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Expression of Laminin During the Differentiation of F9 Teratocarcinoma Stem Cell

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In order to investigate the retinoic acid induced-differentiation of F9 teratocarcinoma stem cell, we have analyzed the change of cell morphology and laminin expression after exposure to retinoic acid and cyclic AMP. It is shown that undifferentiated F9 stem cells grow as closely packed colonies, and it is difficult to distinguish cell-cell boundaries. After retinoic acid and dibutyryl cyclic AMP treatment, F9 cells assume a flat morphology characterized by perinuclear granules and arrest growth. According to Northern blot analysis, laminin expression was increased markedly after retinoic acid treatment. Laminin B1 gene expression was increased at least 30-fold and laminin B2 gene expression was increased approximately 20-fold during differentiation process. Employing immunofluoresence analysis, it was proved that the synthesis of laminin protein was low level in F9 stem cell whereas it became high level in retinoic acid treated F9 cell and the laminin protein was largely accumulated in the cell surface.

Our results suggest that induction of laminin B1 and B2 genes in F9 cells is retinoic acid-mediated control, and morphological change and differentiation of F9 cells might be associated with laminin gene expression.

KEY WORDS: F9 Teratocarcinoma stem cell, Retinoic acid, Differentiation, Laminin

The tumorigenic stem cells of murine teratocarcinoma or embryonal carcinoma cells, which resemble the inner cell mass cells of the early mouse embryo, have the capacity to differentiate into multiple types of cell products (Stevens, 1983; Martin, 1980). Embryonal carcinoma cells have proved to be a good model for studies of events during early embryo development (Martin, 1980). One of these cell lines, F9, shows very low spontaneous differentiation *in vivo* or *in vitro* (Grover and Adamson, 1986). However, it can be induced to differentiate into a homogeneous population of primitive or parietal endoderm in the presence of retinoic acid and dibutyryl cyclic AMP (Strickland *et al.*, 1980). If F9 cells are treated with retinoic acid and allowed to aggregate, they differentiate into the alternate cell type, visceral endoderm characterized by the synthesis of α-fetoprotein (Grover *et al.*, 1983). Therefore, the differentiation may involve molecular mechanism on the cell surface (Joukoff *et al.*, 1986). The differentiation response of F9 cells is irreversible and is accompanied by morphological alteration and increased synthesis and secretion of proteins characterized of differentiated phenotype such as laminin, tyep IV collagen, β2-microglobulin and protease plasminogen activator (Eriksson *et al.*, 1986; Strickland *et al.*, 1980). Besides, differentiation of F9 cells results in production of insulin-like growth factor II (Nagarajan *et al.*, 1985), an increase in the specific activity of UDP-galactose: β-D-galactosyl α-1,3-galactosyl transferase (Cummings and Mattox, 1980), lack of surface antigen SSEA-1 (Griep and Deluca, 1986) and change of colonization pattern (Leonicini *et al.*, 1988).

The retinoids are a class of compounds that can modify processes of cellular proliferation and differentiation (Sporn and Roberts, 1983). Retinol (vitamin A) and its natural analog, retinoic acid,

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are essential for normal development of epithelial tissues and have been found to play a role in the prevention of epithelial carcinogenesis and suppress the development of the malignant phenotype *in vitro* (Lotan, 1983). More recently, it has been shown that retinoids can exert effects on certain fully transformed, invasive, neoplastic cells, leading in certain instances to a suppression of proliferation, and in other instances to terminal differentiation of these cells, resulting in a more benign, nonneoplastic phenotype (Strickland, 1978; Strickl and Madhavi, 1981). Retinoic acid can promote differentiation of cultured cell types including teratocarcinoma cells (Strickland *et al.*, 1980), human promyelocytic leukemia cells (Daenen *et al.*, 1986), and human myeloblast (Fontana *et al.*, 1986).

