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RETINOIC ACID AMBIVALENTLY REGULATES THE EXPRESSION OF MYOD1 IN THE MYOGENIC CELLS IN THE LIMB BUDS OF THE EARLY DEVELOPMENTAL STAGES

TAKASHI MOMOI[1,#], SACHIKO MIYAGAWA-TOMITA[1,2], SEIJI NAKAMURA[3], ICHIROU KIMURA[4] AND MARIKO MOMOI[5]

[1]Division of Development and Differentiation, National Institute of Neuroscience, NCNP, Ogawahigashi-machi, Kodaira, Tokyo 187, Japan

[2]Department of Pediatric Cardiology, The Heart Institute of Japan, Tokyo Women's Medical College, Kawadacho, Shinjuku-ku, Tokyo 162, Japan

[3]Department of Pharmacology, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

[4]Faculty of Human Science, Waseda University, Tokorozawa Saitama 359, Japan

[5]Department of Pediatrics, Jichi Medical School, Minamikawachi-machi, Kawachi-gun, Tochigi 329-04, Japan

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The expression of MyoD1 in myogenic cells located in the muscle prospective region of the limb bud at stage 20-22 was highly sensitive to retinoic acid. Unlike RAR-β, the expression of MyoD1 mRNA in the muscle precursor cells was significantly increased by retinoic acid at lower concentrations (0.1-10 nM), but inhibited by it at higher concentrations (0.1-1 μM). The ambivalent modulation of MyoD1 expression suggested that MyoD1 expression is regulated by not only the retinoic acid receptor and its response element, but also by other factors. Retinoic acid may be involved in the differentiation of the myogenic cells during early development.

Studies on the molecular mechanism of myogenic cell differentiation have been greatly facilitated by the discovery of the MyoD1 gene that is involved in the determination of myogenic cell differentiation and the activation of the promoter of the muscle-specific genes (1,2). On the other hand, although all-trans-retinoic acid (RA), the most biologically active natural metabolite of vitamin A, has an ability to induce the differentiation of various cells (3-5), there is yet little

To whom correspondence should be addressed.

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information about the physiological roles of RA in the differentiation of myogenic cells, with the exception of observations that RA induces the differentiation of P19 embryonal carcinoma cells into myogenic cells (6).

The limb bud is a good model system for investigating the development of muscle and cartilage primordia from a mixture of precursor cells (7-11). The somitic cells, which migrate into the prospective region in the proximal part of the limb bud during stages 15-18 (10,11), are already destined to differentiate into myogenic cells; they begin to differentiate into myoblasts with muscle-specific myosin at stage 25 (7), and myoblast fusion begins at stage 26 (8). On the other hand, undifferentiated mesenchymal cells are steadily proliferating in the distal tip of the limb bud, progress zone, and differentiate autonomously according to their individual positional values into chondrocytes and to form cartilages (9).

The distributions of cellular retinoic acid binding proteins (CRABPs) and retinoic acid receptors (RARs), which are considered to mediate the biological action of RA, have been investigated in the limb buds (12-16); CRABP-II, an isoform of CRABP that is specifically expressed in the muscles during the embryonal stages (17-19), is located in not only the progress zone but also found in the muscle prospective region in the limb bud at stage 23 (12). RAR-α is distributed throughout the limb bud (13), while RAR-β is located in the proximal region of the limb buds (15), in which somitic cells differentiate into myogenic cells. Furthermore, the concentration of RA (20-50 nM) in the limb bud at stage 21-23 (20) is sufficient enough to induce the expression of various genes via RARs. These results suggested that RA is involved in the normal myogenic cell differentiation during development (19).

In this study, to understand the physiological roles of RA in myogenic cell differentiation, we examined whether RA regulates MyoD1 mRNA expression in the myogenic cells in chick limb buds during the early developmental stages.

Materials and Methods

Preparation of the limb bud cells from four different regions

The limb buds during Hamburger and Hamilton stages 20-22 (21) were carefully dissected into four parts (Figure 2A); Part P including the progress zone, in which the precursors of chondrocytes are proliferating and differentiating; Part Z including ZPA that is postulated to produce the morphogen

determining the anteroposterior axis of the limb bud; and Parts M1 and M2 including the muscle prospective region, in which somitic cells are already destined to differentiate into myogenic cells (10,11).

