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Growth Inhibition of Refractory Human Gallbladder Cancer Cells by Retinol, and Its Mechanism of Action

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Among the constituents of the essential nutrient vitamin A, retinol is a potent suppressor of refractory cancer cell growth linked to tumor progression, showing greater efficacy than retinoic acid (RA). However, the mechanisms of retinol action on human refractory cancer are not known well. In the current study, we examined the actions of retinol on proliferation of human gallbladder cancer NOZ C-1 cells. Retinol and RA inhibited the proliferation of human NOZ C-1 cells in dose-dependent manner, while RA was less potent than retinol. Cell incorporation of RA was approximately two-fold higher than retinol and was not correlated with anti-proliferative activity. Retinol did not affect caspase-3 activity or mRNA expression of Bax and Bel-2, which are associated with apoptosis. In addition, protein expression of phosphorylated extracellular signal-regulated kinase (p-ERK)/ERK and p-Akt/Akt were not significantly changed by retinol treatment. In contrast, retinol treatment significantly increased the mRNA expression of endoplasmic reticulum (ER) stress factors (heme oxygenase 1 (HMOX1)), CCAAT/enhancer-binding protein homologous protein (CHOP), 78kDa glucose-regulated protein (GRP78), and DnaJ (Hsp40) homolog, subfamily B, member 9 (DNAJB9). Furthermore, the number of cells in the G0/G1 phase was increased, while the number of cells in the S phase were decreased by retinol treatment. Retinol increased expression of the autophagy-associated protein, LC3-II. These results indicate that retinol is a potent suppressor of gallbladder cancer cell growth by mechanisms that involve ER stress, which results in autophagy and cell cycle delay. This suggests that retinol might be useful for anticancer prevention and therapy in the clinic.

Key words vitamin A; retinol; retinoic acid; retinyl palmitate; anticancer

Vitamin A plays an important role in many essential biological processes. Since the body is unable to synthesize quantities sufficient for daily needs, vitamin A (in a narrow sense: retinol) and pro-vitamin A (a carotenoid) are dietary requirements. Among vitamin A variants (wide sense: retinol, retinal, retinoic acid (RA), retinyl ester (RE)), the main function of retinol in nature is to serve as a chromophore in sight.1) RA, which is an active form of vitamin A, shows a range of effects, including promotion of muco cutaneous formation, growth,2) apoptosis,3) and embryonic development as well as induction of cell differentiation,4) and immune regulation.5) Retinol is either esterified into RE for storage, or oxidized into retinal for vision regulation, and further oxidation to RA in cells.

Previous studies have shown that RA exhibits anti-cancer effects. In particular, the importance of RA is exemplified by its strong cellular differentiation-inducing capability and its utility in the therapy of acute promyelocytic leukemia.8–10) RA also shows anti-proliferative activities against melanoma,11) prostate cancer,12,13) lung cancer,14,15) and breast cancer,16,17) but not against refractory cancers. None-the-less, it is not known whether other forms of vitamin A, such as retinol and REs, in particular retinyl palmitate (RP), exhibit anti-cancer effects. Recently, Park et al. have shown that retinol inhibits the growth and the invasion of RA-resistant colon cancer cells in vitro.18–20) In addition, we have shown that retinol is a more potent suppressor of cancer cell growth and adhesion of several cancer cell lines including refractory cancers (pancreatic, gallbladder, breast, or prostate cancer, and cholangiocarcinoma) than RA or RP in vitro.21)

Most vitamin A actions other than vision are considered to be mediated by RA, which can regulate gene expression by binding to nuclear RA receptors (RAR) or retinoid X receptors (RXR).22) Retinol binds to RA receptors extremely weak as compared with RA.23,24) In addition, retinol inhibits both the proliferation and adhesion of refractory cancer cells to a greater extent than RA.21) These results suggest that retinol may affect cancer cell growth directly independent of activating RAR/RXR pathways.

Our current study examines the mechanism of the anti-proliferative activity by retinol on human gallbladder cancer NOZ C-1 cells, which is one of the most drug-resistant refractory cancers known. Our work shows for the first time that retinol suppresses cell growth of gallbladder cancer along and that it induces endoplasmic reticulum (ER) stress, resulting in autophagy and cell cycle delay, in a fashion that is not dependent on RAR/RXR.

MATERIALS AND METHODS

Chemicals and Cells Retinoic acid (all-trans, RA), retinol, RP, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and all chemicals were of reagent grade. Human gallbladder cancer NOZ C-1 cells were supplied by Dr. Tomokazu Matsuura, Jikei University School of Medicine (Tokyo, Japan).25) Cell Culture NOZ C-1 cells were grown in William’s Medium E medium (Invitrogen, Carlsbad, CA, U.S.A.) containing 2mm l-glutamine (Invitrogen) and 10% fetal bovine serum (FBS, Invitrogen). Cells were sub-cultured every week
and attached cells were removed with trypsin–ethylenediaminetetraacetic acid (EDTA) (Invitrogen). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Cell numbers were estimated using an electric particle counter (Coulter Electronics, Hialeah, FL, U.S.A.).

**Cell Growth**

Cells were trypsinized and suspended in appropriate medium containing 10% FBS. Cells (1×10⁶ cells/mL) were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. After 1 d, various concentrations of retinol or RA were added to the cultures. Control cells were treated with 0.1% DMSO or ethanol without chemicals. Cells were incubated for 72 h, and then viable cell number was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously. Values for percent net cell growth were calculated with the following formula: ([absorbance of experimental cell density]−[absorbance of initial cell density])×[absorbance of control cell density]−[absorbance of initial cell density]×100.

