Hinokitiol Induces Differentiation of Teratocarcinoma F9 Cells

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Hinokitiol, a constituent of the wood of *Chamaecyparis taiwanensis*, was found to induce differentiation of teratocarcinoma F9 cells. When examined by the agar-overlay method, in which expression of plasminogen activator as a differentiation marker protein was detected, this compound exhibited a dose- and time-dependent induction. Induction of differentiation by hinokitiol occurred irreversibly and required its addition for more than 12 h. Among its structure-related compounds tested, tropolone and two colchicine-related compounds exerted potent activities comparable to that of hinokitiol. These findings indicate that free tropolone structure in the molecules plays an essential role in inducing differentiation of F9 cells. Hinokitiol showed a strong inhibitory effect on DNA synthesis in very early stages of culture, suggesting that this effect may be responsible for triggering differentiation of F9 cells.

Key words hinokitiol; differentiation; teratocarcinoma; plasminogen activator; tropolone

Murine F9 cells, the malignant stem cells derived from a teratocarcinoma,¹⁾ can be induced to differentiate to endoderm-like cells by exposure to retinoic acid.²⁾ It causes differentiation of F9 cells into a tissue type that resembles extraembryonic primitive endoderm of the embryo. This differentiation is characterized by several phenotypic changes such as morphological alterations and expression of differentiated cell-specific proteins including laminin, type IV collagen and plasminogen activator (PA).^{3,4)} Such dramatic changes of F9 cells have also been demonstrated by the treatment of various inducers such as sodium butyrate,^{5,6)} anticancer drugs^{5,7)} and genetical approaches.^{8,9)} Although recent reports describe that some nuclear proto-oncogenes are rapidly and transiently expressed in the cells by some inducers,¹⁰⁾ none of the induction mechanisms are clearly dissolved. To date, these stem cell lines have been used as a model system for studying biological aspects and pharmacological effects of teratogenic agents during the process of differentiation. It is still important to search for other agents able to induce differentiation of F9 cells, leading to the development of a promising new drug for differentiation chemotherapy.

In this study, we investigated the differentiation-inducing abilities of naturally-occurring substances with respect to PA production in F9 cells, and found that hinokitiol is a potent inducer. Hinokitiol is a constituent of the wood of *Chamaecyparis taiwanensis* with a chemical structure comprising a tropolone ring.¹¹⁾ This compound has been known as a long-standing antimicrobial agent¹²⁾ and its unique inhibitory effects on the growth of tumor cells have been reported.^{13,14)} However, there has been no significant study of its differentiation-inducing activity. Our results show that hinokitiol can irreversibly induce differentiation of F9 cells and its activity may be due to the inhibition of DNA synthesis.

MATERIALS AND METHODS

Materials Hinokitiol, tropolone, colchicine were ob-

tained from Tokyo Kasei Kogyo (Tokyo, Japan) and demecolcine and trimethylcolchicinic acid were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) (Chart 1). Eagle's minimum essential medium (MEM) was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum was a product of Filtron (Brooklyn, Australia) and used after heat inactivation. Plasminogen was purchased from Japan Chemical Research (Kobe, Japan) and skim milk was from Difco Labs. (Detroit, MI, U.S.A.). [6-Methyl-³H]thymidine was obtained from ICN Biomedical Inc. (Costa Mesa, CA, U.S.A.). All other chemicals employed in this study were commercially available products of the highest grade.

Cell Culture F9 cells were obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan) and cultivated in MEM supplemented with 5 mM glutamine, 1 mM sodium pyruvate and 10% fetal bovine serum at 37°C in an atmosphere of 5% CO₂-air. Cells were passaged at confluence by treatment with 0.2% trypsin-0.02% EDTA-20 mM phosphate buffered saline (pH 7.4,

Chart 1. Chemical Structures of the Compounds Tested in This Study

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PBS) at 37 °C for 10 min and used for the experiment at passages 3 to 7. About 120 cells were seeded in gelatin-precoated culture dishes (35 mm, Nunc) and cultivated for 3 d to form colonies.

Agar-Overlay Method for Detecting PA-Producing Colony Differentiation of F9 cells was evaluated by the production of PA, a marker protein of differentiation, from the colonies according to the method of Kosaka *et al.*¹⁵⁾ The medium was aspirated from the cultured dishes and 1 ml of fresh medium was added. Each compound was dissolved in distilled water or ethanol and small volumes of sterile concentrated solutions were added at the final ethanol concentration of less than 1%. Ethanol had no effect on the colonization below this concentration. After cultivation for the indicated period, the colonies were macroscopically counted, washed with PBS and further incubated with 1 ml of prewarmed (43 °C) agar-overlay solution which contained 0.75% agar, 2.5% skim milk and 0.3 CU/ml bovine plasminogen in MEM at 37 °C for 24 h. In this assay culture, PA produced from the colony catalyzes the conversion of plasminogen to plasmin, which finally digests casein to make a clear plaque spot. These spots were counted as PA-producing colonies and determined for their PA production rate (%) by dividing that value by the total number of colonies tested.

