Induction of apoptosis by hinokitiol, a potent iron chelator, in teratocarcinoma F9 cells is mediated through the activation of caspase-3

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Abstract. Hinokitiol, a potent iron chelator, has been reported to induce differentiation in teratocarcinoma F9 cells with a reduction of viable cells. In this study, we examined the steps leading to eventual cell death by hinokitiol during differentiation. Hinokitiol induced DNA fragmentation of F9 cells in a concentration- and time-dependent manner. This effect was also observed in a cell-free system using the nuclei from intact cells and the cytosols from hinokitiol-treated cells. In contrast, hinokitiol methyl ether and hinokitiol-Fe (III) complex, which are deficient in iron-chelating activity, showed no DNA fragmentation activity in both cell culture and cell-free systems. These results suggest that iron deprivation by hinokitiol may be involved in the induction of apoptosis of F9 cells. Caspase-3, one of the key enzymes in the apoptotic cascade, was specifically activated by hinokitiol treatment, but not by the other two derivatives. In addition, its specific inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, strongly blocked hinokitiol-induced DNA fragmentation. These results indicate that iron deprivation by hinokitiol can induce apoptosis of F9 cells through the activation of caspase-3.

INTRODUCTION

F9 cells, mouse embryonal carcinoma cells, cloned by Berstine (Berstine *et al.* 1973), can be induced to differentiate into primitive endoderm-like cells by treatment with retinoic acid (Strickland *et al.* 1980, Hogan *et al.* 1981), sodium butyrate (Nishimune *et al.* 1983, McCue *et al.* 1984), anticancer drugs (Nishimune *et al.* 1983, Doi *et al.* 1995) and several chelators such as hinokitiol (Muto *et al.* 1995), dithizone (Tanaka *et al.* 1995) and deferoxamine (Tanaka *et al.* 1997). Differentiation of F9 cells induced by these agents is associated with the disappearance of stage-specific embryonic antigen-1, the change in cell morphology and the expression of plasminogen activator, laminin and collagen type IV. Because of these properties, F9 cells are thought to be useful for studying the early stage of mammalian

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development, differentiation or teratogenesis by various kinds of agents (Muto *et al.* 1995, Tanaka *et al.* 1995).

In a previous paper (Tanaka *et al.* 1997), we have demonstrated that a therapeutic iron chelator, deferoxamine, causes a reduction in viable cell number during the process of differentiation induction in F9 cells and that it is partly due to the occurrence of apoptotic cell death. Deferoxamine and some other iron chelators have also been reported to induce apoptosis in proliferating lymphocytes and leukaemic cells by the mechanism of iron deprivation (Haq *et al.* 1995, Hileti *et al.* 1995). In addition, many anti-tumour agents have been known to induce not only necrotic cell damage but also apoptotic cell death in tumour cells, supporting the evidence that many chemicals can induce apoptosis at lower concentrations than their grossly toxic ones. Although the intracellular apoptotic cascade mediated by physiological death signals such as Fas and tumour necrosis factor receptor has been considerably elucidated, the mechanism of apoptosis triggered by these xenobiotic agents remains to be elucidated.

In this study, we examined an apoptogenic activity of hinokitiol, a potent iron chelator, in F9 cells under both cell culture and cell-free conditions. Apoptosis by this chelator was found to be due to its iron-chelating ability resulting in subsequent iron deprivation and to be mediated by the activation of caspase cascade

MATERIALS AND METHODS

Materials

Hinokitiol was a product of Tokyo Kasei Kogyo (Tokyo, Japan). Hinokitiol methyl ether was synthesized according to the method of Yamato *et al.* (1984) and its structure and purity were confirmed by the spectrophotometrical analysis and HPLC method. Hinokitiol-Fe (III) complex was synthesized as a reddish precipitate by mixing 10 mM hinokitiol and 3.3 mM iron (III) chloride and its structure was confirmed spectrophotometrically. Eagle's minimum essential medium (MEM) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan) and fetal bovine serum (FBS) was from Filtron (Brooklyn, Australia). Diphenylamine, proteinase K and benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Acetyl Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO) were purchased from Takara Shuzo Co. (Kusatsu, Japan). Acetyl Tyr-Val-Ala-Asp-*p*-nitroanilide (Ac-YVAD-*p*NA), acetyl Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-*p*NA) and acetyl Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) were obtained from Biomol Research Laboratories (PA, USA). Other chemicals used in this study were obtained from Nacalai Tesque (Kyoto, Japan).

