ANTICANCER RESEARCH *25*: 2639-2644 (2005)

Growth Inhibitory Effect of D-allose on Human Ovarian Carcinoma Cells *In Vitro*

LI SUI[1,3], YOUYI DONG[1], YASUO WATANABE[1], FUMINORI YAMAGUCHI[1], NAOYA HATANO[1], KEN IZUMORI[2] and MASAAKI TOKUDA[1,2]

[1]*Department of Cell Physiology, Faculty of Medicine,*
[2]*The Rare Sugars Research Center and*
[3]*Kagawa Industry Support Foundation, Kagawa University, Kagawa, Japan*

Abstract. *Background: D-allose is a rare sugar found in nature and, because of its very limited amount and of the high cost associated with its synthesis, its physiological functions remain virtually unknown. The aim of the current study was to investigate the effect of D-allose on the proliferation of human ovarian carcinoma cells in vitro. Materials and Methods: Human ovarian carcinoma cells (OVCAR-3 cell line) were exposed to rare sugars including D-allose, D-altrose, D-psicose and D-talitol. Cell growth was evaluated by MTT assay. Cell cycle analysis was carried out by flow cytometric assay. The expression of cell cycle regulatory proteins was determined by Western blot analysis. TUNEL assay was employed for the detection of apoptotic cells. Results: D-allose had a significant inhibitory effect on ovarian cancer cell proliferation in a dose-dependent manner, and caused a moderate G2/M arrest in the cell cycle, up-regulation of Cdk inhibitors p21 and p27 levels, and the induction of apoptosis in OVCAR-3 cells. Conclusion: Our results show, for the first time, that D-allose inhibits the growth of ovarian carcinoma cells in vitro. Although the exact mechanisms remain unclear, these findings suggest that D-allose possesses a novel inhibitory property on ovarian carcinoma cell proliferation, and may represent a new class of compounds with possible therapeutic potential.*

D-allose, the C-3 epimer of glucose (Figure 1), is a type of monosaccharide rarely distributed in nature. Because their synthesis is laborious, time-consuming and inefficient (1, 2), the biological effects of rare sugars have not been investigated in detail. Recently, a simple method to produce rare sugars on a large scale was demonstrated using L-rhamnose isomerase (L-RI) from D-psicose (3), while D-psicose has been produced using D-tagotose 3-epimerase (D-TE) from D-fructose (4). An effective strategy for the mass production of rare sugars has been developed by the discovery of Izumoring in Kagawa, Japan (5). This novel synthesis may facilitate many research areas, including medicine. The purpose of the current study was to investigate the physiological activity of rare sugars. It has been recently reported that D-allose could substantially inhibit segmented neutrophil production and, to a lesser degree, platelet formation, without other detrimental clinical effects, and may play a pivotal role in organ or tissue transplantation as an immunosuppressant (Arnold *et al.* US patent. No. 5620960, 1997). Murata *et al.* discovered that D-allose has a unique activity to inhibit the production of reactive oxygen species (ROS) and has scavenging activity against ROS (6). The effect of rare sugars on cell proliferation *in vitro* using an ovarian carcinoma cell line is described below.

Correspondence to: Masaaki Tokuda, MD, Ph.D., Department of Cell Physiology, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-Cho, Kita-Gun, Kagawa, 761-0793. Japan. Tel: +81-87-891-2095, Fax: +81-87-891-2096, e-mail: tokuda@med.kagawa-u.ac.jp

Key Words: D-allose, cell proliferation, ovarian carcinoma.

Materials and Methods

Cell culture. The human ovarian adenocarcinoma cell line OVCAR-3 (ATCC, Manassas, VA, USA) was maintained at 37˚C under a humidified atmosphere of 5% CO_2 in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO, USA), supplemented with 10% heat-inactivated fetal calf serum, 10 μg /ml insulin and antibiotics (100 IU/ml penicillin, 100 μg /ml streptomycin), as recommended by the supplier.

