoic acid (RA) on the proliferation of acute myeloblastic leukemia (AML) cells were studied. AML samples were divided into three groups. Namely, RA stimulated blast colony formation by AML samples in group A and inhibited that by the samples in group B, regardless of added growth factors. For the samples in group C, RA inhibited the colonies formed by granulocyte colony-stimulating factor (G-CSF) but stimulated those by granulocyte macrophage CSF (GM-CSF). To investigate the mechanism involved, the effects of RA on growth factor receptors on AML cells were examined by flow cytometry using fluorolabeled ligands. For the samples in groups A and B, RA affected neither G-CSF receptor (GR) nor GM-CSF receptor (GMR). For the samples in group C, exposure to 10^{-7} *M* RA for 1 day clearly increased GMR, but did not affect GR. This finding supports the hypothesis that the increase of GMR is one of the causes of the stimulative effects of RA on cells cultured with GM-CSF in group C.

Key words: Retinoic acid — Acute myeloblastic leukemia — Growth factor receptor

Retinoic acid (RA) induces neutrophilic differentiation of acute promyelocytic leukemia (APL) cells,[1,2] which have a rearranged retinoic acid receptor (RAR) α gene.[3] However, the effects of RA on the proliferation of normal hemopoietic cells and acute myeloblastic leukemia (AML) cells are little known. We showed that RA had three types of effects on the colony formation by AML cells.[4] Namely, for some samples, RA stimulated the colony formation regardless of added growth factors. For other samples, RA inhibited it regardless of the factors. In a third group, RA inhibited the colonies formed by granulocyte colony-stimulating factor (G-CSF), but stimulated those by granulocyte macrophage colony-stimulating factor (GM-CSF). We supposed that RA might induce the production of some growth factors in AML cells, but attempts to detect such factors were unsuccessful. Interestingly, we found that RA inhibited the colony formation of normal bone marrow cells exposed to G-CSF and stimulated that of cells exposed to GM-CSF.[4] Therefore, the third type of response of AML cells to RA may reflect that of normal hemopoietic cells.

In this paper, in order to clarify the mechanism of the findings described above, we examined the effects of RA on growth factor receptors of AML cells.

MATERIALS AND METHODS

Blast cells Fresh leukemic cells were obtained with informed consent from eight AML patients, each assigned a unique identifying number. The clinical data of the patients are summarized in Table I. Leukemic blast cells were separated from the peripheral blood by two Ficoll-Hypaque centrifugations, as described previously.[5] Cells were cultured immediately or preserved until use in liquid nitrogen in the presence of 50% fetal calf serum (FCS), 10% dimethylsulfoxide and α-minimal essential medium (MEM).

Cell lines Four cell lines were used. OCI/AML-4, OCI/AML-5 and OCI/AML-6 were established from the peripheral blood of AML (FAB M4) patients. These lines are growth-factor responsive and their characteristics were reported previously.[6,7] NB4 is a factor-independent cell line established from APL (FAB M3) cells with t(15;17) by Lanotte *et al.*[8]

RA and growth factors All-*trans*-RA (Sigma Chemical, St. Louis, MO) was prepared just before use in 100% ethanol and diluted into growth medium at desired concentrations. The final concentration of ethanol in the cultures did not affect cell growth. All the procedures involving RA were performed in subdued light and culture plates containing RA were covered with aluminium foil.

Recombinant human G-CSF and GM-CSF were gifts from Drs. S. Clark and G. Wong (Genetics Institute, Cambridge, MA). G-CSF and GM-CSF were used at final concentrations of 100 and 500 units/ml respectively, which supported optimal growth of AML cells.

Cell culture assay Cells were plated in 96-microwell plates (Linbro, Flow Lab, McLean, VA) in 0.1 ml of α-MEM containing 20% FCS, 0.8% methylcellulose

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with G-CSF or GM-CSF or without added CSF, in the absence or presence of increasing concentrations (10^{-9} to 10^{-6} *M*) of RA. The cells were incubated in a moist atmosphere at 37°C with 5% CO_2. After 5–7 days, colonies containing more than 20 cells were counted using an inverted microscope.

For some samples, cells were cultured in suspension under the same conditions described above. After 5–7 days, cell numbers were counted.

