Differential-inducing factor-1 (DIF-1), a chemical compound (1-[3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl]-1-hexanone) discovered from Dictyostelium discoideum, is a putative morphogen required for undifferentiated cells of Dictyostelium to differentiate to stalk cells. To find a novel antineoplastic agent, the effect of DIF-1 on mammalian tumor cells has been examined, because in structure DIF-1 was found to be similar to differanisole A, which was identified from Chaetomium and induces differentiation in immature tumor cells. DIF-1 was found to inhibit proliferation and induce differentiation in mammalian cells, such as murine erythroleukemia (B8) and human leukemia (K562) cell lines. In the human myeloid leukemia cell line HL-60, DIF-1 inhibits proliferation and promotes retinoic acid-induced differentiation. However, the precise mechanisms underlying its antiproliferative and differentiation-inducing effects remain unknown. Not only tumorigenesis but also atherogenesis involves abnormality in cell proliferation and differentiation. Vascular smooth muscle cells (VSMCs) found in atheromatous plaques and restenotic lesions are characterized by mitogenicity and a dedifferentiated phenotype. Therefore, substances that prevent proliferation and phenotypic modulation of VSMCs may be useful for the treatment of atherosclerosis. In this study, we examined the effects of DIF-1 on the proliferation and differentiation of VSMCs to explore whether DIF-1 or its derivatives could be applied to the treatment of atherosclerotic vascular diseases.

To elucidate the mechanism for the antiproliferative effect of DIF-1, we examined the effects of DIF-1 on cell cycle events that occur between G₀ and S phases, including the expression of cyclins and cyclin-dependent kinases (Cdks), Cdk activation, the expression of Cdk inhibitor proteins, the phosphorylation of the retinoblastoma protein (pRb), and DNA synthesis. Most of the antiproliferative substances so far studied seem to exert their effects by upregulating Cdk inhibitors. However, we report here that the antiproliferative effect of DIF-1 may not result from the induction of Cdk inhibitors but probably from the strong suppression in the expression levels of the D-type cyclins, making DIF-1 unique among antiproliferative agents. Moreover, we show that this

**Key Words:** differentiation-inducing factor-1 vascular smooth muscle cell cell cycle cyclin differentiation
compound is able to prevent phenotypic modulation and induce differentiation of VSMCs.

Materials and Methods

Chemicals
DIF-1 was purchased from Affiniti Research Products. Cycloheximide (CHX) and l-a-phosphatidylinositol (PI) were from Sigma. Wortmannin was from Wako Pure Chemicals Industries. LY294002 (2-[4-morpholinyl]-8-phenyl-4H-1-benzopyran-4-one) was from Calbiochem.

Cell Culture
VSMCs obtained from the media of human umbilical arteries by explant were cultured as described8,9 and used within 3 passages. G∞ synchronization was achieved by serum starvation for 48 hours.

DNA Synthesis Assay
DNA synthesis was assessed by the level of thymidine (TdR) incorporation as described.8,9

Flow Cytometry
Cells dispersed by trypsinization and suspended in PBS were stained with propidium iodide using the Cycle TEST PLUS DNA Reagent Kit (Becton Dickinson). The fluorescence of DNA was measured using a flow cytometer (FACSCalibur, Becton Dickinson), and the cell cycle was analyzed by computer software (ModFit LT, Becton Dickinson).

Immunoprecipitation and Western blotting
Cell lysates were immunoprecipitated and analyzed by Western blotting as described.8,9

Cdk Assay
Cdk activities were measured using glutathione S-transferase–fused murine pRb carboxyl terminal (GST-Rb) (Santa Cruz Biotechnology) as described.8,9 Phosphorylated proteins were visualized and quantified using a bioimage analyzer (BAS-2500, Fuji Photo Film Co).

