The effects of retinoic acid on reversing the adipocyte differentiation into an osteoblastic tendency in ST2 cells, a murine bone marrow-derived stromal cell line

Jin Ding, Je-Tae Woo & Kazuo Nagai*

Department of Bioengineering, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan

(* Author for correspondence; E-mail: knagai@bio.titech.ac.jp; Fax: +81-45-9245820)

Received 18 November 2000; accepted 16 January 2001

Key words: adipocyte, alkaline phosphatase, bone, mouse ST2 cells, osteoblast, retinoic acid, stromal cell line

Abstract

Although the mouse bone marrow stromal cell line ST2 has been known to be differentiated into osteoblasts, the differentiation characteristics of the cell into adipocyte and the concerned relationship between its adipogenesis and osteogenesis remains unknown. The adipogenic induction medium which is made up of insulin, dexamethasone (DEX) and 3-isobutyl-1-methylxanthine (IBMX), stimulated the expression of an early adipogenic marker PPAR γ and a late marker GPDH in ST2 cells. The triglyceride accumulation and lipid stain level generated by the induction medium in ST2 cells was inhibited by RA with IC₅₀ at about 1 nM. The induction medium up-regulated expression of PPAR γ and GPDH was also inhibited by RA whereas RA (30 nM) exerted no effect on the cell growth. Interestingly, treatment of the cells with induction medium in the presence of RA caused a 3- or 10-fold higher in ALP activity respectively as compared to those treated with RA or the induction medium alone. RT-PCR analysis showed that such a synergistic effect of RA and the induction medium paralleled the process of inhibition on adipogenesis. Additional experiments showed that IBMX played a key role in increasing the effect of RA and ALP activity. Our results suggested that the relationship between adipogenesis and osteogenesis in ST2 cells was reciprocally interrelated and the process of adipogenesis could be potentially reversed into an osteoblastic tendency. This is the first report demonstrating that RA transforms adipogenic potential into an osteoblastic tendency.

Abbreviations: ALP, alkaline phosphatase; Dex, dexamethasone; DTT, ($\pm$)-Dithiothreitol; GAPDH, glyceraldehyde phosphate dehydrogenase; GPDH, α -glycerol-3-dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; PBS, phosphate buffer saline; PMSF, phenylmethylsulfonyl; PPAR γ , peroxisome proliferator activated receptor γ ; RA, retinoic acid; RT-PCR, reverse transcription-PCR; SDS, sodium dodecylsulfate; TG, triglyceride.

Introduction

Some bone marrow stromal precursor cells are capable of differentiating into either an osteoblast or an adipocyte. An imbalance between the plasticity of adipogenesis and osteoblastogenesis of these bipotential precursor cells results in an increase of adipocytes which occurs at the expense of osteoblast. This was postulated as one of the mechanisms to explain why morphological increase of bone marrow adipose tis-

sue in conjunction with a decrease of bone volume in patients with osteoporosis. Several studies have been conducted to elicit the reciprocal linkage and capabilities of both osteogenic and adipogenic differentiation by utilizing such bipotential types of bone marrow-derived stromal cells (Grigoriadis et al., 1988; Bennet et al., 1991; Beresford et al., 1992; Jilka et al., 1996; Rickard et al., 1996; Diascro et al., 1988); Thomas et al., 1999; Park et al., 1999).

ST2 cell line, a clone of stromal cells, was isol-

ated from the bone marrow of BC8 mice and was referred to as preadipocyte (Ogawa et al., 1988). It was reported that ST2 cells were able to differentiate into osteoblast-like cells (Yamaguchi et al., 1996; Natsume et al., 1997) or show characteristics typical of osteoblast (Otsuka et al., 1999) by induction with bone morphogenetic proteins (BMPs) or ascorbic acid respectively. However, though a preadipocyte, little evidence was obtained on the development of ST2 cell line from preadipocyte to adipocyte. Our study indicated that treatment of ST2 cells with the classical adipogenic induction medium in which insulin, dexamethasone (Dex) and 3-isobutyl-1-methylxanthine (IBMX) were combined in the form of a cocktail, was sufficient for the differentiation of ST2 into the adipocyte exhibiting the characteristic feature of the adipose phenotype.

All-trans retinoic acid (RA), a structural derivative of Vitamin A, is known to be an effective inhibitor of adipocyte differentiation (Stone et al., 1990; Kamei et al., 1994; Xue et al., 1996; Hida et al., 1998). Whereas RA also is a potent inducer of osteogenic enzyme alkaline phosphatase (ALP) which acts solely (Ng et al., 1988; Zhou et al., 1991, 1994; Heath et al., 1992) or concertedly with other growth factors e.g. TNF- α , TGF- β , IGF-I, bFGF, PTH, and PGE₂ (Ng et al., 1989; Benayahu et al., 1994; Manji et al., 1995; Freyria et al., 1999) in regulating the expression of ALP in a variety of models of cells isolated from bone especially the undifferentiated mesenchymal cell line. To investigate the concerned relationship between its adipogenesis and osteogenesis in ST2 cells, we examined the tendency of differentiation of ST2 cells into adipocytic and osteoblastic cells by employing the adipogenic induction medium and retinoic acid. We report here that the induction medium-stimulated adipocyte differentiation of ST2 cells was inhibited by RA which occurred simultaneously with an induction on the ALP activity under the adipogenic condition.