Cells and cell culture

Mouse embryonal carcinoma F9 cells were provided by Dr Y Nishimune (Research Institute for Microbial Diseases, Osaka University). F9 cells were maintained in MEM supplemented with 10% heat-inactivated FBS, 5 mM L-glutamine and 1 mM sodium pyruvate, and grown in an atmosphere of 5% CO₂ at 37°C. Cells were detached from the surface of dishes by 0.2% trypsin-0.02% EDTA in calcium- and magnesium-free phosphate-buffered saline, washed and seeded again at a density of $1-2 \times 10^5$ cells/10 ml/10 cm² dish every 2-3 days. In our standard culture conditions, the population doubling time of the logarithmically growing F9 cells was 14.8 h. For cell growth and DNA fragmentation analyses, cells (1×10^6 /10 cm² dish) were cultured for 48 h in the culture medium, and then treated with

various concentrations of compounds for 24 h or the periods shown in the respective figures. Cells were harvested and centrifuged at 1500 *g* for 10 min. Cell pellets collected in microtubes were lysed in 400 μ l of lysis buffer (0.2% Triton X-100–10 mM Tris-HCl (pH 8.0)–1 mM EDTA) at 4°C for 15 min. Cell lysates were centrifuged at 13 000 $\times$ *g* for 20 min and the supernatants containing fragmented DNAs were subjected to gel electrophoresis and DNA determination. The unfragmented DNAs in the precipitate were also subjected to DNA determination. Fragmented DNA in the samples (200 μ l aliquot) were precipitated by isopropyl alcohol. DNA content was determined by the diphenylamine reaction (Burton 1968).

DNA fragmentation analysis

The samples (200 μ l) for gel electrophoresis were treated with 100 μ g/ml DNase-free RNase at 37°C for 1 h and 1 mg/ml proteinase K at 50°C for 45 min. DNAs were precipitated by adding 50 μ l of 5 M NaCl and 250 μ l of isopropyl alcohol. After centrifugation at 12 000 $\times$ *g* for 10 min, DNA pellets were resolved in 8.9 mM Tris-HCl (pH 8.0)–8.9 mM borate–1 mM EDTA (TBE buffer) at the equivalent concentration to total (unfragmented plus fragmented) DNA. Two μ l of 1 mg/ml ethidium bromide was added to 18 μ l of DNA samples. Fragmented DNA was analysed by electrophoresis on a 2% agarose gel in TBE buffer. The gels were photographed under UV transilluminator.

Isolation of nuclei and cytosols

Isolation of nuclei and cytosols was performed by the method of Yoshida *et al.* (1996) with slight modifications. Briefly, intact and hinokitiol-treated F9 cells were resuspended in 0.25 M sucrose–50 mM Tris-HCl (pH 7.5)–25 mM KCl–5 mM MgCl₂–0.25% Triton X-100 (STKM buffer) at a density of 2.5×10^8 cells/ml. After incubation for 15 min on ice, the cell lysates were centrifuged at 800*xg* for 5 min at 4°C. The pellets were resuspended in STKM buffer at the same cell density of 2.5×10^8 cells/ml and used as the nucleus suspensions. The supernatants were further ultracentrifuged at 105 000 $\times$ *g* for 60 min at 4°C and the resultant supernatants were used as the cytosol fractions.

