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Functional modes of retinoic acid in mouse osteoblastic clone MC3T3-E1, proved as a target cell for retinoic acid

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Mouse osteoblastic clone MC3T3-E1 was proved as a target cell for retinoic acid (RA) in bone tissues through the demonstration of RA-receptor gene expression by the northern blot analysis. The effect of RA on the cell growth of MC3T3-E1 was repressive for both subconfluent and confluent growth, whereas RA enhancement of alkaline phosphatase expression was observed at the confluent stage. This implies that RA is a regulatory factor leading osteogenesis of the cells after the confluent stage. RA exhibited simultaneously the stage-dependent effects on EGF-dependent mitogenesis: promotive at the subconfluent, but repressive at the confluent stage.

Retinoic acid; Retinoic acid receptor gene; Epidermal growth factor; Alkaline phosphatase; Osteoblastic clone; (Mouse, Calvaria)

1. INTRODUCTION

Retinoids play important roles in mammalian bone development [1]. Retinoids, in vitro, stimulate bone resorption in embryonic rat long bones [2] and inhibit collagen synthesis in chick and mouse calvaria [3]. These effects of retinoids suggest bone as a target tissue. Recently, we demonstrated by in situ hybridization for mRNAs of retinoic acid receptor (RAR) that the genes are expressed intensively and specifically in calcifying fronts of mouse finger bones during the development [4]. Thus, the specific target cells for retinoic acid (RA) and the action mechanism in bone tissues remain to be clarified.

In the present study, we examined the expression of mRNA for RAR in mouse osteoblastic clone MC3T3-E1 by the northern blot analysis. Furthermore, we surveyed the primary effects of RA on the growth and alkaline phosphatase (ALP) expression, as an essential step leading the osteogenesis, and the growth stage-dependent effects of RA on the epidermal growth factor (EGF)-dependent mitogenesis.

2. EXPERIMENTAL

2.1. *Cell culture*

Osteoblastic cells, clone MC3T3-E1 derived from C57BL/6 newborn mouse calvaria, were donated by Dr H. Kodama. The cell maintenance and the cell cultures for Northern blot analysis were performed with the procedure of Sudo et al. [5]. The preculture of MC3T3-E1 cells (4.5×10^4) was performed in 35 mm ∅ plastic dish containing 1.5 ml of α-modified minimum essential medium (α-MEM)/10% FCS for 2 days to the subconfluent growth and for 4 or 5 days to the confluent growth. The cells thus precultured were transferred into fresh α-MEM/2% FCS containing the varied RA (Sigma) concentration. All cultures were conducted at 37°C in 5% CO_2.

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2.2. *RNA preparation and Northern blot analysis*

Total RNA of MC3T3-E1 cells were prepared by the guanidinium thiocyanate/CsCl method [6]. The RNA sample (10 μg per track) was size-fractionated on 1% agarose/0.66 M formaldehyde gel and capillary-blotted on nylon membrane. The membrane was hybridized at 42°C overnight with the ^{32}P-labeled probe (10^6 dpm/ml). The membrane was washed in 2 × SSC/1% SDS for 10 min at room temperature, 1 × SSC/1% SDS for 10 min at 68°C, 2 × SSC/RNase A (1 μg/ml) for 5 min at 37°C, and finally 0.1 × SSC/1% SDS for 10 min at 68°C. Autoradiography with intensifying screen was performed for 1 day at −70°C. Drs M. Petkovich and P. Chambon donated the human RARα cDNA clone (ligand-binding region E) [7], from which the labeled RNA probe (sp. radioact. 5×10^8 dpm/μg RNA) was prepared with [α-^{32}P]UTP (Amersham).

