

Arctiin induces cell growth inhibition through the down-regulation of cyclin D1 expression

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Received July 30, 2007; Accepted September 24, 2007

Abstract. Arctiin is a major lignan constituent of *Arctium lappa* and has anti-cancer properties in animal models. It was recently reported that arctiin induces growth inhibition in human prostate cancer PC-3 cells. However, the growth inhibitory mechanism of arctiin remains unknown. Herein we report that arctiin induces growth inhibition and dephosphorylates the tumor-suppressor retinoblastoma protein in human immortalized keratinocyte HaCaT cells. We also show that the growth inhibition caused by arctiin is associated with the down-regulation of cyclin D1 protein expression. Furthermore, the arctiin-induced suppression of cyclin D1 protein expression occurs in various types of human tumor cells, including osteosarcoma, lung, colorectal, cervical and breast cancer, melanoma, transformed renal cells and prostate cancer. Depletion of the cyclin D1 protein using small interfering RNA-rendered human breast cancer MCF-7 cells insensitive to the growth inhibitory effects of arctiin, implicates cyclin D1 as an important target of arctiin. Taken together, these results suggest that arctiin down-regulates cyclin D1 protein expression and that this at least partially contributes to the anti-proliferative effect of arctiin.

Introduction

The lignans are a group of compounds formed by the condensation of pairs of phenylpropane structures and are

ubiquitously present in a variety of seeds, beans, fruits and vegetables (1). They influence several biological functions, such as hormone metabolism, cell proliferation, transformation and differentiation (1). Arctiin is a lignan constituent of *Arctium lappa* and exhibits anti-cancer effects in animal models. Takasaki *et al* reported that arctiin has a remarkable anti-tumor-promoting effect as seen in a two-stage carcinogenesis test of mouse skin tumors induced by 7,12-dimethylbenz[α]anthracene (DMBA) as an initiator and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) as a promoter by topical and oral administration (2). Hirose *et al* showed that arctiin has a protective effect on 2-amino-1-methyl-6-phenylimidazo[4,5- β]pyridine (PhIP)-induced carcinogenesis particularly in the mammary gland during the promotion period (3). In addition, it was reported that arctiin induces growth inhibition in human prostate cancer PC-3 cells and that it is associated with up-regulation of the anti-adhesion mucin MUC-1 gene (4). However, the growth inhibitory mechanisms of arctiin remain unknown.

Cell cycle progression is regulated by proteins called cyclins and cyclin-dependent kinases (CDKs), which associate with each other (5). Cyclins activate their partners, CDKs and direct their enzymes to specific substrates. Cyclin D1/D2/D3-CDK4/6 and cyclin E/A-CDK2 play important roles in promoting the G1-to-S phase transition of the cell-cycle by phosphorylating the tumor-suppressor retinoblastoma protein (RB) (5). The process of cyclin-dependent activation of CDKs is counterbalanced by CDK inhibitors (6). Two families of CDK inhibitors were identified in mammalian cells and each has a different mode of action. One group, composed of related proteins known as p21^{WAF1}, p27^{Kip1} and p57^{Kip2}, inhibit cyclin E/A-CDK2 complexes (7). The second family of CDK inhibitors is also called the INK4 protein family and its members, p15^{INK4b}, p16^{INK4a}, p18^{INK4c} and p19^{INK4d}, directly bind to CDK4/6 and are specific inhibitors of cyclin D-dependent kinases (8).

In the present study, we analyzed the growth inhibitory mechanisms of arctiin using human malignant tumor cells. We showed that arctiin down-regulates the cyclin D1 protein expression and that this is at least partially related to the anti-proliferative effect of arctiin.