Materials and methods

Materials

All chemicals unless otherwise indicated were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). RPMI 1640 medium was purchased from Life technologies (Grand Island, NY, U.S.A.) and Fetal Bovine Serum was obtained from JRH Biosciences (Lenexa, U.S.A.). All culture dish and assay

plate were purchased from Corning Inc. (Corning, NY, U.S.A.)

Cell culture and differentiation

ST2 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Cells were maintained in the medium termed 'standard medium' of which RPMI 1640 medium was supplemented with 10% fetal bovine serum, 50 μ M β -mercaptoethanol, 40 μ g ml⁻¹ kanamycin, and 8 μ g ml⁻¹ tylosin tartrate. Cells were passaged every 2 or 3 days after reaching 70% confluence. Passages 4 through 14 were used in all experiments. Confluent cells which were about 24 hr after the cells' passage and replated at a density of 1×10^4 cm⁻² were induced to differentiate by 3 days' incubation with the 'induction medium; which contained 10 μ g ml⁻¹ insulin 0.25 μ M Dexamethasone (Dex), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) in the standard medium. After this '3-day-incubation' in the induction medium, the medium was replaced with the standard medium and the cells were maintained in the standard medium for another 3-day period. More than 30% of the cells acquired the adipocyte morphology on the 6th day after exposure of the cells to the adipogenic induction medium. To study the effects of *all trans*-retinoic acid (RA) on the induction of differentiation, retinoic acid in different concentrations was added along with the induction medium or various combinations of the components which consisted of the induction medium. When the induction medium was replaced by standard medium after the first 3 days' incubation, no further retinoic acid was added.

Cell counting

Cells were replated in a 96-well-plate treated with either RA or the induction medium alone or both. On the 6th day after inoculation, the cells were trypsinized and the total number of the cells was determined by counting after appropriate dilutions on a haemocytometer grid. Adipocyte formation were quantified by counting the lipid droplet contained cells in 30 contiguous observed fields per well under the light microscope of original magnification $\times 10$.

Assay of triglycerid (TG) content

Cells were seeded in a 24-well-plate in the standard medium and were treated with the induction medium alone or combining with various concentrations of retinoic acid. On the 6th day after induction, the cells

were washed with PBS(–) twice and fixed by methanol and then dried. The triglyceride content in the monolayer cells was determined by using colorimetric Triglyceride E-Test Wako Kit (Wako Pure Chemical Industries, Ltd. Osaka, Japan) in accordance with the manual provided by the manufactures. The protein content was determined with protein assay reagent (Bio-Rad Lab., Tokyo, Japan). The results are expressed as micrograms of TG per milligram of protein per well.

Oil Red O staining

To measure the extent of adipocyte differentiation, the cells were subcultured in a 6-well-plate in the induction medium with or without 30 nM. RA. Cells were stained with Oil Red O according to Kasturi and Joshi (1982). For histochemistry photograph, the stained cells counterstained with hematoxylin (Chayen et al., 1991).

Preparation of nuclear extracts

Nuclear proteins were extracted from the cells subcultured in 50 mm dish. Cells were scraped off and washed twice with PBS(–) and then solubilized in 100 μ l buffer A (1% Triton-X, 1.5 mM MgCL, 10 mM KCL, 0.5 mM DTT, 200 mM sucrose, 0.5 mM PMSF, 10 μ g ml^{–1} leupeptin and 10 mM Hepes-NaOH, pH 8.0) on the ice for 10 min. After centrifugation at 4000 rpm $\times$ 10 min, at 4 °C, the cell pellets were resuspended in the same volume of buffer A and centrifuged repeatedly. The clarified pellets retained were the nuclei which were resuspended in buffer B (20 mM Hepes-NaOH, 0.1 M KCl, 20% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 1 mM EDTA and 10 μ g ml^{–1} leupeptin) and were lysed by sonicating the debris on the ice for 2 min (high speed, 30 sec interval). The content of the nuclear protein was measured and rapidly frozen in liquid nitrogen and then stored at –80 °C.

Western blotting analysis

20 micrograms of nuclear protein were fractionated by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membrane. The membrane was incubated in PBS-Tween (–), 0.5% Tween 20) containing 5% skim milk at 4 °C over night. The blots were then incubated in PBS-Tween containing 5% skim milk with primary antibody, polyclonal anti-PPAR γ antibody (1:500 dilu-

tion; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, U.S.A.) for 2 hr. After being washed with PBS-Tween, the membrane was incubated with secondary antibody of horseradish peroxidase-conjugated anti-rabbit IgG (1:200 dilution, Santa Cruz Biotechnology, Inc.) in a similar conditions to that of the primary antibody for 2 hr. After being washed the blots were developed and visualized by using ECL Western blotting detection reagent (Amersham Life Sciences) following the manufacturer's instruction.

Assay of α -glycerophosphate-3-dehydrogenase activity

Cells were seeded in a 6-well-plate and the GPDH activity was determined on the base of the method of Kozak and Jensen (1974). Results are expressed as units per milligram of protein. One unit of enzyme activity corresponds to the oxidation of 1 nmol of NADH/minute.