Reagents. Rare sugars including D-allose, D-altrose, D-psicose, D-talitol, and non-rare sugars including D-glucose and D-fructose were supplied from The Rare Sugars Research Center, Kagawa University, Kagawa, Japan. All sugars were dissolved at a concentration of 1 M in sterile phosphate-buffered saline (PBS) and were further sterilized by filtration. 3-[4,5-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), and RNase A was purchased from Roche Diagnostics Corporation (Indianapolis, IN, USA).

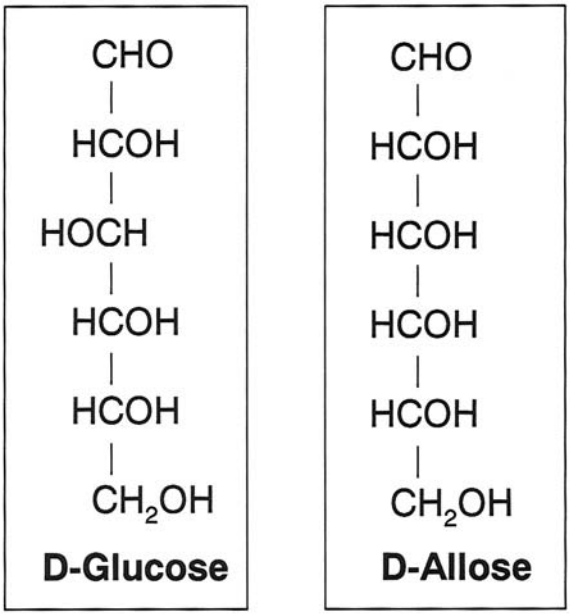

Figure 1. *The structures of D-allose and D-glucose.*

MTT assay. The growth assay was initiated, when OVCAR-3 cells were on the logarithmic growth phase (crowded 70% confluent). Cells were seeded onto 96-well plates with $6.0x10^3$ cells/well in 0.1 ml medium and cultured for 24 h. The culture medium was then removed and fresh medium containing 50 mM of various sugars was added (0 day). After exposure to the sugars for 1-5 days, 10 μl MTT solution (5 mg/ml in PBS) was added to each well and the plates were incubated for an additional 4 h at 37˚C. To examine a dose-dependent effect, D-allose was added at different concentrations (1, 5, 10, 20, 50 mM). After 1-3 days, the MTT assay was performed. To achieve solubilization of the formazan crystal formed in viable cells, dimethylformamide (DMF)-20% sodiumdodecyl sulfate (SDS) (pH 4.7) was added to each well. The absorbance was measured by a micro plate reader at 595 nm.

Cell cycle analysis. Untreated (control) and D-allose-treated cells were harvested using trypsinization (0.25% trypsin / 1 mM EDTA) after 5 days of treatment, washed twice with ice-cold PBS (pH 7.2) and fixed in 1 ml of 70% ethanol ($1x10^6$ cells/sample) for 2 h at 4˚C. The cells in each of these ethanol solutions were washed twice with ice-cold PBS and incubated in 1 ml PBS containing 50 μg propidium iodide and 200 μg RNase A for 30 min at 37˚C in the dark. Flow cytometric analysis was performed with a FACSEpics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). The effect on cell cycle was determined by changes in percentage of cell distribution at each phase of the cell cycle and analyzed using System II software (Beckman Coulter).

Western blot analysis. After different treatments (untreated and treated by D-glucose, D-psicose, D-allose) for 5 days, cells were scraped into lysis buffer (1% NP40, 150 mM NaCl, 50 mM NaF, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM Na_3VO_4, 10 μM Na_2MnO_4, 1 mM PMSF, 10 μg/ml leupeptin, 1% aprotinin). Cells were then disrupted by sonication, after they were centrifuged for 1 h at 100,000 x g, and the protein concentration in the supernatant was determined using a protein assay kit based on the Bradford (Bio-Rad Laboratories, Tokyo, Japan). Proteins were separated on a 12% SDS-PAGE gel and transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA). The membranes were blocked with 5% (w/v) nonfat dried milk and immunoblotting was performed using anti-cyclin D1, anti-cyclin E, anti-cyclin B1, anti-Cdk2 and anti-Cdk4 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cyclin A, anti-Cdk1, anti-p21 and anti-p27 antibodies (NeoMarkens, Fremount, CA, USA) and anti-β-actin antibody (Sigma, St. Louis, MO, USA). Membranes were probed with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham, Tokyo, Japan), and proteins were detected by an enhanced chemiluminescence system (Amersham).