2.3. *Assays of cell growth and ALP activity*

The cell number of MC3T3-E1 cells was counted by a Coulter counter after the treatment with 0.1% trypsin and 0.02% EDTA. For the ALP activity measurement, the cells were solubilized in 1 ml of 0.2% Nonident P-40 and sonicated in an ice bath for 5 min, the lysates were centrifuged for 10 min at 3000 rpm, and the supernatants were used for the assay with solution (pH 9.8) containing 10 mM *p*-nitrophenyl phosphate at 37°C. An aliquot of each cell lysate was subjected to the protein assay using the Bradford's method [8]. All control cells were cultured with the vehicle (0.1% (v/v) ethanol).

2.4. *Assays of thymidine incorporation and EGF binding*

To estimate the mitogenesis of MC3T3-E1 cells incubated in the absence and presence of 10 ng/ml mouse EGF (Collaborative Research), the [^{3}H]thymidine (0.25 μCi per dish, Amersham) incorporation of the cells was measured by pulsing for 1 h. For the EGF binding of MC3T3-E1 cells cultured without added EGF, the cells were fixed with 0.2% paraformaldehyde and the binding assay with ^{125}I-EGF (sp. radioact. 168.2 dpm/fmol, iodinated by the chloramine-T method [9]) was performed with the method of Kawamoto et al. [10].

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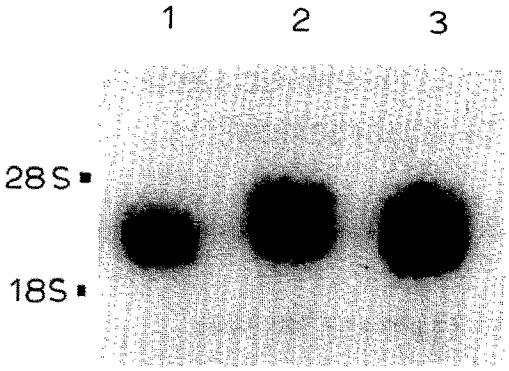

Fig.1. Northern blot analysis of RAR mRNA expressed in MC3T3-E1 cells cultured in α-MEM/10% FCS. Ribosomal RNAs (28 S and 18 S) are indicated as size markers. (1) 5th day; (2) 7th day; (3) 21st day.

3. RESULTS

3.1. *Expression of RAR gene*

The RAR mRNA (single band at about 3.1–3.3 kbp) was detected by the Northern blot analysis in MC3T3-E1 cells cultured up to the multilayer phase (21st day) (fig.1).

3.2. *Effects of RA on growth and ALP expression*

The growth of MC3T3-E1 cells proceeds, after the inoculation, from monolayer phase to multilayer phase around 4–5th day where the cells finally differentiated into osteocytes and mineralized in the culture, as observed under the basal culture conditions [5]. The ALP specific activity stayed at a very low level at the subconfluent stage in the monolayer phase, but was enhanced intensively after the cells reached the confluent stage.

The exposure of MC3T3-E1 cells to 10^{-6} M RA brought about 50% inhibition of the monolayer growth (for 72 h from 2nd day), but about 180% enhancement of the ALP specific activity at the confluent stage (for 48 h from 5th day) (fig.2). However, the exposure of the subconfluent cells (for 48 h from 2nd day) to 10^{-6} M RA did not affect the ALP specific activity significantly, and the cell growth at the confluent stage (for 72 h from 5th day) was repressed upon exposure to 10^{-6} M RA.

3.3. *Effects of RA on EGD-dependent growth and EGF binding*

As shown in figs.3A and 4, the mitogenic effect of EGF on the growth of MC3T3-E1 cells without added RA was observed at both the subconfluent (for 16 h