PI 3-Kinase (PI3K) Assay
PI 3-kinase was immunoprecipitated with an anti-phosphotyrosine antibody (PY20, Transduction Laboratories). PI (chloroform solution) was dried under nitrogen and suspended in water by sonication. After 3 washes with the kinase buffer (in mmol/L, Tris/HCl [pH 7.8] 50, NaCl 50, MgCl2 2, and EDTA 0.5), the precipitates were suspended in the same buffer containing 0.5 mg/mL PI micelles and 37 kBq [γ-32P]ATP and incubated at 30°C for 10 minutes. The reaction was terminated by the addition of 0.5 mL of 1 mol/L HCl and 2 mL of chloroform/methanol (2:1, vol/vol). The lower organic phase was dried under nitrogen, dissolved in a small volume of chloroform, spotted on a silica-gel thin-layer plate (Silica gel 60, Merck), and developed with chloroform/methanol/28% NH3/water (70:100:15:25, vol/vol). Radioactive spots on the plate were visualized by autoradiography after electrophoresis on 2% agarose gel and visualization by staining with ethidium bromide. Amplified DNAs were identified by sequencing. The levels of ethidium bromide fluorescence of DNAs obtained in every PCR cycle were plotted on a semilogarithmic graph to determine an appropriate PCR cycle number at which all of the samples were plotted within a linear range of the graph. The amounts of DNAs were quantified at the cycles thereby determined.

Statistics
Results are expressed as the mean±SD of the number of observations. Statistical significance was assessed by the Student t test for paired or unpaired values.

Results

DIF-1 Inhibited VSMC Proliferation
First, we tested whether DIF-1 has an antiproliferative effect in VSMCs as reported in tumor cells. To examine the effect of DIF-1 on DNA synthesis, G∞-synchronized cells were labeled with [3H]TdR by stimulating with growth medium (DMEM containing 20% FBS and 5 ng/mL basic fibroblast growth factor) for 30 hours in the presence of various concentrations of DIF-1 (Figure 1A). DIF-1 inhibited DNA synthesis in a dose-dependent manner, and the effect was maximal at 30 μmol/L. However, DNA synthesis was quickly recovered when DIF-1 was removed after 30-hour incubation (Figure 1B). The increase in cell number was also reversibly suppressed by DIF-1 (Figure 1C). Therefore, the antiproliferative effect of DIF-1 is unlikely to be due to cytotoxicity.

To determine where in the cell cycle the action point for DIF-1 is located, the cell cycle distribution was analyzed by flow cytometry (Figure 2A). After DIF-1 was added to exponentially growing cells, the cell population in S and G2/M phases significantly decreased, whereas that in G0/G1 phase increased. Therefore, DIF-1 was likely to inhibit the cell cycle in G0/G1 phase but unlikely to interrupt S and G2/M phases. As shown in Figure 2B, DIF-1 did not cause cell detachment, and it had no apparent effect on the cell shape, except that the number of cells under mitosis decreased after the treatment with DIF-1.

DIF-1 Inhibited Cdk Activities
The phosphorylation of pRb is a milestone in the cell cycle at which the final decision is made whether the cell should advance to S phase to begin DNA synthesis.10 We precipitated pRb with an antibody that recognizes both the hypophosphorylated and hyperphosphorylated forms and fractionated the precipitates by SDS-PAGE (Figure 3A). Stimulation with growth medium elevated the amount of hyperphosphorylated pRb without increasing that of hypophosphorylated pRb (110 kDa). The effect of DIF-1 was small at 30 μmol/L, but 30 μmol/L of DIF-1, which strongly suppressed DNA synthesis, completely inhibited the increase in the amount of hyperphosphorylated pRb.

To determine the mechanism by which DIF-1 inhibits pRb phosphorylation, we assayed the activities of Cdk2, Cdk4, and Cdk6, because pRb is considered to be phosphorylated in vivo by these Cdks activated during G1 phase. Figure 3B shows the effect of DIF-1 (30 μmol/L) on their activities.
Stimulation with growth medium for 12 to 24 hours markedly elevated the levels of Cdk2-induced phosphorylation of GST-Rb, but DIF-1 strongly inhibited these activities. The activities of Cdk4 and Cdk6 were maximally elevated at 12 hours (middle to late G1). They were also strongly suppressed in the presence of DIF-1. However, the expressions of Cdk2, Cdk4, and Cdk6 were not affected by DIF-1, indicating that the DIF-1–induced inhibition of Cdk activities was unlikely to be due to reduction in their amounts (Figure 3C).