TUNEL assay. The TUNEL assay was performed with the Apoptosis Detection System, Fluorescein Kit (Promega, Madison, WI, USA). In brief, cells were spread on a poly-L-lysine slide (Sigma), treated with D-allose for 5 days, fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. The cells were incubated in 50 μl of TdT incubation buffer (nucleotide mix [fluorescein-12-dUTP] and TdT enzyme prepared as per the manufacturer's protocol), incubated for 60 min at 37˚C inside the humidified chamber. The reaction was stopped by washing the cells in 2 x SSC followed by washing twice in PBS. The cells were counterstained with 1 μg/ml propidium iodide and washed in distilled water. Staining was observed with a fluorescence microscope. Cells that displayed green fluorescence were indicative of DNA fragmentation due to labelling by fluorescein-12-dUTP.

Statistical analysis. Data are expressed as mean±standard deviation (SD), and the significances of differences were evaluated using the Student's *t*-test.

Results

Effect of D-allose on cell proliferation. The effect of rare sugars on cell proliferation was assessed by the MTT assay. The inhibitory effect of D-allose on cancer cell proliferation was shown from day 1, and became more significant at day 5, in contrast to untreated cells ($p<0.01$, Figure 2A); however, the inhibitory effect was not observed for the other sugars. Furthermore, studies on the concentration effect of D-allose indicated that D-allose inhibits cells proliferation in a dose-dependent manner (Figure 2B).

Effect of D-allose on the cell cycle progression. To investigate whether D-allose exerts its inhibitory effect by perturbing the cell cycle, flow cytometry was used to determine the effect on cell cycle distribution. In control cultures, 12.6% cells were in G2/M-phase, however, in D-allose-treated cells (50 mM for 5 days) this percentage increased to 24.6%, and a moderate G2/M-phase arrest was observed (Figure 3).

Effect of D-allose on cell cycle regulatory proteins. Cell cycle progression is tightly regulated through a complex network