Immunostaining

Limb bud cells at stages 20-22 were cultured on a gelatin-coated plate (Corning N.Y.). The cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, and then endogenous peroxidase activities in the cells were abolished by soaking the plated cells in 5 mM $NaIO_4$ for 10 min. After removing the $NaIO_4$, the plates were then preincubated with PBS containing 0.5 % skim milk, 2 % bovine serum albumin and 5 % heat-inactivated goat serum and then incubated with anti-RAR-χ for about 72 hrs at 4 ^{0}C. The frozen sections of limb bud at stage 23 and 26 were prepared for the immunostaining of myosin. The sections were incubated with anti-muscle-specific myosin (MF-20) for 72 hrs at 4 ^{0}C. After washing with PBS containing 0.5 % skim milk, the immunoreactivities on the limb bud sections and the limb bud cells were detected with on ABC kit (Vecstastain, Vector, USA) using diaminobenzidine as the substrate to detect peroxidase activity. The specificity of the immunostaining was examined by replacing the primary antibody with preimmune serum.

Northern Blotting

Chick RAR-β cDNA was isolated by screening the λgt10 cDNA library of the neural tube of chick embryos (stage 23) with a mouse RAR-β cDNA (a gift from Dr. P.Chambon) as a probe. The chick MyoD cDNA probe was produced by the reverse transcription-polymerase chain reaction according to the published sequence (22). The sense primer, 5'-CATGGACTTACTGGGCCCCA-3' (positions 156-175) was combined with the antisense primer 5'-TGCATGTCCGACGTGTTGAA-3' (position 259-240) in the polymerase chain reaction to generate a putative 105 base pair cDNA fragment. The DNA sequences of the fragment of chick MyoD1 and cDNA of chick RAR-β were identical to the published sequences of chick MyoD1 and RAR-β, respectively (15,22).

Cells were incubated without or with different concentrations of RA for 24 hrs. Total RNA was prepared from the cells by the guanidine thiocyanate method. The total (20 μg) was subjected to formaldehyde agarose (1 %) gel electrophoresis and then transferred to a nitrocellulose filter. The filter was hybridized with the chick RAR-β cDNA probe and chick MyoD1 cDNA probe, which were ^{32}P-labeled by a multiprimer labeling kit (Takara, Kyoto). The MyoD1 and RAR-β mRNA on the filters were assigned arbitrary numerical values based on densitometric scanning (TIAS-100, ACI Japan) of the autoradiograms.

Results

As shown in Figure 1A, the myogenic cells were myosin-positive cells in the limb bud at stage 26, but at stage 23, myosin-positive cells could not be observed anywhere in the limb buds except for the myotome (Figure 1B). The expressions of RAR-β and MyoD1 mRNA were examined in the limb buds during stages 20-26 (Figure 1C). MyoD1 mRNA was not detected during stages 20-24, and it faintly appeared at stage 25; however RAR-β mRNA was already,

