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Effects of DIF-1, an Anti-Tumor Agent Isolated from *Dictyostelium discoideum*, on Rat Gastric Mucosal RGM-1 and Leptomeningeal Cells

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ABSTRACT—DIF-1 is a putative morphogen that induces stalk cell formation in the cellular slime mold *Dictyostelium discoideum*. We have previously discovered that DIF-1 suppresses cell growth and induces cell differentiation *in vitro* in some tumor cells, and also that relatively low concentrations of DIF-1 promote retinoic acid-induced cell differentiation in the human myeloid leukemia HL-60 cells. In this study, to verify cell biological and therapeutic potential of DIF-1, we have examined whether and how DIF-1 affects normal mammalian cells *in vitro*, using rat leptomeningeal (RLM) cells and rat gastric mucosal RGM-1 cells. In growing phase of both cells, DIF-1 at 5-40 μM suppressed cell growth in a dose-dependent manner. High concentrations (15-40 μM) of DIF-1 were toxic to the growing cells so that the cells showed unusual morphology, but many of them were still alive even at Day 3-4. Withdrawal of DIF-1 allowed the cells to grow. In confluent phase of the cells, DIF-1 at more than 15 μM promoted medium acidification that resulted in cell death, but DIF-1 itself did not markedly affect cell viability for 3 days. DIF-1 increased $[Ca^{2+}]_i$ in RLM cells but did not affect β-trace secretion (an index of cell function in RLM cells). A low concentration (5 μM) of DIF-1 in combination with retinoic acid (0.1 μM) showed no marked effects on cell growth, cell viability, cell morphology and β-trace secretion in RLM cells. The present results indicate that DIF-1 may be utilized as a tool for cell biology. Also, since these concentrations of DIF-1 kill some tumor cells within 2-3 days, the present results suggest that DIF-1 might be utilized in the treatment of some sorts of cancer with a bearable degree of side-effects.

INTRODUCTION

The cellular slime mold *Dictyostelium discoideum* is a simple eukaryote that forms a fruiting body consisting of spores and stalk cells at the end of its development. DIF-1 (differentiation-inducing factor-1), 1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-1-hexanone (Fig. 1) (Morris *et al*., 1987), is a putative morphogen that induces stalk cell formation (Kay *et al*., 1989). This signal molecule has been suggested to function at least partly via an increase in $[Ca^{2+}]_i$ in *D. discoideum* (Kubohara and Okamoto, 1994; Schaap *et al*., 1996; Azhar *et al*., 1997).

Recently, it was found that DIF-1 suppresses cell growth and induces erythroid differentiation in murine and human leukemia (B8 and K562) cells (Asahi *et al*., 1995). It was also found that DIF-1 raises $[Ca^{2+}]_i$ and suppresses cell growth in rat pancreatic tumor, AR42J cells (Kubohara *et al*., 1995b), and that high concentrations of DIF-1 induce apoptosis in the tumor cells (Kubohara *et al*., 1995a). Moreover, it was shown with the human myeloid leukemia, HL-60 cells (Collins *et al*., 1977), that DIF-1 suppresses cell growth at least partly by arresting the cells in G1 phase of cell-cycle, and that relatively low concentrations of DIF-1 promote retinoic acid-induced cell differentiation (Kubohara, 1997). These findings indicate that DIF-1 may have therapeutic potential in the treatment of a wide range of cancer. In particular, since retinoic acid has already been utilized in differentiation-therapy for human acute promyelocytic leukemia (APL) (Huang *et al*., 1988), co-administration of retinoic acid and DIF-1 (or its artificial analogs) may improve chemoprevention/differentiation-therapy for APL and the other types of leukemia. However, it has not been examined if/how DIF-1 affects normal mammalian cells. Detailed analyses on the effects of DIF-1 on normal cell functions should be necessary for the therapeutic use of this agent. It should also be of great importance to verify the

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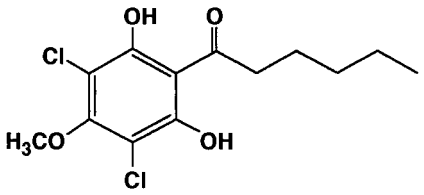

Fig. 1. Chemical structure of DIF-1.

cell biological potential of DIF-1 in many types of cells, in terms of basic cell science.

In our laboratory, Ishikawa and co-workers have established an *in vitro* primary culture system with rat leptomeningeal cells and have been analyzing the functions of the cells (Ishikawa *et al*., 1995a,b). Leptomeninges are the cerebrospinal fluid-blood barrier covering the brain parenchyma (Smith and Shine, 1992; Tanno *et al*., 1993). *In vitro* studies have revealed that these cells secrete many interesting proteins such as β-trace protein (prostaglandin D synthase), IGF-II, IGF-binding protein-2, apolipoprotein E, β2-microglobulin and many others (Ishikawa *et al*., 1995b; Ohe *et al*., 1996) which are seen in cerebrospinal fluid *in vivo*, suggesting that meningeal cells play pivotal roles at least as an origin of the cerebrospinal fluid proteins. Since the meningeal cells can be cultured *in vitro* and also because the assay system for cell biological function (β-trace secretion) have been established (Kabeya *et al*., 1998), these cells should provide an ideal *in vitro* system suitable for the analysis of the effects of DIF-1 on normal cell functions.