The mechanism by which retinoic acid induces differentiation is largely unknown. One hypothesis suggests that retinoic acid may act in a manner analogous to the steroid hormones and its effects in controlling gene expression are mediated by intracellular binding proteins (Sporn and Roberts, 1983). Specific cellular binding proteins for retinol (CRBP) and for retinoic acid (CRABP) have been found (Grippo and Gudas, 1987, Stoner and Gudas, 1989). Recently, retinoic acid receptor (RAR) has also been identified and cloned from human cDNA library and was homologous to the receptors for steroid hormones, thyroid hormones and vitamin D3, suggesting that the molecular mechanisms of the retinoic acid are similar to those described for other members of this nuclear receptor family (Petkovich *et al.*, 1987). Therefore, CRABP and CRBP may act as shuttles of the retinoids from cytoplasm to nucleus, then the complex of RAR and retinoids ultimately results in modulation of transcription of specific genes.

Laminin is the first basement membrane protein to appear during embryonic development and is detected between cells in the 16-cell (morula) embryo. Current models suggest that each laminin molecule contains A (400-KDa), B1 (230-KDa) and B2 (220-KDa) chains, which form a cross-shaped structure by inter- and intrachain disulfide bonds (Sasaki *et al.*, 1987). Laminin had diverse biological functions including promotion of cell attachment, neurite outgrowth, mitogenesis and differentiation (Ogawa *et al.*, 1988; Pikkarainen *et al.*, 1988). Roles for laminin in induction of polarity, intracellular flattening, formation of specialized junction and as an auto- antigen have been also suggested (Timple *et al.*, 1984). For F9 cells, it is known that laminin may affect the differentiation direction, basement membrane production, and epithelial organization (Grover and Adamson, 1985).

We examined the change of morphology, steady-state mRNA levels of laminin B1 and B2, and the production and distribution of laminin protein in F9 cells during retinoic acid-induced differentiation.

Materials and Methods

Cell Culture and Induction of Differentiation

F9 cells were obtained from ATCC (USA) and grown on gelatinized plasticware in Dulbecco's Modified Eagle's Medium (Sigma Chemical Co., Mo. USA) supplemented with 10% calf serum, penicillin (100U/ml) and streptomycin (100 μg/ml) (Gibco, Grand Island, N. Y. USA) in humidified atmosphere of 5% CO_2 at 37°C. Stock culture were passed by trypsinization every 2 to 3 days.

Differentiation was induced by the addition of 1×10^{-6}M all trans-retinoic acid, 5×10^{-4}M dibutyryl cyclic AMP and 2.5×10^{-4}M theophylline. Theophylline serves as a cyclic AMP stabilizer. Retinoic acid was prepared as 10^{-3}M stock in ethanol, dibutyryl cyclic AMP was prepared as 100mM aqueous solution, asnd theophylline was 100mM stock in 0.1N NaOH. Stock solution was diluted directly in the medium.

Isolation of Total RNA from F9 Cell

Total cellular RNAs were isolated from undifferentiated and differentiated F9 cells by acid-guanidinium thiocyanate-phenol-chloroform (AGPC) extraction method (Chomczynski and Sacchi, 1987).

Transfer of Formaldehyde-denatured RNA to Nitrocellulose Membrane

The total RNAs isolated were electrophoretically separated in a formaldehyde-agarose gel as described by Lehrach *et al.* (1977). Consequently

the RNAs were transferred to a nitrocellulose membrane and then the membrane was baked for 4-5 hours at 80°C.

Preparation of Radiolabeled DNA Probes

The 464 base pair EcoRI fragment of 5′ end of mouse laminin B1 cDNA (Kim and Choi, 1989), the 30 base pair synthetic laminin B2 oligonucleotide and the 600 base pair PstI fragment of mouse actin cDNA were used as DNA probes. The sequence of laminin B2 oligonucleotide was 5′-GGC CTC CCT TGT CTT CTC ATT GGC TTC AGC-3′ and was obtained from L. J. Gudas, Department of Cancer Genetics, Dana-Farber Cancer Institute, Boston, U.S.A. Laminin B1 and actin cDNA were labeled by random primer method (Feinberg and Vogelstein, 1984) and laminin B2 oligonucleotide was labeled by 5′ end labeling method (Davis *et al.*, 1986).

