Induction of in Vitro Differentiation of Mouse Embryonal Carcinoma (F9) Cells by Inhibitors of Topoisomerases

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ABSTRACT

To investigate the possible involvement of topoisomerases in embryonal differentiation, we examined the effect of topoisomerase inhibitors on the in vitro differentiation of mouse embryonal carcinoma F9 cells. We found that camptothecin, teniposide (VM-26), or genistein, specific inhibitors of topoisomerases, induced morphological as well as biochemical changes (production of tissue plasminogen activator, synthesis of laminin, and disappearance of stage-specific embryonic antigen 1) specific to F9 cell differentiation. Since these changes were indistinguishable from those observed in F9 differentiation induced by retinoic acid (plus dibutyryl cyclic AMP), it was suggested that inhibition of cellular topoisomerase activities triggered F9 cell differentiation into parietal endoderm-like cells in the same manner as retinoic acid (plus dibutyryl cyclic AMP). Experiments using differentiation-resistant mutant F9 cell lines, however, indicated that the molecular cascade involved in topoisomerase inhibitor-induced differentiation involves different steps from those functioning in the retinoic acid-induced differentiation cascade.

INTRODUCTION

F9, an established mouse embryonal carcinoma cell line, differentiates into endoderm-like cells in vitro in the presence of retinoic acid (1). Dibutyryl cyclic AMP further differentiates the primitive endoderm-like cells into those which exhibit biochemical and morphological characteristics similar to those of parietal endoderm cells (2, 3). These endoderm-like cells can also be converted to visceral endoderm-like cells when exposed to aggregation conditions (3). Because of these distinct and versatile responses to differentiation, F9 cells have been widely used as a model for studying the mechanism of embryonic differentiation. Despite numerous studies, however, the molecular mechanism of differentiation, particularly the nature of the intracellular cascade leading to differentiation, is yet to be understood. Therefore, we attempted to identify compounds with known biological functions which affect retinoic acid-induced F9 differentiation or induce differentiation by themselves.

Recently, considerable attention has been focused on topoisomerases which catalyze configurational changes in DNA and which are believed to be involved in the dynamic behavior of chromatin. Several compounds specifically inhibit topoisomerases, among which is camptothecin, isolated from Camptotheca acuminata (4) and a specific inhibitor of topoisomerase I (5). VM-26 and genistein inhibit topoisomerase II rather than topoisomerase I (6–8). The former is a semisynthetic derivative of podophyllotoxin (6), and the latter was isolated from subterranean clover (9) as well as Pseudomonas spp. (10). These inhibitors affect a number of fundamental cellular functions associated with chromatin structures which include DNA replication (11), RNA transcription (12), cell division (13), sister chromatid exchange, and chromosome stability (14). Specific gene expression in several cultured mammalian cell lines induced by topoisomerase inhibitors has been reported recently. Camptothecin induced gene expression specific to HL-60, U937, ML-1, K562, and M1 cell differentiation (15). Novobiocin, a topoisomerase II inhibitor, also induced differentiation of HL-60 (16). Mouse erythroleukemia (Friend) cells were differentiated into erythroid cells by genistein in vitro (17, 18). Here we report that a series of compounds which specifically inhibit topoisomerases effectively triggered embryonal differentiation in F9 cells, and the differentiated cells exhibited morphological and biochemical characteristics of parietal endoderm cells, indicating that inhibition of topoisomerases can trigger F9 cell differentiation into parietal endoderm-like cells. We also present evidence that the cascade leading to differentiation induced by topoisomerase inhibitors includes steps which differ from those in retinoic acid-induced differentiation. Possible mechanisms of F9 cell differentiation by topoisomerase inhibitors are discussed.