Effect of DIF-1 on the Expression of Cyclins

Cyclins are activated by associating with Cdns. Cdk4 and Cdk6 associate with the D-type cyclins, and Cdk2 combines with cyclins E and A. Therefore, these cyclins are considered to be essential for the progression of G1 phase and the transition from G1 to S phase. We examined the effects of DIF-1 on the mRNA expression of various cyclins by Northern blotting (Figure 4A). DIF-1 (30 μmol/L) elevated the level of cyclin G mRNA, which was expressed in quiescent cells and decreased after mitogenic stimulation. DIF-1 significantly inhibited the expression of cyclin C mRNA, which was normally elevated in early G1 phase and sustained until 24 hours. The mRNA levels of the D-type cyclins began to increase soon after growth stimulation. They were maximally elevated at 12 hours and sustained until 24 hours. However, DIF-1 strongly suppressed these expressions. DIF-1 also inhibited the expressions of cyclins E and A, which normally increased from late G1 phase.

Consistently with the result of Northern blotting, DIF-1 strongly inhibited the protein expressions of cyclins D1 and D2 (Figure 4B), although we did not clearly detect cyclin D3 protein (not shown).

Effect of DIF-1 on the Expression of Cdk Inhibitors

To test whether Cdk inhibitor proteins are involved in the antiproliferative effect of DIF-1, we examined the effects of DIF-1 on the expression of Cdk inhibitors by Western blotting. Figure 5A demonstrates the effect of DIF-1 on p21<sup>Cip1/Waf1/Sdi1</sup>. Although low concentrations (2.5 to 10 μmol/L) of DIF-1 significantly upregulated the expression of p21, DNA synthesis was not inhibited at these concentrations (Figure 1A). In contrast, high concentrations (20 to 40 μmol/L) of DIF-1 that inhibited DNA synthesis reduced the expression of p21 (Figure 5A). p21 expression was not upregulated by DIF-1 (30 μmol/L) throughout the course of G1/S progression. The expressions of other Cdk inhibitor p27<sup>Kip1</sup>, p57<sup>Kip2</sup>, and p15<sup> Ink4B</sup> were not significantly changed by DIF-1 (Figure 5B). The expressions of p16<sup> Ink4A</sup> and p18<sup> Ink4C</sup> were upregulated by mitogenic stimulation, but they were
suppressed by this compound. We also examined the effects of DIF-1 on the levels of p21 and p27 associating with Cdks (Figure 5C). DIF-1 reduced the amounts of p21 associating with Cdk2, Cdk4, and Cdk6, whereas it did not change the levels of p27 associating with these Cdks. It was unlikely, therefore, that these Cdk inhibitors were responsible for the antiproliferative effect of DIF-1.

**Effect of DIF-1 on the Early Mitogenic Signals**

The above results suggested that DIF-1 inhibits the cell cycle by suppressing the expressions of the D-type cyclins in early G1 phase. To determine the upstream signals involved in this effect, we examined whether protein synthesis is required for the cyclin D1 suppression by DIF-1 (Figure 6A). CHX (5 μmol/L) inhibited the expression of cyclin D1 mRNA induced by mitogenic stimulation. However, CHX did not prevent DIF-1 from inhibiting the expression of cyclin D1. Therefore, mitogens may require de novo protein synthesis to induce cyclin D1, but DIF-1 may not require newly synthesized proteins to inhibit the induction.

Mitogen-activated protein kinase (MAPK) and PI3K are well known to be activated after growth factor receptor stimulation. Evidence suggests that these pathways are involved in mitogen-induced cyclin D1 expression. Therefore, we examined whether these pathways are involved in the DIF-1–induced effect on cyclin D1 expression. Mitogenic stimulation rapidly phosphorylated p44/42 MAPK, but DIF-1 had no significant effect on the phosphorylation (Figure 6B). DIF-1 did not influence the expression of p42 MAPK, either. Wortmannin (300 nmol/L) and LY294002 (25 μmol/L), PI3K inhibitors, significantly inhibited cyclin D1 mRNA expression induced by mitogenic stimulation, suggesting that PI3K is involved in cyclin D1 induction (Figure 6C). These PI3K inhibitors indeed inhibited mitogen-stimulated PI3K activity, whereas DIF-1 was not able to inhibit the activity, whether it was added to the kinase reaction mixtures or to living cells (Figure 6D).