RGM-1 is an epithelial cell line established from gastric mucosa of adult Wistar rats and is well characterized (Hassan *et al*., 1996; Okada *et al*., 1996). This cell line also provides a useful model system for the analysis of the effects of DIF-1 on normal cells.

In this study, in order to verify both cell biological and therapeutic potential of DIF-1 and thus to elucidate if/how this agent affects normal cells, we examined the *in vitro* effects of DIF-1 on RGM-1 and rat meningeal cells. We show here that DIF-1 suppresses cell growth but is less toxic to the normal cells than to some tumor cells. It is also shown that cell growth, viability and β-trace secretion were not markedly affected in the presence of both retinoic acid and a low concentration of DIF-1, an expected combination for leukemia therapy. The potential of DIF-1 in mammalian cells will be discussed.

MATERIALS AND METHODS

Chemicals

DIF-1 was purchased from both Molecular Probes (USA) and Affiniti Research Products (UK) and stored as a 10 mM solution in EtOH at –20°C. All-*trans* retinoic acid was from Wako Pure Chemical Industries (Osaka, Japan) and stored as a 100 mM solution in EtOH at –20°C.

Examination of the effects of DIF-1 on growing cells

Rat leptomeningeal (RLM) cells and rat gastric mucosa RGM-1 cells were used in this study. Meningeal cells were prepared from the brains of 1-2-day-old rats as described previously (Ishikawa *et al*., 1995a,b; Ohe *et al*., 1996). The RLM cells and RGM-1 cells were plated on poly-D-lysine-coated multi(12)-well plates at a density of 2-4 $\times 10^3$ cells/cm^2, and incubated with 1 ml of DMEM-FBS (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum) at 37°C (5% CO_2) for 1-2 days. The incubation media were replaced each with 1ml of fresh DMEM-FBS containing 0-40 μM DIF-1 and/or 0.1 μM RA, and incubated at 37°C (5% CO_2). Cell growth and morphology were observed by a phase-contrast microscope. At 24 hr of incubation, cell density was counted; living cell numbers in 3-4 different microscopic fields of each well were counted and the mean of the numbers was used as a cell density. Round cells detached from the bottom of the wells were regarded as dead cells.

Examination of the effects of DIF-1 on confluent state of cells and β-trace secretion

Cells were incubated at 37°C (5% CO_2) in multi(12)-well plate as described above for 5-7 days until they reached a confluent state. The incubation media were replaced each with 1ml of fresh DMEM-FBS containing 0-40 μM DIF-1 and/or 0.1 μM RA, and cells were incubated at 37°C (5% CO_2) continuously, or during incubation, the media were replaced with fresh ones containing the reagents at every 24 hr. For the assay for β-trace secretion, confluent RLM cells were incubated with DIF-1/RA-containing media, and the incubation media were collected and replaced with 1 ml of fresh DMEM-FBS containing the reagents at every 12 or 24 hr. β-trace contents of the media thus collected were assayed using anti-β-trace protein antibody as described by Kabeya *et al*. (1998).

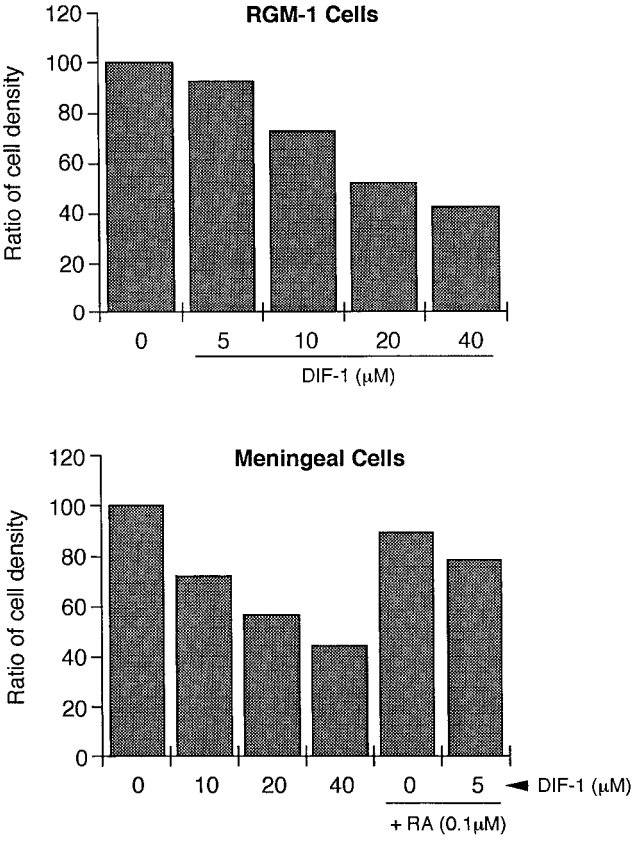

Fig. 2. Effects of DIF-1 on cell growth in RGM-1 and RLM cells. Cells were incubated for 1 day without or with 5-40 μM of DIF-1 and/or 0.1 μM of retinoic acid (RA), and cell density was counted microscopically.

