Korean J. Zool. 38:483-489, 1995

Effect of Retinoic Acid on Membrane Fusion and Expression of Fibronectin in Chick Embryonic Myoblasts

Hye Sun Kim[1], Pil Joong Chung[1], Man-Sik Kang, Chin Ha Chung*, and Doo Bong Ha

Department of Molecular Biology and SRC for Cell Differentiation, College of Natural Sciences, Seoul National University, Seoul 151-742; [1]Department of Biological Science, College of Natural Sciences, Ajou University, Suwon 442-749, Korea

Retinoic acid was found to block membrane fusion of chick embryonic myoblasts in culture. This effect was dose-dependent and could be reversed upon removal of the agent from the culture medium. Furthermore, the retinoic acid-mediated inhibition of membrane fusion was observed with the fusion competent cells but not with the cells that had already been committed for fusion, indicating that the effect of RA is differentiation stage-specific. However, retinoic acid showed little or no effect on the ability of the cells to form bipolar shape and to align along their axes. Neither the cell proliferation nor accumulation of muscle specific proteins, such as creatine kinase and tropomyosin, was impaired significantly. On the other hand, retinoic acid blocked the differentiation time-dependent loss of fibronectin, whose process is prerequisite for myoblast fusion. These results suggest that retinoic acid acts as a specific inhibitor of membrane fusion by preventing the loss of fibronectin from the differentiating myoblasts.

KEY WORDS: Myogenesis, Fusion, Fibronectin, Retinoic Acid, Chick Myoblasts

Myogenic differentiation of skeletal muscle cells is characterized by the fusion of mononucleated myoblasts into myotubes (Bischoff and Holtzer, 1969; O'Neill and Stockdale, 1972). Concurrent with the morphological changes, a large number of muscle specific proteins, such as -actin, myosin heavy chain, creatine kinase and acetylcholine receptor, are synthesized (Konigsberg *et al.*, 1978; Nadal-Ginard, 1978). The proliferation and differentiation of muscle cells, both *in vivo* and *in vitro*, depend on their environment, in particular, extracellular diffusible molecules, such as growth factors, neuromediators and hormones (Florini *et al.*, 1991).

Retinoic acid (RA), a major metabolite in the physiological pathway of retinol metabolism, has been shown to alter the growth and differentiation of many cell types and tissues (Lotan, 1980; Sporn and Roberts, 1983). A number of reports have demonstrated that RA exerts its effect by altering gene expressions of proteins, including cAMP-dependent protein kinase and extracellular matrix proteins (Ludwig *et al.*, 1980; Bolmer and Wolf, 1982; Kenney *et al.*, 1986; Niles *et al.*, 1986). However, its effect is rather diverse with the cell types (Griep and Deluca, 1986; Strickland and Mahdavi, 1978; Jetten, 1980) and relatively little is known about its effect on embryonic muscle cells.

Fibronectin is one of the major extracellular matrix components in muscle (Stenman and Vaheri, 1978; Walsh *et al.*, 1981). The function

*To whom correspondence should be addressed.

of fibronectin in muscle tissues is not known yet, but has been suggested to be related with promotion of cell migration, regulation of cell shape, proliferation, and guidance of cell migration (Hynes, 1981). In addition, it has been reported that addition of exogenous fibronectin to rat myoblast cultures inhibits the formation of myotube (Podleski *et al.*, 1979) and the level of fibronectin in chick embryonic myoblasts decreases during the course of myogenic differentiation (Chung and Kang, 1990). Thus, it seems likely that fibronectin molecules play a key role in the regulation of the myogenic process.

In an attempt to explore the mechanism that regulates the myogenic differentiation, we examined the effect of RA on the proliferation and membrane fusion of myoblasts as well as the accumulation of muscle specific proteins in chick embryonic muscle cells in culture. We also examined the effect of RA on the expression of fibronectin in the cultured cells.