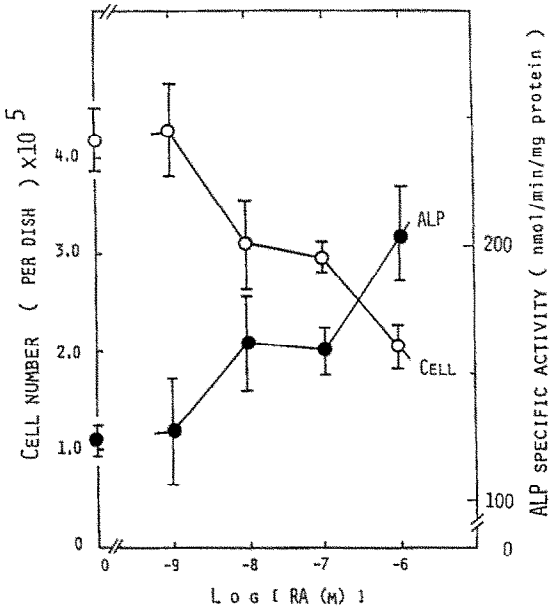

Fig.2. Effects of RA on the subconfluent growth (○) and the ALP activity (●) at the confluent stage of MC3T3-E1 cells. The data are mean values from 4 or 5 samples with the indicated SDs.

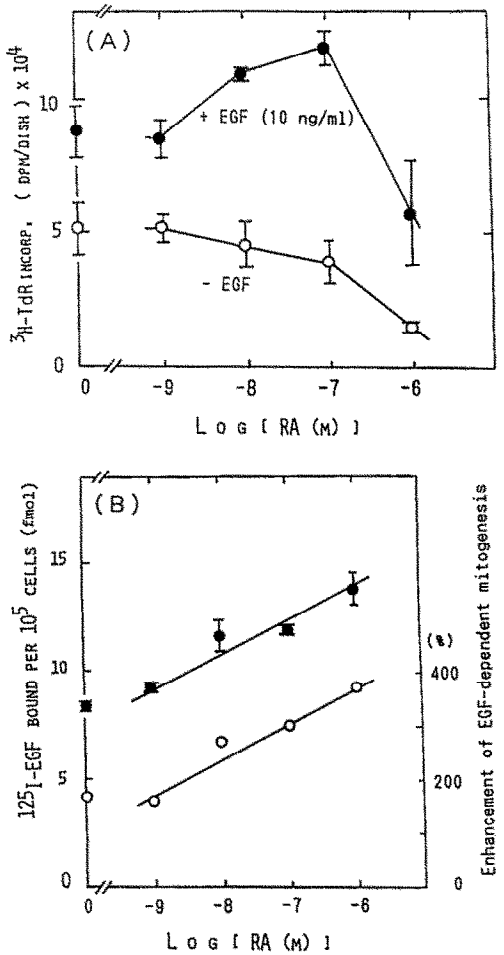

Fig.3. (A) Effect of RA on the EGF mitogenicity of MC3T3-E1 cells in the subconfluent growth. With (●) and without (○) 10 ng/ml of EGF. The data are means of duplicate cultures. (B) Dosage effect (●) of RA on ^{125}I-EGF binding capacity of MC3T3-E1 cells at the subconfluent stage. The non-specific binding level in the presence of excess cold EGF was subtracted from the total binding to give the specific binding. The stimulative effect of EGF on DNA synthesis (○) estimated from fig.3A is also inserted.

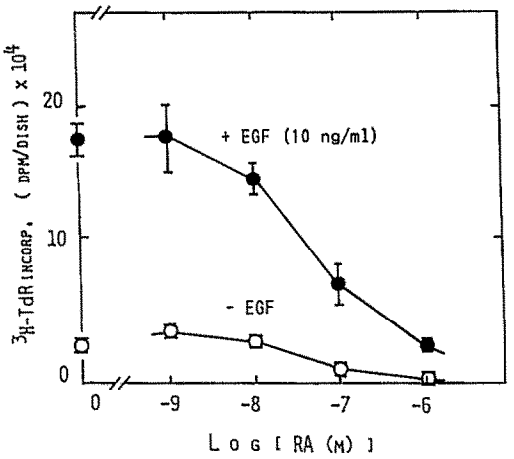

Fig.4. Effect of RA on the EGF mitogenicity of MC3T3-E1 cells in the confluent growth. With (●) and without (○) 10 ng/ml of EGF. The data are means of duplicate cultures.