RT-PCR analysis

Total RNA was isolated from cell cultures using TRIzol reagent (Life Technologies, N.Y. U.S.). Single-strand cDNA was reverse transcribed from 5 μ g total RNA using the Ready-To-GoTM T-Primed First-Strand Kit (Amersham Pharmacia Biotech, Inc, Piscataway, U.S.) following the procedure provided by the manufacturer. 50 μ g cDNA was amplified in 50 μ l KOD-Plus-DNA Polymerase mixture (TOYOBO CO, LTD, Osaka, Japan) which contained 0.3 μ M primer designed as follows: Mouse α -glycerol-3-dehydrogenase (GPDH) sense primer 5'-CCATCACCATCTTCC AGGAG-3' and antisense primer 5'-GCATGGACTGTGGTCATGAG-3' (expected size = 600 bp); Mouse tissue-non-specific alkaline phosphatase (TNSALP) sense primer 5'-CCCTGAAACTCC AAAAGCTC-3' and antisense primer 5'-TCTGGTGGCATCTCGTTATC-3' (expected size = 466 bp); House-keeping gene of mouse glyceraldehyde phosphate dehydrogenase (GAPDH) was adopted as an internal control with the correspondent sense primer 5'-CCATCACCATCTTCCAGGAG-3' and antisense primer 5'-GCATGGACTGTGGTCATGAG-3' (expected size = 322 bp). The total reaction cycles for ALP detection was 30 and each cycle consisted of 94 °, 60 sec; 57 °C, 60 sec and 72 °, 60 sec. The amplification of GPDH amounted to 35 reaction cycles of each including 94 °, 30 sec; 62 °C, 45 sec and 72 °C, 60 sec.

Assay of alkaline phosphatase activity

Cells were subcultured in 96-well-plate in the standard medium and then treated with the various combinations of the components of the induction medium in the absence or presence of 30 nM retinoic acid. The ALP activity was determined according to Lowry et al. (1954) with some modifications. Briefly, the cell layers were washed with PBS twice and fixed with EtOH for one minute. Then the cells were incubated at 37 °C for 30 min with 2 mM *p*-nitrophenyl phosphate in 200 μ l buffer (0.1 M glycine, 1 mM MgCl₂ and 0.1 M ZnCl₂, pH 10.5). The reaction was terminated by an addition of 100 μ l 0.05N NaOH. Results are expressed as units per microgram of protein per minute. One unit equals to the amount of enzyme that will produce 1 nmol of *p*-nitrophenol min⁻¹. The protein content was determined with protein assay reagent (Bio-Rad Lab., Tokyo, Japan).

Statistics

Data are presented as mean values $\pm$ S.D. The statistical significance of differences among groups (control and treated) was tested by one-way ANOVA. Any difference was considered significant at $p < 0.05$. Student's *t*-test were used to analyse statistically significant differences at $p < 0.05$ for time course.

Results

The inhibitory effect of retinoic acid (RA) on the differentiation of ST2 cells into adipocytes

ST-2 cells grown in the standard medium displayed a typical elongated fibroblastic morphology of a preadipocyte (Figure 1A). However, when ST-2 cells were cultured in the 'induction medium' containing a combination of insulin, IBMX and Dex, namely 'cocktail', the cells acquired rounded appearance with a microscopic accumulation of oil droplets, indicating an adipocyte phenotype appeared in the cells (Figure 1C). When all-trans RA was simultaneously added with the induction medium at the concentration of 30 nM, the cultured cells maintained fibroblastic-like shapes and the accumulation of oil droplets was dramatically inhibited (Figure 1E and F). The induction medium induced accumulation of triglyceride was inhibited by treatment with RA in a dose-dependent manner (0.03–30 nM). Its IC₅₀ was at about 1 nM and triglyceride accumulation was inhibited by about

70%. These results corresponded to the microscopic observation on the number of adipocytes developed and the density of the adipocyte clusters dispersed among the fibroblastic-like cells.

The effect of RA on the PPAR γ and GPDH expression in ST2 cells

The adipocyte differentiation program is a sequential event fulfilled with expression of stage-specific adipocyte markers. Among these markers we examined the effect of RA on the expression of PPAR γ , an early transcriptional factor and a terminal adipocyte specific marker GPDH. In Figure 3A, the results of western blotting showed that ST2 cells expressed PPAR γ in those of control cells which was activated in the presence of induction. After the co-treatment with RA, the up-regulated expression of PPAR γ was attenuated from the 2nd day throughout the induction period. As shown in Figure 3B, Treatment of ST2 cells with the induction medium stimulated the GPDH enzyme activity which was inhibited by co-treatment with RA. The inhibitory effect of RA exhibited in a dose-dependent manner with the RA concentrations ranging from 30 nM to 0.03 nM. The activity was inhibited by 90% on the 6th day after the treatment with RA at 30 nM. RT-PCR analysis also indicated that the induction medium stimulated mRNA expression of GPDH was inhibited by RA. Such an inhibitory effect corresponded to the alteration of enzyme activities from the 4th to 6th day after addition of RA (Figure 3C and D). These results suggested that the inhibitory effect exerted by RA on the ST2 cell differentiation at the early stage through antagonizing the induction medium induced up-regulation of transcriptional factor PPAR γ , which would take turns to direct and trigger the expression of other adipocyte associated markers including GPDH.