Materials and Methods

Cell culture

Myoblasts from breast muscle of 12-day-old chick embryos were prepared as described previously (Kwak *et al.*, 1989; Kim *et al.*, 1992). The cells were plated on collagen-coated culture dishes at a concentration of 5×10^5 cells/ml in Eagle's minimum essential medium (E-MEM) containing 10% (v/v) horse serum, 10% (v/v) chick embryo extract, and 1% (v/v) antibiotic/antimycotic solution. One day after cell seeding, the culture medium was changed with the same medium but containing 2% embryo extract. All-trans-RA (Sigma) was added to the culture medium at the time of medium change. At appropriate time of culture, the cells were washed with ice-cold phosphate buffered saline (PBS) and immediately fixed using a mixture of 95% ethanol, 40% formaldehyde and acetic acid (20:2:1 by volume). The fixed cells were stained with hematoxilin solution and observed under a microscope. Degree of myoblast growth and fusion was determined as described previously (Kwak *et al.*, 1989).

[^{3}H]Thymidine incorporation

DNA synthesis was measured by incorporation of [^{3}H]thymidine into trichloroacetic acid (TCA)-precipitable material. At 24 and 48 hr of culture, 1 Ci/ml of [^{3}H]thymidine was added to the culture medium and further incubated for the next 24 hr. After the incubation, the cells were freed from unincorporated radioisotopes by washing three times with ice-cold PBS and then extracted with 10% TCA. The TCA pellets were solubilized in 1% sodium dodecyl sulfate (SDS) and counted for their radioactivity using a scintillation counter.

Immunoblot analysis

Creatine kinase and tropomyosin were purified from adult chick muscle as described (Eppenberger *et al.*, 1967; Smillie, 1982). Antisera against the purified proteins were prepared by injecting into albino rabbits. Anti-fibronectin antibody was obtained from Dr. Moon (Moon *et al.*, 1994).

Myoblasts that had been cultured in the presence and the absence of RA were washed three times with ice-cold PBS, harvested by centrifugation, and disrupted by sonication for 30 sec in 50 mM Tris-HCl (pH 7.4) containing 5 mM $MgCl_2$ and 0.5 mM EDTA. The cell lysates were then subjected to electrophoresis on 10% (w/v) polyacrylamide gels containing SDS (Laemmli, 1970). The proteins in the gels were transferred onto nitrocellulose membranes or polyvinylidene fluoride (PVDF) membranes and reacted with specific antisera and then with anti-rabbit IgG conjugated with alkaline phosphatase or horseradish peroxidase (Towbin *et al.*, 1979). The immunoreactive proteins were visualized using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) or enhanced chemiluminescence system (ECL; Amersham). Proteins were assayed as described by Bradford (1976).

Results

Effect of RA on the membrane fusion

To investigate the effect of RA on myogenic differentiation, myoblasts that had been cultured for 24 hr were treated with increasing amounts of

the agent and observed under a microscope 48 hr after the treatment. The untreated, control cells fused normally to form multinucleated myotubes (Fig. 1A), while the cells treated with RA (5×10^{-5} M) were unable to form thick myotubes (Fig. 1B). This inhibition of the fusion occurred in a dose-dependent manner, and half-maximal inhibition was obtained at approximately $2\text{-}3 \times 10^{-5}$ M of RA (Fig. 2). Therefore, the effective concentration of RA seems to be much higher than the physiological concentration of RA, which is about 10^{-8} M in human plasma (DeRuyter *et al.*, 1979). However, it has been reported that more than 10^{-6} M of RA is typically needed to exert their effect in many other cultured cells (Yen *et al.*, 1984; Griep and Deluca, 1986; Raynaud *et al.*, 1988). In addition, ethyl alcohol (final conc. 1%) alone, which was used as the solvent of RA, did not show any influence on the myoblast fusion (data not shown). Thus, it appears unlikely that the effect of RA is simply due to its nonspecific toxicity.

Noteworthy is the finding that the RA-treated cells can change their morphology to bipolar shape and align their bipolar axes. Furthermore, addition of the agent to the cells at 48 hr of culture, which had already been committed for membrane fusion, showed relatively little effect on the fusion (Fig. 1C) as compared to the cells treated at 24 hr (Fig. 1B). These results suggest that the effect of RA is differentiation stage-specific. We also examined whether the inhibitory effect of RA is reversible. The cells that had been treated with the agent for the first 24 hr were transferred to the fresh medium and cultivated further for the next 24 hr. As shown in Fig. 1D, the cell fusion resumed within 24 hr. These results again indicate that the inhibition of myoblast fusion by RA is not due to a cytotoxic effect.