DNA fragmentation analysis in a cell-free system

DNA fragmentation analysis in a cell-free system was carried out according to the method of Yoshida *et al.* (1996). Fifty μ l of the nucleus suspensions isolated from untreated F9 cells equivalent to 1.25×10^7 cells were mixed with 100 μ l of the cytosols isolated from untreated or hinokitiol-treated cells equivalent to 2.5×10^7 cells. The reaction mixture was incubated at 37°C for 1 h and subsequently centrifuged at 12 000 $\times$ *g* for 10 min. The fragmented DNAs were extracted from the pellets by adding 200 μ l of lysis buffer for 15 min at 4°C and subjected to agarose gel electrophoresis as described above.

Caspase assay

Intact and hinokitiol-treated F9 cells lysed in STKM buffer at a density of 2.5×10^8 cells/ml for 15 min on ice were centrifuged at 800 *g* for 5 min at 4°C. Cell lysates were further centrifuged at 900 *g* for 10 min at 4°C and the supernatants were used as the enzyme samples. Caspase activities were colorimetrically measured using Ac-YVAD-pNA and Ac-DEVD-pNA as substrates (Gurtu, Kain & Datta *et al.* 1996, Datta *et al.* 1997, Gurtu *et al.* 1997). The assay was performed in 96-well microtitre plates by incubating 20 μ l of cell lysates with 178 μ l of reaction buffer (100 mM HEPES (pH 7.5)–20% (v/v) glycerol–5 mM dithiothreitol–0.5 mM EDTA) and 2 μ l of 10 mM substrate at 37°C for 2 h. *p*-Nitroaniline

formed by the enzyme reaction was spectrophotometrically monitored by a microplate reader (Bio-Rad model 450) and determined for its amount on the basis of its standard curve.

RESULTS

Detection of DNA fragmentation in F9 cell treated with hinokitiol and its derivatives

When F9 cells were treated with various concentrations of hinokitiol for 24 h, F9 cells showed internucleosomal DNA fragmentation in a concentration-dependent manner (Fig. 1A). This effect was weakly observed at 2 $\mu\text{g/ml}$ hinokitiol and clearly shown at concentrations of 10 and 50 $\mu\text{g/ml}$. Since hinokitiol has been reported to induce differentiation of F9 cells at the same concentration range (Muto *et al.* 1995), these findings indicate that hinokitiol may induce simultaneously both differentiation and apoptosis in the

Figure 1. Detection of DNA fragmentation in F9 cells treated with hinokitiol by agarose gel electrophoresis. (A) F9 cells were treated with various concentrations of hinokitiol for 24 h. lane 1, 100 bp ladder marker; lane 2, DNA from control cells; lane 3, 4, 5 and 6, DNA from cells treated at 0.4, 2, 10 and 50 $\mu\text{g/ml}$, respectively. (B) F9 cells were treated with 10 $\mu\text{g/ml}$ hinokitiol for indicated times. lane 1, DNA from control cells; lane 2, 3 and 4, DNA from cells treated for 6, 12 and 24 h, respectively. (C) F9 cells were treated with 10 $\mu\text{g/ml}$ hinokitiol (lane 1), 11.2 $\mu\text{g/ml}$ hinokitiol-Fe (III) complex (lane 2) and 25 $\mu\text{g/ml}$ hinokitiol methyl ether (lane 3). After each treatment, fragmented DNAs were analysed by agarose gel electrophoresis.

same population of F9 cells. In addition, F9 cells treated with 10 $\mu\text{g/ml}$ hinokitiol showed a weak DNA fragmentation within 12 h and an apparent one after 24 h (Fig 1B). The time-course of the hinokitiol-induced DNA fragmentation was similar to the time-course of the hinokitiol-induced differentiation, the percentage of the plasminogen positive colonies in hinokitiol-treated (10 $\mu\text{g/ml}$) F9 cells were 1–2% for 6 h, 11–12% for 12 h and 45–46% for 24 h, respectively (Muto *et al.* 1995).

