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Teratocarcinoma F9 Cells Induced to Differentiate With Sodium Butyrate Produce Both Tissue-Type and Urokinase-Type Plasminogen Activators

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Abstract Sodium butyrate (NaB) can induce teratocarcinoma cell differentiation as retinoic acid (RA). However, the function of these two agents seems to be a little different [Kosaka et al., Exp Cell Res, 192:46–51, 1991]. F9 cells treated with NaB synthesize both tissue-type (tPA) and urokinase-type (uPA) plasminogen activator, though RA induces only tPA production. Urokinase-type PA is demonstrated to exist in association with membrane and to localize its activity to the close environment of the cell surface. This may cause the specific cell morphology and characteristics of differentiated F9 cells induced with NaB. © 1992 Wiley-Liss, Inc.

Key words: embryonal carcinoma, stem cell, retinoic acid

Teratocarcinoma contains malignant stem cells which resemble early embryonic cells in some respects, and these stem cells have been used as a model system for studying various aspects of the process of differentiation [Martin, 1980]. F9 is one of the best studied mouse teratocarcinoma stem cell lines. Treatment with retinoic acid (RA) causes differentiation of F9 cells into a tissue type that resembles extraembryonic primitive endoderm of the embryo [Strickland and Mahdavi, 1978].

It is known that sodium butyrate (NaB) induces a wide variety of effects on mammalian cells in culture, including a decrease in DNA replication leading to an arrest of cell division, modification of cell morphology, and alteration in the level of certain gene products [Kruh, 1982; Scott et al., 1982; Toscani et al., 1988]. Recently, NaB was shown to induce F9 cell differentiation [Nishimune et al., 1983]. The addition of NaB into F9 cell cultures results in some of the same phenotypic changes that result from the induction of differentiation with RA, *i.e., morphological* alteration and increase in the synthesis and secretion of proteins characteristic of the differentiated phenotypes such as laminin, endoA, and plasminogen activator (PA). However, F9 cells treated with NaB undergo a morphological change from the typical stem cell type to a flat polygonal shape, having a process at each angle [Kosaka et al., 1991], which is a bit different from differentiated F9 cells induced with RA [Strickland and Mahdavi, 1978]. Furthermore, the expression of specific differentiation markers, especially PA, is more remarkable and rapid in response to NaB than to RA [Kosaka et al., 1991].

PA catalyzes the proteolytic activation of plasminogen to the broad-specificity protease plasmin which can activate or degrade extracellular proteins. It is reasonable to suggest that PA functions in the interaction between cells and their surrounding matrix, and that the enzyme participates in processes such as tissue remodeling, cellular morphology, and migration [Saksela et al., 1988; Danø et al., 1985]. In mammalian tissues, two different types of PAs have been recognized: the urokinase-type (uPA) and the tissue-type (tPA). In mouse embryogenesis, parietal endoderm is characterized by high level of tPA production and visceral endoderm makes uPA at the same stage [Marotti et al., 1982].

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Both PAs should play some important roles in differentiation steps of mouse embryo.

In NaB-induced F9 cell differentiation, high level of PA activity was observed [Kosaka et al., 1991]. It would influence a morphology and some other phenotypes of differentiated F9 cells. To obtain further understanding of NaB induction, especially to know the difference between NaB and RA induction of teratocarcinoma cell differentiation, we studied the production of tPA and uPA during F9 cell differentiation induced with NaB.

METHODS

Cell Culture

The stock culture of F9 cells was on gelatin-coated culture dishes in Eagle's minimal essential medium (MEM) containing 5 mM glutamine, 1 mM sodium pyruvate, and 10% fetal calf serum (FCS) at 37°C. For experimental cultivation the stock culture was treated with 0.125% trypsin and 0.5 mM EDTA in phosphate-buffered saline (PBS) at 37°C for 10 min. The cells were then seeded at 1–2 × 10^6 per 60-mm culture dish. Treatment of cells with sodium butyrate (NaB) was accomplished by adding small volumes of sterile concentrated solutions into the media more than 18 h after cell inoculation.

Samples for Zymographic Assay

Cells were seeded at 1–2 × 10^6 per 60-mm culture dish. On the next day, the medium was changed to 1 ml of fresh medium containing NaB. After various intervals, the conditioned medium was collected and centrifuged at 10,000*g* for 5 min to remove cellular debris and stored at −30°C. The cell-layer was washed with PBS three times and collected by pipetting in PBS containing 1 mM EDTA. The pellet was resuspended in PBS at 1 × 10^7 cells/ml and stored at −30°C.

