ELSEVIER

European Journal of Pharmacology 421 (2001) 93–100

ejp

www.elsevier.nl/locate/ejphar

Caspase-independent apoptosis induced by differentiation-inducing factor of *Dicytostelium discoideum* in INS-1 cells

Shuku Fujimaki [a,b], Yuzuru Kubohara [b], Isao Kobayashi [a], Itaru Kojima [b,*]

[a] *Department of Laboratory Medicine, Gunma University School of Medicine, Maebashi 371-8512, Japan*
[b] *Institute for Molecular and Cellular Regulation, Gunma University, Maebashi 371-8512, Japan*

Received 18 January 2001; received in revised form 26 April 2001; accepted 30 April 2001

Abstract

Differentiation-inducing factor (DIF) is a lipophilic hormone of *Dicytostelium discoideum* and has been shown to exert diverse effects in mammalian cells. We investigated the effect of DIF on cell viability in insulin-secreting INS-1 cells. DIF induced cell death in a dose-dependent manner. In DIF-treated cells, nuclear condensation and shrinkage of the cell body were observed. After 6 h of DIF treatment, cells became Tdt-mediated dUTP-biotin nick end-labeling-positive, and DNA ladder formation was detected, indicating that DIF induced apoptosis in these cells. DIF did not activate caspase-3, a key enzyme mediating apoptotic signals generated by various agents. Furthermore, DIF-induced cell death was not affected by Z-asp-2, 6-dichlorobenzoyloxymethylketone, a broad inhibitor of the caspases. As is the case in other types of cells, DIF increased cytoplasmic free calcium concentration in INS-1 cells. However, DIF-induced cell death was not affected by chelating intracellular free calcium by 1, 2-bis(2-aminoophenoxy)ethane-*N*, *N*, *N*, *N*-tetra acetic acid (BAPTA). These results indicate that DIF induces apoptosis in INS-1 cells by a mechanism independent of caspase-3. DIF-induced elevation of cytoplasmic calcium does not mediate the effect of DIF on cell death.

Keywords: DIF (differentiation-inducing factor); Apoptosis; Caspase; Ca^{2+}; Insulin

1. Introduction

The DIF (differentiation-inducing factor) is a small lipophilic molecule capable of inducing differentiation in the slime mold *Dicytostelium discoideum* (Kay et al., 1989). Upon starvation, vegetatively growing amoebae of this organism start morphogenesis to form a multicellular body consisting of two cell types, spores and stalk cells. DIF is synthesized in developing *D. discoideum* and is involved in stalk cell differentiation (Kay and Jermyn, 1983).

Oka et al. (1985) isolated a factor, named differanisole A, from conditioned medium of the soil microorganism *Chaetomium* spp. This molecule induces differentiation of erythroleukemia cells into hemoglobin-producing erythrocytes and interestingly, this factor closely resembles DIF in its chemical structure (Oka et al., 1985). Moreover, it has now been shown that DIF induces differentiation of murine and human erythroleukemia cells (Asaka et al., 1995) and conversely, differanisole A induces stalk cell differentiation in *D. disciodeum* (Kubohara et al., 1993). These results suggest the existence of a common mechanism in the differentiation of *D. discoideum* and mammalian erythroleukemia cells. In addition, DIF provides a tool to study the mechanism of differentiation of mammalian cells and, furthermore, DIF may have therapeutic potential in the treatment of cancer.

Recent studies have shown that DIF suppresses cell growth in all the mammalian cells tested including pancreatic AR42J cells (Kubohara et al., 1995a), various types of leukemia cells (Kubohara, 1997, 1999) and some normal mammalian cells (Kubohara et al., 1998; Miwa et al., 2000). DIF also promotes differentiation in such cells as HL60 myeloid leukemia (Kubohara, 1997) and vascular smooth muscle cells (Miwa et al., 2000). At high concentrations, DIF induces cell death in many types of cells

* Corresponding author. Tel.: +81-27-220-8835; fax: +81-27-220-8893.
E-mail address: ikojima@showa.gunma-u.ac.jp (I. Kojima).

0014-2999/01/$ - see front matter
PII: S0014-2999(01)01034-2

(Kubohara et al., 1995a,b; Kubohara, 1997). Hence, DIF is a unique factor capable of inducing growth arrest, differentiation and cell death in various types of normal and tumor cells.

The mechanism(s) by which DIF exerts its diverse actions in various cells is unclear. Since DIF exerts effects in different types of cells, it is likely that such cells have a common target molecule(s) which is critical in intracellular signaling. In this regard, DIF has been shown to elevate cytoplasmic free calcium concentrations in all types of cells studied so far by mobilizing Ca^{2+} from an intracellular pool (Kubohara et al., 1995a, 1998; Kubohara, 1997, 1999; Miwa et al., 2000). DIF also activates phosphoinositide (PI) 3-kinase and thereby activates a serine–threonine kinase Akt in K562 leukemia cells (Kubohara and Hosaka, 1999). However, it is not clear at present whether or not DIF induces its diverse effects solely by the activation of these pathways. With regard to the regulation of cell-cycle, DIF reduces the expression of G1-cyclins and inhibits the activity of cyclin-dependent kinases: cdk2, cdk4 and cdk6 (Miwa et al., 2000). Again, it is not certain how DIF reduces the expression of G1-cyclins.

In the present study, we further studied the mechanism by which DIF induces cell death in mammalian cells. Herein we used INS-1 cells, glucose-responsive pancreatic β-cells (Asfari et al., 1992). These are endocrine cells but resemble in many respects, neuronal cells. Since many factors induce cell death in β-cells (Efanova et al., 1998), we studied the effect of DIF in these cells.