The synergistic stimulation effect of RA and the induction medium on ALPase activity in ST2 cells

Although ST2 cells are known to be preadipocytes, they have recently been reported to be differentiated into osteoblasts. To investigate the possible potentiality of RA in inducing ALP, which is one of the osteoblastic differentiation markers, we analysed ALP enzyme activity in ST2 cells which were treated with the induction medium and 30 nM RA alone or both respectively throughout the induction interval. The treatment of ST2 cells with RA alone induced ALP activity whereas the induction medium failed (Figure

Figure 1. Effects of retinoic acid (RA) on adipocyte differentiation of ST2 cells induced by the induction medium. ST2 cells were cultured in the standard medium for 1 day to approach confluence. Then the cells were switched to the induction medium in the absence or presence of 30 nMRA. For control cells, the vehicle of methanol (1%) was added to the culture of cells which was maintained in the standard medium. After 3 days' exposure to inducers, all the cultures were replaced with the standard medium. Before inoculation and 6 days after induction, cells were stained with Oil Red O and photographed as described in Materials and Methods. (A) Preadipocytes before treatment. (B) No treatment. (C) Cocktail. (E) Cocktail + 30 nMRA. Original magnification $\times 10$. (D, F) are the high magnifications of (C, E) respectively. Original magnification $\times 40$. The arrow indicated the lipid droplets stained with Oil Red O.

4A). The inducible effect of RA reached the maximal level on the 3rd day after treatment and sustained even after the RA was washed out (Figure 4A). However, when the cells were simultaneously treated with the induction medium in the presence of RA, ALP activity was synergistically induced by a 3 fold greater amount than those of RA alone (Figure 4A and 5B). Continuous treatment with RA in the induction medium sustained such synergistic inducible effect for at least 6 days comparing with those continuously treated with RA alone, which also maintained the induction effect on ALP activity (Figure 5B). The results of RT-PCR showed that mRNA level of ALP in ST2 cells was induced after 12 hr exposure to RA alone and reached the maximum within the 24 hr while no bands were detected in the following five days. In the cells of the control and those exclusively treated with the cocktail, the amount of mRNA could not be detected either. When the cells were treated with both 30 nM RA and the induction medium, the correspondent mRNA level was first detected 6 hr earlier than those treated with RA alone and reached the maximum after the 24 hr post-exposure, there were still certain, albeit

weaker, amounts of mRNA steadily detectable in the following 3 days (Figure 4B). Although there was a time lag (about 2 days), the inducible effect of RA on ALP activity corresponded to the transcriptional up-regulation of mRNA level in ST2 cells. These results suggested that the characteristic inhibitory effect of RA on the adipocyte differentiation of ST2 cells coincided with a promotion of osteoblastic tendency rather than just a simple block of adipogenesis.

Further response of ST2 cells to the treatments with RA and the induction medium

To better understand the simultaneous functions of the RA and the induction medium in adipogenesis and osteogenesis, we measured the ALP activity paralleling the triglyceride level in ST2 cells under different conditions varying in the time of treatment with RA or the induction medium. When the induction medium was added following the pre-incubation of ST2 cells with 30 nM RA for 1–3 days, the inducible effects of RA on the ALP activity was proportional to the days of pre-incubation with RA (data not shown) and the maximal effect was revealed in the cells pre-

Figure 2. Effect of retinoic acid (RA) on the triglyceride level and the formation of adipocyte in ST2 cells induced by the induction medium. ST2 cells were cultured in the standard medium for 1 day and then the confluent cells were switched to the induction medium in the presence of graded concentrations of RA (0.03, 0.1, 0.3, 1, 3, 10 and 30 nM) or as control, still maintained in the standard medium with treatment of vehicle (methanol) replaced with the standard medium for additional 3 days period. (A) The triglyceride content was estimated and (B&C) the number of both total cells and adipocytes was counted on the 6th day after the exposure of the cells to the induction medium and 30 nMRA as described in Materials and Methods. The results represent the means $\pm$ SD of triplicate determinations of two independent experiments. $P < 0.001$ vs. control.

incubated with RA for 3 days (Figure 5A and B). Vice versa, when RA was treated following the pre-incubation of ST2 cells with the induction medium for 3 days there was no inducible effect on ALP (Figure 5A and B) observed. The longer the length (within 3 days) of pre-incubation with the induction medium was, the weaker the correspondent inducible effects were observed (data not shown). On the other hand, the paralleling TG accumulation was inhibited when ST2 cells were pre-incubated with RA for 1–3 days following a replacement with the induction medium, which the extent of inhibition was approximated to the days of pre-incubation with the RA (data not shown). Pre-incubation with RA alone or both RA and the induction medium for 3 days showed no significant inducible effect on TG production was explored (Figure 5C and D). Such inhibitory effect of RA on TG production was exerted at the early stage of the first 3 days, whereas the RA treatment following the cells pre-incubated with the induction medium for 3 days did not inhibit the triglyceride accumulation (Figure 5C and D). These results suggested that in ST2 cells, similar to the effects which the induction medium ex-

erted in inducing adipocyte differentiation, the effects exerted by RA was at the early stage (at least 1–3 days), which inhibited the induction medium stimulated triglyceride accumulation and also paralleled a synergistic induction in ALP expression. This possibly resulted from the fact that the induction medium rendered ST2 cells more susceptible to the effects of RA which was initiated from the early stages.