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Abbreviations: RB, retinoblastoma protein; CDK, cyclin-dependent kinase; CKI, CDK inhibitor; siRNA, small interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Key words: arctiin, lignan, cyclin D1, retinoblastoma protein

Materials and methods

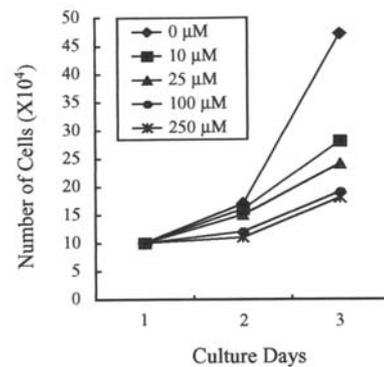
Cell culture and reagents. The human immortalized keratinocyte cell line HaCaT (a gift from Dr N.E. Fusenig, German Cancer Research Center, Heidelberg, Germany), the human osteosarcoma cell line MG63, the human lung cancer cell line A549, the human colorectal cancer cell line HCT116, the human cervical cancer cell line HeLa, the human breast cancer cell line MCF-7 and the human transformed renal cell line 293T were maintained in DMEM with 10% fetal bovine serum. The human melanoma cell line UACC-62 and the human prostate cancer cell line DU145 were maintained in RPMI-1640 medium with 10% fetal bovine serum. All of these cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. Arctiin (Alps Pharmaceutical, Japan) was dissolved in dimethyl sulfoxide (DMSO).

Analysis of cell cycle progression. Cells were treated with Triton X-100 and their nuclei were stained with propidium iodide before DNA content was measured using a Becton-Dickinson FACSCalibur (Becton-Dickinson, Mountain View, CA, USA). At least 10,000 cells were counted and the ModFit LD ver. 2.0 software package (Becton-Dickinson, Franklin Lakes, NJ, USA) was used to analyze the data.

Protein isolation and Western blot analysis. Cells were lysed by lysis buffer (50 mM Tris-HCl, pH 7.5 and 0.1% SDS). The protein extract was then boiled for 5 min and loaded onto a 12% polyacrylamide gel for p15^{INK4b}, p18^{INK4c}, p19^{INK4d}, p21^{WAF1/Cip1}, p27^{Kip1}, p57^{Kip2}, CDK2, CDK4, CDK6, cyclin A, cyclin D1, cyclin D3, cyclin E and α -tubulin detection or 7% polyacrylamide gel for RB detection, electrophoresed and transferred to a nitrocellulose membrane. A mouse monoclonal antibody against human cyclin D1 (DCS-6, MBL, Nagoya, Japan) and CDK6 (DCS-83, MBL) and rabbit polyclonal antibodies against human p15^{INK4b} (C-20, Santa Cruz Biotechnology, CA, USA), p18^{INK4c} (N-20, Santa Cruz Biotechnology), p19^{INK4d} (C-20, Santa Cruz Biotechnology), p21^{WAF1/Cip1} (C-19, Santa Cruz Biotechnology), p27^{Kip1} (C-19, Santa Cruz Biotechnology), p57^{Kip2} (P-0357, Sigma, MO, USA), CDK2 (M-2, Santa Cruz Biotechnology), CDK4 (H-22, Santa Cruz Biotechnology), cyclin A (C-19, Santa Cruz Biotechnology), cyclin D3 (C-16, Santa Cruz Biotechnology), cyclin E (HE-12, Santa Cruz Biotechnology), RB (PM-14001A, Pharmingen, NJ, USA), phospho-RB (Ser⁷⁸⁰) (#9307, Cell Signalling, MA, USA) and α -tubulin (Oncogene Research Product, CA, USA) were used as primary antibodies. Signals were then detected with an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech, NJ, USA).

RNA isolation and Northern blot analysis. Total RNA was isolated from HaCaT cells treated with or without 100 μ M arctiin for the indicated times using a Sepasol RNA isolation kit (Nakalai Tesque Inc., Kyoto, Japan) according to the manufacturer's instructions. Total RNA was fractionated on 1% agarose gels, transferred to nylon filters and hybridized with a cyclin D1 cDNA probe (a gift from Dr R.G. Pestell, Georgetown University School of Medicine, Washington, DC, USA) according to standard procedures (9). The same filter was