To determine the effect of RA on proliferation of myoblast, the cells were incubated with [^{3}H] thymidine at two different period of culture in the presence of increasing amounts of RA (Table I). This agent slightly reduced the incorporation of [^{3}H]thymidine for both of the cells cultured 24-48 hr and 48-72 hr at all concentrations tested (*i.e.*, inhibition ranged from 10 to 35%). And the most significant inhibition was observed only upon treatment of RA at 5×10^{-5} M to the cells cultured for 48-72 hr, whose incorporation of [^{3}H] thymidine was inhibited by about 35%. Because 5×10^{-5} M RA treated at 24 hr nearly completely blocked myoblast fusion while the same concentration of RA at 48 hr showed relatively

Fig. 1. Inhibition of myoblast fusion by RA and its reversal. Myoblasts were treated with 5×10^{-5} M RA at 24 hr (*i.e.*, the time of media change) (B) or 48 hr of culture (C). The cells treated with RA at 24 hr were freed of the drug at 48 hr (D). As a control, myoblasts were also cultured for 72 hr without any treatment (A). All of the photographs were obtained at 72 hr of culture.

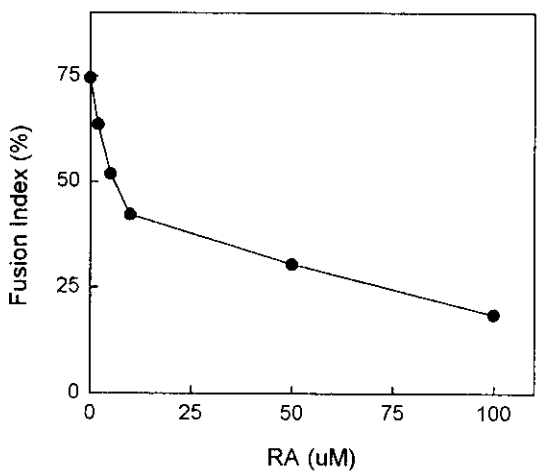

Fig. 2. Dose-dependent inhibition of myoblast fusion by RA. Myoblasts that had been cultured for 24 hr were treated with increasing amounts of RA and further cultivated for the next 48 hr. The cells were then fixed, stained, and observed under a microscope. Fusion index is defined as the percentage of nuclei in myotubes compared to total nuclei.

Table I. Effect of RA on the incorporation of [^{3}H] thymidine.

RA	[^{3}H]Thymidine incorporated 24-48 hr	48-72 hr
(M)	cpm/well (%)	cpm/well (%)
0	2919 252 (100)	4177 450 (100)
5×10^{-6}	2930 277 (100)	3416 333 (82)
1×10^{-5}	3109 298 (107)	3427 276 (82)
5×10^{-5}	2524 223 (87)	2673 252 (64)

Myoblasts that had been cultured for 24 hr were further cultivated in the presence increasing amounts of RA. To each of these cells, 1 Ci/ml of [^{3}H]thymidine was added at the time of RA-treatment or 24 hr after the treatment. They were then further incubated for the next 24 hr. After the incubation, the unincorporated radioisotopes were removed and the TCA-precipitable materials were counted by scintillation counter.

little effect on the fusion (see Fig. 1B and 1C), the differentiation stage-specific inhibition by RA must not be due to its insignificant effect on the cell proliferation.