Assay for DNA Synthesis One ml of F9 cell suspension (1×10^5 cells/ml) was cultivated in 24-well microplates (CORNING) for 24 h at 37 °C in an atmosphere of 5% CO₂-air and then incubated with various concentrations of hinokitiol for another 24 h. Cells were pulsed with 37 kBq/well of [³H]thymidine for the last 1 h of incubation. Cells were washed with PBS, treated with 0.2% trypsin–0.02% EDTA–PBS for 10 min, and collected on a glass filter paper. The filters were washed with 5% trichloroacetic acid and ethanol and their radioactivities were counted in a liquid scintillation counter (Aloka). DNA contents were fluorometrically determined by the method of Hinegardner¹⁶⁾ using salmon DNA as a standard.

RESULTS

Differentiation-Inducing Activity of Hinokitiol Among several plant-originated compounds tested in this study, hinokitiol was found to have a potent inducing activity of PA production in F9 cells. As shown in Fig. 1, hinokitiol exhibited a dose-dependent PA induction at the concentrations ranging from 6 to 60 μ M, and about half the colonies were changed to the differentiated cells at 60 μ M. At these concentrations, hinokitiol showed no inhibitory effect on the colony numbers. This activity was the same as that of 10 mM sodium butyrate used as a positive inducer (data not shown). However, the differentiation by hinokitiol slightly decreased at 120 μ M with the reduction of total colony number. Therefore, differentiation induction of F9 cells by hinokitiol was evaluated at the concentrations of 60 μ M in the following experiments. Its induction profile was subsequently examined with respect to the incubation time. Figure 2 illustrates that PA production was apparently observed within 12 h

after addition of hinokitiol and reached a plateau between 24 and 48 h after addition.

Characteristics of Hinokitiol-Induced Differentiation of F9 Cells The monolayer culture of undifferentiated F9 cells formed tightly packed colonies with a morphology characteristic of embryonal carcinoma cells, as shown in Fig. 3(a). Hinokitiol treatment of these cells at 60 μ M for 24 h caused a slightly dispersed arrangement of cells exhibiting a flat polygonal shape (Fig. 3(b)). Such morphological change was similar to that observed in the cells incubated with 10 mM sodium butyrate for 24 h (Fig. 3(c)). To compare the induction profiles of hinokitiol and butyrate, the reversibility of their differentiation-inducing potencies was determined. Cells were incubated with 60 μ M hinokitiol or 10 mM sodium butyrate for 24 h, followed by further incubation in a fresh medium without inducer for

Fig. 1. Differentiation-Inducing Activity of Hinokitiol as a Function of Concentration

The colonies of F9 cells were incubated with various concentrations of hinokitiol for 24 h. After washing of the culture dishes, PA-producing colonies were estimated by the agar-overlay method in which the formation of caseinolytic plaques were detected. PA production rate (%) was expressed as the percentage of the total colonies. All data represent mean $\pm$ S.D. from triplicate culture dishes.

Fig. 2. Differentiation-Inducing Activity of Hinokitiol as a Function of Time

The colonies of F9 cells were incubated with 60 μ M hinokitiol for the indicated periods and then PA-producing colonies were estimated by the agar-overlay method. PA production rate (%) was expressed as the percentage of the total colonies. All data represent mean $\pm$ S.D. from triplicate culture dishes.

Fig. 3. Morphological Changes of F9 Cells Incubated with Hinokitiol and Sodium Butyrate

Undifferentiated F9 cells (a) were treated with 60 μ M hinokitiol (b) or 10 mM sodium butyrate (c) for 24 h and microscopically examined.

Fig. 4. Irreversibility of Differentiation Induction by Hinokitiol

The colonies of F9 cells were incubated with 60 μ M hinokitiol (a) or 10 mM sodium butyrate (b) for 24 h. After washing, the cells were incubated without agents for an appropriate time as indicated. PA production was then assessed by the agar-overlay method and expressed as mean $\pm$ S.D. from triplicate culture dishes.

Fig. 5. Inhibitory Effect of Hinokitiol on DNA Synthesis in F9 Cells

(a) F9 cells were incubated with various concentrations of hinokitiol for 24 h and pulsed with [3 H]thymidine for the last 1 h. (b) Cells were exposed to 60 μ M hinokitiol for an appropriate time and pulsed with [3 H]thymidine for the last 1 h. DNA contents were separately determined and the relative value (%) of DNA synthesis (cpm/ μ g DNA) was calculated. Each value represents mean $\pm$ S.D. from triplicate culture dishes.

Fig. 6. Differentiation-Inducing Activity of Hinokitiol-Related Compounds

The colonies of F9 cells were incubated with various compounds for 24 h and PA production was determined by the agar-overlay method. $\circ$, colchicine; $\bullet$, cholchicine; $\square$, demecolcine; $\blacksquare$, trimethylcolchicinic acid. All data represent mean $\pm$ S.D. from triplicate culture dishes.