a



b

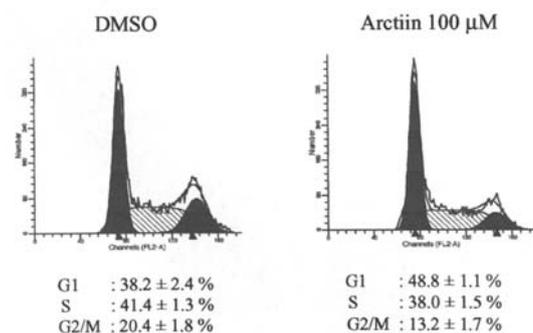


Figure 1. The effect of arctiin on the growth and cell cycle progression of HaCaT cells. (a) HaCaT cells were plated at 1×10^5 cells in 25-mm-diameter dishes. Twenty-four hours after plating, various concentrations of arctiin were added to the culture medium. From the first to the third day after plating, the number of viable cells was counted by the trypan blue dye exclusion test. (b) Unsynchronized HaCaT cells were exposed to 100 μ M arctiin for 48 h and the DNA content of the cells was determined by flow cytometry. The data represent the means of triplicate experiments and are shown as the means \pm SD (n=3).

rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe for internal control and mRNA levels were determined using a Fuji Image Analyzer Bas 2000 (Fujix, Tokyo, Japan).

siRNA transfection. Cyclin D1 siRNA#1 (#16810, Ambion, TX, USA) and siRNA#2 (#118854, Ambion) and a negative control siRNA (#4611, Ambion) were used. The targeted exon of cyclin D1 siRNA#1 or #2 is exon 3 or 5, respectively. MCF-7 cells were plated at 5×10^4 cells in 24-well plates and transfected with 0.5 μ M of siRNA using lipofectamine 2000 reagent (Invitrogen, CA, USA).

Results

Arctiin induces growth inhibition in HaCaT cells. We examined the effect of arctiin on the proliferation of human immortalized keratinocyte HaCaT cells. Arctiin inhibited the growth of HaCaT cells in a dose-dependent manner. The number of viable cells treated with 10, 25, 100 or 250 μ M arctiin for 48 h was 60, 51, 40, or 38% of the number of viable cells treated with vehicle only for 48 h, respectively (Fig. 1a).

SPANDIDOS PUBLICATIONS treated with 100 μ M arctiin were close to being arrested in G₁ phase. Arctiin at 100-250 μ M exhibited a maximum growth inhibitory effect, although it did not completely inhibit cell growth (Fig. 1a). To investigate the effect of arctiin on cell cycle progression, the DNA content of cell nuclei was measured by flow cytometric analysis. Treatment with 100 μ M arctiin for 48 h significantly ($P < 0.05$) increased the percentage from 38.2 to 48.8% in the G₁ phase (Fig. 1b).

Arctiin decreases cyclin D1 protein levels in HaCaT cells.

Cyclin-CDKs play important roles in promoting the G₁-to-S phase transition of the cell cycle by phosphorylating the RB protein (5) and the process of cyclin-dependent activation of CDKs is counterbalanced by CDK inhibitors (6). We screened for the expression of CDK inhibitors to clarify the molecular mechanisms underlying arctiin-induced growth inhibition in HaCaT cells. Whole-cell lysates were isolated after 0, 6, 12, 24 and 48 h of culturing with 100 μ M arctiin and were then subjected to immunoblotting. As shown in Fig. 2a, treatment with arctiin had no effect on the expression of the p57^{Kip2} protein. Arctiin slightly affected the expression of p15^{INK4b}, p18^{INK4c} and p19^{INK4d} and down-regulated the expression of the p21^{WAF1/Cip1} protein after 48 h of treatment. Weak down-regulation of the p27^{Kip1} protein was also observed from 24 to 48 h after exposure to arctiin. The amount of p16^{INK4a} protein was below the detection limit (data not shown). The G₁-to-S phase transition is also regulated by CDKs (5). We also examined whether the expression of CDKs can be altered by treatment with arctiin. The expression of the CDK4 protein was not altered by the treatment and arctiin slightly changed the expression of CDK2 and CDK6 (Fig. 2a). Then, we investigated the effect of arctiin on the expression of cyclins. Among the cyclins examined, the amount of cyclin D1 started to decrease after 24 h of culturing and further decreased to undetectable levels after 48 h. Weak down-regulation of the cyclin D3 and A protein was observed 48 h after treatment with arctiin. However, arctiin did not affect the expression of the cyclin E protein (Fig. 2a) and we could not detect the cyclin D2 protein.