The role of IBMX in the exertion of synergistic stimulation effect of RA and the induction medium on ALPase in ST2 cells

To investigate detailed mechanism of the synergistic stimulation effect of RA and the induction medium on the expression of ALP in ST2 cells, we measured the ALP activity in ST2 cells which the three components of the induction medium: IBMX, insulin and Dex were supplemented in an assorted way with or without 30 nM RA for 3 days, the period that such synergistic stimulation effects could be apparently observed. In Figure 6, upon treating ST2 cells with various combinations of components of the induction medium in

Figure 3. Effect of retinoic acid (RA) on the expression of PPAR γ protein and GPDH in ST2 cells induced by the induction medium. ST2 cells were cultured in the standard medium for 1 day to approach confluence. The cells were switched to induction medium in the absence (Cocktail) or presence of 30 nM RA (RA + Cocktail). The vehicle of methanol (1%) was added to the culture of cells which were still maintained in the standard medium (Control). After incubation for 3 days, all cultures were replaced with the standard medium. (A) The nuclear protein extracts prepared from each culture at different days of differentiation process were analysed by western blotting revealed by ECL technique as described in Materials and Methods. (B) GPDH activity was analysed at the indicated days and the (C) dose effects of RA (0.03 nM, 0.3 nM, 3 nM, and 30 nM) on the GPDH activity was also investigated on the 6th day after inoculation. Results represent the Mean $\pm$ SD of two separate experiments performed in triplicate. $P < 0.05$ vs. control. * $P < 0.001$ vs. control. (D) Paralleling the activity assay, total RNA was isolated and mRNA levels of GPDH and GAPDH were detected by reverse transcription-PCR as described in Materials and Methods.

the absence of RA, the ALP activity observed was at a considerably lower level with the exception that a minor stimulation generated after IBMX paired up with insulin as a supplement. However, noticeably, when in the presence of RA, dramatic simulations on ALP activity were observed among those combinations in which IBMX was involved as a component. In regards to the dexamethasone and insulin, the treatment with either one or the combination of both in the presence of RA antagonized the minor stimulation on the ALP activities arisen by treating the cells with RA alone. Therefore, the stimulation effect of RA on

the ALP activity in ST2 cells which was amplified by co-treating the cells with the induction medium, was possibly an absolute result due to the combination of RA with the respective three components of the induction medium esp. IBMX.

Discussion

ST2 cell is a preadipocyte line, which was derived from mouse bone marrow stromal cells, having potential to differentiate into osteoblasts (Ostuka et al.,

A **B**

Figure 4. The amplified stimulation effect of Retinoic acid (RA) on gene expression of ALPase and the correspondent activity in ST2 cells induced by the induction medium. Cells were cultured in the standard medium for 1 day to approach confluence. Then the confluent cells shifted into the standard medium treated with 30 nM RA (30 nM RA) alone or the induction medium containing 30 nM RA (30 nM RA + Induction medium). For the control cells, vehicle of methanol (1%) was added to the standard medium. After 3 days' incubation, all cultures were replaced with the standard medium and (A) the ALP activity was assayed at the indicated time after inoculation as described in Materials and Methods. Results are expressed as Mean $\pm$ SD of two independent experiments performed in triplicate. $P < 0.05$ vs. control. (B) Total RNA was extracted and the mRNA level of ALP was detected by reverse transcription-PCR as described in Materials and Methods. Mouse glyceraldehydes phosphate dehydrogenase (GAPDH) primers were used for the correction of equal loading of the DNAs.

1999). In the present study, we found that co-treatment with RA and the induction medium not only inhibited the adipogenesis in ST2 cells which was achieved by the induction medium (Figure 1), but also synergistically induced an osteoblastic tendency reflected on the stimulation of ALP activity (Figure 4). Our observations provided further evidence that the relationship between adipogenesis and osteogenesis in ST2 cells is reciprocally linked and the process of adipogenesis could be potentially reversed into an osteoblastic tendency. Bone marrow stromal cells include fibroblasts, osteogenic cells, and adipocytes. These cells

are known to be derived from multipotential stem cells in the bone marrow (Owen, 1988). Several studies have been conducted to investigate the reciprocal relationship of osteogenic and adipogenic differentiation by using multipotential cell types of bone marrow-derived stromal cells (Beresford et al., 1992; Park et al., 1999; Nuttallet et al., 1998). In our studies, treatment with the induction medium at an early stage (the first 3 days of the total 6days interval) was essential for the commitment of ST2 cells into adipogenic differentiation. The TG accumulation was inhibited by RA addition which synchronized or preceded the

Figure 5. The parallel comparisons between the ALP activity and triglyceride level which reflect the response of ST2 cells to the treatments with RA and the induction medium under different incubation conditions. Cells were cultured in the standard medium for 1 day to approach confluence. Then the cells were treated with RA and cocktail in various ways. The left side Table E listed the culture conditions representing in the upper graph A and C. The period of pre-incubation was 3 days (3d) and the followed replacement incubation also lasted 3 days (3). The right side Table F listed the culture conditions of continuous treatment with RA or the induction medium or both, which represented in the upper graph B and D. The ALP activity and the correspondent triglyceride level were assayed as described in Materials and Methods on the indicated days. Results are expressed as Mean $\pm$ SD of two independent experiments performed in triplicate. $P < 0.05$ vs. control.

treatment with induction medium (Figure 5 C and D). Such inhibitory effects were not observed when RA was added following the 3 days' pre-treatment with the induction medium (Figure 5C). On the other hand, paralleling the effects of RA on TG production, RA induced ALP activity in ST2 cells especially with the induction medium when both were treated at the early stage (the first 3 days) simultaneously (Figure 4A) or the replacement of the induction medium followed the