Assay for $[Ca^{2+}]_i$

RLM cells were incubated in culture flasks until cells reached a confluent state. The cells were collected by trypsin treatment (Ohe *et al.*, 1996), and incubated with FuraPE3/AM (Wako Pure Chemical Industries, Osaka, Japan). FuraPE3 fluorometry was performed as described previously (Kubohara, 1997).

RESULTS

Effects of DIF-1 on RGM-1

We first examined the effects of DIF-1 on growing phase of the rat gastric mucosa cell line RGM-1 (Figs. 2 and 3). In the absence of DIF-1, RGM-1 cells grew as shown in Fig. 3

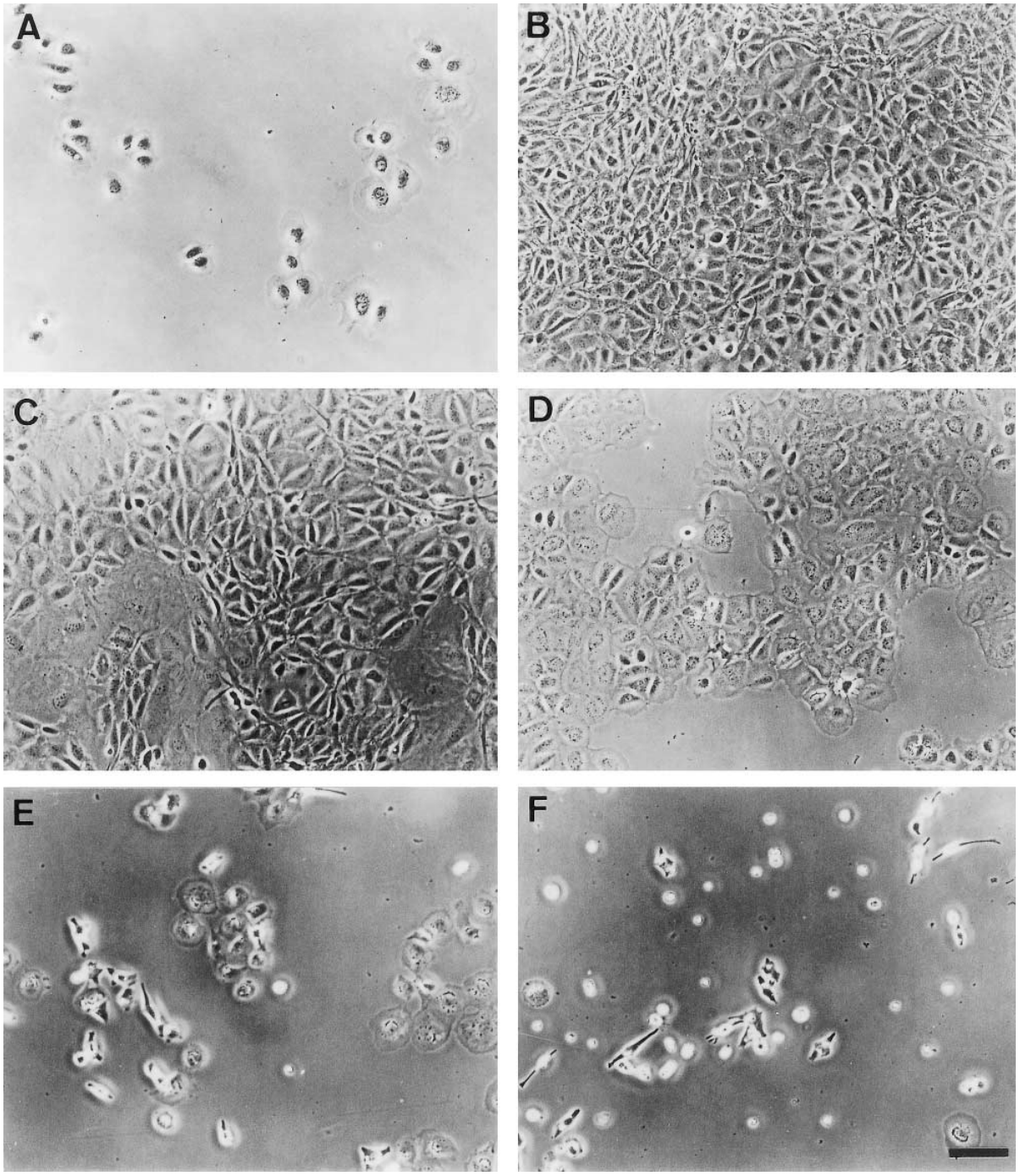

Fig. 3. Effects of DIF-1 on growing RGM-1 cells. RGM-1 cells (**A**: Day 0) were incubated without (**B**) or with 5 (**C**), 10 (**D**), 20 (**E**), and 40 μM (**F**) of DIF-1, and cell growth and morphology were observed microscopically at Day 3. Control cells grew and reached a confluent state in 3-4 days. In the presence of 5 μM DIF-1, cell growth and morphology were not markedly affected. DIF-1 at 10 μM slightly suppressed cell growth but did not affect morphology. In the presence of 20-40 μM DIF-1, cell growth was greatly suppressed, and there appeared some vesicle-containing cells and bi-polar cells. Bar; 100 μm.