2. Materials and methods

DIF-1 (1-(3, 5-dichloro-2, 6-dihydroxy-4-methoxyphenyl)hexan-1-one) was purchased from Affinity Research Products (Mamhead, Exeter, UK). Unless otherwise mentioned, DIF means DIF-1 in this study. 1-(2, 4, 6-trihydroxyphenyl)-hexan-1-one (THPH) was kindly provided by Dr. R.R. Kay (MRC, Cambridge, UK). 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) was purchased from Calbiochem (La Jolla, CA, USA) and 1, 2-bis(2-aminoophenoxy)ethane-*N*, *N*, *N*, *N*-tetra acetic acid acetoxymethyl ester (BAPTA/AM) was from Research Biochemicals International (Natick, MA, USA). Anti-caspase-3 antibody, anti-Bcl-2 antibody and anti-Bax antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and RPMI 1640 and fetal calf serum were purchased from GIBCO BRL (Gland Island, NY, USA). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), ionomycin, and wortmannin were purchased from Sigma (St. Louis, MO, USA). The apoptosis detection kit and 2-mercaptoethanol were purchased from Wako (Osaka, Japan). Z-Asp-2, 6-dichlorobenzoyloxymethylketone (Z-Asp-CH_2-DCB) was obtained from Funakoshi (Tokyo, Japan).

2.1. Cell culture

INS-1 cells were maintained in a "complete medium" (CM) composed of RPMI 1640 supplemented with 10 mM HEPES/NaOH (pH 7.4), 10% serum, 5 mM $NaHCO_3$, 1 mM sodium pyruvate, 50 μM 2-β-mercaptoethanol and antibiotics (penicillin and streptomycin) under humidified conditions of 95% air and 5% CO_2 at 37°C (Asfari et al., 1992). Cells were harvested every 3–4 days.

2.2. Measurement of viable cells

The viability of cells was determined by staining with acrydine orange/ethidium bromide (Kanzaki et al., 1994). The number of viable cells was assessed by using MTT (Carmichael et al., 1987). Cells were suspended at a density of 5×10^5 cells/ml in CM, dispensed into 24-well plates and incubated for 24 h at 37°C. Cells were incubated for 24 h in medium containing various concentrations of serum as specified in the legends. Then DIF was added and cells were further incubated for 24 h. Then, 1/10 volume of MTT solution was added to each well and incubated for 3 h. The supernatant was then removed, 200 μl of 0.04 N HCl–isopropanol was added and each sample was measured by a microplate reader.

2.3. Measurement of DNA ladder formation

Cells were cultured at 1×10^5 cells/ml for 24 h. Either DIF or ionomycin was added and cells were further incubated for 6 h. Then, cells were scraped off and centrifuged at $800 \times g$ for 5 min at 4°C. The pellet was suspended in 200 μl phosphate-buffered saline (PBS) and centrifuged at $800 \times g$ for 10 min at 4°C. The pellet was suspended with 100 μl lysis buffer (1 M Tris/HCl (pH 7.4), 0.5 M EDTA (pH 8.0), 10% Triton X-100) for 10 min on ice and centrifuged at $15{,}000 \times g$ for 5 min at 4°C. The supernatant was incubated with RNase for 1 h at 37°C and then with proteinase K for 30 min at 50°C. Samples were incubated with 20 μl 5 M NaCl and 120 μl isopropanol overnight at −20°C, and centrifuged at $15{,}000 \times g$ for 15 min at 4°C. The pellet was used as a sample for the DNA ladder, separated by electrophoresis using 2% agarose gel and visualized with ethidium bromide.

2.4. Detection of apoptosis

Apoptosis was assessed by a terminal deoxynucleotidyl transferase technique (TUNEL method) (Gravriedi, 1992). Cells were seeded at a density of 1×10^7 cells/ml on uncoated glass coverslips, incubated for 24 h in CM, and then incubated for 24 h in medium containing 1% serum. The medium was changed to one containing 1% serum, with or without 25 μM DIF and cells were further incubated for 6 h at 37°C. The cells were then fixed in 4%

formalin at room temperature and washed twice with PBS. After rinsing with PBS, the cells were covered with 1% sodium citric acid–0.1% Triton X-100 for 2 min on ice and rinsed twice with PBS. Then, the cells were covered with a terminal deoxynucleotidyl transferase reaction mixture for 10 min and rinsed with PBS three times. After the terminal deoxynucleotidyl transferase reaction, the cells were inactivated by 3% H_2O_2, washed twice with PBS, covered with peroxidase-conjugated antibody for 10 min under humidified conditions at 37°C, and washed again with PBS three times. The cells were then covered with a 3-3′-diaminobentizine solution for 5 min, washed with water and counterstained with a methylgreen solution for 5 min.

2.5. Measurement of cytoplasmic free Ca^{2+} concentration

The cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) was monitored by measuring fura-2 fluorescence in a basal buffer containing: 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 2.5 mM $CaCl_2$ and no glucose. Cells were seeded on uncoated glass coverslips at a density of 5×10^5 cells/ml. The cells were incubated with 2 μM fura-2/AM for 30 min at room temperature. Fura-2 fluorescence was then measured using microfluorometry as described elsewhere (Kojima et al., 1992). The ratio of the emissions excited by 340 and 380 nm (340:380 ratio) was used as an index of cytoplasmic free calcium concentration (Kojima et al., 1992). To chelate cytoplasmic calcium, cells were loaded with BAPTA by incubating them for 1 h with 10 μM BAPTA/AM.