RA pre-treatment for 3 days (Figure 5A). Although continuous treatment (the thorough interval of 6 days) of the cells with both RA and the induction medium could produce a maintained stimulation on ALP activity (Figure 5B) comparing with those correspondent treatment only at the early stage (the first 3 days) (Figure 4A), treatment of ST2 cells with RA following the 3 days' pre-incubation with the induction medium failed to induce ALP activity (Figure 5A). These res-

Figure 6. The amplified stimulation effect of Retinoic acid (RA) on ALP activity in ST2 cells induced by various re-sorted components of the induction medium. Cells were cultured in the standard medium for 1 day to approach confluence. Then the cells were shifted into the RPMI + 10% FBS medium containing the indicated combinations of the components of the induction medium respectively. Meanwhile RA (30 nM) was added into the corresponding indicated culture. For the control cells, vehicle of methanol (1% v/v) was supplemented with the standard medium. On the 3rd day after inoculation, ALP activity in different treatments was determined as described in Materials and Methods. Results are expressed as Mean $\pm$ SD of two independent experiments performed in triplicate. $P < 0.05$ vs. control.

ults suggested that the length of first 3 days ought to be considered as a turning point for both adipogenic differentiation induced by cocktail and ALP expression in ST2 cells which was induced by RA and heightened by simultaneous treatment with cocktail. In addition, it also seemed that if ST2 cells reached the commitment of differentiation into one direction (either osteogenic or adipogenic tendency), the cells were unable to differentiate into any directions other than the determined one.

Peroxisome proliferator activated receptor γ (PPAR γ), which acts sequentially and cooperatively to trigger adipocyte-specific gene expression such as LPL, aP2, adipsin, is an essential transcriptional factor for the differentiation of adipocyte progenitors into adipocytes. This transcriptional factor could only be modulated by heterodimerizing with retinoid-X receptor (RXR). RXR acts a permissive partner in the PPAR γ -RXR heterodimer, however a nonpermissive inactive partner in heterodimers with retinoic acid receptor (RAR) (Fajas et al., 1998). RA is known to block the expression of PPAR γ stimulated by transcriptional factor such as CEBP γ (Schwarz et al., 1997) and thus inhibit adipogenic differentiation. In

our studies the treatment of ST2 cells with RA inhibited adipogenic differentiation stimulated by the induction medium through blocking the expression of PPAR γ . This is supported by the fact that RA is unable to inhibit the adipogenic differentiation at the later stage when the cells have already been committed due to the induction medium treatment. Moreover the effect of RA was also influenced by relative availability of RAR and RXR receptor in the cell. The up-regulation of RAR expression by RA treatment may permit RXR to form inactive heterodimers with RAR rather than with PPAR γ hence, weaken the adipocyte induction.

Retinoic acid is able to induce the increase of ALP activity in a variety of cells (Gianni et al., 1991, 1993a, 1993b; Scheibe et al., 1991; Keidel et al., 1993; San Miguel et al., 1998, 1999). Among all these cells studied, the induction of ALP was observed at least 48 hr after the treatment with retinoic acid, identical with present results with ST2 cells (Figure 5B). In our experiments, treatment of ST2 cells with RA alone at concentration of 30 nM slightly elevated the ALP activity. However, simultaneous treatment with RA and the induction medium synergistically increased

the activity of ALP in ST2 cells. Several combination experiments of each component of the induction medium showed that the three components of the induction medium exerted different effects on the ALP activity. Among these components, IBMX was necessary to produce this synergistic ALP induction. IBMX is a cAMP phosphodiesterase inhibitor that increases the intracellular cAMP level by inhibiting the degradation of the cAMP produced within the cell. Several studies undertaken ensured that ALP activity could be up-regulated by treatment with cAMP phosphodiesterase inhibitor including IBMX in mouse L cells and neonatal mouse calvarial bone cells (Firestone et al., 1981, YEE et al. 1986, Brown et al., 1990). In L929 mouse fibroblasts, RA is incapable of inducing ALP activity at lower concentrations (less than 1 mM), but co-treatment of the cells with cAMP synergistically increased the activity of ALP. This synergistic effect in the induction of ALP may result from that cAMP sensitised the cells to the action of RA. In ST2 cells, at the concentration of 0.5 mM, IBMX alone exerted no effects on the ALP activity and the synergistic effect of RA and IBMX on ALP expression was observed when the cells were co-treated with both agents, thus the synergistic effects of RA and IBMX may result from which IBMX sensitise the ST2 cells to the action of RA. Though the level of ALP mRNA was not accurately quantified or qualified, co-treatment with the induction medium and RA generated the appearance of detectable mRNA 6 hr earlier and 3 days longer than those treated with RA alone. Such modulation of ALP gene expression implied the possibility that the transcriptional rate was elevated so that more nascent ALP mRNA was produced. Because the combination effects of two agents prolonged steady level of ALP mRNA was detected, we could not rule out a possibility in increasing the stability of the mature mRNA, which perhaps was mediated by the de novo expression of a mRNA stabilizing factor (Kyeyune-Nyombi et al., 1991). Taken together, the synergistic effects of RA and IBMX on the expression of ALP in ST2 cells might be in part regulated at the transcriptional or post transcriptional level.

Acknowledgements

We thank Dr S. Takeshita (Washington University), Dr M. Mie and Miss E. Otsuka (Tokyo Institute of Technology) for their helpfulness in preparing the experiment procedures. Thanks to Dr M. Takami (Showa

University) and all the members in the Bone Group of our lab for their kind helpfulness in designing this works. This work was supported in part by Grant-in-Aid for Scientific Research on Priority Areas (B), Grant-in-Aid for Scientific Research (C) and the Ministry of Education, Science, Sports and Culture of Japan.