It is known that hinokitiol is capable of chelating iron (II) or iron (III) ion with high affinity (Bryant *et al.* 1953) and that cellular iron deprivation produced by hinokitiol results in the inhibition of DNA synthesis (Muto *et al.* 1995). Next, we examined the involvement of its chelating ability in inducing apoptosis of F9 cells, by using hinokitiol derivatives which are deficient in metal-chelating activity. Two hinokitiol derivatives, hinokitiol methyl ether and hinokitiol-Fe (III) complex, shown no differentiation-inducing efficacy in the concentration ranges of 1–200 $\mu\text{g/ml}$ and 2.8–112 $\mu\text{g/ml}$, respectively. The cells treated with hinokitiol methyl ether and hinokitiol-Fe (III) complex for 24 h at concentrations of 25 and 11.2 $\mu\text{g/ml}$, respectively, which were equimolar with 10 $\mu\text{g/ml}$ hinokitiol, showed no DNA fragmentation (Fig 1C).

Detection of hinokitiol-induced DNA fragmentation in a cell-free system

As the first step to clarify whether cytosolic factor(s) in hinokitiol-induced DNA fragmentation are involved or not, we carried out experiments in a cell-free system. Hinokitiol-induced DNA fragmentation was further examined by incubating nuclei isolated from untreated cells with cytosols isolated from hinokitiol-treated F9 cells. The cytosols isolated from F9 cells treated with 10 $\mu\text{g/ml}$ hinokitiol for 24 h had an apparent DNA fragmentation-inducing activity toward the intact nuclei, whereas those isolated from cells treated with hinokitiol methyl ether or hinokitiol-Fe (III) complex showed no activity beyond the background levels (Fig 2). Faint DNA ladders shown in lanes 2, 4 and 5 commonly occurred in intact nuclei prepared in this study. This result obtained in a cell-free system coincided with that observed in directly treated F9 cells, suggesting the occurrence of cytosolic factor(s) mediating apoptotic cascade in the cytosols of hinokitiol-treated F9 cells.

Activation of caspase in F9 cells by hinokitiol

Although caspase family proteinases, particularly caspase-1 and caspase-3, have been reported to be involved in the apoptotic cascade regulated by Fas and TNF receptor, their participation in chelator- or iron deprivation-induced apoptosis is as yet unknown. We measured caspase-1 and caspase-3 activities in hinokitiol-treated F9 cells using their substrates, Ac-YVAD-pNA and Ac-DEVD-pNA, respectively. Hinokitiol at 10 $\mu\text{g/ml}$ showed a time-dependent increase of caspase-3 activity after 12 h of treatment, whereas caspase-1 activity showed little activation even after 24 h (Fig 3). Hinokitiol-stimulated (24 h) Ac-YVAD-pNAase activity was inhibited 33.8% by 50 μM Ac-YVAD-CHO, a caspase-1-specific inhibitor. Hinokitiol-stimulated (24 h) Ac-DEVD-pNAase activity was inhibited 97.1% by 50 μM Ac-DEVD-CHO, a caspase-3-specific inhibitor.

To clarify whether chelating activity of hinokitiol is involved in the activation of caspase family proteinase, the effects of hinokitiol, hinokitiol methyl ether and hinokitiol-Fe (III) complex on caspase activities in F9 cells were compared. When treated for 24 h at concentrations equimolar with 10 $\mu\text{g/ml}$ hinokitiol, all three compounds hardly showed the alteration in Ac-YVAD-pNAase activity, but hinokitiol only increased Ac-DEVD-pNAase activity (Fig 4). Two hinokitiol derivatives with no

Figure 2. DNA fragmentation in F9 cells treated with hinokitiol and its derivatives in cell-free system. Nuclei from untreated F9 cells were incubated at 37°C for 1 h with cytosols from F9 cells treated with 10 $\mu\text{g/ml}$ hinokitiol (lane 3), 11.2 $\mu\text{g/ml}$ hinokitiol-Fe (III) complex (lane 4) and 25 $\mu\text{g/ml}$ hinokitiol methyl ether (lane 5) for 24 h. Lane 1, DNAs from control cells; lane 2, DNA prepared from nuclei of untreated F9 cells. Fragmented DNAs were analysed by agarose gel electrophoresis.

metal-chelating ability could not activate Ac-DEVD-*p*NAase, activity, indicating that the activation of caspase-3 may be regulated by cellular iron deletion. These results indicate that hinokitiol-induced activation of caspase-3 is an essential trigger of subsequent apoptotic cell death.