MATERIALS AND METHODS

Materials. Camptothecin and genistein were obtained from Sigma Chemical Co. (St. Louis, MO) and Funakoshi Co. (Tokyo, Japan), respectively. VM-26 (teniposide) was a generous gift from Dr. T. Kamiya (Kyowa Hakko Co., Tokyo, Japan). Retinoic acid and dibutyryl cyclic AMP were purchased from Sigma. These compounds were stored in the following solvents and concentrations at −20°C before use: camptothecin (DMSO3, 1 mM); genistein (ethanol, 37 mM); VM-26 (DMSO, 15 mM); retinoic acid (ethanol, 1 mM); and dibutyryl cyclic AMP (H2O, 50 mM). No effects of the solvents on F9 cell differentiation were observed at the solvent concentrations which were carried over to the medium with the drugs. FITC-conjugated goat anti-rabbit IgG and rabbit anti-mouse IgG were purchased from Cappel Laboratories (West Chester, PA) and Sigma, respectively. Peroxidase-conjugated donkey anti-rabbit IgG and ECL detection kit were obtained from Amersham International (Bucks, United Kingdom). Anti-laminin antibody was obtained from Bioscience Products AG (Emmenbrucke, Switzerland) and Sigma. Mouse monoclonal anti-SSEA-1 antibody was kindly provided by Dr. D. Solter (Max Planck Institute, Freiburg, Germany). Skim milk, gelatin, and agar noble were supplied by Difco (Detroit, MI). All other agents were reagent grade. ES medium was purchased from Nissui Seiyaku (Tokyo, Japan). UCS medium was obtained from Dr. Y. Nishimune (Osaka University, Osaka, Japan). The cells were cultured at 37°C in a CO2 (5%) incubator in ES medium supplemented with FCS (10%, v/v) in plastic tissue culture dishes (Falcon) which had been coated with gelatin (0.4%, w/v).

Assays for Tissue Plasminogen Activator, Laminin, and SSEA-1. Tissue plasminogen activator was assayed as described by Nishimune et al. (19). In essence, F9 cells were overlaid with ES medium containing Noble agar (0.75%, w/v), skim milk (2.5%, w/v), and human plasminogen (0.2 casein units/ml). After 24 h of incubation at 37°C in a CO2

1 The abbreviations used are: DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; SSEA-1, stage-specific embryonic antigen 1; ES medium, Eagle’s medium supplemented with 1-glutamine, L-asparagine, L-aspartic acid, L-alanine, L-glutamic acid, glycine, L-proline, L-serine, sodium pyruvate, and cytosocobalamin; UCS, fetal calf serum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MNNG, N-methyl-N’-nitro-N-nitrosoguanidine; PA+ colonies, plasminogen activator-producing colonies.

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Laminin was assayed by either Western blotting or staining the cells with FITC-conjugated antibody against laminin. For Western blotting, the cells were cultured for 5 days, after which culture fluids were subjected to SDS-PAGE (3% stacking gel and 5% separation gel). The separated bands were transferred to Hybond-C (Amersham) by semidry blotting and fixed with glutaraldehyde (1%). The samples were first treated with rabbit anti-laminin IgG and incubated with the second antibody, peroxidase-conjugated donkey anti-rabbit IgG, as described previously (17). A commercial kit (ECL detection system) was then used to detect peroxidase activity. For fluorescent staining, the cells were fixed in the dishes with a methanol:acetic acid mixture (20:1) and washed with 0.05% (v/v) Tween 20 in phosphate-buffered saline. Anti-laminin antibody was then added, followed by FITC-conjugated goat anti-rabbit IgG. After adding one drop of 90% (v/v) glycerol in Na2CO3-NAHCO3 (5 mM) buffer (pH 9.5), the cells were photographed using a Nikon Optiphot epifluorescence microscope with a B-2 filter equipped with a Microflex UFX-II photomicrographic attachment.

SSEA-1 was detected by fluorescence staining in the same manner as laminin, except that anti-SSEA-1 antibody and FITC-conjugated goat anti-mouse IgG were used instead of anti-laminin antibody and FITC-conjugated goat anti-rabbit IgG, respectively.