(Control) and reached a fully confluent state in 3-4 days. In the presence of 5 μM DIF-1 (Fig. 3), cell growth and cell morphology were not much affected so that cells reached a confluent state as control cells did. DIF-1 at 10 μM suppressed cell growth slightly but did not affect cell morphology (Fig. 3). Cell growth was greatly suppressed in the presence of 20-40 μM of DIF-1 where the cells showed characteristic morphology; there appeared some flattened vesicle-containing cells and bi-polar cells (Fig. 3). The cells started growing after withdrawal of DIF-1 (data not shown), which indicates that the effect of DIF-1 is reversible.

We then examined the effects of DIF-1 on cell viability

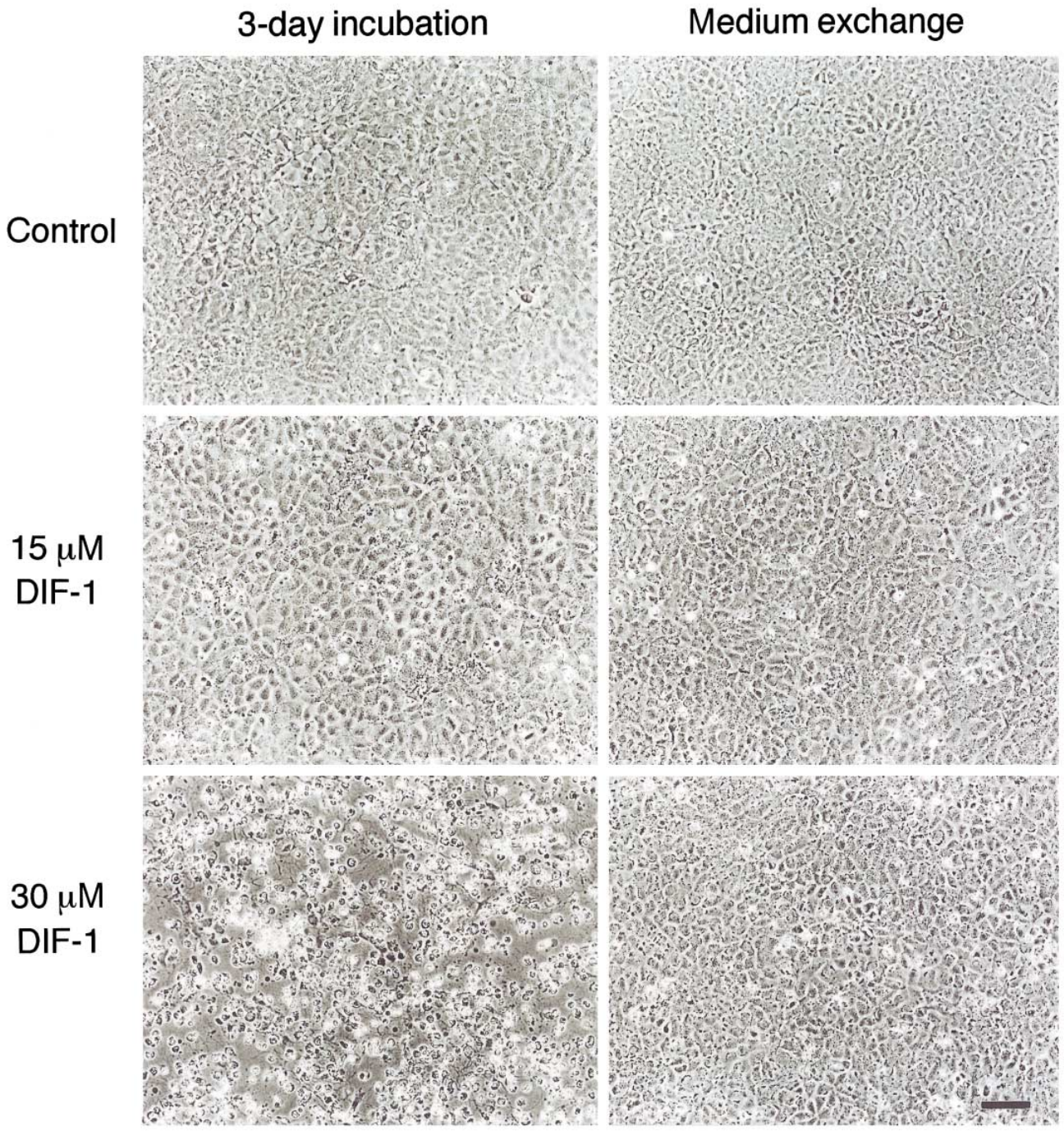

Fig. 4. Effects of DIF-1 on confluent RGM-1 cells. Confluent RGM-1 cells were incubated without (control) or with 15-30 μM DIF-1 continuously (left photos), or with exchanging the incubation media with fresh ones at every 24 hr (right photos), and at Day 3, cell morphology and viability were observed microscopically. DIF-1 at 15 μM was not markedly toxic to the cells. Continuous incubation with 30 μM DIF-1 caused medium acidification, which was toxic to the cells so that most died in 2-3 days. However, confluent RGM-1 cells survived for 3 days when incubation medium was exchanged with fresh one at every 24 hr. Bar; 100 μm.