Receptor assay The difference in the ligand-binding capacity of G-CSF receptor (GR) and GM-CSF receptor (GMR) on the cells untreated and treated with RA was examined. Leukemic cells were cultured in α-MEM containing 20% FCS with or without 10^{-7} *M* RA for 1 day. The cells were washed twice in RFD1 wash buffer (R and D systems, Minneapolis, MN) and incubated with 1 μg/ml of phycoerythrin(PE)-conjugated G-CSF or GM-CSF (R and D systems) for 60 min at 4°C, according to the manufacturer and Lehn *et al.*[9] As a control, streptoavidin-PE was used. The cells were washed and analyzed by FACScan (Becton Dickinson, Mountain View, CA) using the Consort 30 system program. To check the specificity of the binding of PE-conjugated CSF, we confirmed that the pretreatment of the cells with a 100-fold molar excess of unlabeled CSF blocked the binding of PE-conjugated CSF to the cells.

We confirmed that there was no significant difference in morphological appearance, viability, or cell density between the cells treated with 10^{-7} *M* RA for 1 day and untreated cells.

Detection of mRNA for GM-CSF receptor Total cellular RNA was extracted from cells by the acid-phenol technique,[10] and Northern blot analysis was performed according to the standard method.[11] The probe was *Kpn*I-*Eco*RI fragment of human GMR α chain cDNA (a gift from Dr. D. P. Gearing).[12]

In order to detect faint mRNA, reverse-transcriptase polymerase chain reaction (RT-PCR) was done. The first strand cDNA was synthesized from 1 μg of total RNA using Moloney-mouse leukemia RT and oligo$(dT)_{18}$ primer (Clontech Inc., Palo Alto, CA). The cDNA was amplified by 34 cycles of PCR using *Taq* polymerase. The primers were oligonucleotides with the antisense sequence of GMR α chain cDNA 131–147, and with the sequence of 676–660.[12] As a control, 25 cycles of PCR using G3PDH primers (Clontech Inc.) were done. The PCR products were electrophoresed on an agarose gel,

Table I. Clinical Data of the AML Patients

Patient No.	FAB classification	WBC (/μl)	Blast cells (%)
328	M4	4,600	39
276	M2	47,500	23
440	M2	30,100	91
272	M2	22,300	28
365	M1	19,200	81
520	M2	82,300	25
232	M2	124,000	56
417	M1	5,000	36

Table II. Effects of Retinoic Acid on Colony Formation of AML Cells

Group	Patient No. Cell line	Colony number per well					
		No CSF		G-CSF		GM-CSF	
		Control	RA	Control	RA	Control	RA
A	328	0±0	0±0	70±9	110±14[a]	11±2	31±5[a]
B	276	25±3	4±1[a]	201±27	42±6[a]	120±15	75±9[a]
	440	0±0	0±0	21±4	6±1[a]	66±8	46±5[a]
	272	0±0	0±0	10±2	6±0[a]	50±4	34±4[a]
	OCI/AML-4	5±1	1±0[a]	7±1	2±1[a]	99±12	20±3[a]
	OCI/AML-5	8±2	0±0[a]	70±9	4±0[a]	111±15	15±2[a]
	OCI/AML-6	0±0	0±0	28±2	4±1[a]	25±3	5±1[a]
	NB4	301±41	31±5[a]	314±37	25±4[a]	308±44	29±4[a]
C	365	0±0	0±0	46±6	33±4[a]	49±6	81±10[a]
	520	5±1	4±1	149±11	90±9[a]	22±4	70±9[a]
	232	11±3	4±2[a]	41±6	11±3[a]	66±5	130±18[a]
	417	0±0	0±0	70±6	24±4[a]	13±3	29±4[a]

Values indicate colony number formed by blast cells without added growth factor or with G-CSF or GM-CSF, in the absence or presence of 10^{-7} *M* RA. Cells from patients, OCI/AML-4, OCI/AML-5, OCI/AML-6, and NB4 cells were plated at the density of 10^4/well, 4×10^3/well, 2×10^3/well, 10^4/well, and 3×10^3/well, respectively. Values show mean±SD of five wells. Samples were divided into three groups according to the response to RA.

a) Significantly different from the control ($P<0.05$).

stained with ethidium bromide, transferred to a nylon membrane, and hybridized with a labeled GMR probe described above. We confirmed that the number of cycles used did not reach the plateau level of PCR products.
Statistics Data are shown as mean±SD. Group data were compared by using Student's *t* test. Calculated *P* values of less than 0.05 were regarded as significant.