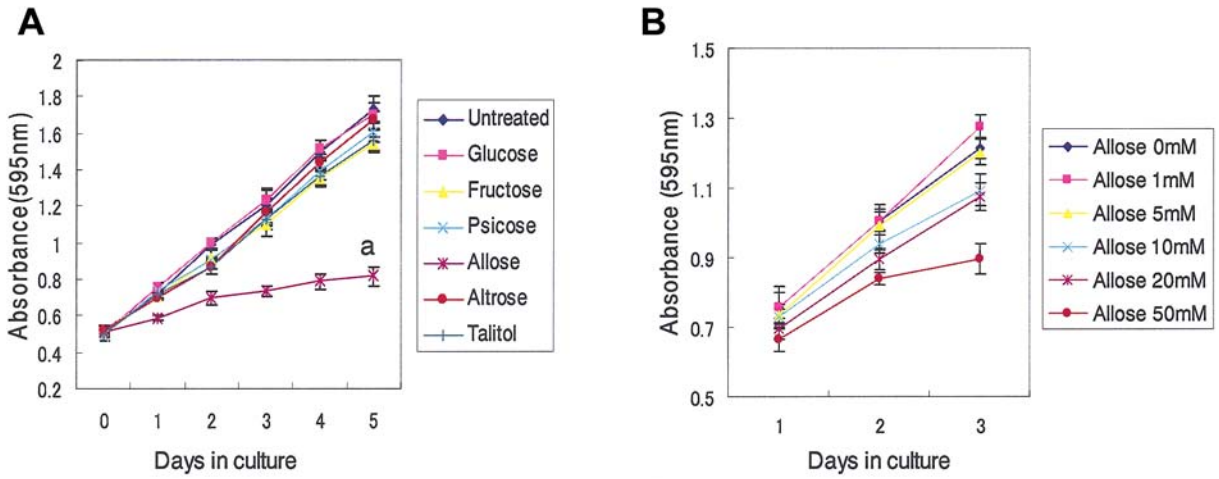

Figure 2. *Cell growth as determined by MTT assay. An obvious cell growth inhibitory effect of D-allose was observed (A), and the inhibitory effect of D-allose was dose-dependent (B). Values are the average of eight wells ±SD.* $^{a}p<0.01$ *vs. control.*

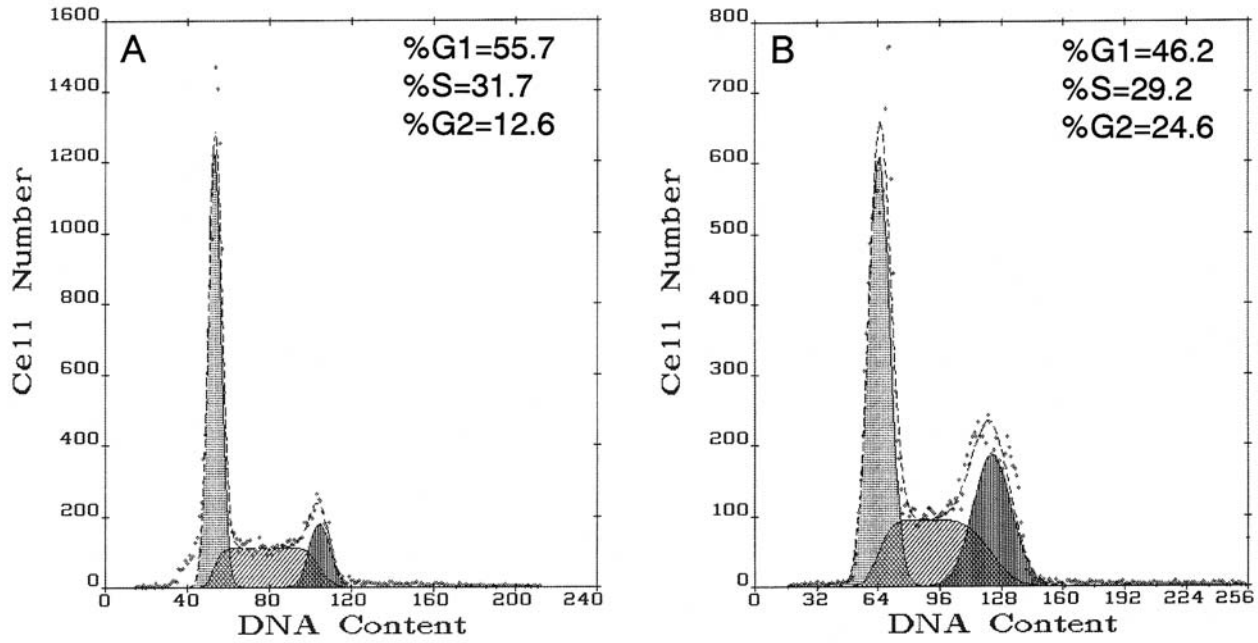

Figure 3. *Effect of D-allose on cell cycle. OVCAR-3 cells were cultured for 5 days in the absence (A) or in the presence of 50 mM D-allose (B). Increased G2/M-phase cells in D-allose-treated OVCAR-3 cells (24.6%) in contrast with control cells (12.6%) can be observed.*

of positive and negative cell cycle regulatory molecules, such as cyclins, cyclin-dependent kinases (Cdks) and Cdk inhibitors. In order to elucidate the specific cell cycle regulatory proteins responsible for the growth inhibitory effect of D-allose in OVCAR-3 cells, protein extracts were prepared from cells treated with 50 mM D-glucose, D-psicose and D-allose. Western blot analysis was performed using antibodies specific for cyclin A, cyclin B1, cyclin D1, cyclin E, Cdk1, Cdk2, Cdk4, p21 and p27. No alteration of cyclins or Cdks was observed on any sugars examined, however, Cdk inhibitors p21 and p27 expression levels were increased in D-allose-treated cells in contrast to control (Figure 4).