The time course study indicated that arctiin strongly decreased the cyclin D1 protein in a time-dependent manner. To investigate whether arctiin down-regulates the cyclin D1 protein in a dose-dependent fashion, whole-cell lysates were isolated after 24 or 48 h of culturing with 0, 10, 25, 100 μ M arctiin and then subjected to immunoblotting. After 24 h of culturing, the expression of the cyclin D1 protein was significantly repressed by 100 μ M arctiin (Fig. 2b). After 48 h, arctiin strongly down-regulated the cyclin D1 protein expression in a dose-dependent manner and arctiin at 25 and 100 μ M decreased the amount of cyclin D1 protein to undetectable levels (Fig. 2b). These results indicated that arctiin down-regulates the expression of cyclin D1 protein in a time- and dose-dependent manner. Taken together with the results presented in Figs. 1a and 2b, we suggest that arctiin-induced growth inhibition is correlated with the arctiin-induced down-regulation of cyclin D1 protein. Then, we examined the effect of arctiin on the cyclin D1 mRNA level. Total RNAs were isolated after 0, 6, 12, 24 and 48 h of culturing with or without 100 μ M arctiin and then subjected to Northern blot analysis. As shown in Fig. 2c, arctiin slightly affected the

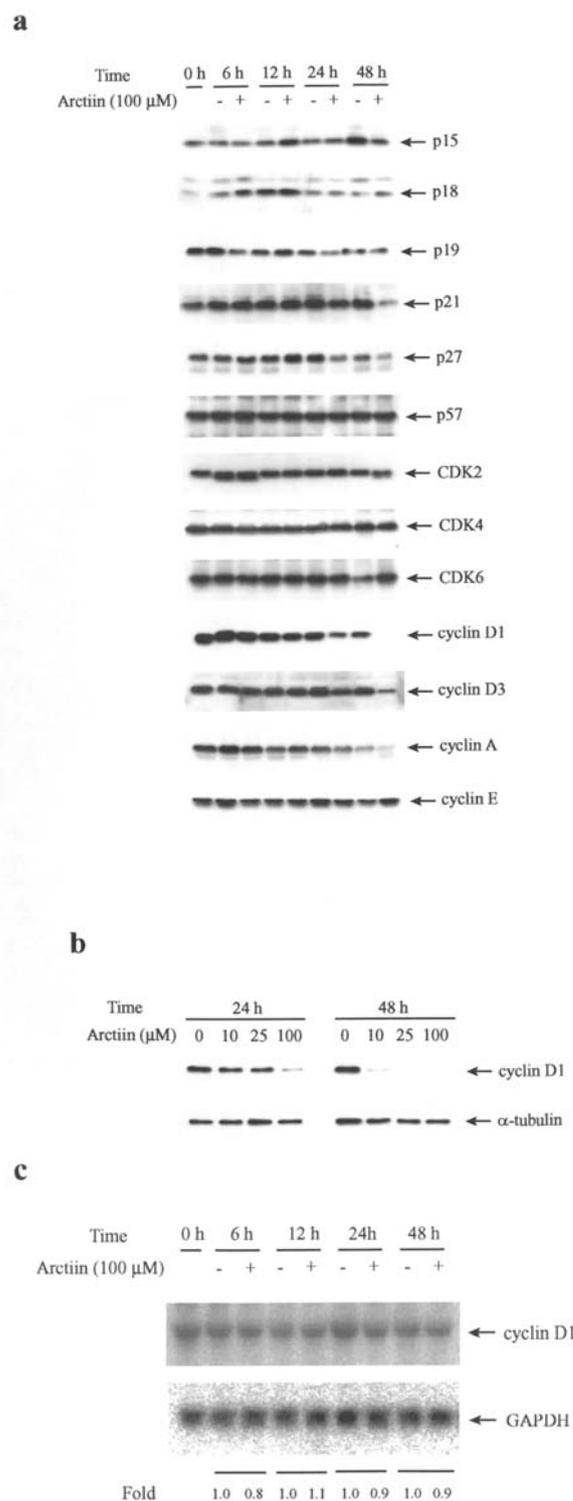


Figure 2. The effect of arctiin on the expression of CDK inhibitors, CDKs and cyclins in HaCaT cells. (a) HaCaT cells were exposed to DMSO (-) or 100 μ M arctiin (+) and were lysed at the indicated times after treatment. Western blotting was performed to examine the expression of p15^{INK4b}, p18^{INK4c}, p19^{INK4d}, p21^{WAF1/Cip1}, p27^{Kip1}, p57^{Kip2}, CDK2, CDK4, CDK6, cyclin