RESULTS

Effect of RA on blast colony formation Blast cells from patients and cell lines were tested in clonogenic assay. Table II shows the mean colony numbers formed without added factors or with G-CSF or GM-CSF, in the absence or presence of RA. The table contains the colony number only at 10^{-7} *M* RA because this concentration has a sufficient effect on colony formation.[4] As we had reported previously,[4] the samples could be divided into three groups according to the effects of RA on their colony formation. Group A: RA increased the colony number regardless of added growth factor (Pt. 328). Group B: RA decreased the colony number regardless of the factor (Pts. 276, 440, 272 and all the cell lines tested). Group C: RA decreased the colony number in the presence of G-CSF, but increased it in the presence of GM-CSF (Pts. 365, 520, 232, 417).

To confirm the finding in group C, cells were cultured in suspension for 6 days and cell numbers were counted. Fig. 1 shows the results from pt. 365. The outcome was the same as that in colony assay.

Effect of RA on GM-CSF receptor The ligand-binding capacity of receptors was examined by flow cytometry using fluorolabeled ligands. Fig. 2 shows typical histo-

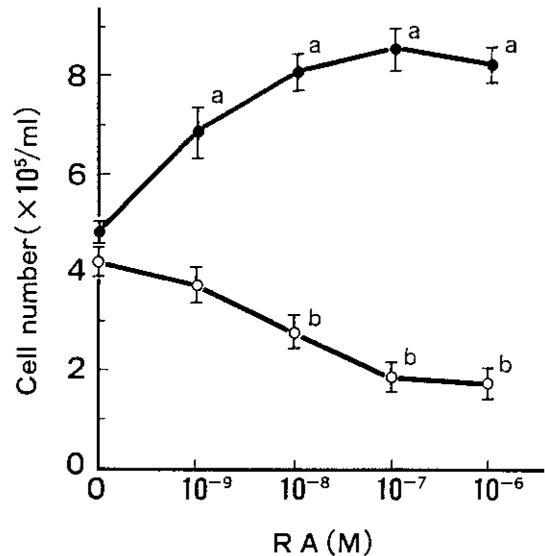

Fig. 1. Retinoic acid dose-response curve for blast cells from patient No. 365 cultured (2×10^5 cells per ml) with GM-CSF (●) or G-CSF (○) in suspension. After 6 days, the cell number was counted. a, b: $P<0.05$, compared to the value without added RA in the presence of GM-CSF or G-CSF, respectively.