References

- Benayahu D, Fried A, Shamay A, Cunningham N, Blumberg S & Wientroub S (1994) Differential effects of retinoic acid and growth factors on osteoblastic markers and CD10/NEP activity in stromal-derived osteoblasts. *J Cell Biochem* 56: 62–73.
- Bennett JH, Joyner CJ, Triffitt JT & Owen ME (1991) Adipocytic cells cultured from marrow have osteogenic potential. *J Cell Sci* 99: 131–139.
- Beresford JN, Bennett JH, Devlin C, Leboy PS & Owen ME (1992) Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. *J Cell Sci* 102: 341–351.
- Brown NA, Stofko RE & Uhler MD (1990) Induction of alkaline phosphatase in Mouse L cells by overexpression of the catalytic subunit of cAMP-dependent protein kinase. *J Biol Chem* 265: 13181–13189.
- Chayen J & Bitensky L (1991) *Practical Histochemistry* (pp. 114–115) Wiley and Sons Ltd., Chichester, U.K.
- Diascro DD, JR, Vogel RL, Johnson TE, Witherup KM, Pitzenger SM, Rutledge SJ, Prescott DJ, Rodan GA & Schmid A (1998) High fatty acid content in rabbit serum is responsible for the differentiation of osteoblasts into adipocyte-like cells. *J Bone Miner Res* 13: 96–106.
- Firestone GL & Heath EC (1981) The cyclic AMP-mediated induction of alkaline phosphatase in mouse L-cells. *J Biol Chem* 256: 1396–1403.
- Freyria AM, Ronzière, Roche S, Rousseau C & Herbage D (1999) Regulation of growth, protein synthesis, and maturation of fetal bovine epiphyseal chondrocytes grown in high-density culture in the presence of ascorbic acid, retinoic acid, and dihydrocytochalasin. *J Biol Chem* 274: 84–98.
- Gianni M, Studer M, Carpani G, Terao M & Garattini E (1991) Retinoic acid induces liver/bone/kidney-type alkaline phosphatase gene expression in F9 teratocarcinoma cells. *Biochem J* 274: 673–678.
- Gianni M, Zanotta S, Terao M, Garattini S & Garattini E (1993a) Effects of synthetic retinoids and retinoic acid isomers on the expression of alkaline phosphatase in F9 teratocarcinoma cells. *Biochem Biophys Res Commun* 196: 252–259.
- Gianni M, Terao M, Sozzani S & Garattini E (1993b) Retinoic acid and cyclic AMP synergistically induce the expression of liver/bone/kidney-type alkaline phosphatase gene in L929 fibroblastic cells. *Biochem J* 296: 67–77.
- Grigoriadis AE, Heersche JNM & Aubin JE (1988) Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. *J Cell Biol* 106: 2139–2151.
- Heath JK, Suva LJ, Yoon K, Kiledjian M, Martin TJ & Rodan GA (1992) Retinoic acid stimulates transcriptional activity from the alkaline phosphatase promoter in the immortalized rat calvarial cell line, RCT-1. *Mol Endocrinol* 6: 636–646.