D1, cyclin D3, cyclin A and cyclin E. An antibody to α -tubulin was used as a loading control. (b) HaCaT cells were treated with various concentrations of arctiin for 24 or 48 h and the expression of cyclin D1 protein was then examined in Western blotting. (c) HaCaT cells were treated with 100 μ M arctiin for the indicated times and the expression of cyclin D1 mRNA was examined by Northern blot analysis. The transcript is ~4.3 kb. The mRNA level of cyclin D1 was standardized against that of GAPDH. Data shown below the blots represent fold induction in the mRNA expression and each value was compared with that of the control without arctiin which was estimated as 1.0.

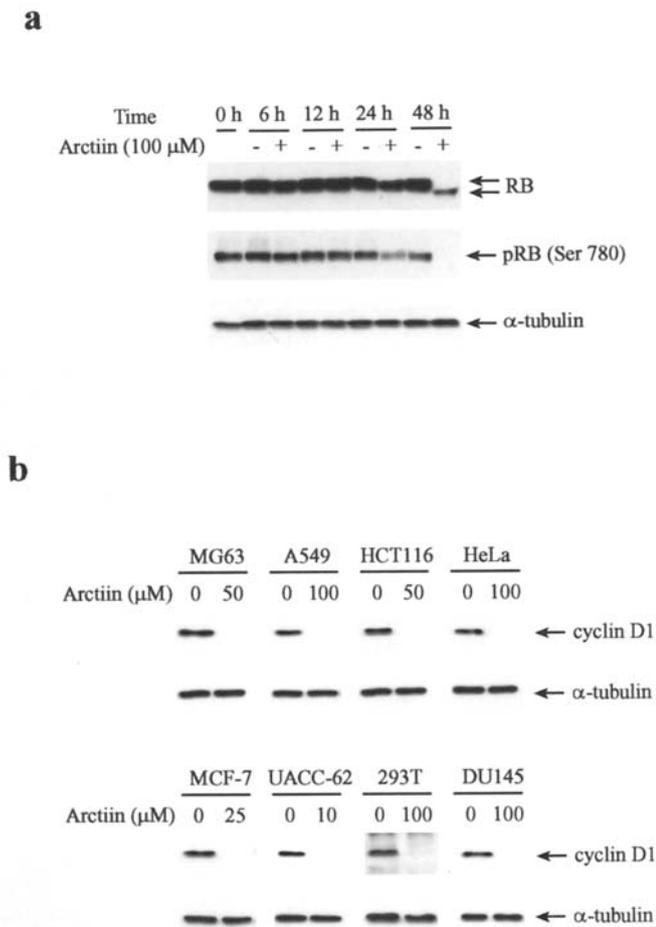


Figure 3. The effect of arctiin on the expression of RB and phosphorylated RB in HaCaT cells. (a) HaCaT cells were exposed to DMSO (-) or 100 μ M arctiin (+) and were lysed at the indicated times after treatment. Western blotting was then carried out using antibodies against RB and phospho-RB (Ser 780). (b) The human osteosarcoma cell line MG63, the human lung cancer cell line A549, the human colorectal cancer cell line HCT116, the human cervical cancer cell line HeLa, the human breast cancer cell line MCF-7, the human melanoma cell line UACC-62, the human transformed renal cell line 293T and the human prostate cancer cell line DU145 were treated with various concentrations of arctiin for 48 h and the expression of cyclin D1 protein was then examined by Western blotting.

amount of cyclin D1 mRNA. This result suggested that arctiin post-transcriptionally down-regulates the cyclin D1 protein expression.