Effect of D-allose on induction of apoptosis. In order to determine if D-allose treatment could induce apoptosis, the TUNEL assay was carried out on those OVCAR-3 cells continuously exposed to 50 mM D-allose for 5 days. No

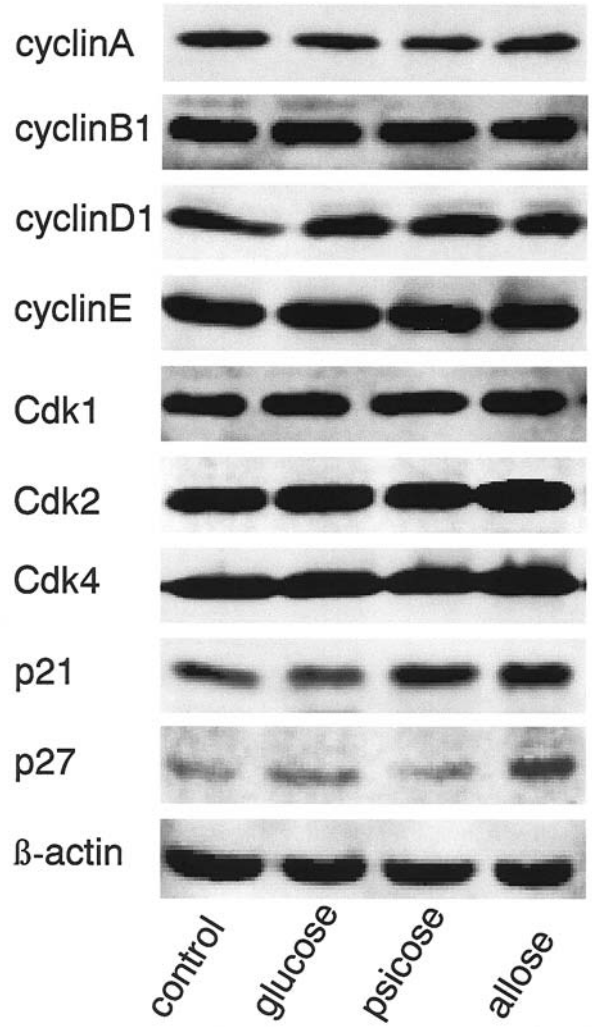

Figure 4. *Western blot of cyclin, Cdk and Cdk inhibitor protein levels in untreated and sugar-treated cells. None of the sugars tested induced any significant change in cyclin and Cdk levels. However, the expression of Cdk inhibitors p21 and p27 increased in D-allose-treated OVCAR-3 cells in contrast with the control (β-actin).*

apoptotic cells were observed in untreated cells, while a few apoptotic cells (8±3%) were found among D-allose-treated cells (Figure 5). Subsequently, the expression of Bax and Bcl-2 proteins involved in the control of programmed cell death was also examined. However, no significant change was observed in Bax and Bcl-2 protein levels after D-allose treatment (data not shown).

Discussion

D-allose has received very scant research attention, because of its rarity and the high cost involved in its synthesis, and thus its physiological functions are virtually unknown. However, a novel process for the mass production of all rare sugars, Izumoring , has been developed (3-5). As a result, research in the medical field on rare sugars has become possible, and this is, to our knowledge, the first study on the biological effect of rare sugars on cell proliferation *in vitro*.