2.6. Western blot analysis

After incubation with the respective substances, the cells were harvested in PBS by trypsinization, equilibrated in hypotonic buffer (20 mM HEPES/NaOH (pH 7.4), 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA) and centrifuged at $800 \times g$ for 5 min. The resulting pellet was then dissolved in hypotonic buffer by addition of 0.1 mM phenylmethysulfonyl fluoride and incubated on ice for 15 min. The cells were homogenized by passing it through a syringe (20 gauge) for approximately 10 times. The supernatant of the second centrifugation ($10{,}000 \times g$ at 4°C for 10 min) was used as cytosolic extract. The protein concentration was determined by using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Then, 40 μg of cytosolic protein from each sample was separated on a 15% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis (SDS-PAGE) and blotted onto a polyvinylidine difluoride membrane (Nihon Millipore, Yonezawa, Japan) by electroblotting. To reduce nonspecific antibody binding, the membrane was blocked with 10% Blockade/Tris–buffered saline (TBS) for 3 h at room temperature, then incubated overnight with rabbit polyclonal antibodies to caspase-3 in 1:200 dilution and washed with TBS. After

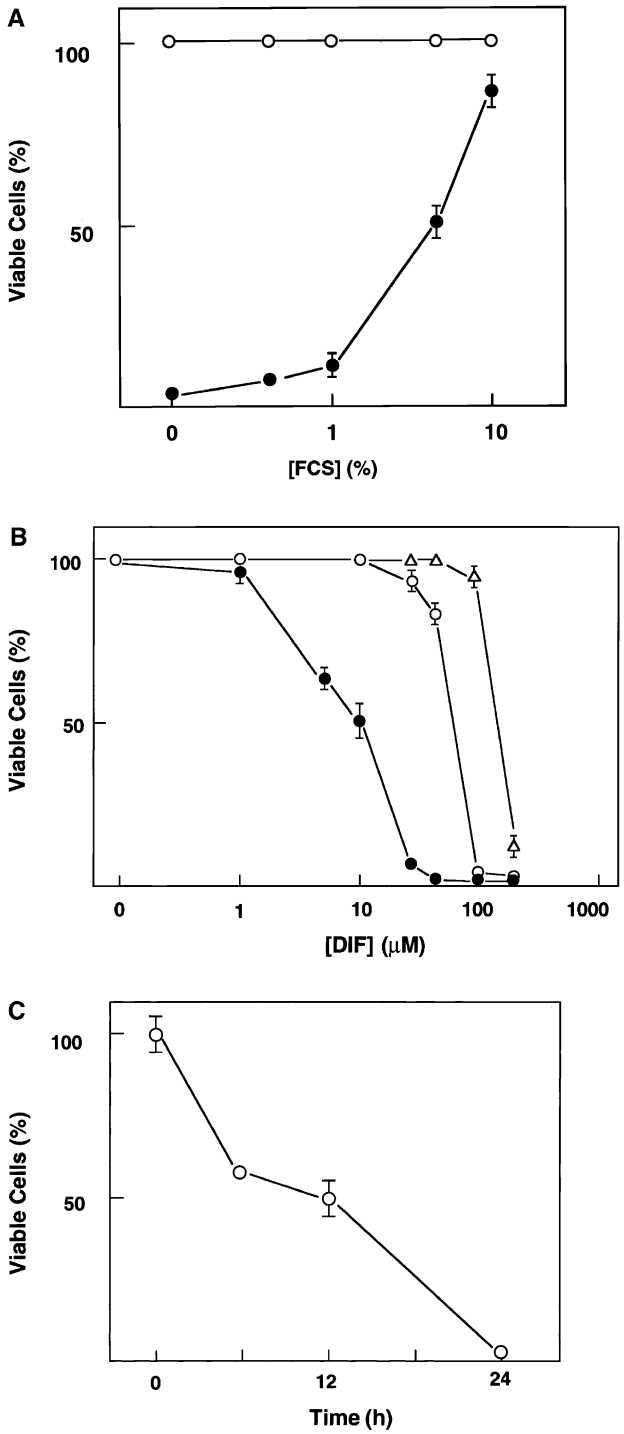

Fig. 1. Effect of DIF on the number of viable cells. (A) INS-1 cells were incubated for 24 h in medium containing 25 μM DIF and various concentrations of serum in the presence (●) and absence (○) of 25 μM DIF. Changes in the number of viable cells were assessed by using MTT. Values are the means ± S.E. for four experiments. (B) INS-1 cells were incubated for 24 h in medium containing 1% (●), 5% (○) or 10% (△) serum and various concentrations of DIF. The number of viable cells was assessed by using MTT. Values are the means ± S.E. for four experiments. (C) INS-1 cells were incubated for various times in the presence of 25 μM DIF and 1% serum. The number of viable cells was counted by using MTT. Values are the means ± S.E. for four experiments.