0, 4, 8 and 12 h. PA production of each culture was, then, determined by the agar-overlay method. Hinokitiol-treated cells exhibited the same level of differentiation even after 12 h-incubation in the fresh medium, whereas the inducing activity of butyrate disappeared just after 4 h (Fig. 4). The latter cells were observed to revert to a morphology similar to the undifferentiated phenotype following further cultivation.

Inhibitory Effect on DNA Synthesis Cell proliferation of F9 cells was inhibited by hinokitiol in a dose-dependent manner, as shown in Fig. 5(a). This inhibition was observed even at 0.6 μ M and no proliferation occurred at more than 30 μ M. In fact, cell proliferation was completely depressed after 4 h, when cells were incubated with 60 μ M hinokitiol (Fig. 5(b)).

Differentiation-Inducing Activity of Hinokitiol-Related Compounds Hinokitiol is one of the derivatives of tropolone and, in addition, colchicine-related compounds also include tropolone ring in their molecules. Therefore, the differentiation-inducing activities of tropolone, colchicine, colchicine, demecolcine and trimethylcolchicinic acid were compared to that of hinokitiol. When treated

at 10 $\mu\text{g/ml}$ for 24 h, tropolone (82 μM) exhibited the same inducing activity as hinokitiol (60 μM), but four colchicine-related compounds showed no induction (Fig. 6). However, trimethylcolchicinic acid and colchicine exerted their potentials to induce differentiation of F9 cells at 25–100 $\mu\text{g/ml}$ (73–292 μM) and 50–100 $\mu\text{g/ml}$ (130–260 μM), respectively.

DISCUSSION

In the present study, we detected a potent activity of hinokitiol in inducing differentiation of F9 cells by evaluating both the morphological change and the production of differentiation marker protein. This effect was observed in a dose-dependent fashion, showing a maximum activity at 60 μM . Several reports have dealt with its cytotoxic effect on mammalian cells *in vitro*. Yamato *et al.*^{13,17)} have described its cytopathogenic concentration (IC_{50}) against some tumor cell lines to be around 3.6 μM , while Inamori *et al.*¹⁴⁾ have recently reported that it effectively inhibited cell growth of both tumor and blastic cells at 1.8–3.6 μM . Compared with these values, a relatively high concentration was required for induction of differentiation of F9 cells. Although hinokitiol caused no reduction of viable colony numbers up to 60 μM in the present study, it is suggested that hinokitiol-induced differentiation of F9 cells may be associated with its strong cytotoxicity.

Hinokitiol-induced differentiation required incubation for at least 12 h, resulting in irreversible change into a differentiated phenotype. Such an induction profile resembled those of various agents known as differentiation inducers,⁵⁾ but not that of sodium butyrate.¹⁵⁾ The latter, a potent differentiating agent for a wide range of neoplastic cells *in vitro*, is known to induce differentiation *via* the inhibition of DNA synthesis resulting from the inhibition of histone deacetylase,^{6,18)} whereas most other inhibitors cause differentiation through the direct inhibition of DNA synthesis.⁵⁾ Such a difference is considered to be associated with the reversibility of differentiation induction. Since rapid and strong inhibition of DNA synthesis by hinokitiol was observed, this irreversible effect might be essential in the initiation process for differentiation. In fact, Yamato *et al.*¹⁷⁾ have suggested that tropolone and its derivatives may inhibit ribonucleotide reductase, an obligatory enzyme in DNA synthesis.¹⁹⁾ They proposed a hypothesis that these compounds chelate with Fe ions present in the catalytic moiety to inactivate the enzyme. In addition, Kontoghiorghes *et al.*²⁰⁾ reported the cytotoxic and DNA-inhibitory effects of some iron chelators. We must further clarify how inhibition of DNA synthesis by hinokitiol is linked to induction of differentiation of F9 cells.

Tropolone was also found to be as potent as hinokitiol and, in addition, two colchicine-related compounds which comprise free tropolone structure in their molecules showed comparable activities. Judging from the molar

basis, these four agents possessed almost the same inducing activities at around 100 μM . The results indicate that no substitution on the tropolone structure is essential to induce differentiation effectively, even though the membrane permeability of these compounds remains to be elucidated. The finding that colchicine showed no activity indicates that its biological alterations (such as microtubular depolymerization²¹⁾) do not participate in this differentiation process of F9 cells. From these results, it is concluded that hinokitiol could be a unique differentiation inducer which functions via a mechanism distinct from those of retinoic acid and sodium butyrate. As differentiation of F9 cells mimics embryonal development, a more detailed profile of hinokitiol-induced differentiation is needed to gain some insights into embryogenesis. Furthermore, it would be of great interest to evaluate the capability of hinokitiol in inducing differentiation of other undifferentiated cells such as human myeloid leukemia cells.

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