Arctiin dephosphorylates RB protein in HaCaT cells. Since cyclin D1 activates CDK4/6, we investigated whether the phosphorylation status of RB is altered in arctiin-treated HaCaT cells. RB is a key substrate for various cyclin-CDK complexes and specific sites on it are phosphorylated by distinct cyclin-CDK complexes (10,11). Therefore, we used an anti-phospho-RB (Ser 780) antibody, which specifically recognizes CDK4/6-phosphorylated residues Ser 780 on RB (10,11). Western blot analysis showed that from 6 to 12 h after treatment with arctiin, the phosphorylation status (Ser 780) of RB was not changed (Fig. 3a). However, the expression of phosphorylated RB protein recognized by anti-phospho-RB

(Ser 780) started to decrease after 24 h of culturing and further decreased to undetectable levels after 48 h (Fig. 3a). Then, we used an anti-RB antibody, which recognizes the RB protein regardless of its phosphorylation status. Western blot analysis indicated that 48 h after treatment with arctiin, the amount of RB protein slightly decreased and the RB protein band shifted from a hyperphosphorylated form to a hypophosphorylated form (Fig. 3a). These results indicated that arctiin dephosphorylates RB protein in HaCaT cells.

Arctiin decreases cyclin D1 protein levels in various types of human tumor cells. To clarify whether the down-regulation of cyclin D1 by arctiin is a general event, we subsequently examined the effect of arctiin on the cyclin D1 expression in other human tumor cell lines. For this investigation, we used the osteosarcoma cell line MG63, the lung cancer cell line A549, the colorectal cancer cell line HCT116, the cervical cancer cell line HeLa, the breast cancer cell line MCF-7, the melanoma cell line UACC-62, the human transformed renal cell line 293T and the prostate cancer cell line DU145. As shown in Fig. 3b, arctiin inhibited the expression of cyclin D1 protein in all of these cell lines, suggesting that the arctiin-induced down-regulation of cyclin D1 protein is an ubiquitous event in human tumor cells.

Depletion of cyclin D1 protein renders MCF-7 cells insensitive to the growth inhibitory effect of arctiin. Finally, we examined whether cyclin D1 is involved in cell growth inhibition by arctiin in MCF-7 cells. As shown in Fig. 3b, arctiin down-regulates the cyclin D1 protein expression in MCF-7 cells. However, the amount of cyclin D3 protein was not altered by arctiin (data not shown) and the cyclin D2 protein was not detected in MCF-7 cells. If cyclin D1 is one of the important targets of arctiin, cyclin D1-depleted MCF-7 cells are expected to be insensitive to the action of arctiin. To test this hypothesis, we used cyclin D1 siRNAs to specifically deplete cyclin D1. As shown in Fig. 4a, treatment with cyclin D1 siRNA#1 or siRNA#2, respectively, almost depleted the cyclin D1 protein. However, the cyclin D1 siRNAs had no effect on the expression of cyclin D3 protein (Fig. 4a). As shown in Fig. 4c, cyclin D1 siRNA-treated cells proliferate more slowly than mock- or control siRNA-treated cells. To characterize the cyclin D1 siRNA-treated cells further, we performed FACS analysis (Fig. 4b). The cyclin D1 siRNA-treated cells showed a slightly but significantly ($P < 0.05$) increased percentage of G1 phase compared to mock- or control siRNA-treated cells. Then we examined the effect of arctiin on cyclin D1-depleted cells. As shown in Figs. 4c and d, arctiin inhibited growth in mock- or control siRNA-treated MCF-7 cells. On the other hand, arctiin did not induce growth inhibition in cyclin D1 siRNA-treated cells (Figs. 4c and d). Collectively, along with the fact that arctiin down-regulates the cyclin D1 protein in MCF-7 cells (Fig. 3b), suggest that cyclin D1 is one of the key targets of arctiin for its growth inhibitory function.

Discussion

Arctiin is a major lignan of *Arctium lappa*. Our results indicated that arctiin inhibited cell growth and dephosphorylated