Inhibition of hinokitiol-induced DNA fragmentation by caspase inhibitor

Z-VAD-FMK is widely used as an inhibitor of caspase families including caspase-3 (Hirsch *et al.* 1997, Shao *et al.* 1997, Shimizu & Pommier 1997). To confirm the involvement of caspase-3 in the apoptosis of F9 cells induced by hinokitiol, its effect on hinokitiol-induced DNA fragmentation was examined. F9 cells were treated with 10 $\mu\text{g/ml}$ hinokitiol for 24 h in the absence or presence of 12.5, 25 and 50 μM Z-VAD-FMK. Z-VAD-FMK exhibited a dose-dependent inhibition of DNA fragmentation induced by hinokitiol (Fig. 5). In particular, hinokitiol-induced DNA fragmentation was hardly observed in the presence of 50 μM Z-VAD-FMK.

Figure 3. Time-dependent activation of caspases in cytosols from F9 cells treated with hinokitiol. Cell lysate from F9 cells treated with 10 $\mu\text{g/ml}$ hinokitiol for indicated times were assayed for caspase-1 (open circle) and caspase-3 (closed circle) activities using their specific substrates. Values represent means $\pm$ SD from triplicate assays.

DISCUSSION

It has been known that hinokitiol is capable of chelating metal ions, in particular ferric ion with high affinity (Bryant *et al* 1953). In this study, hinokitiol was found to induce both internucleosomal DNA fragmentation and caspase-3 activation in F9 cells in similar concentration- and time-dependent manners, but hinokitiol methyl ether and hinokitiol-Fe (III) complex, which had no metal-chelating activity, showed no effect on these two biochemical events. These results suggest that the metal chelation by hinokitiol may be a critical step to trigger apoptosis of F9 cells. It has been reported that cellular iron deprivation caused by gallium and chelators such as deferoxamine induce apoptosis in human leukaemic CCRF-CEM cells (Haq *et al* 1995), proliferating lymphocytes and HL-60 promyelocytic cell line (Hileti *et al* 1995) and F9 cells (Tanaka *et al* 1997). Several studies have shown that such iron chelators and gallium appear to inhibit DNA synthesis by action on ribonucleotide reductase, resulting in an arrest in cell division (Lederman *et al* 1984, Chitambar *et al* 1991). Ribonucleotide reductase, a key enzyme of DNA synthesis, contains iron (III) ions in its active site. In fact, we have previously shown that hinokitiol inhibits DNA synthesis of F9 cells at the same concentration (Tanaka *et al* 1997). This effect of hinokitiol may be sufficient to commit F9 cells in certain phases of the cell cycle to apoptotic cell death.

Figure 4. Measurement of caspase activities in cytosols from F9 cells treated with hinokitiol and its derivatives. F9 cells were treated with 10 $\mu\text{g/ml}$ hinokitiol, 11.2 $\mu\text{g/ml}$ hinokitiol-Fe (III) complex and 25 $\mu\text{g/ml}$ hinokitiol methyl ether for 24 h. Then, caspase-1 (open column) and caspase-3 (closed column) activities of the individual cytosols were measured using their specific substrates. Values represent means $\pm$ SD from triplicate assays.