The conditioned medium of PYS cells, parietal yolk sac carcinoma cell line [Lehman et al., 1974], and LLC cells, Lewis lung carcinoma cell line [Skriver et al., 1984], were used for the standard of tPA and uPA, respectively.

Preparation of Membrane Fraction

Subcellular fractionation was performed according to the procedure of Quigley [1976]. Briefly, F9 cells treated with NaB for 24 h were washed with PBS containing 1 mM EDTA three times and then collected with a rubber policeman. The cell pellet was then resuspended in 1 ml of 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and homogenized in a Daunce type homogenizer. The homogenate was centrifuged at 600*g* for 8 min at 4°C to remove nuclei, and further centrifuged at 100,000*g* for 90 min at 4°C. The supernatant was used as a cytosol fraction. The pellet, containing plasma membrane and lysosomal granules, was resuspended in the same buffer of the same volume of cytosol fraction and used as a membrane plus granule fraction.

Sucrose gradient centrifugation was used to subfractionate the membrane plus granule fraction. The fraction was layered over a discontinuous sucrose gradient composed of equal volumes of 60%, 40%, and 20% sucrose (wt/vol) and centrifuged at 100,000*g* for 3 h. Discrete bands of subcellular components were removed from the gradient, diluted with homogenizing buffer, and washed by centrifugation at 100,000*g* for 1 h. The individual pellets were resuspended in the same volume of homogenizing buffer and are referred to as gradient fractions A, B, C, and D from the top, respectively. Each fraction was stored at −30°C.

SDS-PAGE

Each 24 μl of the conditioned medium and subcellular and sucrose gradient fractions was mixed with 6 μl of ×5 SDS sample buffer, and 15 μl of the membrane plus granule fraction was mixed with 15 μl of ×2 SDS sample buffer (final concentration of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% glycerol, and 0.002% bromophenol blue) were electrophoresed through a 10% polyacrylamide gel with a 4% stacking gel as described by Laemmli [1970]. Gels were run at 4°C at constant current.

Zymographic Assay for PA

After electrophoresis, the gel was shaken for 30 min in 2.5% Triton X-100 in 10 mM Tris-HCl (pH 8.0) and washed four times in H_2O to remove SDS and Triton X-100 from the gel, respectively, and then placed on the indicator gel containing 1.0% purified agar (Difco), 2.0% casein, 0.1 unit/ml bovine plasminogen, and 0.05% NaN_3, sealed in a plastic container, and incubated at 37°C for 24–48 h. Photographs were taken using dark-field illumination.

Antibodies

Mouse uPA was purified from the serum-free culture medium of Lewis lung carcinoma (LLC) cells producing the enzyme [Skriver et al., 1984], by the method of Rijken and Collen [1981] with a small modification. Briefly, phosphate-cellulose column chromatography was used for concentration of culture fluid and the eluate with 0.5 M NaCl was applied to affinity column chromatography on zinc-chelate agarose followed by gel filtration on Sephadex G-150.

To raise antibodies against mouse uPA, rabbits were immunized with the purified enzyme fraction in complete Freund's adjuvant followed by boost injections several times. A high titer serum was used as anti-uPA antibody. Goat antibody to human tPA was purchased from American Diagnostica Inc.

By using fibrin plate assay [Astrup and Mullertz, 1952], we could detect that both anti-human tPA and anti-mouse uPA antibodies specifically in ×200 dilution blocked plasminogen-dependent mouse tPA and uPA activity, respectively. In order to check the type of PA, we have demonstrated the neutralization of the enzyme activities with specific antibodies on zymogram.

RESULTS

Morphological Observation and Type of Plasminogen Activator of Differentiated F9 Cells Induced With NaB or RA

The monolayer culture of F9 cells shows the typical morphology of EC stem cells as tightly packed colonies (Fig. 1A). Soon after the addition of NaB, it changes to a slightly dispersed arrangement of cells, each cell changing to a flat polygonal shape having a process at each angle (Fig. 1C). Furthermore, this change is rapid and becomes apparent within 5–6 h after the addition of NaB and is accomplished in the majority of cells within 24 h after the addition. In contrast, morphology of cells induced with RA is known as endodermal [Strickland and Mahdavi, 1978], which is quite a different shape from the cells induced with NaB (Fig. 1B). Moreover, this change is relatively slow and gradual, requiring more than a few days [Strickland and Mahdavi, 1978].