and morphology in a confluent state of RGM-1 cells. In the absence of DIF-1, cells kept confluent and they were alive at Day 3 (Fig. 4); cell morphology did not change for more than a week (data not shown). In the presence of 5-15 μM DIF-1, they showed normal morphology at Day 3 (data not shown). In contrast, confluent cells were killed within 3 days when 30 μM of DIF-1 was present in the media (Fig. 4). However, repeated experiments showed that in the presence of 30 μM DIF-1, the culture media became yellow (indication of acidification) before cells died (data not shown). This seemed to indicate that DIF-1 would promote cell metabolism in some way resulting in the accumulation of metabolites and the acidification of the culture media, which might cause cell death; that is, the cytotoxic effect of DIF-1 was exerted at least in part via the acidification of the media and/or the accumulation of cytotoxic metabolites. To assess this possibility, the culture media were exchanged with fresh ones containing DIF-1 at every 24 hr and cell viability was observed. As expected, cells were viable at Day 3 even in the presence of 30 μM of DIF-1 (Fig. 4).

Effects of DIF-1 on rat leptomeningeal cells

The effects of DIF-1 on growing phase of the rat leptomeningeal (RLM) cells were examined (Figs. 2 and 5). In the absence of DIF-1, RLM cells grew as shown in Fig. 5 (control) and reached a full confluent state in 3-4 days. In terms of the inhibitory effect of DIF-1 on cell growth, we obtained almost the same results with RLM cells as seen with RGM-1 (Figs. 2, 3 and 5). Again, withdrawal of DIF-1 allowed the cells to grow (data not shown). It should be noted however that in the presence of 20-40 μM DIF-1, there appeared many bi-polar cells with long cytoplasmic projections distinct from normal (Fig. 5).

We then examined the effects of DIF-1 on cell viability and cell morphology in confluent RLM cells. We obtained almost the same results as with RGM-1, except that DIF-1 at 15 μM was much toxic to RLM cells (data not shown) than to RGM-1 (Fig. 4); confluent RLM cells were killed by continuous incubation with 15-30 μM DIF-1. But this toxicity was again reduced by medium exchange (data not shown).

Combinatory effects of DIF-1 and retinoic acid on rat leptomeningeal cells

Retinoic acid (RA) has been used in differentiation-therapy for APL (Huang *et al*., 1988). Recently, we have shown with myeloid leukemia HL-60 that relatively low concentrations of DIF-1 promote RA-induced cell differentiation, suggesting that co-administration of RA and DIF-1 may improve differentiation-therapy for leukemia (Kubohara, 1997). We therefore examined the effects of RA and DIF-1 on RLM cells (Figs. 2 and 5). RA at 0.1 μM showed no marked effects on both cell growth and morphology in RLM (Figs. 2 and 5). RA (0.1 μM) in combination with 5 μM DIF-1 slightly suppressed cell growth but did not affect cell morphology in RLM (Figs. 2 and 5).

Neither RA (0.1 μM) nor the combination of RA (0.1 μM) and DIF-1 (5 μM) affected cell viability and morphology in confluent RLM cells at least for 3 days (data not shown).

Effects of DIF-1 on β-trace secretion and $[Ca^{2+}]_i$

To assess if DIF-1 affects normal cell functions, we examined the effects of DIF-1 on the secretion of β-trace protein in confluent RLM cells, using the medium-exchange-system described above. When the culture media were exchanged at every 24 hr, low concentrations (5-10 μM) of DIF-1 and/or RA (0.1 μM) did not affect β-trace secretion but high concentrations (20-30 μM) of DIF-1 inhibited β-trace secretion (data not shown). In this case however, since the culture media had become yellow in 24 hr in the presence of high concentrations of DIF-1, it seemed possible that the medium acidification and/or the accumulation of some metabolites affected β-trace secretion although the cells were alive. We therefore assessed β-trace secretion by exchanging the culture media with fresh ones at every 12 hr (Fig. 6). Under the conditions, DIF-1 and/or RA did not affect β-trace secretion at least for 2 days (Fig. 6).

In order to elucidate the mechanism of DIF-1-action in RLM cells, we examined the effect of DIF-1 on $[Ca^{2+}]_i$. Expectedly, DIF-1 (20 μM) increased $[Ca^{2+}]_i$ in RLM cells, suggesting that DIF-1 might exert its effects at least in part via an increase in $[Ca^{2+}]_i$ (Fig. 7).

DISCUSSION

We have previously shown that DIF-1, a putative morphogen in *D. discoideum*, has anti-tumor activities *in vitro* in several tumor cells tested so far (Asahi *et al*., 1995; Kubohara *et al*., 1995a,b; Kubohara, 1997). However, it has not been verified whether and how DIF-1 affects normal cells. In the present study, to assess this question, we have examined the effects of DIF-1 on normal cell functions, using RGM-1 and RLM cells; in this study, we have observed the DIF-1-treated cells for 3-4 days, since DIF-1 shows cytotoxic effects on tumor cells within 2-3 days (Kubohara *et al*., 1995a,b; Kubohara, 1997). Table 1 summarizes the present and previous results on the effects of DIF-1.