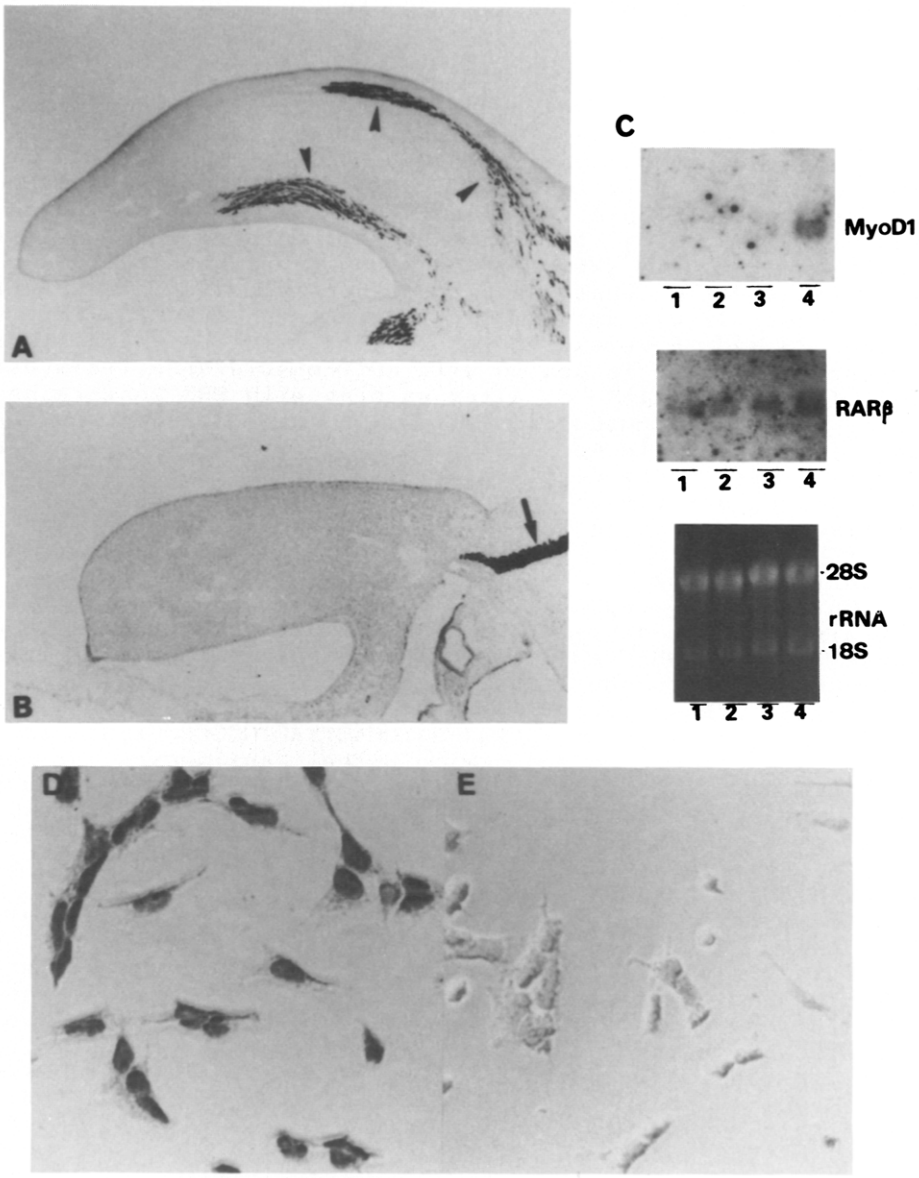

Figure 1.
The differentiation of myogenic cells during the development of limb buds.
(A) Limb bud at stage 26 was immunostained with anti-myosin. The muscle cells become myosin-positive in the limb bud. Arrowheads indicate the myosin-positive regions. (B) Limb bud at stage 23 was immunostained with anti-myosin. Myosin was positive in the myotome, but negative in the limb bud. Arrow indicates myosin-positive region. (C) The expression of MyoD1 and RAR-β mRNA during the development of limb buds. Lane 1, stage 20-22; lane 2, stage 23-24; lane 3, stage 25 and lane 4, stage 26. (D) The limb bud cells (stage 20-22) were immunostained with anti-RAR-α antiserum. RAR-α was positive in the nuclei of all the limb bud cells. (E) The limb bud cells (stage 20-22) stained with preimmune-serum.