Effect of RA on the synthesis of muscle-specific proteins

During myogenic differentiation, membrane fusion is known to occur concurrently with the induction of muscle-specific proteins, such as myosin, -actin, tropomyosin, creatine kinase, and acetylcholine receptor (Konigsberg *et al.*, 1978; Nadal-Ginard, 1978). A number of reports have demonstrated that the morphological differentiation of cultured myoblasts can be uncoupled from the synthesis of muscle-specific proteins (Paterson and Prives, 1973; Emerson and Becker, 1975; Merlie and Gros, 1976). To examine whether RA may also affect biochemical differentiation of myoblasts, the cells were cultured with and without the agent followed by immunoblot analysis using the anti-creatine kinase and anti-tropomyosin antisera. As shown in Fig. 3, both creatine kinase and tropomyosin were accumulated in the RA-treated cells although their levels were slightly lower than those of the untreated control cells. Thus, the drug appears to block membrane fusion with relatively little effect on the synthesis of muscle specific proteins. These

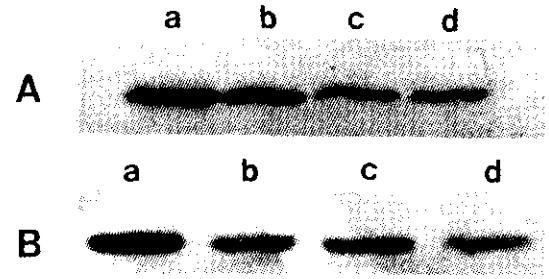

Fig. 3. Effect of RA on the synthesis of creatine kinase (A) and tropomyosin (B). Myoblasts cultured for 24 hr were incubated in the absence (lane a) and presence of 5×10^{-6} M (b), 5×10^{-5} M (c), 1×10^{-4} M (d) of RA. The cells were then further cultured for the next 48 hr followed by immunoblot analysis using anti-creatine (A) and anti-tropomyosin antisera (B) as described under "Materials and Methods".

results suggest that the inhibition of myoblast fusion by RA occurred via a different pathway from the induction of muscle specific gene expression.

Effect of RA on the level of fibronectin

Fibronectin has been suggest to play a key role in the regulation of myoblast fusion (Podleski *et al.*, 1979; Hynes, 1981; Chung and Kang, 1990). In addition, retinoids are known to regulate the production of extracellular matrix proteins including fibronectin (Patt *et al.*, 1978; Bolmer and Wolf; 1982, Kenney *et al.*, 1986). Therefore, we examined whether RA also influence the expression of fibronectin in differentiating myoblasts. Myoblasts that had been cultured for 24 hr were incubated for the next 48 hr in the presence and absence of RA followed by electrophoretic analysis on 10% polyacrylamide gels containing SDS. As shown in Fig. 4 (lanes a-c), the level of fibronectin dramatically decreased as the myogenic differentiation proceeded (*i.e.*, At the time of 72 hr culture, fibronectin could hardly be detected in the gel). In contrast, the cells that had been treated with either 10^{-5} or 5×10^{-5} M RA retained fibronectin to nearly the same level seen in the control, 24 hr cultured cells (lanes d and e, respectively). To confirm the Coomassie-stained doublet indeed represent fibronectin molecules, the protein obtained from the 24 hr cultured cells was also subjected to immunoblot

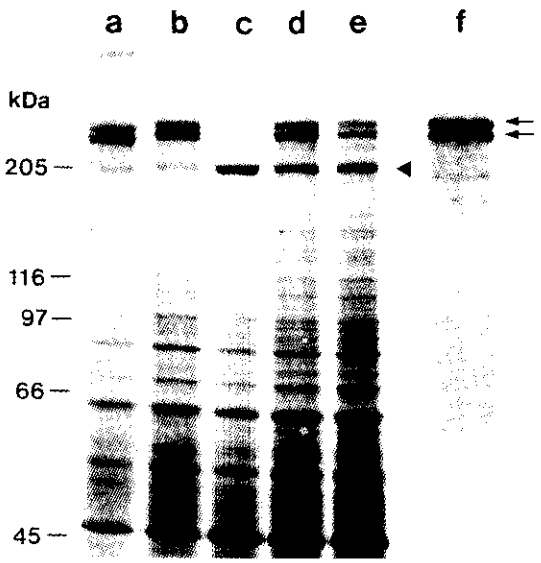

Fig. 4. Effect of RA on the synthesis of fibronectin. Myoblasts were cultured for 24 (lane a), 48 (b), and 72 hr (c). At 24 hr, the cells cultured in parallel were treated with 10^{-5} (d) and 5×10^{-5} M RA (e) and further cultivated for the next 48 and 24 hr, respectively. The cells cultured for the indicated periods were disrupted by sonication, and their lysates were subjected to electrophoresis on 10% polyacrylamide slab gels containing SDS. Proteins in the gel were visualized by staining with Coomassie R-250. The lysate obtained from the cells cultured for 48 hr were also subjected to immunoblot analysis using anti-fibronectin antibody (f) as described under "Materials and Methods". The arrows indicate the fibronectin molecules, and the arrowhead shows myosin heavy chain.