Northern and Slot Blot Hybridization

The Northern blotted nitrocellulose membrane was hybridized with ^{32}P-labeled cDNA probe and with ^{32}P-labeled oligonucleotide (Sambrook *et al.*, 1989). The filter was exposed to X-ray film at -70°C for several days. The expressions of the genes were analyzed by microdensitometer.

For slot blot analysis, nitrocellulose membrane was prepared and then applied to slot blot apparatus according to the recommended protocol (Bio-Rad #170-6543). Each slot contained 10 μg of total RNAs. The membranes were baked for 4-5 hours at 80°C, then hybridized with ^{32}P-labeled DNA probe, washed and exposed to X-ray films.

Immunofluoresence

Rabbit antilaminin antibody and fluorocein isothiocyanate (FITC) conjugated goat-antirabbit Ig were obtained from Sigma.

Cells were grown on 22 × 22mm coverslips placed individually into 35mm tissue culture dishes. The coverslips were pre-coated with 0.3% gelatin and air dried prior to use. Differentiation was induced as described above. The coverslips were washed twice for 30 seconds each time in a solution of 1:1 PBS and alpha MEM (PBS-MEM). Then they were incubated with primary antibody, rabbit antilaminin antibody, diluted into 60 μl of PBS-MEM per coverslip for 45 minutes at room temperature in a humidified chamber. They were washed three times for 5 minutes each time in 3ml of PBS-MEM and then incubated with secondary antibody, FITC-conjugated goat-antirabbit IgG, diluted into 60 μl of PBS-MEM per coverslip for 45 minutes at room temperature in a humidified chamber. The coverslips were washed as above and cells were fixed by immersing the coverslip in freshly-prepared methanol: acetic acid (95:5, v/v) for 15 minutes at −20°C. The coverslips were air-dried and the cells were rehydrated under PBS for 15 minutes. The coverslips were mounted onto a slide and photographed.

Results

Morphological Observations

F9 cells grew in culture as closely packed colonies, and it was difficult to distinguish cell-cell boundaries (Fig 1A). After addition of differentiation inducers, retinoic acid-dibutyryl cyclic AMP-theophylline (RACT), the cell growth was ceased and differentiation was occurred. After 3 days of treatment, more than 50% of cells acquired the differentiated morphology characterized by flat, perinuclear granules (Fig. 1B). After 6 days of treatment, most of cells acquired the differentiated morphology (Fig. 1C).

Comparision of Steady State Level of Laminin B1 mRNA in F9 Cells Before and After Differentiation

For this study, we used a 464 bp 5′ end of laminin B1 cDNA which was previously cloned from mouse F9 teratocarcinoma stem cell line in our laboratory. Restriction map and electrophoresis of EcoRI digested laminin B1 cDNA is shown in Fig. 2. The relative level of laminin B1 mRNA was determined by Northern and slot blot analysis (Fig. 3), and then the degree of the gene expression was analyzed by microdensitometer.

According to these results, laminin B1 gene expression was 30 times higher in differentiated F9 cells than in undifferentiated F9 stem cells. In contrast, actin gene expression remained the same degree.