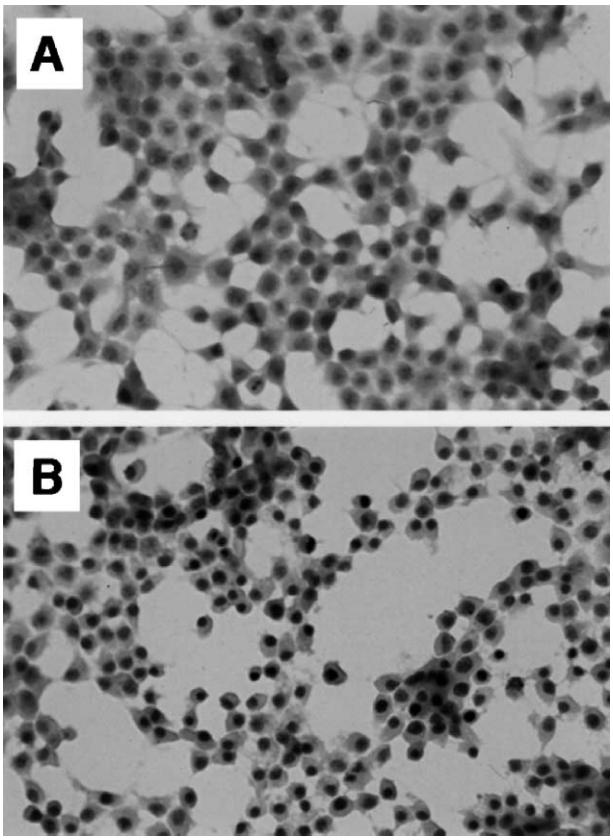

Fig. 2. Effect of DIF on the morphology of INS-1 cells. INS-1 cells were incubated for 6 h in medium containing 1% serum in the presence and absence of 25 μM DIF. Cells were then stained with hematoxylin. Results are representative of three independent experiments.

incubation with peroxidase-labeled anti-rabbit immunoglobulin G antibody for 1 h at room temperature, the membrane was washed with TBS and analyzed by exposure to X-ray film using ECL Western blotting detection reagent (Amersham, Bucks, UK).

3. Results

3.1. Effect of DIF on viability of INS-1 cells

We studied the effect of DIF on cell viability in INS-1 insulinoma cells. First, cells were incubated in medium containing various concentrations of serum in the presence or absence of 25 μM DIF for 24 h, then the number of viable cells was assessed by using MTT. In the absence of DIF, cells were alive for 24 h irrespective of the serum concentration. As shown in Fig. 1A, 25 μM DIF reduced the number of living INS-1 cells depending upon the serum concentration. Most of the cells died, as assessed by acrydine orange/ethidium bromide staining when the serum concentration was less than 1%. Fig. 1B shows the dose–response relationship for DIF-induced cell death in the presence of various concentrations of serum. DIF induced cell death in a concentration-dependent manner. In the presence of 1% serum, most of the cells died after incubation with 25 μM DIF. The ID_{50} was approximately 12 μM. In the presence of 5% serum, the effect of DIF was observed at 25 μM. In the presence of 10% serum, DIF-induced cell death was observed at 200 μM. DIF-induced cell death was therefore attenuated by an increase in serum concentration. It should be noted that THPH, the dechlorinated analogue of DIF, was ineffective in inducing cell death under the same conditions (data not shown). Therefore, the following experiments were done using

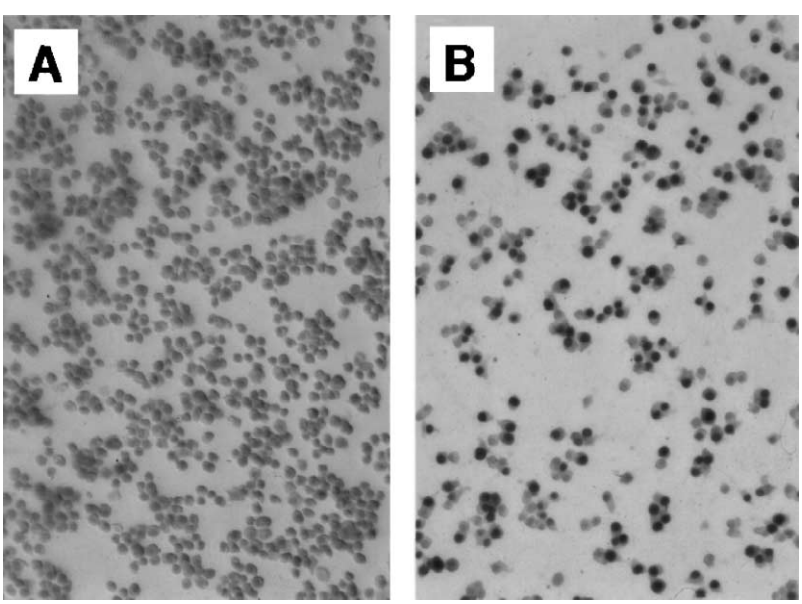

Fig. 3. Effect of DIF on apoptosis. INS-1 cells were incubated for 6 h in medium containing 1% serum in the presence (B) or absence (A) of 25 μM DIF. Apoptotic cells were detected by the TUNEL method. Results are representative of two independent experiments.

medium containing 1% serum. Fig. 1C depicts the time course of cell death induced by DIF. Cell death was detected after 6 h of DIF treatment.

We then investigated whether or not DIF-induced cell death was apoptotic. First, we observed the changes in the morphology of the cells after treatment with DIF. Cells were incubated in medium containing 1% serum in the presence and absence of 25 μM DIF for 6 h (Fig. 2). The cells shrunk after DIF treatment and the nuclei were condensed in DIF-treated cells. Most of the DIF-treated cells became TUNEL positive (Fig. 3). To further confirm that DIF induced apoptotic cell death in INS-1 cells, we observed the ladder formation of DNA. As shown in Fig. 4, DIF induced DNA ladder formation in INS-1 cells as did ionomycin, a calcium ionophore. These results indicate that DIF-induced cell death can be defined as apoptosis.