Effects of DIF-1 on normal cells

DIF-1 at 5-40 μM suppressed cell growth in a dose-dependent manner (Figs. 2, 3 and 5) in both RLM and RGM-1 cells. High concentrations (20-40 μM) of DIF-1 affected cell morphology but the cells survived at Day 3-4; there appeared many bi-polar cells with long cytoplasmic projections, indicating that DIF-1 induced re-arrangement of cytoskeletal proteins possibly via a signalling system (Figs. 3 and 5). Characterization of the bi-polar cells should be of interest for the elucidation of DIF-1-signalling system (if any) in the mammalian cells.

In confluent RLM and RGM-1 cells, high concentrations of DIF-1 promoted the acidification of the culture media, which might result in cell death. Although it is unknown how DIF-1 promotes medium acidification and what triggers cell death under the *in vitro* conditions, it should be of great importance to note that both RLM and RGM-1 cells survived at least for 3

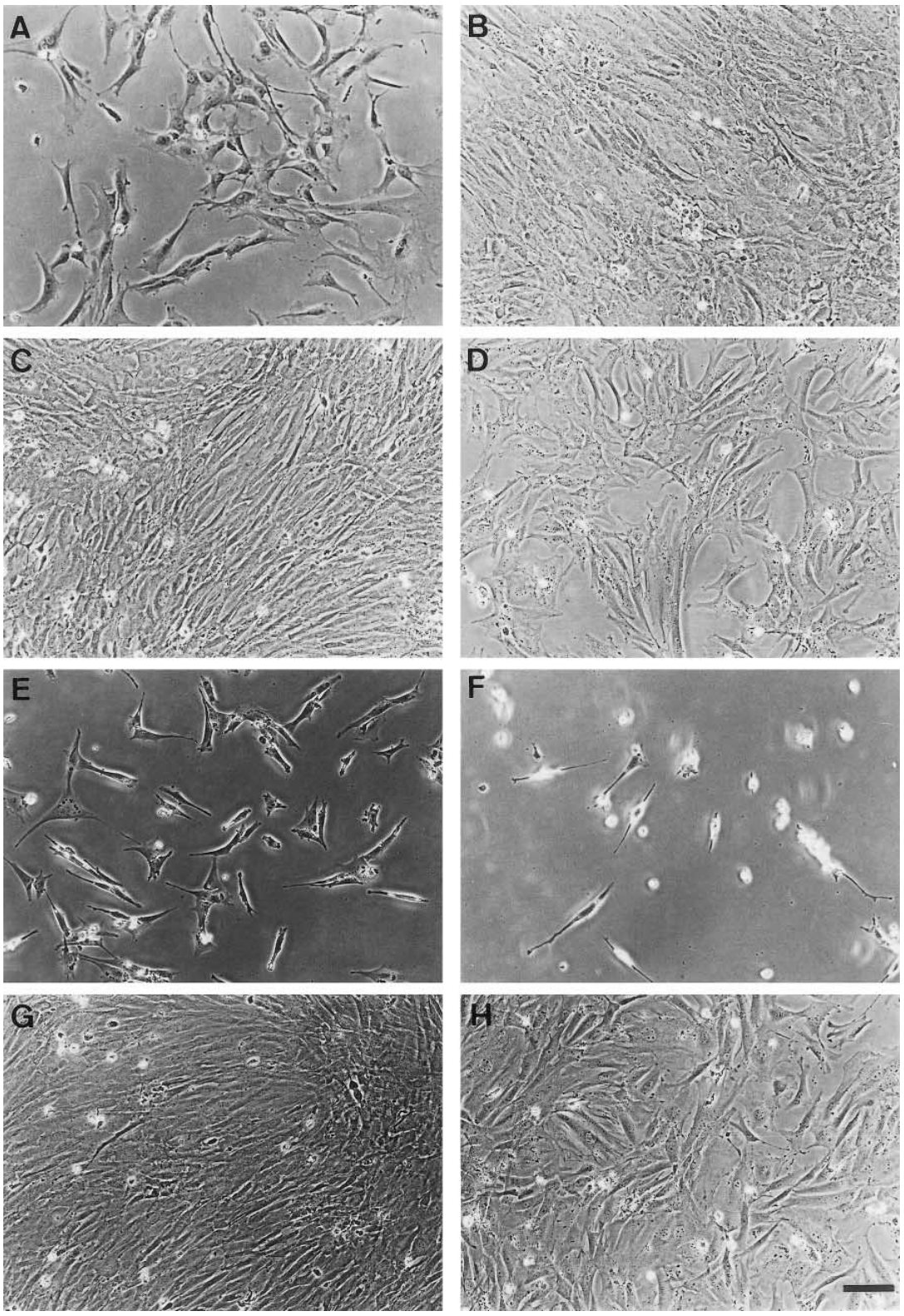

Fig. 5. Effects of DIF-1 and retinoic acid on growing RLM cells. Growing RLM cells (**A**: Day 0) were incubated without or with 5-40 μM of DIF-1, or 0.1 μM retinoic acid (RA), and cell growth and morphology were observed microscopically at Day 4 (**B**: control, **C**: 10 μM DIF-1, **D**: 20 μM DIF-1, **E**: 40 μM DIF-1, **G**: 0.1 μM RA, **H**: 0.1 μM RA and 5 μM DIF-1). Control cells grew to reach a confluent state in 3-4 days. DIF-1 at 5 μM showed no marked effect on both cell growth and morphology. DIF-1 at 10 μM slightly suppressed cell growth but did not affect morphology. At 20-40 μM, DIF-1 suppressed cell growth greatly and converted many cells to bi-polar cells with long cytoplasmic projections. RA showed no marked effect on both cell growth and morphology. RA in combination with 5 μM DIF-1 slightly suppressed cell growth but did not affect cell morphology. Bar; 100 μm.