Therefore, it was concluded that RACT treat-

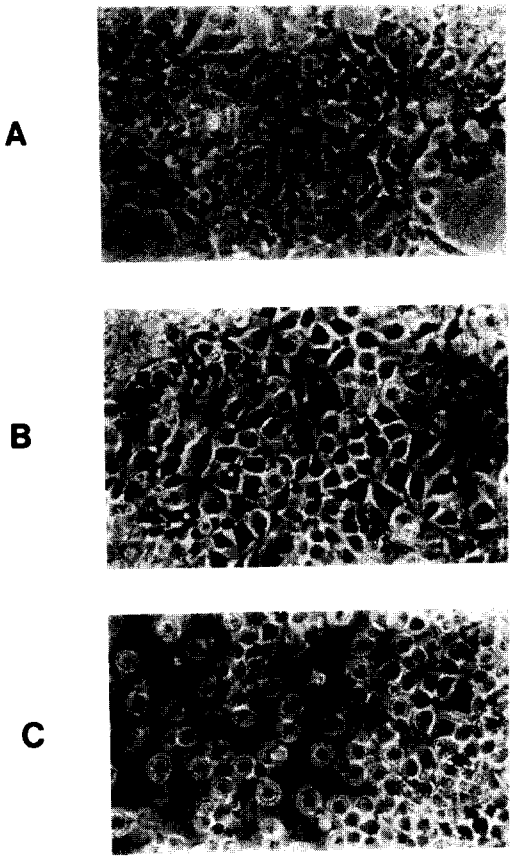

Fig. 1. Phase-contrast photomicrographs of live cultures of F9 cells before and after exposure to retinoic acid--dibutyryl cAMP-theophylline. (A) F9 cells growing exponentially in the undifferentiated state. (B) F9 cells cultured for 3 days in 1 μM RA, 500 μM dibutyryl cyclic AMP and 250 μM theophylline (RACT). (3) F9 cells cultured for 6 days in RACT.

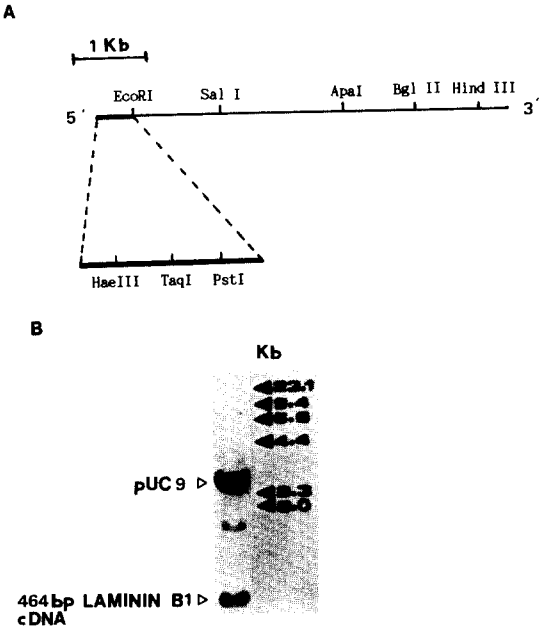

Fig. 2. Restriction map of laminin B1 cDNA(A) and electrophoresis of EcoRI digested laminin B1 cDNA on 1% low melting point agarose gel(B). The 5′ end of laminin B1 cDNA (464 bp EcoRI fragment) is indicated by solid bar and used as a probe. λ Hind III-molecular size markers(Kb) are indicated on the right.

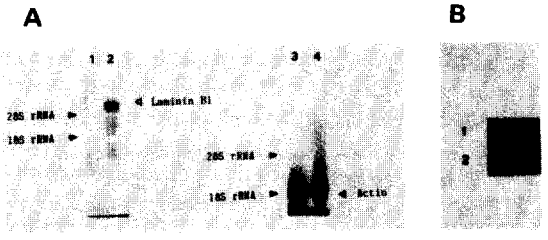

Fig. 3. Comparision of the steady state levels of laminin B1 mRNA in F9 cells before and after exposure to RACT. (A) Total cellular RNAs from F9 stem cells (lane 1,3) and RACT-treated F9 cells (lane 2, 4) were isolated and 20 μg of RNAs per lane were electrophoresed on 1% agarose-formaldehyde gel. After electrophoresis, RNAs were transferred to nitrocellulose membrane and hybridized with the 464bp laminin B1 cDNA probe (lane 1, 2) and 600bp actin cDNA probe(lane 3, 4) respectively. The expression of laminin B1 gene was quantitated by microdensitometer. (B) Total cellular RNAs were isolated from F9 stem and differentiated cells as described in (A). 10 μg of the RNA per slot was blotted onto nitrocellulose paper. Then, hybridized with 5′ end of laminin B1 cDNA probe and analyzed by microdensitometer. Lane 1, F9 undifferentiated stem cells; lane 2, F9 cells treated with RACT.

ment caused a marked increase in the steady state level of laminin B1 mRNA in differentiated F9 cell.