3.2. Involvement of the caspase in the action of DIF

We then studied the involvement of the caspase system in the action of DIF. Among various types of caspases, caspase-3 is a key enzyme regulating apoptosis induced by various agents (Villa et al., 1997). Caspase-3 is activated by members of the death receptor superfamily and the mitochondrial pathways associated with cytochrome *c* release (Hengartner, 2000). As shown in Fig. 5, treatment

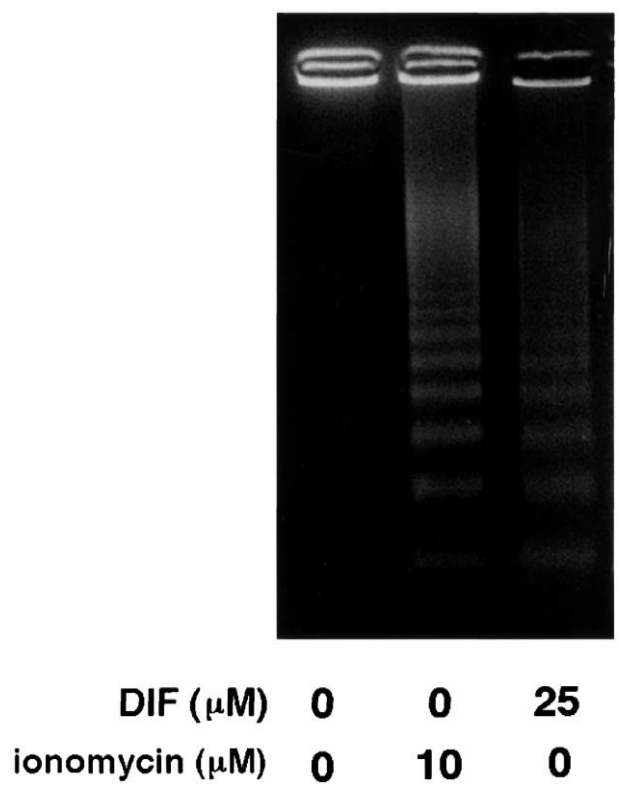

Fig. 4. Effect of DIF on the formation of DNA ladder. INS-1 cells were incubated for 6 h in medium containing 1% serum in the presence or absence of either 10 μM ionomycin or 25 μM DIF. DNA was extracted and separated by agarose gel. The result is representative of four independent experiments.

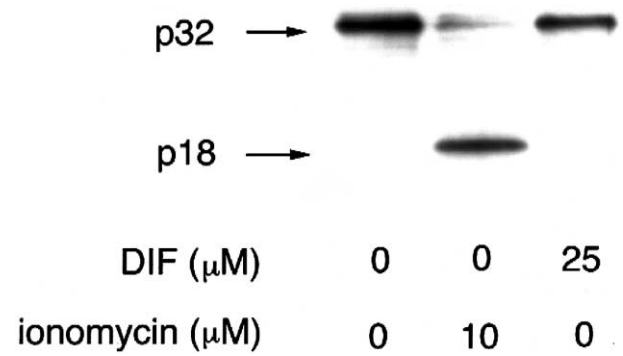

Fig. 5. Proteolytic activation of caspase-3. Cells were incubated for 6 h with 100 nM ionomycin or 25 μM DIF. Western blotting was performed using anti-caspase-3 antibody. The results are representative of three independent experiments.

with ionomycin induced proteolysis of caspase-3, indicating that caspase-3 was activated by the proteolytic cleavage. In contrast, DIF did not cause proteolysis of caspase-3 (Fig. 5). Consistent with this observation, DIF-induced apoptosis was not attenuated by a broad inhibitor of caspases, Z-Asp-CH_2-DCB (Mashima et al., 1995) (Fig. 6). It should be noted that this inhibitor attenuated cell death induced by transforming growth factor-β (TGF-β) (Fig. 6).

The Bcl family controls apoptosis in many cell systems (Hengartner, 2000). We determined whether or not DIF induced changes in the expression of the Bcl family. As shown in Fig. 7, DIF increased the expression of Bax, a proapoptotic member of the Bcl family, at 24 h. In contrast, the expression of Bcl-2 was already significantly reduced by DIF at 6 h (Fig. 7).

3.3. Role of Ca^{2+} and phosphoinositide 3-kinase in the action of DIF

DIF increases $[Ca^{2+}]_c$ in many types of cells (Kubohara et al., 1995a,b; Kubohara, 1997, 1999). Since excessive

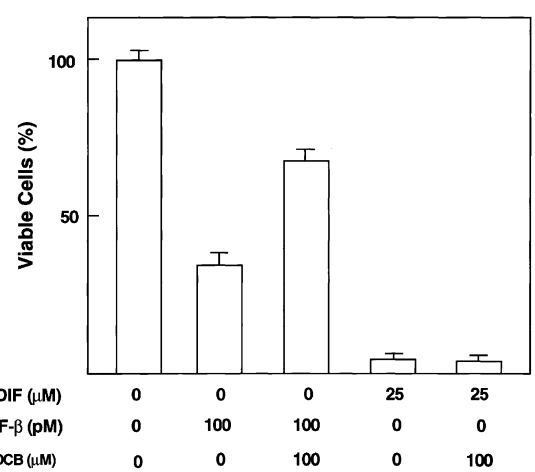

Fig. 6. Effect of caspase Inhibitor on cell viability. Cells were incubated for 24 h with 100 pM TGF-β or 25 μM DIF in the presence and absence of 100 μM Z-Asp-DCB. The number of variable cells was assessed by MTT. Values are the means ± S.E. for four experiments.