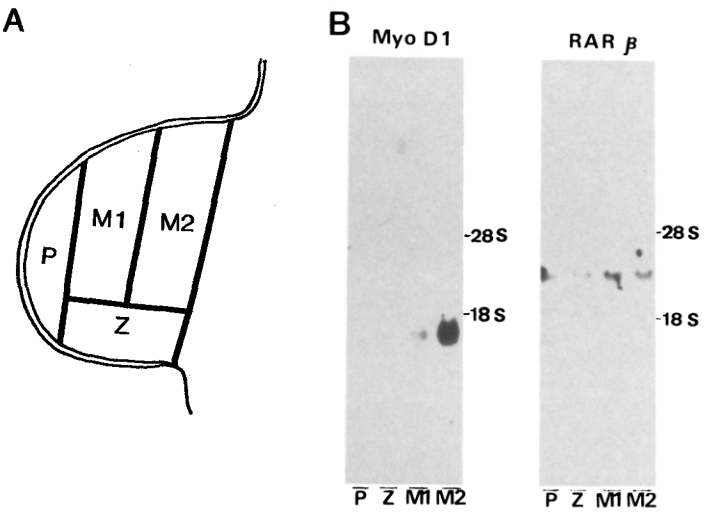

Figure 2.
The expression of MyoD1 and RAR-β mRNA in the various regions of limb buds at stage 20-22.
(A) Four regions, Part P, Z, M1 and M2, were carefully dissected from 100 wing limb buds. P, region including progress zone; Z, region including ZPA; M1, muscle prospective region and M2, muscle prospective region located in the proximal part of limb bud. (B) The expressions of MyoD1 and RAR-β mRNA in these four regions were examined by northern blot analysis.

but faintly, expressed during stages 20-22, and its content increased during development. RAR-α was positive in the all cells in the limb buds during stages 20-22 (Figure 1D).

The limb buds during stages 20-22 were carefully dissected into four regions, Part P, Part Z, Part M1 and Part M2, as shown in Figure 2A. A high amount of MyoD1 mRNA and a relatively smaller amount of it was detected in the cells in Parts M2 and M1 after 24 hrs incubation, respectively, but not observed in Parts P and Z (Figure 2B). On the other hand, RAR-β was preferentially expressed in Parts M1 and M2 rather than Parts P and Z, but the amount of RAR-β mRNA in the regions were only slightly different.

The responses of the expression of RAR-β and MyoD1 to RA were entirely different in the various regions of the limb bud (Figure 3). The expression of MyoD1 mRNA was increased in the cells in Parts M1 and M2 by the incubation with 0.1-10nM RA; In particular, the maximum level of MyoD1 mRNA expression was achieved in Part M1 at 0.1nM RA, but incubation with higher concentrations of RA(0.1-1μM) had the opposite effect, decreasing the expression (Figure 3A and C). Unlike the expression of MyoD1 in Parts M1 and M2, the expressions of MyoD1 in the cells of Parts P and Z were not induced by RA. On the other hand, the expressions of RAR-β in the cells of the four regions were