analysis using anti-fibronectin antiserum. Fig. 4 (lane f) clearly shows that the doublet bands correspond to fibronectin. These results suggest that RA involves in the continuous expression of fibronectin, which may in turn result in the prevention of myoblast fusion.

Noteworthy is the finding that myosin heavy chain molecules accumulate as the myogenic process proceeds whether or not RA was treated (Fig. 4, marked by the arrow-head). These results further indicate that RA-mediated fusion block occurs independently from the synthesis of muscle specific proteins.

Discussion

The present studies have demonstrated that RA is capable of preventing myoblast fusion with insignificant effect on the synthesis of muscle specific proteins. This inhibitory effect of RA on the fusion could be reversed upon removal of the drug. In addition, RA showed relatively little effect on the proliferation of myoblast. Moreover, the cells treated with RA retained their ability to form bipolar shape and to align their bipolar axes. These results clearly suggest that RA acts as a specific inhibitor of membrane fusion of embryonic myoblasts. Of interest was the finding, however, that RA showed much less significant effect on the fusion when treated to the cells that had already committed for fusion, unlike its effect on the fusion competent cells. Thus, it seems likely that the effect of RA on the fusion is differentiation stage-specific.

In general, the functions of fibronectin include promotion of cell adhesion, regulation of cell shape, and guidance of cell migration (Hynes, 1981). These functions are also important for the myogenic differentiation of myoblasts. However, it has been suggested that the loss of fibronectin from the surface of myoblasts is prerequisite to myoblast fusion and that its continued presence is inhibitory to myogenesis (Podleski *et al.*, 1979; Chung and Kang, 1990). In addition, it has been demonstrated that the loss of fibronectin at the time of myoblast fusion is due to the decrease of fibronectin receptor on the cell surface, especially the matrix assembly receptor (Chung and Kang, 1990, Moon *et al.*, 1994). Importantly, retinoids are known to affect the synthesis and cell surface content of fibronectin (Patt *et al.*, 1978; Bolmer and Wolf, 1982).

In the present study, we have also shown that RA dramatically alter the level of fibronectin in cultured myoblasts. In the control cells, the level of fibronectin fell to a level that could hardly be detected during the course of myogenic differentiation in accordance with the previous reports (Chung and Kang, 1990). On the other hand, the RA-treated myoblasts retained fibronectin almost to a level that was seen with the 24 hr-cultured cells. It has been reported that the

disappearance of fibronectin from the surface of fusing myoblast may not be due to a change in the rate of synthesis of fibronectin but due to a gradual loss from the cells with unknown mechanisms (Gardner and Fambrough, 1983). Therefore, it is not clear whether the treatment of RA resulted in an increased synthesis of fibronectin and/or of its receptor or in the prevention of the loss of fibronectin. However, it seems that the changes in the level of fibronectin in myoblasts is critical for membrane fusion and RA inhibits myoblast fusion perhaps by controlling the level of fibronectin in the surface of differentiating myoblasts.

Acknowledgements

This work was supported by the Ajou University Research Fund, the Ministry of Education through Basic Science Research Program, and Korea Science and Engineering Foundation through Research Center for Cell Differentiation.

References

Bischoff, R. and H. Holtzer, 1969. Mitosis and process of differentiation of myogenic cells *in vitro*. *J. Cell Biol.* **41:** 188-200.

Bolmer, S.D. and G. Wolf, 1982. Retinoids and phorbol esters alter release of fibronectin from enucleated cells. *Proc. Natl. Acad. Sci. USA* **79:** 6541-6545.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.