Comparision of Laminin B2 Gene Expression in Undifferentiated and Differentiated F9 Cells

Thirty base pair laminin B2 oligomer described in Materials and Methods was used as a probe. Then, the relative level of laminin B2 mRNA was determined by Northern blot analysis. After addition of RACT, steady state level of laminin B2 mRNA (8Kb) increased markedly as indicated in Fig. 4. Therefore, we concluded that RACT treatment caused increase in the laminin B2 gene expression as in the case of laminin B1 gene ex-

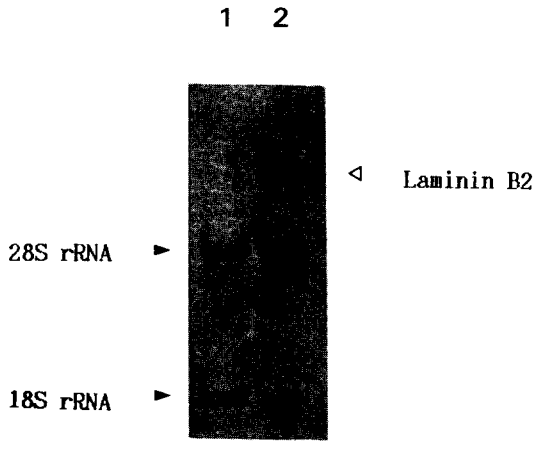

Fig. 4. Northern blot analysis of laminin B2 mRNA expression in F9 cells before and after exposure to RACT. Total cellular RNAs from F9 cells were treated as described in Fig. 3(A). 20 μg of total RNAs were separated in 1% agarose-formaldehyde gel, transferred to nitrocellulose membrane and hybridized with 30 bp laminin B2 oligonucleotide. Lane 1, F9 undifferentiated stem cells; lane 2, F9 cells treated with RACT.

pression.

Immunofluorescence

In immunofluorescence test, it was shown that F9 cells cultured in the presence of RACT produced large quantities of laminin protein as a thick deposit around outer layers of the cell, whereas, it was scarcely detected in the cytosol of F9 stem cell (Fig. 5). This result indicates that the increased level of laminin mRNA is directly related with the increase in the production of laminin protein during F9 cell differentiation.

Discussion

It has been known that retinoids, a family of compounds comprising both the natural and synthetic analogues of retinol, are potent agents for the control of both differentiation and proliferation (Sporn and Roberts, 1983). Therefore, at the gene level, it appears that retinoids affect the expression of genes involved in both differentiation and proliferation. As for a evidence of this hypothesis, retinoic acid receptor (RAR) was recently identified and its function seems very similar to that of the steroid hormone superfamilies (Petkovich *et al.*, 1987). The proteins of this superfamilies suggested to have DNA binding functions and control the expression of their responsive genes (Beato, 1989). The alternative to a steroid-like mechanism for retinoic acid is to suggest that they control gene expression via interactions with protein kinases, both cyclic AMP-dependent and cyclic AMP-independent (Plet *et al.*, 1982). It was also reported that differentiation response of F9 cells is accompanied by both morphological alterations and an increase in the synthesis of constituents of the cytoskeleton and basement membrane (BM). Basement membrane including laminin may convey regulatory information through binding interaction with cytoskeletal proteins. For instances, laminin binds to the cell surface laminin-binding protein (or laminin receptor), and the laminin-binding protein successively binds to actin filaments directly. These BM-cytoskeletal linkages are most likely critical for morphogenesis. In addi-