Another possible action site of hinokitiol in inducing apoptosis of F9 cells is the mitochondrial respiratory chain, which includes many iron-containing enzymes. Recently, it has been reported that apoptosis is accompanied by a mitochondrial permeability transition (PT) (Marchetti *et al.* 1996, Zamzami *et al.* 1996a,b, Kroemer 1997, Zamzami *et al.* 1997). PT causes the formation of pores, probably by apposition of inner and outer mitochondrial membrane proteins, followed by the mitochondrial transmembrane potential disruption. These events trigger the release of proteins of mitochondrial intermembrane space, such as cytochrome *c* and apoptosis-inducing factor, into the cytoplasm. Finally, those proteins released play an important role in activating caspase-3 (Liu *et al.* 1996, Susin *et al.* 1996, Golstein 1997, Kluck *et al.* 1997, Kluck *et al.* 1997, Yang *et al.* 1997). So, we speculate that hinokitiol may inhibit the function of iron-containing enzyme(s) involved in the mitochondrial respiratory chain *via* its iron-chelating activity, resulting in mitochondrial dysfunction. However, we have no direct evidence to support this hypothesis at present. Further studies are needed to characterize more clearly the action site or protein in the process of apoptosis induced by hinokitiol.

Recently, a cell-free system has come into common use to study apoptotic cascade (Yoshida *et al.* 1996, Newmeyer *et al.* 1994, Enari *et al.* 1995, Shimizu & Pommier 1996, Anjum & Khar 1997, Shao *et al.* 1997, Shimizu & Pommier 1997). Using this system, we have found that hinokitiol specifically activates cytosolic factor(s) which mediates subsequent apoptotic cascade. Caspase family proteinases are noted as general mediators of the apoptotic cell death signal. In this study, caspase-3, but not caspase-1, was shown to be activated by treatment with hinokitiol. Furthermore, hinokitiol-induced apoptosis was efficiently inhibited by Z-VAD-FMK, an inhibitor of caspase family proteinase, in a concentration-dependent manner. These results indicate that hinokitiol-induced apoptosis is triggered by the activation

Figure 5. Inhibition of DNA fragmentation in hinokitiol-treated F9 cells by Z-VAD-FMK. F9 cells were treated with 10 $\mu\text{g}/\text{ml}$ hinokitiol in the presence of various concentrations of Z-VAD-FMK. Fragmented DNAs were analysed by agarose gel electrophoresis. Lane 1, DNA from control cells; lane 2, 3, 4 and 5, DNA from cells treated with 10 $\mu\text{g}/\text{ml}$ hinokitiol in the presence of 0, 12.5, 25 and 50 μM Z-VAD-FMK, respectively; lane 6, 7 and 8, DNA from cells treated with 12.5, 25 and 50 μM Z-VAD-FMK, respectively.

of caspase-3, which is strictly dependent on the iron-chelating ability of hinokitiol. Recently, several studies have revealed the late steps of apoptotic cascade from caspase-3 activation to considerable DNA fragmentation (Liu *et al.* 1997, Enari *et al.* 1998, Sakahira *et al.* 1998). Both DNA fragmentation factor and caspase-activated DNase are specifically activated by caspase-3, and then these activated components can induce DNA fragmentation. Our results also indicate that the marked activation of caspase-3 was demonstrated by treatment with hinokitiol within 12 h of incubation followed by the activation of DNase involved in DNA fragmentation.

The three phenomena (inhibition of cell growth, induction of cell differentiation and apoptosis) caused by hinokitiol are strongly inter-related. By hinokitiol treatment, we can observe inhibition of DNA synthesis within 4 h (Muto *et al.* 1995). Subsequently, differentiation and apoptosis occur in parallel. The cell cycle arrest may be one of the important triggers

causing differentiation and apoptosis. In our view, the differentiation-committed cells and apoptosis-committed cells are not same. The direction of the two types of fate in growth arrested F9 cells may be determined by random probability.

In conclusion, hinokitiol, a potent iron chelator, induced a concentration and time-dependent apoptosis in F9 cells at the same concentrations as it induced differentiation. This indicated that cellular differentiation and apoptosis could occur simultaneously in the same population of F9 cells. Furthermore, our findings demonstrated that cellular iron deletion became a key factor to induce apoptotic cell death. The elucidation of the mechanism of hinokitiol-induced apoptosis will be useful to understand the apoptosis and/or necrosis caused by other xenobiotic compounds.

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