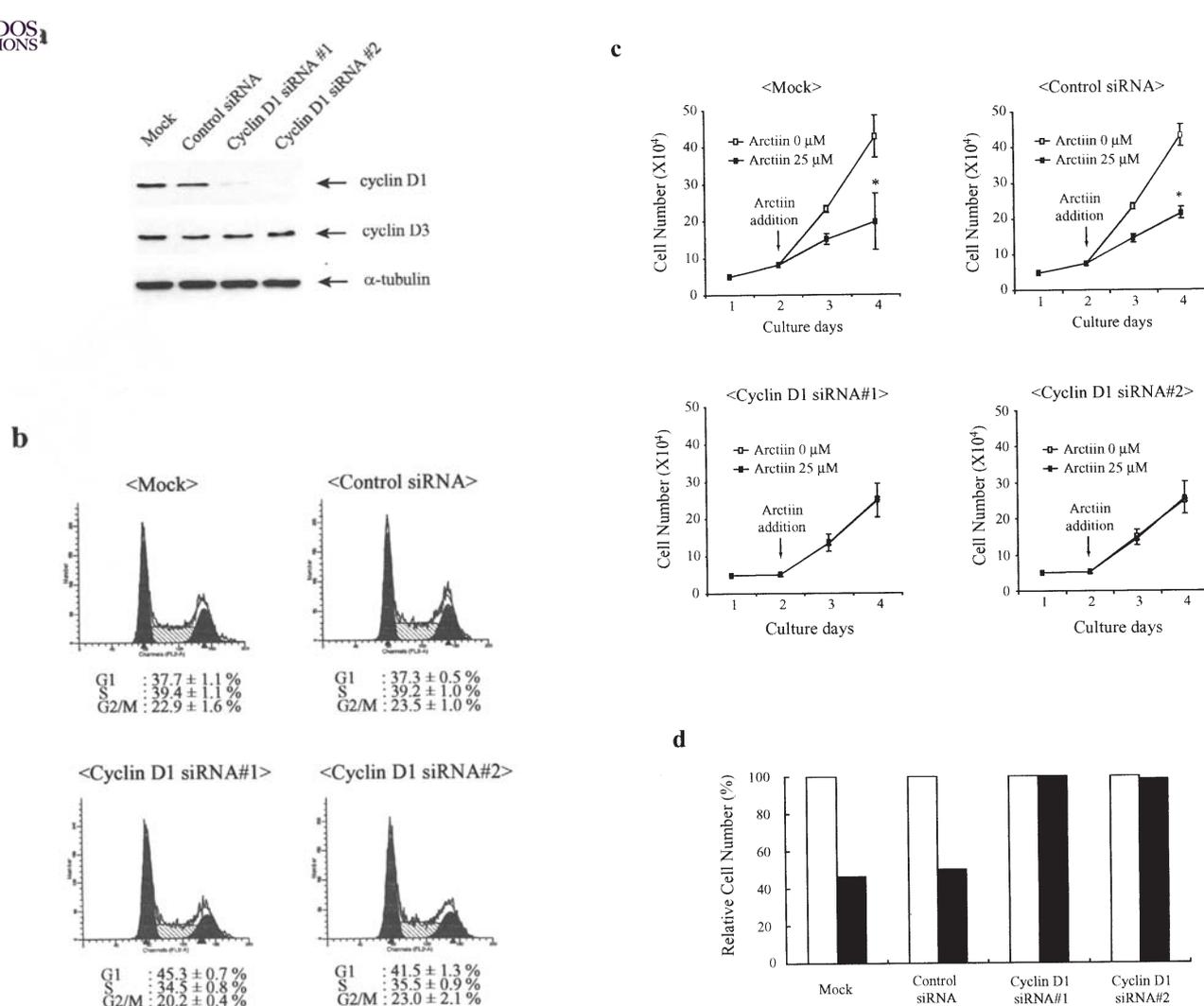


Figure 4. Depletion of cyclin D1 protein renders MCF-7 cells insensitive to the growth inhibitory effect of arctiin. (a) MCF-7 cells were plated at 5×10^4 cells in 24-well plates and transfected with $0.5 \mu\text{M}$ of siRNA. Cyclin D1 siRNA#1 and siRNA#2 and a negative control siRNA were used. Mock, MCF-7 cells treated with lipofectamine 2000 reagent only. Twenty-four hours after transfection, cell lysates were prepared and the expression of cyclin D1 or D3 protein was examined by Western blotting. (b) Forty-eight hours after transfection the DNA content of the cells was determined by flow cytometry. The data represent the means of triplicate experiments and are shown as the means \pm SD ($n=3$). (c) Twenty-four hours after transfection, arctiin at $25 \mu\text{M}$ was added and cell growth was compared with a control culture. The data represent the means of triplicate experiments and are shown as the means \pm SD ($n=3$). * $P<0.05$. (d) Relative cell viability of MCF-7 cells treated with DMSO (□) or $25 \mu\text{M}$ arctiin (■) for 48 h.