In the present study, the effect of rare sugars including D-allose, D-altrose, D-psicose and D-talitol on cell proliferation was evaluated, and a novel antiproliferative effect of D-allose was identified in ovarian carcinoma cells. The inhibitory activity was approximately proportional to the dose and length of the treatment. To our knowledge, this is the first report that D-allose has a significant inhibitory effect on cancer cell proliferation *in vitro*.

Recently, the mechanism of cell cycle progression has been extensively studied. The cell cycle profile was analyzed by flow cytometry in order to study the molecular events involved in the growth inhibition of ovarian cancer cells induced by D-allose treatment. The addition of D-allose to ovarian carcinoma cells increased the percentage of cells in the G2/M-phases. These data suggest that the mechanism of the D-allose anti-proliferative effect may involve cell cycle arrest. The changes in multiple regulatory molecules in the cell cycle progression were also investigated. Since eukaryotic cell proliferation is regulated by expression and sequential activation of cell cycle-dependent cyclins, Cdks, and Cdk inhibitors (7), the expression of these regulatory proteins was examined. It was found that both p21 and p27, important Cdk inhibitors, were up-regulated in D-allose-treated OVCAR-3 cells. It has been reported that p21 and/or p27 play a major role in regulating early and late cell cycle phase-specific Cdks (8, 9). Their over-expression is implicated in the induction of blockade at a specific stage of the cell cycle (9, 10). Our data indicate that G1-phase-regulating proteins cyclin D1, cyclin E as well as associated Cdk2 and Cdk4 were not changed, in agreement with the result of the cell cycle analysis. Furthermore, no change was observed for G2/M-phase-regulating proteins cyclin B1 and Cdk1 in D-allose-treated OVCAR-3 cells, suggesting that these proteins may not be involved in the arrest of the G2/M-phase in OVCAR-3 cells. Taken together, we can speculate that the cell growth inhibitory effect of D-allose might be associated with the up-regulation of Cdk-inhibitor (p21, p27) levels.

The cell growth inhibitory effect of D-allose may also be due to programmed cell death. In the present work, the TUNEL assay led to the observation that there may be a mild induction of apoptosis in D-allose-treated cells. It is known that Bcl-2 and Bax play a major role in determining whether cells undergo apoptosis under the experimental conditions that promote cell death (11, 12). Bcl-2 can protect cells from apoptosis (12), while increased expression of Bax can induce apoptosis (11). The ratio of Bax to Bcl-2 is important for the survival of drug-induced apoptosis in leukemia cell lines (12). In our study, however, no change in