Chung, C.Y. and M.S. Kang, 1990. Correlation between fibronectin and its receptor in chick myoblast differentiation. *J. Cell. Physiol.* **142:** 392-400.

DeRuyter, M.G., W.E. Lambert, and A.P. DeLeenheer, 1979. Retinoic acid: An endogenous compound of hyman blood. unequivocal demonstration of endogenous retinoic acid in normal physiological conditions. *Anal. Biochem.* **98:** 402-409.

Emerson, C.P. and S.K. Becker, 1975. Activation of myosin synthesis in fusing and mononucleated myoblasts. *J. Mol. Biol.* **93:** 431-447.

Eppenberger, H.M., D.M. Dawson, and N.O. Kaplan, 1967. The comparative enzymology of creatine kinase. I. Isolation and characterization from chicken and rabbit tissues. *J. Biol. Chem.* **242:** 204-209.

Florini, J.R., D.Z. Ewton, and K.A. Magri, 1991. Hormones, growth factors, and myogenic differentiation. *Annu. Rev. Physiol.* **53:** 201-216.

Gardner, J.M. and D.M. Fambrough, 1983. Fibronectin expression during myogenesis. *J. Cell Biol.* **96:** 474-485.

Griep, A.E. and H.F. Deluca, 1986. Studies on the relation of DNA synthesis to retinoic acid-induced differentiation of F9 teratocarcinoma cells. *Exp. Cell Res.* **164:** 223-231.

Hynes, R.O., 1981. Fibronectin and its Relation to Cell Structure and Behavior, In: Cell Biology of the Extracellular Matrix (Hay, E.D., ed.). Plenum Publishing Co., New York, pp. 295-333.

Jetten, A.M., 1980. Retinoids specifically enhance the number of epidermal growth factor receptors. *Nature* **284:** 626-629.

Kenney, M.C., L.M. Shih, U. Labermeir, and D. Satterfield, 1986. Modulation of rabbit keratinocyte production of collagen, sulfated glycosaminoglycans and fibronectin by retinol and retinoic acid. *Biochim. Biophy. Acta* **889:** 156-162.

Kim, H.S., I.H. Lee, C.H. Chung, M.S. Kang, and D.B. Ha, 1992. Ca^{2+}/calmodulin-dependent phosphorylation of the 100-kDa protein in chick embryonic muscle cells in culture. *Dev. Biol.* **150:** 223-230.

Konigsberg, I.R., P.A. Sollman, and L.O. Mixer, 1978. The duration of the terminal G1 of fusing myoblast. *Dev. Biol.* **63:** 11-26.

Kwak, K.B., Y.S. Lee, S.W. Suh, C.S. Chung, D.B. Ha, and C.H. Chung, 1989. Purothionin from wheat endosperm reversibly blocks myogenic differentiation of chick embryonic muscle cells in culture. *Exp. Cell Res.* **183:** 501-507.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* **227:** 680-685.

Lotan, R., 1980. Effects of vitamin A and its analogues (retinoids) on normal and neoplastic cells. *Biochim. Biophys. Acta* **605:** 33-91.

Ludwig, K.W., B. Lowey, and R.M. Niles, 1980. Retinoic acids increases cyclic AMP-dependent protein kinase activity in murine melanoma cells. *J. Biol. Chem.* **255:** 5999-6002.

Merlie, J.P. and F. Gros, 1976. *In vitro* myogenesis. Expression of muscle specific function in the absence of cell fusion. *Exp. Cell Res.* **97:** 406-412.

Moon, K.-Y., K.S. Shin, W.K. Song, C.H. Chung, D.B. Ha, and M-S. Kang, 1994. A candidate molecule for the matrix assembly receptor to the N-terminal 29-kDa fragment of fibronectin in chick myoblasts. *J.*

Biol. Chem. **269:** 7651-7657.

Nadal-Ginard, B., 1978. Commitment, fusion and biological differentiation of a myogenic cell line in the absence of DNA synthesis. *Cell* **15:** 855-864.

Niles, R.M.B. Loewy and S. Rogeij, 1986. The effect of retinoic acid on protein phosphorylation in mouse melanoma cells. *Exp. Cell Res.* **164:** 437-448.