the RB protein in human immortalized keratinocyte HaCaT cells. We also found that the growth inhibition induced by arctiin was correlated with the down-regulation of the cyclin D1 protein. Arctiin-induced cyclin D1 suppression occurred in multiple human tumor cells, including osteosarcoma, lung, colorectal, cervical and breast cancer, melanoma, transformed renal cells and prostate cancer. Furthermore, the depletion of the cyclin D1 protein using cyclin D1 siRNAs rendered human breast cancer MCF-7 cells insensitive to the growth inhibitory effects of arctiin, suggesting that cyclin D1 is one of the important targets of arctiin. Taken together, these results suggested that arctiin down-regulates the cyclin D1 protein expression and that this at least partially contributes to the growth inhibition caused by arctiin.

Arctiin induced anti-cancer effects in animal models such as a significant anti-tumor-promoting effect in a two-stage carcinogenesis model of mouse skin tumors induced by DMBA and TPA (2). Hirose *et al* reported that arctiin inhibited

PhIP-induced mammary carcinogenesis, particularly during the promotion period (3). These studies suggested that the anti-cancer effect of arctiin mainly resides in its anti-tumor-promoting function. On the other hand, several studies reported that cyclin D1 is an important molecule for tumor promotion. Robles *et al* showed that cyclin D1 deficiency reduced skin tumor development in TPA-treated ras transgenic mice in a two-stage carcinogenesis model using DMBA and TPA (12). These results suggested that cyclin D1 has a role in promoting tumor growth induced by TPA in mouse skin. Furthermore, Qiu *et al* found that a higher expression of cyclin D1/CDK4 occurred in rat mammary gland carcinomas induced by PhIP (13). Therefore, our present observation that arctiin down-regulates the cyclin D1 protein expression may provide a clue for solving the mechanism of an arctiin-induced anti-tumor promoter function.

We indicated that the depletion of cyclin D1 protein renders MCF-7 cells insensitive to the growth inhibitory effects of

arctiin in Fig. 4. To investigate whether overexpression of cyclin D1 resists the growth inhibitory effect of arctiin, we examined the effect of arctiin on cyclin D1-overexpressing cells using human breast cancer cell line T-47D-based stable clones that overexpress HA-tagged cyclin D1 protein (14). However, the expression of the overexpressed cyclin D1 protein was also down-regulated by arctiin and the endogenous cyclin D1 protein (data not shown). As a result, the growth inhibition rate of cyclin D1-overexpressing cells by arctiin was almost equivalent to that of parental cells (data not shown).

Cyclin D1, a regulator of the cell-cycle transition from G1-to-S phase, forms a holoenzyme with CDK4/6 that phosphorylates RB. Overexpression of cyclin D1 promotes cell-cycle progression through the G1 phase (8,15) and contact-independent growth (16). Genetic alterations in the regulatory components that regulate the G1-to-S phase transition in the cell-cycle frequently occur in human cancers (17). Cyclin D1 is overexpressed in many types of human tumors, including those of the breast, esophagus, lung, head and neck, colon and prostate (18-28). For example, the cyclin D1 gene is amplified and overexpressed in 50% of human breast cancers (22,29,30) and its expression is associated with the early onset of disease and risk of tumor progression and metastasis (27,31). Transgenic mice that overexpress cyclin D1 in mammary glands develop breast cancer, suggesting that cyclin D1 functions as an oncogene (32). On the other hand, mice lacking cyclin D1 are resistant to oncogene-induced tumorigenesis, including ras-induced skin tumors (12) and ErbB2 or ras-induced mammary tumor formation (33). Therefore, regulated agents of the cyclin D1 expression may contribute to new strategies for the prevention or therapy of malignancies, which we term 'gene-regulating chemoprevention or chemotherapy' (34,35). Arctiin, which down-regulates the cyclin D1 protein expression, may be a representative of gene-regulating chemopreventive or chemotherapeutic agents.

Acknowledgements

This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a grant (H11-Seikatsu-018) for Research on Environmental Health from the Ministry of Health, Labor and Welfare of Japan. We are grateful to Yuuki Takaoka for the technical support.

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