**Effect of DIF-1 on the Expression of Myosin Heavy Chain (MHC) Isoforms**

Next, we explored whether DIF-1 regulates differentiation of VSMCs. Smooth muscle (SM) MHC isoforms SM1 and SM2 are specific markers for VSMC differentiation. We examined the effect of DIF-1 on the gene expression of SM1 and SM2 by RT-PCR using a single pair of PCR primers that cover the sequence specific to SM2, because SM1 and SM2...
are produced from a single gene by alternative splicing.\textsuperscript{19} To quantitatively perform RT-PCR, the cycle numbers at which the PCR products were within a linear range were determined from the graphs shown in Figure 7A and used for the following experiments. When synchronized in G0 phase, cells in primary culture expressed SM1 and SM2, although the expression of SM2 was much less than that of SM1 (Figure 7B). Mitogenic stimulation rapidly decreased the expression levels of both. SM2 was no longer detected 24 hours after stimulation. However, DIF-1 (30 μmol/L) delayed the reductions. SM1 only slowly decreased up until 48 hours, but thereafter increased. SM2 remained until 24 hours in the presence of DIF-1. In contrast, DIF-1 significantly reduced the expression of SMemb (also designated MHC-B), a nonmuscle-type MHC expressed in immature cells. To examine the effect of long-term administration of DIF-1, it was added to cells in the third passage, which seemed to be completely dedifferentiated to synthetic type, because they expressed only a small amount of SM1 and no SM2 (Figure 7C). The level of SM1 was markedly elevated as the cells were cultured in the presence of DIF-1, and moreover, SM2 was again expressed after 16 days. In contrast, the level of SMemb was reduced rapidly after addition of DIF-1 but was thereafter sustained.

**Discussion**

Hyperplasia of VSMCs plays a key role in the development of atherosclerosis and restenosis after angioplasty.\textsuperscript{5,6} In VSMCs proliferating in the neointima, the differentiated (mature) phenotype has been converted to a dedifferentiated (immature) one.\textsuperscript{3} Therefore, substances that inhibit proliferation and phenotypic modulation may be useful for prevention and therapy of vascular diseases. Several substances have been reported to inhibit VSMC proliferation, but there is little that is also able to inhibit phenotypic modulation or induce differentiation.

DIF-1 inhibited proliferation in VSMCs, as it did in tumor cells. Our results suggested that DIF-1 arrests the cell cycle by inhibiting the expressions of the D-type cyclins, given that
these cyclins play crucial roles in the progression of G1 phase and eventually for the transition to S phase. DIF-1 also inhibited the expression of cyclins E and A, which are induced subsequently to the D-type cyclins. Cyclin D–Cdk4, cyclin D–Cdk6, and cyclin E–Cdk2 complexes are responsible for the phosphorylation of pRb.21 pRb thereby hyperphosphorylated releases transcription factors of the E2F family, which initiate DNA synthesis.22 Consistently with this scenario, DIF-1 inhibited the activities of Cdk4, Cdk6, and Cdk2 and the phosphorylation of pRb.