Isolation of Differentiation-resistant Mutant Cell Lines. F9 cells were incubated overnight in the presence of MNNG (2 μg/ml). The culture was then divided into several portions, and one of the following compounds was added to each at the indicated concentrations: camptothecin, 75 nM; VM-26, 7.5 nM; genistein, 7.5 μM; and retinoic acid, 1 μM. After a 3-day incubation, MNNG was removed by centrifugation, and the cells were further incubated in the presence of the inhibitors or retinoic acid. Cells which thrived in the presence of these compounds were cloned, and a total of eight cell lines (two for each drug) were used for the experiments described in the text.

RESULTS

Effect of Topoisomerase Inhibitors on Cellular Morphology. When mouse embryonal carcinoma (F9) cells were incubated in the presence of camptothecin for 48 h, a specific inhibitor of topoisomerase I, the morphology of the cells changed considerably. This alteration resembled that induced by retinoic acid plus dibutyryl cyclic AMP in which F9 cells differentiate into parietal endoderm-like cells (2). As seen in Fig. 1, upon incubation with camptothecin, F9 cells were converted from tightly packed colonies (A) into slightly dispersed colonies with flat and refractive morphology-displaying processes (D). Those changes are characteristics of F9 cell differentiation induced by retinoic acid plus dibutyryl cyclic AMP (Fig. 1C). Camptothecin, at the concentration which induced the change (100 nM), had apparently no significant effect on the viability of the cells (assayed by trypan blue staining) and caused less than 10% cell death up to 3-day incubation (data not shown). The growth of the camptothecin-treated F9 cells was approximately 40% of

![Fig. 1. Change in morphology of the drug-treated F9 cells. F9 cells (~10^4 per dish) were plated on gelatin-coated Petri dishes. To the culture, retinoic acid (1 μM), retinoic acid (1 μM) plus dibutyryl cyclic AMP (1 mM), camptothecin (100 nM), VM-26 (15 nM), or genistein (35 μM) was added and, after 2 days of incubation in a CO2 incubator at 37°C, pictures were taken using a microscope (Olympus IMT-2). A, control; B, cells incubated with retinoic acid; C, cells incubated with retinoic acid plus dibutyryl cyclic AMP; D, cells incubated with camptothecin; E, cells incubated with VM-26; and F, cells incubated with genistein. All pictures were taken at the same magnification. Bar, 0.1 mm.](image-url)
that of untreated control cells during the first 3-day incubation with the drug (data not shown).

We also examined the effect of other topoisomerase inhibitors, such as VM-26 and genistein, on F9 cells. Both VM-26 and genistein induced morphological changes similar to those induced by camptothecin (Fig. 1, E and F, respectively). The optimum concentration of some of these topoisomerase inhibitors to induce these morphological changes was much lower (camptothecin, 100 nM, and VM-26, 15 nM) than that of retinoic acid (1 µM). Thus, these inhibitors, regardless of which topoisomerase was targeted, effectively induced morphological changes in F9 cells, resembling the features of parietal endoderm cells.

Effect of Topoisomerase Inhibitors on Biochemical Markers Associated with F9 Cell Differentiation. The effect of the topoisomerase inhibitors on F9 cells was also examined by assaying several biochemical markers which are induced or changed during F9 cell differentiation. First, we examined the production of tissue plasminogen activator, an indicator of endoderm differentiation (1, 2). The cells were incubated with either of the inhibitors for 2 days and overlaid with medium containing skim milk. Then the number of haloes, an indication of activator production, was counted. Incubation of the cells with these compounds considerably increased the number of colonies which produced haloes. The activator production as a function of the drug concentration is shown in Fig. 2. As seen in the figure, at the optimal concentrations for each compound (camptothecin, 200 nM; VM-26, 15 nM; and genistein, 55 µM), which were more or less in the same range as those which induced the morphological changes described above, 60 to 80% of the cells produced the activator. Since retinoic acid (1 µM) with dibutyryl cyclic AMP (1 mM) induced the plasminogen activator in about 60% of the cells under the same culture conditions, these topoisomerase inhibitors were equally or more effective than retinoic acid in producing the activator.