It was demonstrated by the formation of caseinolytic plaques that the production of PA in F9 cells was dramatically induced with NaB in a time-dependent manner [Kosaka et al., 1991]. To determine the type of plasminogen activators produced by F9 cells treated with NaB, a conditioned medium and cells were collected and analyzed by zymographic assay. As shown in Figure 2A, three different caseinolytic zones indicating PA activity are observed; two different sized PAs in the conditioned medium (lane 3) and a single PA in the cell lysate (lane 4). All lytic zones are plasminogen dependent (data not shown). Two PA bands in the conditioned medium with relative molecular masses of 60kd and 32kd (Fig. 2A, lane 3) comigrate with the bands of tPA standard from PYS cells (lane 1) [Lehman et al., 1974; Sabbag et al., 1989], and a single PA band of approximately 37kd in the cell lysate (lane 4) comigrates with the uPA standard from LLC cells (lane 2) [Skriver et al., 1984]. In contrast, F9 cells induced with RA produce 60kd and 32kd lytic zones in the conditioned medium (Fig. 2A, lane 5) and no 37kd PA activity in cell lysate if any (lane 6).

In order to identify the three bands, an anti-tPA or an anti-uPA antibody was used. Addition of anti-tPA antibody to an indicator gel (Fig. 2B) results in disappearance of two lytic zones in the

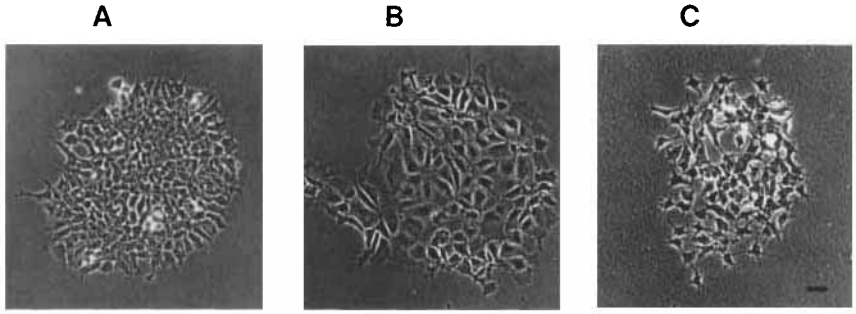

Fig. 1. Changes in morphology of F9 cells in response to NaB or RA. F9 cell cultivation without drugs for 7 days (**A**). One day or 6 days after cell inoculation, cultures were exposed to 10^{-7} M RA for 7 days (**B**) or 5 mM NaB for 24 h (**C**). Bar: 50 μm.

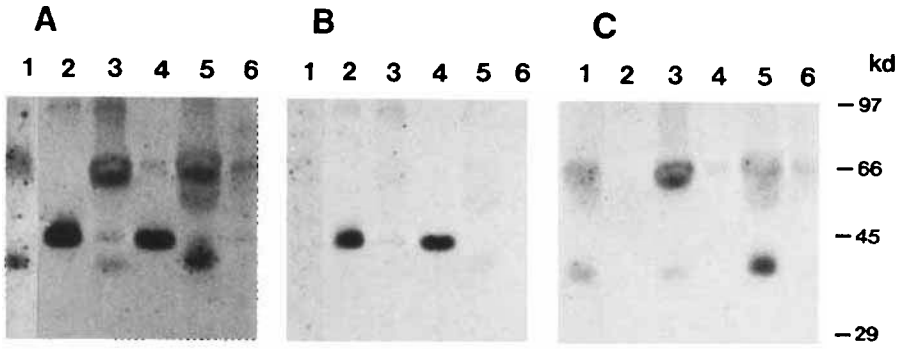

Fig. 2. Zymogram of plasminogen activator of F9 cells induced with NaB: Specific inhibition of plasminogen activator by anti-tPA or anti-uPA antibody. Samples were collected from F9 cells treated with 5 mM NaB for 24 h or 10^{-7} M RA for 7 days and analyzed by SDS-PAGE followed by zymography. Indicator gels contain no antibodies (**A**), antibody to tPA (**B**), or to uPA (**C**) in ×200 dilution. Conditioned medium from PYS cells (lane 1) and LLC cells (lane 2) are used as tPA and uPA standard. Conditioned medium (lanes 3 and 5), and cell lysate (lanes 4 and 6) of F9 cells induced with NaB and RA, respectively. The experiment was repeated four times using separate sample preparations and the results were reproducible.