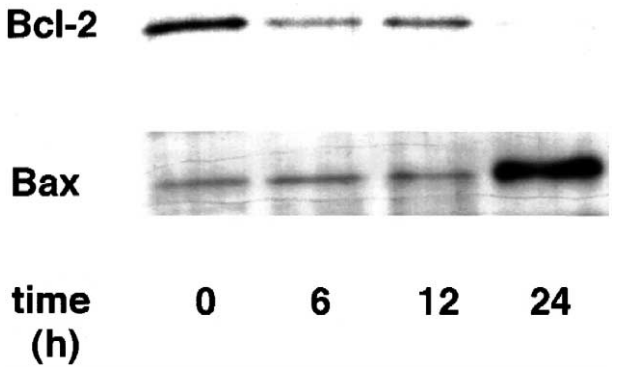

Fig. 7. Changes in the expression of Bcl-2 and Bax. Cells were incubated for the indicated times with 25 μM DIF and changes in the expression of Bcl-2 and Bax were measured by Western blotting. Results are representative of three independent experiments.

elevation of $[Ca^{2+}]_c$ causes apoptosis in many types of cells including β-cells (Efanova et al., 1998), we examined whether DIF induced cell death by increasing $[Ca^{2+}]_c$ in INS-1 cells. To this end, we chelated cytoplasmic Ca^{2+} by adding the membrane-permeable calcium chelator,

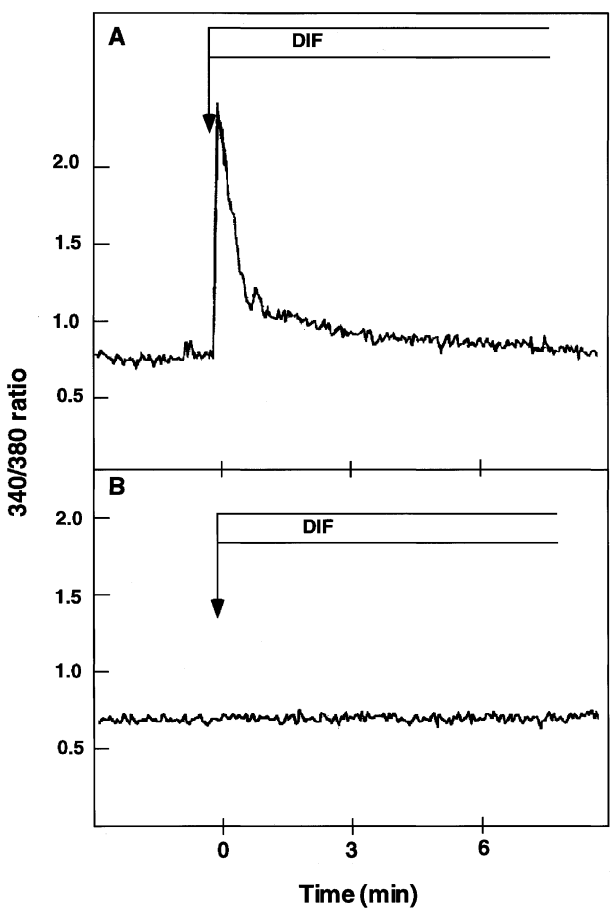

Fig. 8. Effect of DIF on cytoplasmic calcium concentration. BAPTA-loaded (B) or unloaded (A) cells were incubated with fura-2/AM as described in Section 2. Cells were then stimulated by 25 μM DIF, and changes in $[Ca^{2+}]_C$ were measured by monitoring fura-2 fluorescence. Results are representative of six independent experiments.

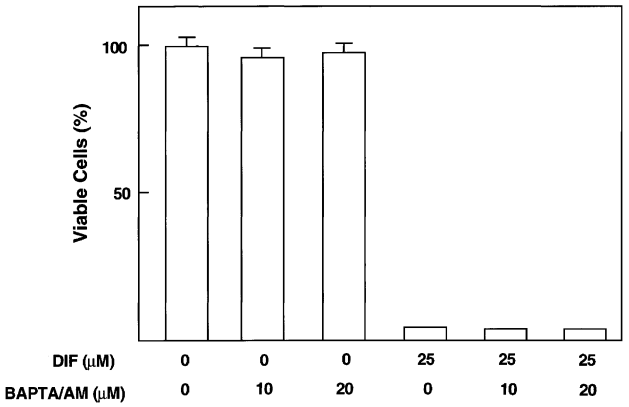

Fig. 9. Effect of DIF on the viability of the BAPTA-loaded and unloaded cells. BAPTA-loaded or unloaded cells were incubated for 24 h in medium containing 1% serum and 25 μM DIF. The number of viable cells was assessed by using MTT. Values are the means ± S.E. for four experiments.

BAPTA/AM. As shown in Fig. 8A, the addition of DIF induced a rapid elevation of $[Ca^{2+}]_c$, as it did in other types of cells. In BAPTA-loaded cells, however, DIF did not increase $[Ca^{2+}]_c$ (Fig. 8B), indicating that BAPTA effectively chelated the changes in $[Ca^{2+}]_c$. Nevertheless, DIF induced cell death even in BAPTA-loaded cells (Fig. 9).

As already mentioned, DIF induces various changes in cellular signaling systems (Kubohara et al., 1995a, 1997, 1998; Kubohara, 1999; Kubohara and Hosaka, 1999). Since DIF was shown to activate phosphoinositide 3-kinase and subsequently activate Akt (Kubohara and Hosaka, 1999), we examined whether the activation of phosphoinoside 3-kinase and Akt is required for DIF-induced apoptosis in INS-1 cells. However, DIF-induced apoptosis was not affected by either wortmannin (Okada et al., 1994) or LY294002 (Vlahos et al., 1994), both of which are inhibitors of phosphoinotide 3-kinase (data not shown). Furthermore, these inhibitors did not affect the dose–response relationship for DIF-induced cell death (data not shown), indicating that the activation of these two kinases is not required for the DIF-induced apoptosis in INS-1 cells. These results are consistent with the general notion that phosphoinoside 3-kinase and subsequent activation of Akt should protect cells from apoptosis-inducing agents, rather than induce apoptosis (Marte and Downward, 1997).