On the other hand, DIF-1 did not induce Cdk inhibitor proteins at antiproliferative concentrations, although p21 was significantly upregulated at lower doses. Therefore, the antiproliferative effect of DIF-1 was unlikely to be mediated by Cdk inhibitors, in contrast to the fact that most of the antiproliferative substances so far examined induce Cdk inhibitors. For example, p21 is induced by nerve growth factor, transforming growth factor-β (TGF-β), interferons,1,25-dihydroxyvitamin D3, retinoids, prostaglandin A2, and nitric oxide.9,23,24 p27 is involved in the negative growth signals for TGF-β and cAMP.25,26 TGF-β also induces p15.27 The manner in which DIF-1 arrests the cell cycle is therefore unique. Substances having similar features are rare. In murine macrophages, cAMP has been shown to inhibit the expression of cyclin D gene (CYL1),28 but this effect has not been reported in other cell species. Recently, the immunosuppressant rapamycin has been reported to reduce cyclin D1 expression in NIH 3T3 fibroblasts.29 In VSMCs, protein kinase C-δ has been shown to mediate an antiproliferative signal by suppressing cyclin D1 expression,30 although in our study, phorbol-myristate-acetate, which activates protein kinase C and has a strong antiproliferative effect on VSMCs, did not inhibit cyclin D1 expression.8 The antiproliferative effect of apolipoprotein E in VSMCs has been also suggested to be caused by suppression of cyclin D1 expression,31 but this effect is not as strong as that elicited by DIF-1.

The MAPK cascade12–15 and the PI3K pathway16,17 have been suggested to mediate the expression of cyclin D1.12–15 However, it was unlikely that DIF-1 suppressed cyclin D1 expression by inhibiting MAPK or PI3K, because DIF-1 did not inhibit their activation induced by mitogenic stimulation. Recently b-catenin, a component of adherens junctions that associates with cadherins, has been suggested to regulate the transcription of cyclin D1.32,33 However, DIF-1 did not influence the expression and subcellular distribution of N-cadherin or β-catenin (not shown).

Whatever the underlying mechanism, it is clear that DIF-1 interrupts a very early stage of the cell cycle, because the expression of the D-type cyclins is one of the earliest events
in G phase. DIF-1 may possibly prevent the G1/G0 transition, namely the entry into the cell cycle. Considering that cell differentiation may be induced after the exit from the cell cycle, the unique nature of DIF-1 led us to speculate that this compound could induce VSMC differentiation. Interestingly, in skeletal myoblasts, forced expression of cyclin D1, but not cyclins E, A, and B, inhibits the ability of MyoD to transactivate muscle-specific genes.34 This suggests that inhibition of cyclin D1 expression per se can lead to differentiation.

Several genes for contractile proteins are sequentially expressed in developing VSMCs, including SM α-actin, SM22α, calponin, h-caldesmon, and SM-MHC isoforms SM1 and SM2.3 We used MHC isoforms as markers for VSMC differentiation, because the pattern of their expression in different phenotypes has been well examined.18–20 SM1 is abundant, and SM2 is exclusively expressed in differentiated VSMCs, whereas SM1 is diminished and SM2 is undetectable in dedifferentiated cells. In our study, DIF-1 reduced the rate of the reduction of SM1 and SM2 in cells in primary culture (partially dedifferentiated cells). Moreover, this compound markedly elevated the level of SM1 and again induced SM2 expression when added to nearly completely dedifferentiated (synthetic-type) cells that had expressed a small amount of SM1 and no SM2. Therefore, DIF-1 may be able to inhibit phenotypic modulation induced by growth stimulation and moreover promote redifferentiation of synthetic VSMCs.

The molecular mechanism of DIF-1 action still remains poorly understood even in *Dictyostelium*. Recently, it has been reported that the activation of a member of the signal transducer and activator of transcription (STAT) factors is involved in the signal transduction of DIF-1 in *Dictyostelium*.35 However, its receptor is still unknown. DIF-1 has similar bioactivities in *Dictyostelium* and mammalian cells. This might mean that mammalian cells have DIF-1 receptors corresponding to those in *Dictyostelium*. It is fascinating to imagine that mammals could produce a bioactive substance corresponding to DIF-1.

In conclusion, DIF-1 induces cell cycle arrest probably by inhibiting the expression of the D-type cyclins. Moreover, DIF-1 may be able to induce differentiation of VSMCs. Assuming that DIF-1 acts in the same way in vivo as it does in vitro, this compound or its derivatives could provide novel strategies for the treatment of vascular diseases.

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Yoshikazu Miwa, Toshiyuki Sasaguri, Chiya Kosaka, Yoji Taba, Akio Ishida, Takeo Abumiya and Yuzuru Kubohara

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