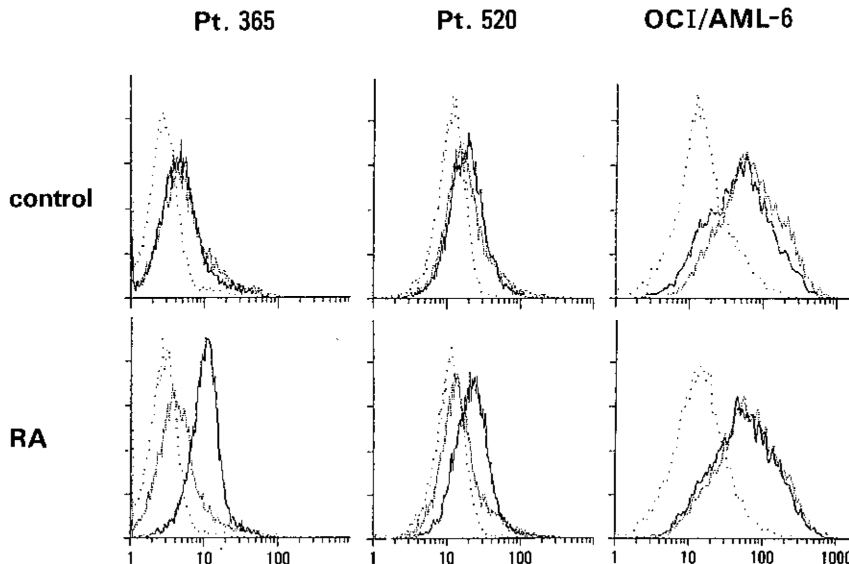

Fig. 2. Flow cytometric analysis of blast cells from pt. 365, pt. 520 and OCI/AML-6, untreated (upper column) or treated with 10^{-7} *M* RA for 1 day (lower column). Cells were incubated with PE-streptoavidin as a control (· · ·), PE-G-CSF (-----), or PE-GM-CSF (——).

Table III. Mean Values of Histograms for PE-conjugated CSF-binding to the Cells Untreated or Treated with RA

Group	Patient No. Cell lines	Mean values of fluorescence intensity					
		Control			Treated by RA		
		Control	G-CSF	GM-CSF	Control	G-CSF	GM-CSF
A	328	15.0	53.6	45.3	17.7	60.0	40.2
B	276	9.7	27.1	31.6	11.7	27.7	25.5
	440	8.1	23.5	20.7	9.2	26.5	25.0
	272	5.2	24.0	25.3	4.0	21.3	24.1
	OCI/AML-4	10.1	10.2	15.4	11.9	12.4	16.0
	OCI/AML-5	11.2	14.3	17.3	12.4	14.6	16.5
	OCI/AML-6	23.2	104.2	96.7	21.6	100.2	98.7
	NB4	27.4	42.8	37.7	28.8	40.4	41.8
C	365	3.8	8.5	7.2	3.5	7.0	13.1
	520	14.8	22.0	24.4	14.8	20.8	34.3
	232	20.8	61.0	48.2	23.3	61.4	66.3

Cells were untreated or treated with 10^{-7} *M* RA for 1 day, washed, incubated with PE-streptoavidin (as a control), PE-G-CSF or PE-GM-CSF, and analyzed by FACScan. Mean values were obtained by using the Consort 30 system program. Cells from pt. 417 were not examined.

grams of the distribution of GMR quantity on cells untreated or treated with RA. For the cells from pts. 365 and 520, which belonged to group C, GMR was increased by RA treatment, but GR was not increased. In OCI/AML-6 cells, which belonged to group B, RA treatment affected neither GMR nor GR.

For the other samples, the mean values calculated by the Consort 30 program are shown in Table III instead of the histograms. In accordance with the results described above, RA increased GMR, but not GR, on the cells in group C. GMR and GR of cells in groups A and B were not markedly affected by RA.

Effect of RA on GM-CSF receptor mRNA To see whether the increase of GMR by RA involved increase of expression of the GMR gene or not, mRNA from cells untreated and treated with RA was examined. As others had reported,[12,13] the mRNA of GMR α chain was barely detectable by Northern analysis (data not shown). Therefore, we used a semi-quantitative RT-PCR to compare the relative amounts of GMR α mRNA. Fig. 3 shows the PCR products from pt. 365's cells, in which GMR was increased by RA according to flow cytometry. As the amounts of the PCR products generated from untreated and treated cells by control primers were similar (lanes A and B, approximately 1,000 bp), it seemed that the concentrations of the cDNA samples were almost the same. The amounts of PCR products generated from the two samples by GMR α primers were almost equal (lanes C and D, approximately 550 bp). Therefore, it seemed that the amounts of GMR α mRNA in the two RNA samples were almost equal. The same result was obtained from the cells from pt. 520 (data not shown).