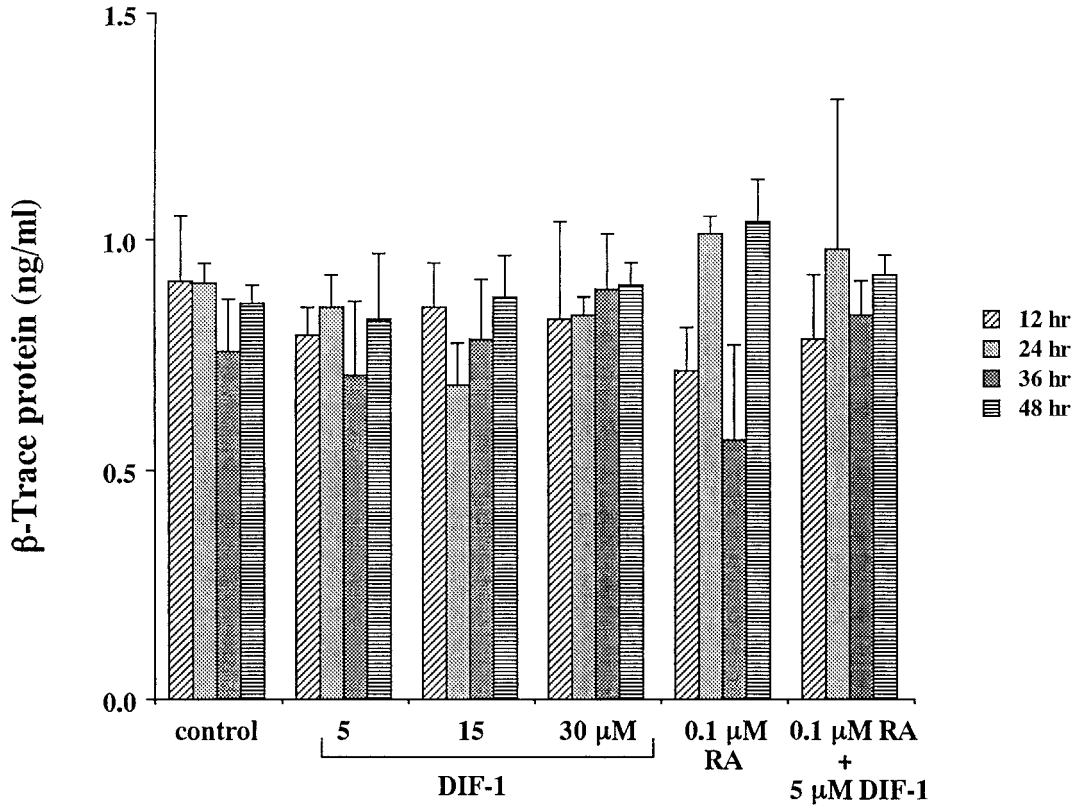

Fig. 6. Effects of DIF-1 on β-trace secretion in RLM cells. Confluent RLM cells were incubated for 2 days without or with 5-30 μM DIF-1 and/or 0.1 μM retinoic acid (RA), and the incubation media were exchanged with fresh ones at every 12 hr. β-trace contents in the withdrawn media were assayed. Data are the means ± S.E. of β-trace proteins secreted during each 12 hr (The experiment was done by 3-well determination (n = 3)).