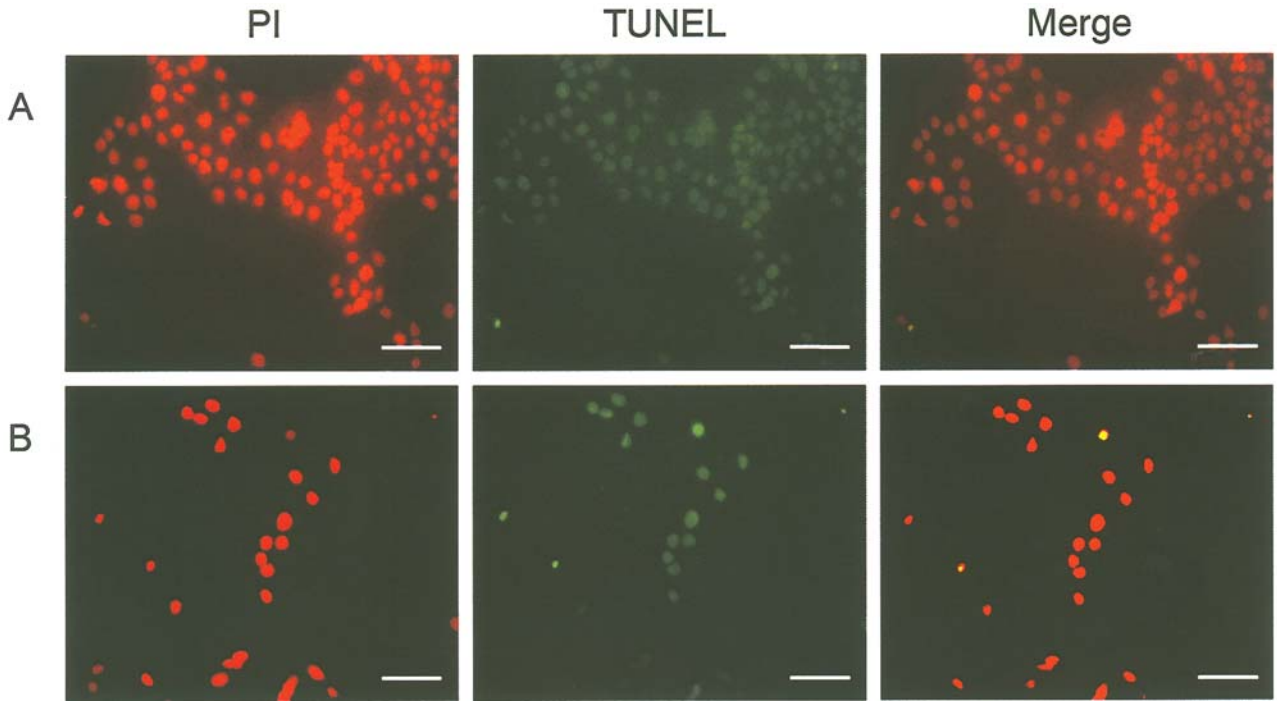

Figure 5. *OVCAR-3 cells examined by the TUNEL assay. Untreated (A) and 50 mM D-allose-treated (B) Nuclei with fragmented DNA were detected by incorporating fluorencence-12-dUTP (green) under fluorescence microscopy. All nuclei were stained with PI (red). The nuclei of the apoptotic cells appear as yellow when merged. A few apoptotic cells were found among D-allose-treated cells (Scale bar: 30 μm).*

the Bax and Bcl-2 protein levels was found after D-allose treatment. This result corroborates our earlier conclusion that the up-regulation of p21 and p27 may be responsible for the potential apoptotic effect of D-allose in OVCAR-3 cells.

It is now accepted that many cell functions are mediated by the carbohydrate moieties present on the cell surface, and that the addition of monosaccharides to the culture medium specifically modifies some of these functions (13-16). Marina *et al.* (17) have reported that the monosaccharides D-Ribose and deoxy-D-Ribose inhibit DNA, RNA and protein synthesis in a variety of cells, thereby inhibiting cell proliferation. Colquhoun *et al.* (18) have also demonstrated that a monosaccharide, 2-deoxy-3-[1-(R) (ethoxycarbony) ethyl]-α-D-*allo*-pyranose, showed a strong inhibitory effect on cell proliferation due to its sugar structure. In addition, Pratt *et al.* (19) have reported that D-allose can reduce glucose transport by modulating the intrinsic activity of the integral membrane carrier protein glucose transporter, and have indicated that D-allose may act either in a tissue-specific and/or glucose transporter1 (Glut1)-specific manner.

Ovarian carcinoma has the lowest overall survival rate of all the gynecological malignancies. Frequently, at the time of diagnosis most patients have advanced disease, that has spread beyond the pelvis and into the peritoneal cavity. Despite the application of new chemotherapeutic drugs in recent decades, the prognosis of ovarian carcinoma remains poor and the chemotherapeutic drugs cause strong side-effects. Therefore, new therapeutic targets and agents are urgently needed. The results of our present study provide experimental evidence that D-allose has an inhibitory activity on the growth of ovarian carcinoma cells. Its action is probably related to its ability to induce G2/M-phase cell cycle arrest, up-regulation of p21 and p27 levels and induction of apoptosis. Although the exact mechanisms need to be investigated further, the novel growth-inhibitory property of D-allose on ovarian carcinoma cell proliferation make it potentially effective and mild therapeutic agent against ovarian carcinoma.