4. Discussion

In the present study, we investigated the effect of DIF in INS-1 cells. As in other types of mammalian cells (Kubohara et al., 1995a,b, 1998; Miwa et al., 2000), DIF induced cell death in INS-1 cells. Results indicated that the DIF-induced cell death can be defined as apoptosis. First, DIF-treated cells shrunk and nuclear condensation was observed (Fig. 2). Second, DIF-treated cells became

TUNEL positive (Fig. 3), suggesting that DIF treatment caused cleavage of genomic DNA. Third, DNA ladder formation was observed in DIF-treated cells (Fig. 4). Like the calcium ionophore, ionomycin and TGF-β, DIF was confirmed to induce apoptosis in INS-1 cells. However, DIF-induced apoptosis was different from that induced by ionomycin or TGF-β. As shown in Fig. 5, ionomycin caused proteolytic activation of caspase-3, whereas DIF did not. Caspase-3 is a key protease involved in apoptosis induced by various agents (Villa et al., 1997; Hengartner, 2000). Tumor necrosis factor-α (TNF-α) and Fas (CD95) ligand activate caspase-3 by signaling through the death receptors, with subsequent activation of caspase-8. Caspase-3 is also activated by caspase-9, which is activated by the release of cytochrome *c* from the mitochondria. However, DIF-induced apoptosis was resistant to an inhibitor of caspases. There are several agents which induce apoptosis by a caspase-3-independent mechanism. For example, toxicity and Jun-N-terminal kinase inducer (TAJ), a member of the TNF-α receptor family, activates c-Jun-N-terminal kinase and induces apoptosis by a caspase-independent mechanism (Eby et al., 2000). Activation of calpain-I leads to apoptosis via a caspase-independent pathway (Lankiewicz et al., 2000). Also, amyloid β-induced neuronal cell death is independent of caspase (Selznick et al., 2000). Furthermore, apoptosis induced by Bax and Bax-like proteins is independent of the caspase system and is resistant to inhibitors of caspases (Xiang et al., 1996; Gross et al., 1998; McCarthy et al., 1997). Apoptosis induced by H_2O_2 is also independent of the caspase system (Kim et al., 2000). Hence, DIF is listed as another example of agents, which cause caspase-independent apoptosis. In this regard, DIF increased the expression of the pro-apoptosis factor Bax and reduced the expression of Bcl-2 (Fig. 7). Since DIF-induced apoptosis was not accompanied by the activation of caspase-3, a decrease in Bcl-2 expression may not be critical for DIF-induced apoptosis. With regard to Bax, apoptosis induced by Bax is resistant to caspase inhibitors. Therefore, it is possible that Bax participates in DIF-induced apoptosis. However, the increase in the expression of Bax was detected at 24 h after the addition of DIF (Fig. 7) while DIF-induced DNA ladder formation was observed within 6 h (Fig. 4). Therefore, DIF may induce apoptosis by a mechanism independent of Bax expression.

DIF affects several intracellular events. Among them, activation of the calcium messenger system, which should be evaluated as a cause of apoptosis. To this end, we chelated cytoplasmic Ca^{2+} by introducing a membrane-permeable calcium chelator BAPTA/AM. As shown in Fig. 9, DIF could induce apoptosis without an elevation of cytoplasmic Ca^{2+}, indicating that Ca^{2+} is not a critical mediator of DIF-induced apoptosis.

In summary, DIF induced apoptosis in insulin-secreting cells through a caspase-3-independent mechanism. The target molecule of the action of DIF remains to be elucidated.

Acknowledgements

The present study was supported by a Grant-in-Aid for Scientific Research from The Ministry of Education, Technology, Science, Sports and Culture of Japan. The authors would like to thank Miss Mayumi Odagiri for the technical assistance.

References

Asaka, K., Sakurai, A., Takahashi, N., Kubohara, Y., Okamoto, K., Tanaka, Y., 1995. DIF-I, morphogen of *D. discoideum*, induces erythroid differentiation in murine and human leukemia cells. Biochem. Biophys. Res. Commun. 208, 1036–1039.

Asfari, M., Janjic, D., Meda, P., Li, G., Halban, P.A., Wollheim, C.B., 1992. Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. Endocrinology 130, 167–178.

Carmichael, J., DeGraff, W.C., Gazdar, A.F., Minnam, J.D., Mitchell, J.B., 1987. Evaluation of tetrazolium-based semiautomated colorimetric assay. Cancer Res. 47, 936–942.

Eby, M.T., Jasmin, A., Kumar, A., Sharma, K., Chaudhary, P.M., 2000. TAJ, a novel member of the tumor necrosis factor-α receptor family, activates the c-Jun-N-terminal kinase pathway and mediates caspase-independent cell death. J. Biol. Chem. 275, 15336–15342.

Efanova, I.B., Zaitsev, S.V., Zhivotovsky, B., Kohler, M., Efendic, S., Orrenius, S., Berggren, P.O., 1998. Glucose and tulbutamide induce apoptosis in pancreatic β-cells. J. Biol. Chem. 273, 33501–33507.

Gravriedi, Y., 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol. 119, 493–501.

Gross, A., Jockel, J., Wei, M.C., Korsmeyer, S.J., 1998. Enforced dimerization of Bax results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J. 17, 3878–3885.