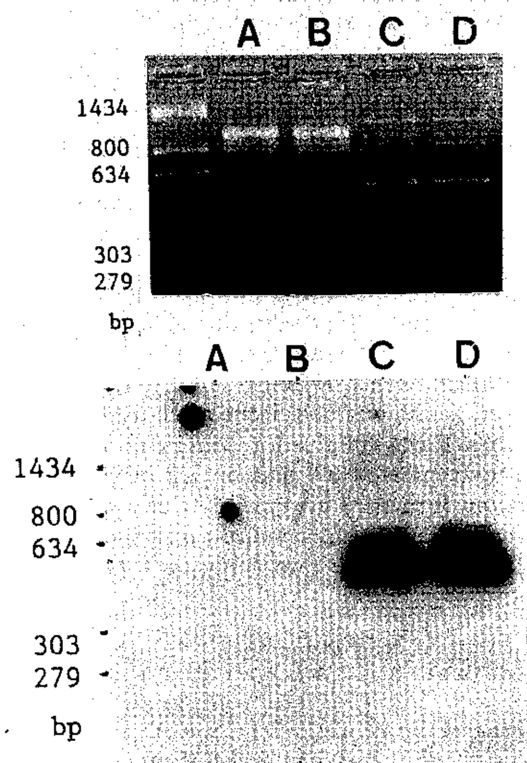

Fig. 3. Ethidium bromide-stained gel containing RT-PCR products (upper) and after Southern blotting to GM-CSF receptor α chain probe (lower). First strand cDNA was synthesized from total RNA of blast cells (pt. 365) untreated (lanes A and C) or treated with RA (lanes B and D). PCR was performed using G3PDH primers as a control (lanes A and B) and GM-CSF receptor α primers (lanes C and D).

DISCUSSION

We have studied the effects of RA on the proliferation of AML cells (not APL). In some blast populations, blasts growing with GM-CSF were stimulated by RA while the same cells growing with G-CSF were inhibited by RA. As a possible mechanism of this phenomenon, we have found that RA increased GMR on the cells which showed the response described above. We therefore tried to examine the induction of GMR α mRNA by RA. Unfortunately, Northern blot analysis with total cellular RNA failed to detect a clear band of GMR mRNA. As the amount of samples available was limited, we used a semi-quantitative RT-PCR method to compare the relative amounts of GMR α mRNA in the cells untreated and treated with RA, but no significant difference was recognized in the amounts of PCR products from the two samples. Therefore, the increase of GMR may not have occurred through the induction of GMR α chain mRNA but through an increase of translation or a prolongation of the half life, although this may require confirmation, as the method we used was not suitable to detect a small difference of quantity. Otherwise, the increase of GMR may have occurred through the induction of GMR β chain[14] mRNA, which was not examined in this study.

To examine the receptors, we used flow cytometry with fluorolabeled ligands. This method can not measure either the number of receptors or the binding affinity, for which Scatchard analysis[15] with radiolabeled ligand would be required. Therefore, we do not know whether the increase of binding capacity of GM-CSF was due to an increase of the number of receptor molecules or to an increase of affinity. However, this method can measure the distribution of the amount of receptor on cells in a sample which seems to consist of heterogeneous cells. Moreover, as this method needs a relatively small number of cells, it was suitable for examining our specimens.

As shown previously[4] and in this paper, RA stimulated colony formation by some AML cell samples regardless of added factor, but samples which showed this kind of response were very rare (only one was available in this study). In this sample, RA did not increase either GR or GMR. Some other mechanism may cause the stimulative effect of RA on this sample.

The proliferation of nine cell lines among those we have examined so far was suppressed by RA regardless of added growth factors.[16] OCI/AML-6 cells were derived from blast cells from pt. 417. The proliferation of OCI/AML-6 cells was suppressed by RA, although that of the original cells cultured with GM-CSF was stimulated by RA. The mechanism of the effect of RA on fresh blast cells seems to be different from that on cell lines.

In this experiment, we have focused on the AML samples whose proliferation was stimulated by RA during GM-CSF stimulation. Because normal hemopoietic precursor cells in bone marrow showed the same pattern of response to RA,[4] normal precursor cells may have the same mechanism as that of AML samples in this group. We tried to enrich the precursor cells in normal bone marrow using anti-CD34 antibody, but could not get a clear result in receptor assay because the obtained cells were few and the binding capacity was weak.

It has been reported that RA has various effects on cells because RA-RAR complex works as a transcriptional factor for various genes.[17] It was also reported that RA increased mRNA for epidermal growth factor (EGF) receptor in fetal rat lung cells through other protein synthesis,[18] although the mechanism was not fully established. We have found three types of response of AML cells to RA. This diversity may be due to differences of target genes of RA-RAR complex as a transcriptional factor. Further investigation of the target genes is required.