- Hida Y, Kawada T, Kayahashi S, Ishihara T & Fushiki T (1998) Counteraction of retinoic acid and 1,25-dihydroxyvitamin D3 on up-regulation of adipocyte differentiation with PPAR γ ligand, an antidiabetic thiazolidinedione, in 3T3-L1 cells. *Life Sci* 62: 205–211.
- Ji XH, Chen D, Xu C, Harris SE, Uundy GR & Yoneda T (2000) Patterns of gene expression associated with BMP-2-induced osteoblast and adipocyte differentiation of mesenchymal progenitor cell 3T3-F442A. *J Bone Miner Metab* 18: 132–139.
- Jika RL, Weinstein RS, Takahashi K, Parfitt AM & Mangolagas SC (1996) Linkage of decreased bone mass with impaired osteoblastogenesis in a murine model of accelerated senescence. *J Clin Invest* 97: 1732–1740.
- Kamei Y, Xu L, Heinzel T, Torchia J, Kurokawa R, Glass B, Lin SC, Heyman RA, Rose DW, Glass CK & Rosenfeld MG (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* 85: 403–414.
- Kasturi R & Johsi VC (1982) Hormonal regulation of stearyl coenzyme A desaturase activity and lipogenesis during adipose conversion of 3T3-L1 cells. *J Biol Chem* 257: 12224–12230.
- Keidel S, Szradenings M & Mueller WH (1993) In vivo biological activity of retinoids partially correlates to their affinity to recombinant retinoic-acid-binding protein 1. *Eur J Biochem* 212: 13–26.
- Kozak LP & Jensen JT (1974) Genetic and developmental control of multiple forms of L-glycerol 3-phosphate dehydrogenase. *J Biol Chem* 249: 7775–7781.
- Kyeyune-Nyombi E, Lau KH, Baylink DJ & Strong DD (1991) 1,25-Dihydroxyvitamin D3 stimulate both alkaline phosphatase gene transcription and mRNA stability in human bone cells. *Arch Biochem Biophys* 291: 316–325.
- Lowry OH, Roberts NR, Wu ML, Hixon WS & Crawford EJ (1954) The quantitative histochemistry of brain II Enzyme measurements. *J Biol Chem* 207: 19–20.
- Manji SS, Zhou H, Findlay DM, Martin TJ & Ng KW (1995) Tumor necrosis factor- α facilitates nuclear actions of retinoic acid to regulate expression of the alkaline phosphatase gene in preosteoblasts. *J Biol Chem* 270: 8958–8962.
- Meunier PJ, Aaron J, Edouard C & Vignon G (1971) Osteoporosis and the replacement of cell populations of the marrow by adipose tissue. *Clin Orthop Res* 80: 147–154.
- Natsume T, Tomita S, Iemura S, Kinto N, Yamaguchi A & Ueno N (1997) Interaction between soluble type I receptor for bone morphogenetic protein and bone morphogenetic protein-4. *J Biol Chem* 272: 11535–11540.
- Ng KW, Gummer PR, Michelangeli VP, Bateman JF, Mascara T, Cole WG & Martin TJ (1988) Regulation of alkaline phosphatase expression in a neonatal rat clonal calvarial cell strain by retinoic acid. *J Bone Miner Res* 3: 53–61.
- Ng KW, Hudson PJ, Power BE, Manji SS, Gummer PR & Martin TJ (1989) Retinoic acid and tumour necrosis factor- α act in concert to control the level of alkaline phosphatase mRNA. *J Mol Endocrinol* 3: 57–64.
- Nuttall ME, Patton AJ, Olivera DL, Nadeau DP & Gowen M (1998) Human trabecular bone cells are able to express both osteoblastic and adipocytic phenotype: implications for osteogenic disorders. *J Bone Miner Res* 13: 371–382.
- Ogawa M, Nishikawa S, Ikuta K, Yamamura F, Naito M & Takahashi K (1988) B cell ontogeny in murine embryo studied by a culture system with the monolayer of a stromal cell clone, ST2: B cell progenitor develops first in the embryonal body rather than in the yolk sac. *EMBO J* 7: 1337–1343.
- Otsuka E, Yamaguchi A, Hirose S & Hagiwara H (1999) Characterization of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid. *Am J Physiol* 277: C132–C138.
- Owen M (1988) Marrow stromal stem cells. *J Cell Sci Suppl* 10: 63–76.
- Park SR, Oreffo ROC & Triffitt JT (1999) Interconversion potential of cloned human marrow adipocytes in vitro. *Bone* 24: 549–554.
- Rickard DJ, Kassem M, Hefferan TE, Sarkar G, Spelsberg TC & Riggs BL (1996) Isolation and characterization of osteoblast precursor cells from human bone marrow. *J Bone Miner Res* 11: 312–324.
- San Miguel SM, Goseki-Sone M, Sugiyama E, Watanabe H, Yanagishita M & Ishikawa I (1998) The effects of retinoic acid on alkaline phosphatase activity and tissue-nonspecific alkaline phosphatase gene expression in human periodontal ligament cells and gingival fibroblasts. *J of Periodontal Res* 33: 428–433.
- San Miguel SM, Goseki-Sone M, Sugiyama E, Watanabe H, Yanagishita M & Ishikawa I (1999) Tissue-non-specific alkaline phosphatase mRNA expression and alkaline phosphatase activity following application of retinoic acid in cultured human dental pulp cells. *Arch Oral Biol* 44: 861–869.
- Scheibe RJ, Moeller-Runge I & Mueller WH (1991) Retinoic acid induces the expression of alkaline phosphatase in P19 teratocarcinoma cells. *J Biol Chem* 266: 21300–21305.
- Schwarz EJ, Reginato MJ, Shao D, Krakow SL & Lazar MA (1997) Retinoic acid blocks adipogenesis by inhibiting C/EBP β -mediated transcription. *Mol Cell Biol* 17: 1552–1561.
- Stone RL & Bernlohr D (1990) The molecular basis for inhibition of adipose conversion of murine 3T3-L1 cells by retinoic acid. *Differentiation* 45: 119–127.
- Thomas T, Gori F, Spelsberg TC, Khosla S, Riggs BL & Conover CA (1999) Response of bipotential human marrow stromal cells to insulin-like growth factors: effects on binding protein production, proliferation, and commitment to osteoblasts and adipocytes. *Endocrinol* 140: 5036–5044.
- Xue JC, Schwarz EJ, Chawla A & Lazar MA (1996) Distinct stage in adipogenesis revealed by retinoid inhibition of differentiation after induction of PPAR γ . *Mol Cell Biol* 16: 1567–1575.
- Yamakuchi A, Ishizuya T, Kintou N, Wada Y, Katagiri, Wozney JM, Rosen V & Yoshiki S (1996) Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3-G2/PA6. *Biochem Biophys Res Commun* 220: 366–371.
- Yee JA, Sutton JK, Shew RL & Olansky L (1986) Parathyroid hormone stimulation of alkaline phosphatase activity in cultured neonatal mouse calvarial bone cells: involvement of cyclic AMP and calcium. *J Cell Physiol* 128: 246–250.
- Zhou H, Hammonds RG, JR, Findlay DM, Fuller PJ, Martin TJ & NG KW (1991) Retinoic acid modulation of mRNA levels in malignant, nontransformed, and immortalized osteoblasts. *J Bone Miner Res* 6: 767–777.
- Zhou H, Manji SS, Findlay DM, Martin TJ, Heath JK & Ng KW (1994) Novel action of retinoic acid. *J Biol Chem* 269: 22433–22439.