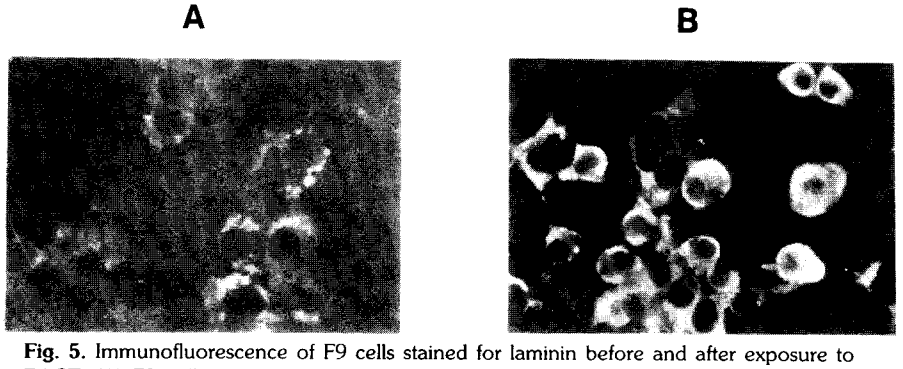

Fig. 5. Immunofluorescence of F9 cells stained for laminin before and after exposure to RACT. (A) F9 cells growing exponentially in the undifferentiated state. (B) F9 cells exposed to RACT.

tion, cytoskeletal alteration can redistribute cytoplasmic mRNAs, alter mobility and function of integral membrane proteins and nuclear structure (Ingber and Folkman, 1989).

In this study, we analyzed the change of morphology and expression of laminin which is differentiation-specific and the major constituent of BM during F9 stem cell differentiation process induced by retinoic acid. We have shown that the morphology of F9 cells, cultured in suspension with RACT, became rounded. Using Northern blot analysis, we also observed that the retinoic acid-treated F9 cells expressed much higher level of laminin B1 (approximately 30 fold) and laminin B2 (approximately 20 fold) compared to F9 stem cells. In addition, by using laminin antibody, we demonstrated that the laminin protein induced by RACT was largerly distributed in the surface of the cells.

These data suggest that the induction of laminin gene is under retinoic acid mediated control. Once laminin protein in produced, it may interconnect with cytoskeleton via laminin receptor or laminin-binding protein, then induces the alteration of cell morphology. Furthermore, laminin-linked changes of cytoskeleton may alter nuclear organization and switch on or off other differentiation-related genes.

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F9 Teratocarcinoma stem cell의 분화에 따른 라미닌의 발현

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본 실험은 레티노익산에 의해 F9 teratocarcinoma stem cell의 분화를 유도하고 이 분화과정에서 세포형태의 변화와 라미닌유전자의 발현을 조사하였다.

분화되지 않은 F9 stem cell은 지속적으로 증식을 하며 세포간의 간격을 구분하기 어려울 뿐만 아니라 불규칙적인 모양을 하고 있으나, 레티노익산과 dibutyryl cyclic AMP처리후의 분화된 F9 세포는 둥글고 평평한 모양을 나타내며 세포성장은 중지되었다. Northern blot분석에 의하여 레티노익산과 cyclic AMP처리 후의 F9 세포에서 라미닌 유전자의 발현은 현저하게 증가하였다. 즉, 라미닌 B1 유전자 발현은 분화과정 동안 최소한 30배, 라미닌 B2 유전자의 발현은 약 20배 증가하였다. 또한 라미닌 항체를 이용한 면역형광 분석결과는 Northern분석결과와 일치하게 분화 후에 라미닌 단백질 합성이 크게 증가되어 있었으며, 생성된 라미닌 단백질은 거의 세포표면에 분포된 것으로 나타났다.

이러한 결과들로부터, 레티노익산에 의해 F9 stem cell의 분화가 유도되며 이 분화과정에서의 형태적인 변화와 분화의 진행은 라미닌의 생성과 밀접한 관련이 있다고 추측된다.