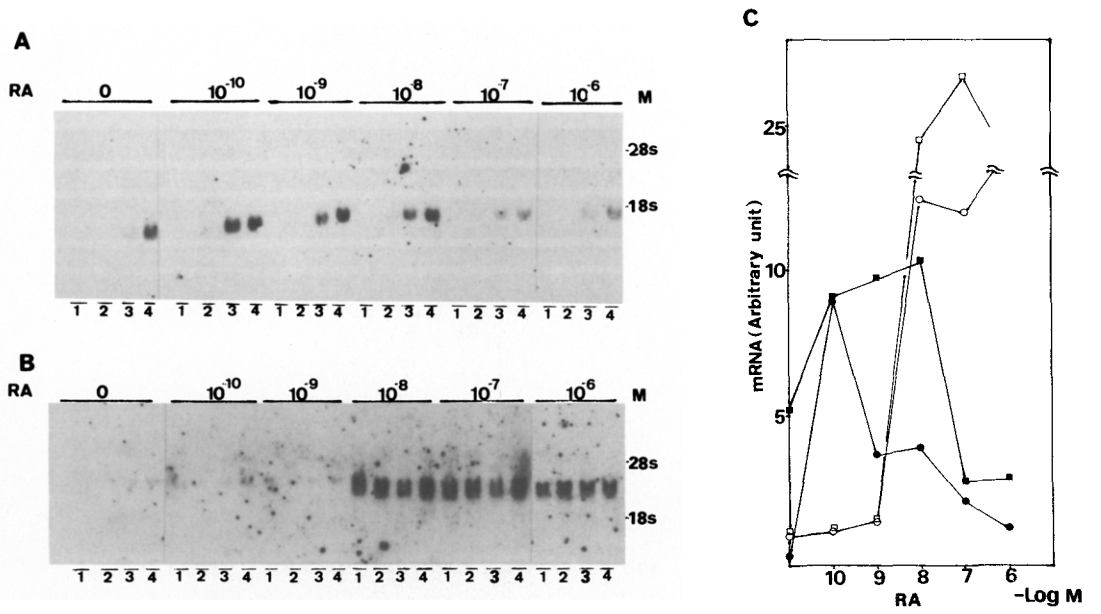

Figure 3.
The expression of the mRNAs of MyoD1 and RAR-β in the cells of the various regions of limb bud at stage 20-22 by the incubation with RA at various concentrations for 24 hrs. The expression of MyoD1 (A) and RAR-β (B) mRNA by the incubation with RA. Lane 1, Part P; lane 2, Part Z; lane 3, Part M1; lane 4, Part M2. (C) The amount of MyoD1 and RAR-β mRNA in the Parts M1 and M2 were assigned arbitrary numerical values based on densitometric scanning of the autoradiograms in (A) and (B). The MyoD1 mRNA in Parts M1 (●—●) and M2 (■—■). The RAR-β mRNA in Part M1 (○—○) and M2 (□—□).

equally sensitive to RA (Figure 3B); They were dose-dependently increased by the incubation with RA (10nM-1μM)(Figure 3C).

Discussion

The limb bud during stages 20-22, which did not express muscle-specific myosin and MyoD1 (Figure 1), was used in this experiment. The cells in Parts M1 and M2 in the limb bud during stages 20-22, spontaneously expressed MyoD1 mRNA after 24 hrs incubation, but the cells in the other parts did not (Figure 2), showing that the cells in Parts M1 and M2 contained most of the muscle precursor cells, which began to differentiate into myogenic cells during the culture period.

The response of the expression of MyoD1 to RA was different in the four limb bud regions, unlike that of RAR-β to RA (Figure 3); RA regulated the expression of MyoD1 mRNA in the cells committed to differentiate into myogenic cells in Parts M1 and M2, but did not in the precursor cells of chondrocytes in the

progress zone, although they also expressed RAR-α protein (Figure 1D) and expressed RAR-β mRNA in response to RA (Figure 3B) These results suggested that RA is involved in the differentiation of the myogenic cells in the early developmental stages via controlling the expression of MyoD1.

However, since RA not only has some stimulatory effects on the differentiation of myogenic cells from chick mesodermal stem cells at stages 3-5, but can also substitute for Hansen's node or the notochord in the induction of cartilage cells (23), and RA has some effects on the chondrogenesis in the distal region of the limb buds (24,25), we can not exclude the possibility that RA is involved in determining of the differentiation from multipotent mesodermal stem cells to monopotent myoblast and chondrocytes, along with other factors.

RA can directly regulate the expression of various genes through three isoforms of RARs, termed α, β and γ (26-28); For instance, the expression of RAR-β is positively autoregulated by RARs and the RA-responsive element (RARE) existing on its 5' flanking sequence (29). However, RARE is not detected in the flanking sequence of all the genes whose expressions are regulated by RA, for instances c-jun (30). Unlike the induction of RAR-β gene by RA, the expression of MyoD1 was ambivalently regulated by RA (Figure 3); The expression of MyoD1 was increased by RA at a low concentration, but was inhibited by RA at a high concentration.

Thus the expression of MyoD1 is regulated in a manner different from that of RAR-β expression (Figure 3), suggesting that the regulation of the expression of MyoD1 by RA is mediated by not only the system of RARs and RARE, but also involved other factors and other response elements. c-Myc and N-myc, which have the same B-HLH motif as MyoD1 and myogenine (31), are negatively regulated by RA (32,33). RA also represses the expression oct-3 via a specific repressor element existing on the 5' flanking sequence and binding proteins different from RARs (34). Furthermore, the expression of MyoD1 is repressed by several factors with mitogenic activity such as FGF and TGF-β (35). The expressions of Id and c-jun, whose gene products inhibit myogenic cell differentiation by suppressing the function of MyoD1 (36,37), are induced in the differentiation of F9 and P19 EC cells by RA (36,38). These factors may be involved in the repression of the expression of MyoD1 induced by RA.

Thus RA may be involved in the differentiation of myogenic cells in the early developmental stages by ambivalently regulating the expression of MyoD1 via both receptor-dependent and -independent processes. The relationship between the biological roles of RA and the differentiation of myogenic cells during devleopment remains to be elucidated.

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