culture medium (lanes 3 and 5) and tPA standard produced by PYS cells (lane 1) but has no effect on cell lysate-mediated caseinolysis (lane 4) and uPA standard (lane 2). On the other hand, anti-uPA antibody [Maekawa et al., 1989] specifically blocked a single lytic zone in cell lysate and uPA standard from LLC cells (Fig. 2C). The data indicate that differentiated F9 cells induced with NaB produce both tPA and uPA, their localization being specific, i.e., tPA is produced in cytoplasm and secreted into culture medium, while uPA exists in cell-associated form. RA could induce the production of only tPA but not uPA (Fig. 2B,C, lanes 5 and 6).

Time Course of PA Production by F9 Cells Treated With NaB

F9 stem cells demonstrated neither tPA nor uPA activity in both culture medium and cell lysate (Fig. 3A,B, lane 1). Six hours after the addition of NaB, a high molecular weight form of tPA was observed at first, which then increased rapidly and reached maximal level by 18 h in conditioned medium. And then, a low molecular weight form of tPA began to appear 12 h after the addition of the drug and reached maximal level by 18 h. Although these two lytic zones were also observed in the culture medium of F9 cells which was induced with RA, they are faint in comparison with NaB induction (Fig. 3A, lanes 9 and 10). The PA activity induced with NaB for 12 h roughly corresponds to that induced with RA for 7 days. Thus the induction of NaB is rapid and remarkable compared with that of RA. On the other hand, uPA was detectable in the lysate of F9 cells treated with NaB for 3 h and increased time-dependently similar to tPAs in the culture medium. The appearance of uPA seems to be a bit earlier than that of tPA in the culture medium. However, the production of uPA was not detected in both culture

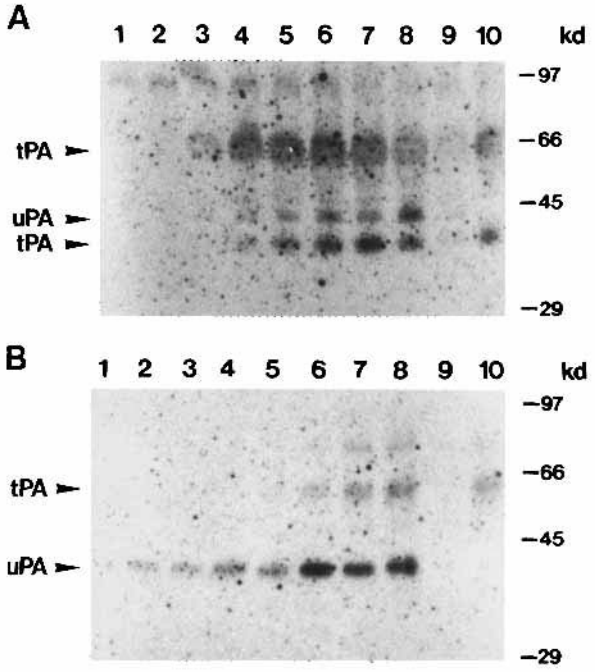

Fig. 3. Time course of plasminogen activator production in the conditioned medium or cell lysate of differentiated F9 cells. F9 cells are treated with 5 mM NaB for 0 h (lane 1), 3 h (lane 2), 6 h (lane 3), 9 h (lane 4), 12 h (lane 5), 18 h (lane 6), 24 h (lane 7), and 36 h (lane 8) or with 10^{-7} M RA for 4 days (lane 9) and 7 days (lane 10). Conditioned medium (**A**) and cell lysate (**B**). Three separate experiments were performed and the results were reproducible.

medium and cell lysate from F9 cells treated with RA for more than 7 days (Fig. 3A,B, lane 10).

Existence of uPA in Plasma Membrane Fraction

Topographical localization of uPA was examined in differentiated F9 cells induced with NaB. As shown in Figure 4A, most of uPA produced by differentiated F9 cells induced with NaB exists in association with the plasma membrane plus granule fraction (lane 2), but little uPA activity, if any, was detected in cytosol fraction (lane 3).