We also examined the effect of the topoisomerase inhibitors on the induction of laminin, a biochemical marker associated with F9 differentiation from endoderm-like into parietal endoderm-like cells, a step which apparently requires cyclic AMP. Since laminin is usually detected in the culture fluid as well as inside the cells, culture fluids after incubation of the cells with the inhibitors for 4 days were electrophoresed and then Western blotted. Typically blotting profiles obtained after incubating the cells with camptothecin or VM-26 as well as with retinoic acid (plus dibutyryl cyclic AMP) are shown in Fig. 3. As seen in the figure, camptothecin or VM-26 induced lammin as effectively as retinoic acid (plus dibutyryl cyclic AMP). Essentially the same result was obtained with genistein (data not shown). The induction of laminin by the topoisomerase inhibitors was also confirmed by staining the cells with FITC-conjugated anti-laminin antibody. One of the results (induction by genistein) is shown in Fig. 4. The induction of laminin production indicated that the cells induced by the topoisomerase inhibitors had characteristics of parietal endoderm cells.

The expression of these biochemical markers (plasminogen activator and laminin) by the topoisomerase inhibitors was irreversible. Even after the inhibitors were removed from the medium after 2 days of incubation, at which very low levels of these markers were detected, the cells continued expressing the markers at almost the same rate as observed with cells which were continuously incubated with the inhibitors (data not shown). We also found that the cells ceased to divide to form colonies after a 24-h incubation with the drugs (data not shown), as observed in retinoic acid-induced differentiation. This phenomenon specific to F9 cell differentiation, probably reflects cellular commitment (to differentiation).

In addition to tissue plasminogen activator and laminin, which are inducibly expressed during F9 cell differentiation into endoderm- and parietal endoderm-like cells, respectively, we also assayed the level of an embryonal cell-specific antigen (SSEA-1) (20). SSEA-1 is present in embryonal cells but disappears during differentiation. Figure 5 shows that SSEA-1,
Fig. 3. Western blotting of laminin. F9 cells were cultured in Petri dishes (35-mm diameter), and the media were replenished 2 days later with fresh ones containing either retinoic acid (1 μM), retinoic acid (1 μM) plus dibutyryl cyclic AMP (1 mM), camptothecin (100 nM) or VM-26 (15 nM). On Day 4 (camptothecin- and VM-26-treated cells) and Day 5 (retinoic acid- and retinoic acid plus dibutyryl cyclic AMP-treated cells), the culture fluids were withdrawn, and the fluid equivalent to that of a 3 x 10⁶ cell culture was subjected to SDS-PAGE and Western blotting as described in “Materials and Methods.” Peroxidase activity was detected using an ECL detection kit with 30-s exposure time. Lane 1, control cells; Lane 2, cells incubated with retinoic acid; Lane 3, cells incubated with retinoic acid plus dibutyryl cyclic AMP; Lane 4, cells incubated with camptothecin; and Lane 5, cells incubated with VM-26.

detected in the cells by FITC-conjugated antibody, disappeared after incubation with camptothecin, VM-26, and genistein, as occurs in retinoic acid-induced cells. This indicates that F9 cells lost the embryo-specific antigen as they underwent the morphological and biochemical changes specific to F9 differentiation.

As far as we investigated, topoisomerase inhibitors induced changes in F9 cells which were almost indistinguishable from those observed in retinoic acid plus dibutyryl cyclic AMP-induced differentiation. We therefore concluded that inhibition of cellular topoisomerase activities by these topoisomerase inhibitors triggered F9 cell differentiation into parietal endoderm-like cells.