from 2nd day) and confluent (for 16 h from 4th day) stage. In the combination of RA and EGF, however, the effect of RA on the EGF-dependent growth appeared to be alternative: a promotive effect in the subconfluent stage (fig.3A) whereas this was a repressive effect in the confluent stage (fig.4). The effect of RA on the binding of ^{125}I-EGF to the MC3T3-E1 subconfluent cells (for 14 h from 2nd day) is demonstrated in fig.3B. The level of specific EGF binding was enhanced in the RA-treated cells in a dose-dependent manner and, as so far tested, reached the highest level at 10^{-6} M RA. Furthermore, the enhancement of EGF binding appears to be in parallel with the RA enhancement of mitogenesis as estimated from fig.3A and illustrated in fig.3B.

4. DISCUSSION

Retinoids regulate growth and differentiation in a variety of cell lines including osteogenic cells such as osteosarcomas and chondrosarcomas [11]. RA differentiation of F9 teratocarcinoma stem cells is accompanied by the increased transcription of the specific genes [12], probably mediated through the specific nuclear RARs [7]. Since 3 subtypes of RAR (α,β,γ) cDNA identified in mouse [13] and human [7,14,15] have high homology to each other, our probe from a cDNA of human RARα possibly cross-hybridizes with those for the other two subtypes of mouse. Judged from the mRNA size estimated from the band of northern blot (fig.1), the detected mRNA are indicated as RARβ and/or RARγ. The RAR gene expression thus demonstrated in MC3T3-E1 cells presents, for the first time, direct evidence to identify the osteoblastic cells as target cells for the RA action, and substantiated the spatial expression pattern of RAR gene in mouse bone development as revealed by means of in situ hybridization [4] with RAR riboprobes as employed in the present study.

Our results with MC3T3-E1 cells showed that the functional mode of RA is remarkably dependent on the culture stage (fig.2): the confluent cells were, as in the subconfluent cells, susceptible to the growth repression effects of RA, whereas the ALP activity only of the confluent cells was enhanced by RA. It is of some interest to note that a similar mode of RA function has been reported in relatively undifferentiated bone mesenchymal cells such as UMR 201 from neonatal rat calvaria [16]. Thus, the RA enhancement of ALP expression in the confluent MC3T3-E1 cells implies that RA is a regulatory factor initiating osteogenesis of osteoblastic cells after the confluent stage. In this respect, what is noteworthy is the recent demonstration that RAR antisense DNAs inhibit ALP induction in malignant keratinocytes [17], suggesting the possibility that the RAR gene expression participates in regulatory expression of ALP likewise in MC3T3-E1 cells.

In MC3T3-E1 cells at the confluent stage, EGF alone exhibits a remarkably promotive effect on mitogenesis but a simultaneously repressive effect on ALP expression [18], as confirmed also in the present study. We elucidated the RA enhancement of the EGF-dependent subconfluent growth and of the EGF binding (fig.3). As reported with some fibroblastic and epidermal cell lines, RA enhances EGF binding with a concomitant increase of the receptor number [19,20]. The present results with subconfluent MC3T3-E1 cells indicate that the amplified mitogenic effect is possibly mediated through the enhancement of EGF binding induced by RA. On the other hand, like the case of confluent mouse fibroblast Balb/c 3T6 cells [21], the repressive effect of RA on the EGF-dependent mitogenesis of the confluent MC3T3-E1 cells was also shown (fig.4).

In conclusion, the stage-dependent functions of RA revealed in MC3T3-E1 cells are physiologically significant in view of the temporal switching of regulatory mode of RA, apparently in favour of osteogenesis after the confluent stage as characterized by RAR gene expression as well as ALP level enhancement.

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