The membrane plus granule fraction was further purified by centrifugation on a discontinuous sucrose gradient (Fig. 4B) [Quigley, 1976]. The most membrane-enriched fraction B has the highest uPA activity of all four fractions (lane 2). The fraction C, which contains membrane, mitochondria, and endoplasmic reticulum components, also has high uPA activity but this is less than that of the fraction B (lane 3). The fractions A and D, which contain little membrane components, have little uPA activity (lanes 1 and 4).

DISCUSSION

In F9 cell differentiation system, it is well known that RA induces the production of only tPA but not uPA, and the differentiation process is irreversible [Strickland and Mahdavi, 1978; Strickland et al., 1980; Rickles et al., 1988]. Furthermore, when F9 cells are treated with RA and grown in suspension for several days, they form an embryoid body consisting of a core of undifferentiated stem cells surrounded by visceral endoderm which synthesizes and secretes both tPA and uPA [Sabbag et al., 1989].

Treatment with NaB markedly enhanced the production of PA and the phenotypic changes are far more rapid than the differentiation induced with RA. We further demonstrated by zymographic assay that differentiated F9 cells induced with NaB synthesized not only tPA but also uPA (Fig. 2) without any formation of visceral endoderm cells on embryoid bodies [Rickles et al., 1988]. Thus, differentiation pathway induced with NaB seems to be different from that induced with RA, though the appearance of various differentiation-specific markers are very similar [Kosaka et al., 1991].

Tissue-type PA has been primarily associated with the fibrinolytic function, while uPA is believed to have a regulatory role in other forms of extracellular proteolysis [Danø et al., 1985]. Normal and neoplastic cells possess a specific uPA receptor which may serve to focus the proteolytic activity of uPA at the cell membrane [Bajpai and Baker, 1985; Vassalli et al., 1985; Blasi et al., 1986; Stoppelli et al., 1986]. It seems likely that treatment with NaB caused a decrease in an ability to cell attach to a collagen-coated culture dish. Urokinase-type PA associated with plasma membrane may play an important role in the morphological changes induced with NaB (Fig. 1). Existence of high uPA activity in membrane-enriched fraction of F9 cells treated with NaB (Fig. 4) strongly indicates that uPA exists in the form of receptor-bound similar to the cases in other cell lines [Saksela et al., 1983; Bajpai et al., 1985; Vassalli et al., 1985]. Moreover, it may be possible that uPA associated with membrane plays some important role in reversibility of differentiation induced with NaB [Kosaka et al., 1991], i.e., the degradation of extracellular matrices including coated collagen by uPA results in changes of the close environment of cell surface and may decrease in the stability of differentiated phenotypes.

Although F9 teratocarcinoma stem cells can be differentiated by treatment with RA or NaB, the differentiated cell character and also the

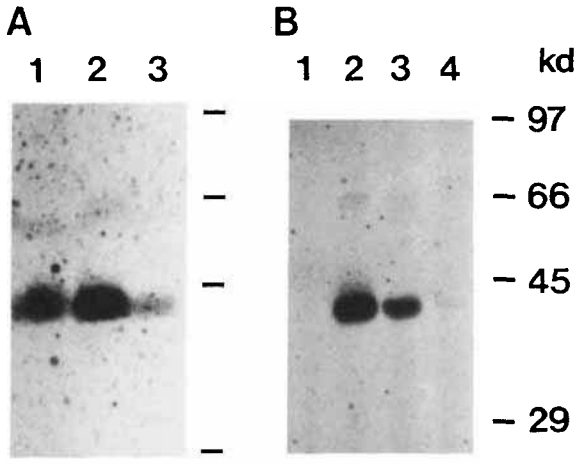

Fig. 4. Urokinase-type plasminogen activator in plasma membrane and cytosol fraction. **A:** F9 cells treated with 5 mM NaB for 24 h were fractionated as described in Methods. Membrane plus granule fraction (lane 2) and cytosol fraction (lane 3) were analyzed by zymographic assay. Lane 1 is mouse uPA standard from LLC cells. **B:** Membrane plus granule fraction was subfractionated by using sucrose gradient centrifugation as described in Methods. Fraction A (lane 1), B (lane 2), C (lane 3), and D (lane 4). The experiment was repeated three times and the results were reproducible.

differentiation pathway in response to these agents has not been defined in detail. It is possible that further studying of various roles of uPA and tPA during differentiation induced with NaB may provide us with more information about the mechanism of F9 cell differentiation induced with RA or NaB, in addition to the reversible and irreversible steps during F9 cell differentiation.

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