O'Neill, M.C. and F.E. Stockdale, 1972. A kinetic analysis of myogenesis *in vitro*. *J. Cell Biol.* **52:** 52-65.

Paterson, B. and J. Prives, 1973. Appearance of acetylcholine receptor in differentiating cultures of embryonic chick breast muscle. *J. Cell Biol.* **59:** 241-245.

Patt, L.M., K. Itaya, and S.I. Hakomori, 1978. Retinol induces density-dependent growth inhibition and changes in glycolipids and LETS. *Nature* **273:** 379-382.

Podleski, T.R., I. Greenberg, J. Schlessinger, and K.M. Yamada, 1979. Fibronectin delays the fusion of L6 myoblasts. *Exp. Cell Res.* **122:** 317-326.

Raynaud, F., C. Liapi, P. Gerbaud, W.B. Anderson, and D. Evain-Brion, 1988. Effect of retinoic acid on cAMP dependent protein phosphorylation in psoriatic fibroblasts. *Biochem. Biophys. Res. Commun.* **156:** 263-268.

Smillie, L.B., 1982. Preparation and identification of α- and β-tropomyosins. *Methods Enzymol.* **85:** 234-241.

Sporn, M.B. and A.B. Roberts, 1983. Role of retinoids in differentiation and carcinogenesis. *Cancer Res.* **43:** 3034-3040

Stenman, S. and A. Vaheri, 1978. Distribution of a major connective tissue protein, fibronectin, in normal tissues. *J. Exp. Med.* **47:** 1054-1070.

Strickland, S. and V. Madhavi, 1978. The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell* **15:** 393-403.

Towbin, H., T. Staehelin, and J. Gordon, 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and applications. *Proc. Natl. Acad. Sci. USA* **76:** 4350-4354.

Walsh, F.S., S.E. Moore, and S. Dhut, 1981. Monoclonal antibody to human fibronectin: Production and characterization using human muscle culture. *Dev. Biol.* **84:** 121-132.

Yen, A., S.L. Reece, and K.L. Albright, 1984. Membrane origin for a signal eliciting a program of cell differentiation. *Exp. Cell Res.* **152:** 493-499.

(Accepted September 14, 1995)

Retinoic Acid가 배양계배 근원세포의 융합과 Fibronectin의 발현에 미치는 영향

김혜선[1] · 정필중[1] · 강만식 · 정진하 · 하두봉(서울대학교 분자생물학과 및 세포분화연구센터, [1]아주대학교 생명과학과)

많은 세포와 조직에서 분화 조절물질로 알려져 있는 비타민 A의 대사산물인 rectinoic acid(RA)가 배양 계배 근원세포의 성장과 분화에 미치는 영향을 조사하였다. RA가 처리된 세포는 그 농도가 증가함에 따라 융합 억제 효과도 증가함을 보였고, 배양액으로부터 RA를 제거해 주면 근원세포의 융합이 재개되는 것으로 보아, RA는 근원세포의 융합을 가역적으로 억제함을 알 수 있었다. 그러나 이미 최종 분화 단계에 들어선 세포에서는 융합 억제 효과를 보이지 않아, RA의 효과는 분화 단계에 따라 특이적으로 작용함을 보여준다. 융합 억제 효과에도 불구하고, RA는 근원세포의 융합 전단계인 방추형으로 신장하는 것과 그들이 가상의 축을 따라 배열하는 데는 영향을 주지 않았다. 또한 세포의 증식과 creatine kimase, tropomyosin과 같은 근특이 단백질의 축적에도 큰 영향을 주지 않았다. 한편, 근원세포는 융합하기 전에 필수적으로 fibronectin의 양이 감소되는 것으로 알려져 있는데, 본 연구에서 RA는 fibronectin의 감소를 억제함을 알 수 있었다. 이상의 결과로 RA는 배양 근원세포의 융합만을 특이하게 억제하는 물질이며, 그 작용은 fibronectin의 감소를 방해하는 것과 밀접한 관계가 있는 것으로 사료된다.