Acknowledgements

This work was supported by a research grant from the Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER) Project of Japan.

References

1 McDonald EJ: A new synthesis of D-psicose (D-ribo-hxulose). Carbohyd Res *5*: 106-108, 1967.
2 Doner LW: Isomerization of D-fructose by base: liquid-chromatographic evaluation and the isolation of D-psicose. Carbohyd Res *70*: 209-216, 1979.

3 Bhuiyan SH, Itami Y, Rokui Y, Katayama T and Izumori K: D-allose production from D-psicose using immobilized L-rhamnose isomerase. J Ferment Bioeng *85*: 539-541, 1998.
4 Itoh H, Sato T and Izumori K: Preparation of D-psicose from D-fructose by immobilized D-tagatose 3-epimerase. J Ferment Bioeng *80*: 101-103, 1995.
5 Izumori K: Bioproduction strategies for rare hexose sugars. Naturwissenschaften *89*: 120-124, 2002.
6 Murata A, Sekiya K, Watanabe Y, Yamaguchi F, Hatano N, Izumori K and Masaaki Tokuda: A novel inhibitory effect of D-allose on production of reactive oxygen species from neutrophils. J Biosci Bioeng *96*: 89-91, 2003.
7 Sherr CJ: The Pezcoller lecture: cancer cell cycles revisited. Cancer Res *60*: 3689-3695, 2000.
8 Beach D: p21 is a universal inhibitor of cyclin kinase. Nature *366*: 701-704, 1993.
9 Harper JW, Adami GR, Wei N, Keyomarsi K and Elledge SJ: The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin idependent kinases. Cell *75*: 805-816, 1993.
10 El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B: WAF-1, a potential mediator of p53 tumour suppression. Cell *75*: 817-825, 1993.
11 Salomons GS, Brady HJ, Verwijs-Janssen M, Van Den Berg JD, Hart AA, Van Den Berg H, Behrendt H, Hahlen K and Smets LA: The Bax alpha:Bcl-2 ratio modulates the response to dexamethasone in leukaemic cells and is highly variable in childhood acute leukaemia, Int J Cancer *71*: 959-965, 1997.
12 Sedlak TW, Oltvai ZN, Yang E, Wang K, Boise LH, Thompson CB and Korsmeyer SJ: Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. Proc Natl Acad Sci USA *92*: 7834-7838, 1995.
13 Rocklin RE: Role of monosaccharides in the interaction of two lymphocyte mediators with their target cells. J Immunol *116*: 816-820, 1976.
14 Baba T, Yoshida T, Yoshida T and Cohen S: Suppression of cell-mediated immune reactions by monosaccharides. J Immunol *122*: 838-841, 1979.
15 Stutman O, Dien P, Wisun RE and Lattime EC: Natural cytotoxic cells against solid tumors in mice: blocking of cytotoxicity by D-mannose. Proc Natl Acad Sci USA *77*: 2895-2898, 1980.
16 Muchmore AV, Decker JM and Blaese RM: Evidence that specific oligosaccharides block early events necessary for the expression of antigen-specific proliferation by human lymphocytes. J Immunol *125*: 1306-1311, 1980.
17 Marini M, Zunica G and Franceschi C: Inhibition of cell proliferation by D-ribose and Deoxy-D-ribose. Proc Soc Exp Biol Med *180*: 246-257, 1985.
18 Colquhoun A, Alaluf S, Bradley A, Gemmell N, Gibbs G, Osborn HM, Harwood LM and Newsholme EA: Novel Monosaccharides as potent inhibitors of cell proliferation. Cell Biochem Funct *15*: 243-249, 1997.
19 Pratt SE, Colby-Germinario S, Manuel S and Germinario RJ: Evidence that modulation of glucose transporter intrinsic activity is the mechanism involved in the allose-mediated depression of hexose transport in mammalian cells. J Cell Physiol *161*: 580-588, 1994.

Received December 20, 2004
Accepted April 25, 2005