Hengartner, M.O., 2000. The biochemistry of apoptosis. Nature 407, 770–776.

Kanzaki, M., Hattori, M.A., Horiuchi, R., Kojima, I., 1994. Coordinate actions of FSH and insulin-like growth factor-I on LH receptor expression in rat granulosa cells. J. Endocrinol. 141, 301–308.

Kay, R.R., Jermyn, K.A., 1983. A possible morphogen controlling differentiation in Dictyotelium. Nature 262, 717–719.

Kay, R.R., Berks, M., Traynor, D., 1989. Morphogen hunting in *D. discoideum*. Development 81–90, suppl.

Kim, D.K., Cho, E.S., Um, H.D., 2000. Caspase-dependent and independent events in apoptosis induced by hydrogen peroxide. Exp. Cell Res. 257, 82–88.

Kojima, I., Mogami, H., Ogata, E., 1992. Oscillation of cytoplasmic free calcium concentration induced by IGF-I. Am. J. Physiol. 262, E307–E311.

Kubohara, Y., 1997. DIF-I, putative morphogen of *D. discoideum*, suppresses cell growth and promotes retinoic acid-induced cell differentiation of HL60. Biochem. Biophys. Res. Commun. 236, 418–422.

Kubohara, Y., 1999. Effects of differentiation-inducing factors of *D. discoideum* on human leukemia K562 cells: DIF-3 is the most potent anti-leukemic agent. Eur. J. Pharmacol. 381, 57–62.

Kubohara, Y., Hosaka, K., 1999. The putative morphogen, DIF-1, of *D. discoideum* activates Akt/PKB in human leukemia K562 cells. Biochem. Biophys. Res. Commun. 263, 790–796.

Kubohara, Y., Okamoto, K., Tanaka, Y., Asahi, K., Sakurai, A., Takahashi, N., 1993. Differanisole A, an inducer of the differentiation of Friend leukemic cells, induces stalk cell differentiation in *D. discoideum*. FEBS Lett. 322, 73–75.

Kubohara, Y., Kimura, C., Tatemoto, K., 1995a. Putative morphogen, DIF, of *Dictytostelium discoideum* induces apoptosis in rat pancreatic AR42J cells. Dev. Growth Differ. 37, 711–716.

Kubohara, Y., Saito, Y., Tatemoto, K., 1995b. Differentiation-inducing factor of *D. discoideum* raises intracellular calcium concentration and suppresses cell growth in rat pancreatic AR42J cells. FEBS Lett. 359, 119–122.

Kubohara, Y., Kobeya, K., Matsui, H., Ishikawa, K., 1998. Effects of DIF-1, an anti-tumor agent isolated from Dictyostelium discoideum, on rat gastric mucosal RGM-1 a.d. leptomeningeal cells. Zool. Sci. 15, 713–721.

Lankiewicz, S., Marc Luetjens, C., Truc Bui, N., Krohn, A.J., Poppe, M., Cole, G.M., Saido, T.C., Prehn, J.H., 2000. Activation of calpain I converts excitotoxic neuron death into caspase-independent cell death. J. Biol. Chem. 275, 17064–17071.

Marte, B.M., Downward, J., 1997. PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. Trends Biochem. Sci. 22, 355–358.

Mashima, T., Naito, M., Kataoka, S., Kawai, H., Tsuruo, T., 1995. Aspartate-based inhibitor of interleukin-1β-converting enzyme prevents anti-tumor agent-induced apoptosis in human myeloid leukemia U937 cells. Biochem. Biophys. Res. Commun. 209, 907–915.

McCarthy, N.T., Whyte, M.K., Gilbert, C.S., Evan, G.I., 1997. Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage or the Bcl-2 homolog Bak. J. Cell Biol. 136, 215–227.

Miwa, Y., Sasaguri, T., Kosaka, C., Taba, Y., Ishida, A., Abumiya, T., Kubohara, Y., 2000. Differentiation-inducing factor-I, a morphogen of *Dicytostelium*, induces G_1, arrest and differentiation of vascular smooth muscle cells. Circ. Res. 86, 68–75.

Oka, H., Asahi, K., Morishima, H., Sanada, M., Shiratori, k., Iimura, Y., Sakurai, T., Uzawa, J., Iwadare, S., Takahashi, N., 1985. Differanisole A, a new differentiation inducing substance. J. Antibiot. 38, 1100–1102.

Okada, T., Sakuwa, L., Fukui, Y., Hazaki, O., Ui, M., 1994. Blockade of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of phosphatidylinositol 3-kinase. J. Biol. Chem. 269, 3563–3567.

Selznick, L.A., Zheng, T.S., Flavell, S., Rakic, P., Roth, K.A., 2000. Amyloid beta-induced neuronal death is bax-dependent but caspase-independent. J. Neuropathol. Exp. Neurol. 59, 271–279.

Villa, P., Kaufmann, S.H., Earnshaw, W.C., 1997. Caspases and caspase inhibitors. Trends Biochem. Sci. 22, 388–393.

Vlahos, C.J., Matter, W.F., Hui, Y., Brown, R.F., 1994. A specific inhibitor of phosphoinositide 3-kinase, 2-(4-morpholinyl)-8-phenyl-4h-1-benzopyran-4-one (LY294002). J. Biol. Chem. 269, 5241–5248.

Xiang, J., Chao, D.T., Korsmeyer, S.T., 1996. Bax-induced cell death may not require interleukin l(-converting enzyme-like proteases. Proc. Natl. Acad. Sci. U. S. A. 93, 14559–14563.