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REFERENCES

1) Huang, M. E., Ye, Y. C., Chen, S. R., Chai J. R., Lu, J. X., Zhoa, L., Gu, L. J. and Wang, Z. Y. Use of all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia. *Blood,* **72,** 567–572 (1988).
2) Castaigne, S., Chomienne, C., Daniel, M. T., Ballerini, P., Berger, R., Fenaux, P. and Degos, L. All-*trans* retinoic acid as a differentiation therapy for acute promyelocytic leukemia. Clinical results. *Blood,* **76,** 1704–1709 (1990).
3) de Thé, H., Chomienne, C., Lanotle, M., Degos, L. and Dejean, A. The t(15;17) translocation of acute promyelocytic leukemia fuses the retinoic acid receptor α gene to a novel transcribed locus. *Nature,* **347,** 558–561 (1990).
4) Tohda, S., Minden, M. D. and McCulloch, E. A. Interactions between retinoic acid and colony-stimulating factors affecting the blast cells of acute myeloblastic leukemia. *Leukemia,* **5,** 951–957 (1991).
5) Minden, M. D., Buick, R. N. and McCulloch, E. A. Separation of blast cell and T-lymphocyte progenitors in

the blood of patients with acute myeloblastic leukemia. *Blood*, **54**, 186–195 (1979).

6) Wang, C., Koistinen, P., Yang, G. S., Williams, D. E., Lyman, S. D., Minden, M. D. and McCulloch, E. A. Mast cell growth factor, a ligand for the receptor encoded by c-kit, effects the growth in culture of the blast cells of acute myeloblastic leukemia. *Leukemia*, **5**, 522–527 (1991).
7) Tohda, S., Yang, G. S., Ashman, L. K., McCulloch, E. A. and Minden, M. D. Relationship between c-*kit* expression and proliferation in acute myeloblastic leukemia cell lines. *J. Cell. Physiol.*, **154**, 410–418 (1993).
8) Lanotte, M., Martin-Thouvenin, V., Najam, S., Ballerini, P., Valensi, F. and Berger, R. NB4, a maturation inducible cell line with t(15;17) marker, isolated from a human acute promyelocytic leukemia (M3). *Blood*, **77**, 1080–1086 (1991).
9) Lehn, M., Kandil, O., Arena, C., Rein, M. S. and Remold, H. G. Interleukin-4 fails to inhibit interferon-γ-induced activation of human colostral macrophage. *Cell. Immunol.*, **141**, 233–242 (1992).
10) Chromczynski, P. and Sacchi, N. Single-step method of RNA isolation by guanidium-thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159 (1987).
11) Maniatis, T., Fritsch, E. F. and Sambrook, J. "Molecular Cloning. A Laboratory Manual" (1982). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
12) Gearing, D. P., King, J. A., Gough, N. M. and Nicola, N. A. Expression cloning of a receptor for human granulocyte-macrophage colony stimulating factor. *EMBO J.*, **8**, 3667–3676 (1989).
13) Brown, M. A., Gough, N. M., Willson, T. A., Rockman, S. and Begley, G. Structure and expression of the GM-CSF receptor α and β chain genes in human leukemia. *Leukemia*, **7**, 63–74 (1993).
14) Hayashida, K., Kitamura, T., Gorman, D. M., Arai, K., Yokota, T. and Miyajima, A. Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor: reconstitution of a high affinity GM-CSF receptor. *Proc. Natl. Acad. Sci. USA*, **87**, 9655–9659 (1990).
15) Scatchard, G. The attraction proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.*, **51**, 660–672 (1949).
16) Tohda, S., Curtis, J. E., McCulloch, E. A. and Minden, M. D. Comparison of the effects of all-*trans* and *cis* retinoic acid on the blast stem cells of acute myeloblastic leukemia in culture. *Leukemia*, **6**, 656–661 (1992).
17) Hashimoto, Y. Retinobenzoic acids and nuclear retinoic acid receptors. *Cell Struct. Funct.*, **16**, 113–123 (1991).
18) Oberg, K. C. and Carpenter, G. Dexamethasone and retinoic acid regulate the expression of epidermal growth factor receptor mRNA by distinct mechanisms. *J. Cell. Physiol.*, **149**, 244–251 (1991).