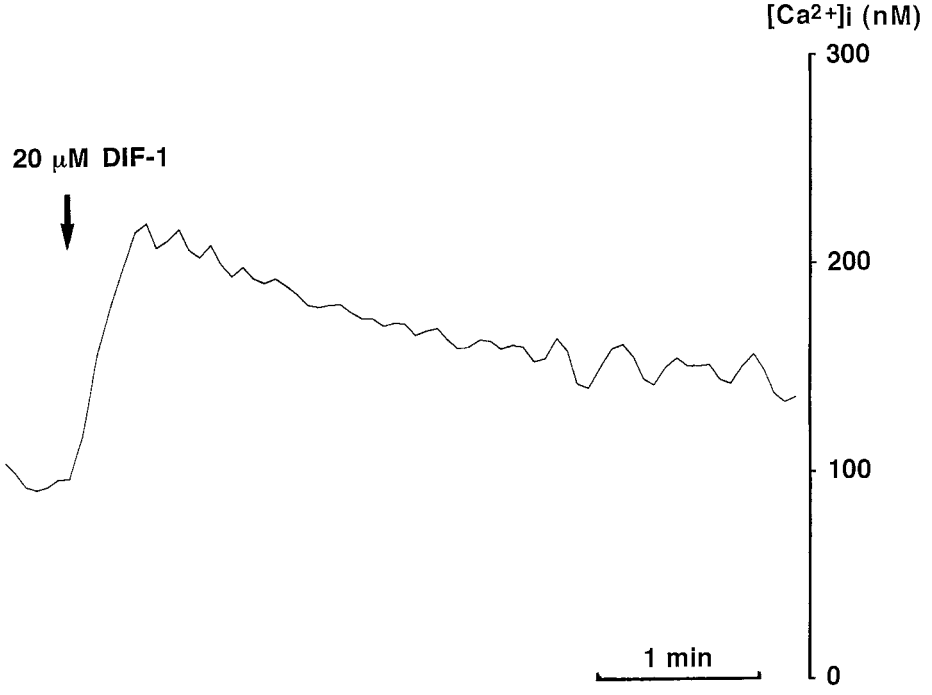

Fig. 7. Effect of DIF-1 on $[Ca^{2+}]_i$ in RLM cells. FuraPE3-loaded RLM cells were stimulated with 20 μM of DIF-1 at the indicated time point. Fluorometry was performed as described under Materials and Methods.

Table 1. Effects of DIF-1 and retinoic acid on RLM and RGM-1 cells

	DIF-1 (μM)							
	5	10	15	20	30	40	RA	RA+ DIF-1
RLM								
Growing cells	–	±	±	+	+	++	–	±
Confluent cells	–		–		±		–	–
β-trace secretion	–		–		–		–	–
RGM-1								
Growing cells	–	±	+	+	++	++	nd	nd
Confluent cells	–		–		–		nd	nd
AR42J (Growing cells)	–	±		++		+++	nd	nd
HL-60 (Growing cells)	–	±	+	++	+++	+++	+,cd	+,cd

Note: Growing cells were incubated with DIF-1 (5-40 μM)/RA (0.1 μM). Confluent cells were incubated with DIF-1/RA and during incubation, the culture-media were exchanged with fresh ones containing the reagents at every 24 hr (for viability test) or every 12 hr (for the assay for β-trace secretion). Cell growth, viability and morphology were microscopically observed for 3 days. β-trace secretion was assayed as described in the text. Data on rat pancreatic tumor AR42J cells and human myeloid leukemia HL-60 cells are from our previous reports (Kubohara *et al*., 1995a,b; Kubohara, 1997).

–, no marked effect; ±, slight inhibition and slight changes in morphology; +, great inhibition and great changes in morphology; ++, great inhibition, great changes in morphology, and partial cell death; +++, great inhibition and a large number of cell death (> 90%); cd, cell differentiation; nd, not determined.

days (Fig. 4 and not shown data) and RLM cells secreted β-trace proteins normally when the culture media were exchanged with fresh ones (Fig. 6). These results would rather reflect *in vivo* situation where the blood-lymph systems circulate continuously, and the present results thus lead to an expectation that DIF-1 may not seriously damage normal confluent cells *in vivo*.

Considering that the high concentrations (20-40 μM) of DIF-1 kill human myeloid leukemia HL-60 (Kubohara, 1997) and rat pancreatic tumor AR42J cells (Kubohara *et al*., 1995a,b) within 2-3 days, DIF-1 seems to be much more toxic to the tumor cells than to normal cells (Table 1).

It seems likely that DIF-1 functions via similar machinery involving increases in $[Ca^{2+}]_i$ in both normal cells (Fig. 7) and tumor cells (Kubohara *et al*., 1995a,b; Kubohara, 1997) as well as in *Dictyostelium* cells (Kubohara and Okamoto, 1994; Schaap *et al*., 1996; Azhar *et al*., 1997). However, the fine mechanisms of DIF-1-action in both mammalian and *Dictyostelium* cells are still unclear.

Combinatory effects of retinoic acid and DIF-1 on normal cells

RA has been used in differentiation-therapy for APL which results in a high score of complete remission (Huang *et al*., 1988). We have recently shown with human myeloid leukemia HL-60 cells that DIF-1 promotes RA-induced cell differentiation (Kubohara, 1997), suggesting that co-administraion of RA and DIF-1 may improve chemoprevention/differentiation therapy for APL and other leukemia. In this study, we have therefore verified the effects of DIF-1/RA on the normal cells.

RA (0.1 μM) or DIF-1 (5 μM) alone showed no marked effect on all the indexes examined here (Table 1). DIF-1 (5 μM) in combination with RA slightly suppressed cell growth, but did not affected cell viability, morphology (Figs. 2 and 5), and β-trace secretion (Fig. 6) in RLM cells (Table 1). These results suggest that RA and DIF-1 may not damage normal cells.

Conclusion

Most importantly, it was suggested here that DIF-1 alone or DIF-1 in combination with RA might be utilized in the treatment of some sorts of cancer cells with a bearable degree of side-effects, although the *in vivo* effects of DIF-1 should be examined in the future. Apart from the therapeutic potential of DIF-1, since DIF-1 affected cell growth and morphology in RGM-1 and RLM cells, it is also expected that DIF-1 may be a useful tool for the analysis of normal mammalian cell functions.

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