Induction in Mutant Cell Lines Resistant to Retinoic Acid-induced Differentiation. We next investigated whether or not the cascade leading to the differentiation by topoisomerase inhibitors is the same as that functioning in retinoic acid (plus dibutyryl cyclic AMP)-induced differentiation. We isolated mutant F9 cells which have become resistant to differentiation by camptothecin, VM-26, genistein, or retinoic acid, respectively. Among them, a pair of mutants resistant to differentiation by each of the compounds (CT'-3 and CT'-6, resistant to camptothecin; VM'-1 and VM'-4, resistant to VM-26; GS'-1 and GS'-2, resistant to genistein, and RA'-6 and ROT-1, resistant to retinoic acid-induced differentiation) were then exposed to the inhibitors as well as to retinoic acid, and production of tissue plasminogen activator by each mutant was examined. The results are summarized in Table 1. Whereas the mutants resistant to differentiation by topoisomerase inhibitors (CR'-3, CT'-6, VM'-1, VM'-4, GS'-1, and GS'-2) resisted differentiation by any of the inhibitors (with the possible exception of CT' cell induction by VM-26), these mutant cells all responded normally to retinoic acid, producing tissue plasminogen activator. Conversely, the mutant cells resistant to differentiation induced by retinoic acid (RA'-6 and ROT-1) responded to all the topoisomerase inhibitors, producing the activator at frequencies almost
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Fig. 5. Immunofluorescence staining of SSEA-1. F9 cells were incubated in the presence of retinoic acid (1 μM) plus dibutyl cyclic AMP (1 mM), camptothecin (100 nM), VM-26 (7.8 nM), or genistein (28 μM). After 5-day incubation, SSEA-1 was stained with FITC-conjugated antibody after reacting the cells with anti-SSEA-1 antibody as described in “Materials and Methods.” The photographs were taken using a Nikon Optiphot epifluorescence microscope with a B-2 filter equipped with a Microflex UFX-II photomicrographic attachment. A, control cells; B, cells incubated with retinoic acid plus dibutyl cyclic AMP; C, cells incubated with camptothecin; D, cells incubated with VM-26; and E, cells incubated with genistein. Bar, 0.1 mm.

The results were essentially the same as those induced by retinoic acid, equivalent to those observed in the parental cell line. The most straightforward interpretation of the results would be that the cascade leading to the differentiation triggered by all three topoisomerase inhibitors has a common step and that the molecular nature of the cascade, or at least some part, differs from that involved in retinoic acid-induced differentiation.

DISCUSSION

In this paper, we reported that incubation of mouse embryonal carcinoma (F9) cells with inhibitors of either topoisomerase I or II induced morphological and biochemical changes whose characteristics were essentially the same as those induced
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Table 1  Induction of tissue plasminogen activator in differentiation-resistant F9 mutant cells

<table>
<thead>
<tr>
<th>Mutant cells</th>
<th>Retinoic acid (1 μM)</th>
<th>Camptothecin (100 nM)</th>
<th>VM-26 (15 μM)</th>
<th>Genistein (35 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT-3</td>
<td>87</td>
<td>&lt;1</td>
<td>15</td>
<td>&lt;1</td>
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<tr>
<td>CT-6</td>
<td>89</td>
<td>&lt;1</td>
<td>12</td>
<td>&lt;1</td>
</tr>
<tr>
<td>VM-1</td>
<td>95</td>
<td>5</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>VM-4</td>
<td>96</td>
<td>8</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>GS'-1</td>
<td>39</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>GS'-2</td>
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<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RA-6</td>
<td>&lt;1</td>
<td>87</td>
<td>42</td>
<td>32</td>
</tr>
<tr>
<td>ROT-1</td>
<td>&lt;1</td>
<td>76</td>
<td>58</td>
<td>13</td>
</tr>
</tbody>
</table>

Cells were treated with the drug concentrations indicated above for 2 days, and PA* colonies were assayed as described in the legend of Fig. 2.

by retinoic acid plus dibutyryl cyclic AMP. These included alterations in cell morphology, production of tissue plasminogen activator and laminin, disappearance of SSEA-1, and cessation of cell division (loss of colony-forming ability). Among them, the production of laminin, which was generally observed in parietal endoderm cells but in neither primitive nor visceral endoderm cells, indicated that the topoisomerase inhibitor-induced cells were equivalent or similar to parietal endoderm cells. In retinoic acid-induced differentiation, parietal endoderm-like cells are differentiated from primitive endoderm-like cells. In retinoic acid-induced differentiation, parietal endoderm-like cells are differentiated from primitive endoderm-like cells in the presence of dibutyryl cyclic AMP. Because of the striking similarity of the changes to those induced during in vitro differentiation by retinoic acid plus dibutyryl cyclic AMP, it seems quite likely that inhibition of topoisomerase activities in the cells triggered F9 cell differentiation into parietal endoderm-like cells.

Induction of embryonal carcinoma cells by inhibitors of not only topoisomerase I (camptothecin) but also of topoisomerase II (VM-26 and genistein) indicates that the induction of differentiation by topoisomerase inhibitors is a more general phenomenon than previously thought. It also showed that inhibition of either topoisomerase I or II can trigger differentiation. Our experiments reported here, however, apparently distinguished the induction process triggered by topoisomerase inhibitors from that functioning in the retinoic acid (plus dibutyryl cyclic AMP)-induced differentiation. In contrast to retinoic acid, these topoisomerase inhibitors triggered differentiation all the way to parietal endoderm-like cells. More interestingly, cells defective in retinoic acid-induced differentiation were susceptible to differentiation by topoisomerase inhibitors. Efforts to obtain mutants resistant to differentiation to both inducers (retinoic acid and topoisomerase inhibitors) have so far been unsuccessful (data not shown). Taken together, inhibition of any topoisomerase activity opens up a new differentiation pathway leading to parietal endoderm cells which bypasses at least a part of the cascade present in retinoic acid (plus dibutyryl cyclic AMP)-induced differentiation. If this is the case, it may be possible to detect other biochemical changes, in addition to those reported here, which are unique to cells differentiated by topoisomerase inhibitors.

Recent studies have indicated that the differentiation cascade induced by retinoic acid is initiated by retinoic acid complexing with retinoic acid receptor proteins, followed by the action of the complex on regulatory sequences of genes whose expression (or repression) is critical for differentiation (21–23). In this context, how does the inhibition of cellular topoisomerases trigger F9 cell differentiation? The topoisomerase inhibitors used here all inhibit the enzyme by blocking the ligation step of the topoisomerase activity, which consists of making double-stranded breaks in DNA and subsequently ligating them. Since topoisomerases are most likely involved in the dynamic behavior of chromatin, inhibition of the enzyme activity should affect a number of intracellular reactions associated with the structure and function of chromatin. Although it cannot be determined from the present study what kind of molecular effect on the chromatin caused the induction of F9 cell differentiation, a likely possibility is that the topoisomerase inhibitors cause a change in the configuration of the chromatin in such a way that the change induced the specific gene expression (or repression) required for F9 differentiation. Such a configurational change itself is similar to that observed in the retinoic acid-induced differentiation, even though the steps leading to its may be quite different as discussed above. If this is the case, the topoisomerase inhibitor-induced F9 differentiation reported here should provide a useful tool to investigate the molecular mechanism of embryonal differentiation, since the primary target of the differentiation inducers (topoisomerase inhibitors) and their effects have been well documented (for a review on topoisomerase inhibitors, see Ref. 11). On the other hand, the possibility that the targets of these compounds were not topoisomerases cannot be excluded, since the optimum concentrations of these compounds which induced F9 differentiation were much lower than those which induced breakages in the cellular DNA. In any event, it is interesting that embryonal differentiation, whose inducers have been considered to be limited to specific compounds such as retinoic acid, is effectively induced by a series of compounds whose biological effects are more general. This suggests that there are several ways to induce embryonal differentiation and that the mechanism of differentiation is much more flexible than previously thought.

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