**Incorporation of Retinol and RA into Cells**

Uptake of retinol and RA into NOZ C-1 cells was examined according to previously described procedures. Briefly, cells (1×10⁶ cells/mL) treated with 10 μM of retinol or RA were incubated for the indicated time at 37°C. Retinol and RA incorporated into cells was extracted with ethyl acetate by mixing. The amounts of retinol and RA in the organic layer were quantified by HPLC with mobile phase solvent (80% methanol, 20% H₂O, and 10 mM ammonium acetate) using a Shimadzu LC-20AD high pressure pump, Shimadzu CTO-10AS column oven including injector, and an SPD-20A UV spectrophotometric detector (Shimadzu Co., Ltd., Kyoto, Japan). A LUNA® 5 μ C18 (2) column (150×4.6 mm, Phenomenex, Rancho Palos Verdes, CA, U.S.A.) was used to separate compounds. The column was eluted with mobile phase solvent at a flow rate of 0.5 mL/min. Retinol and RA were detected with UV monitoring at 325 nm, which is their absorbance maximum. The retention times of retinol and RA were 24.2 and 13.1 min, respectively. Quantitation was performed using the ratios of peak areas to internal standards. Values for cell incorporation were calculated based on the extraction efficiencies of retinol and RA.

**RNA Preparation and Microarray Analysis**

Total RNA was extracted from NOZ C-1 cells treated without or with retinol using an ISOGEN RNA extraction kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Total RNA was purified using the RNeasy® Mini Kit (QIAGEN, Valencia, CA, U.S.A.) according to the manufacturer’s instructions. Microarray analysis was conducted with GeneChip® Human Genome U133 Plus 2.0 ST Array (Affymetrix, Inc., Santa Clara, CA, U.S.A.) at 37°C for 30 min, and then stained with 10 μL of propidium iodide. Cell cycle analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.).

**Cell Synchronization and Flow Cytometry**

NOZ C-1 cells were trypsinized and suspended in appropriate medium without FBS. Cells (1×10⁶ cells/mL) were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. After 24 h, cells were treated with 3 μg/mL aphidicolin (Wako Pure Chemical Industries, Ltd.) for 24 h. Cells were washed with phosphate buffered saline (PBS) and treated with 10% FBS-supplemented media containing 25 μM retinol or DMSO for 24 h. Cells were harvested and fixed in 70% ethanol overnight. Cells were washed with PBS twice, treated with 100 μg/mL RNase A at 37°C for 30 min, and then stained with 10 μL of propidium iodide. Cell cycle analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.).

**Statistical Analysis**

The data are expressed as the mean±standard deviation (S.D.). Data were analyzed using Prism version 6. Statistical significance was assessed using Student’s t-test and one-way ANOVA followed by Dunnett’s multiple comparison’s test. *p<0.05, **p<0.01, and ***p<0.001 vs. control.

**RESULTS**

**Effects of Retinoids on Cell Growth**

Cell growth of human Refractory Gallbladder Cancer We first examined the effects of retinoids (RA, retinol, and RP) on the growth of the human...
refractory gallbladder cancer line NOZ C-1. Cells were treated with various concentrations of retinoids for 72 h, and then cell numbers were measured. The growth of NOZ C-1 cells was inhibited by retinol and RA in dose-dependent manner. Retinol suppressed cell growth approximately 36% at 10 \( \mu \text{M} \) and 78% at 20 \( \mu \text{M} \), and RA inhibited approximately 30% at 20 \( \mu \text{M} \) (Fig. 1). RP did not affect NOZ C-1 cell growth (Fig. 1). These results indicate that retinol suppresses the growth of NOZ C-1 cells greater than RA, and they suggest that retinol is a potent anti-proliferative agent against human refractory gallbladder cancer.

**Incorporation of Retinol and RA into Gallbladder Cancer Cells** Cell growth inhibition may occur through the regulation of existing pathways and/or gene expression associated with signaling of extracellular or intracellular events. Our finding that cell growth inhibition by retinol was greater than by RA, raised the question whether the level of incorporation of retinol and RA into cells affects growth inhibition. Therefore, we examined the incorporation of retinol and RA into NOZ C-1 cells. After cells were incubated at 37°C in the presence of retinol or RA at a concentration of 10 \( \mu \text{M} \), retinoids were extracted from cells and quantified by HPLC. The incorporation of retinoids into NOZ C-1 cells was very rapid (Fig. 2). The uptake of retinol into cells increased with prolongation of incubation time, and achieved near saturation after 15 min. In contrast, the incorporation of RA into cells increased until saturation from 30 to 120 min (Fig. 2). At 30 min, the amount of RA in NOZ C-1 cells was approximately 2.5-fold higher than that of retinol (Fig. 2). These results indicated that cellular incorporation of retinol and RA did not correlate with their anti-proliferative activities against NOZ C-1 cells, since retinol incorporated poorly into NOZ C-1 cells, yet it was more potent cell growth inhibitory effects than RA. This suggested that the mechanism of growth inhibition by retinol is highly dependent on the signaling of intracellular events rather than on extracellular events, and that it did not involve RAR/RXR pathway activated by RA.

**Effects of Retinol on Apoptosis in Gallbladder Cancer Cells** Retinol itself may inhibit NOZ C-1 cell growth by distinct mechanism from RA which act through RAR/RXR pathway. Since the apoptosis is one possibility as the mechanism of retinol action, we examined caspase-3 activity as well as mRNA expression of Bax and Bcl-2 in cells. NOZ C-1 cells were treated without and with retinol for 72 h, and Bax and Bcl-2 mRNA expressions were quantified by real-time PCR. As shown in Fig. 3A, significant changes on caspase-3 activity by retinol treatment were not observed. In addition, neither mRNA expression of Bax nor Bcl-2 showed significant changes in NOZ C-1 cells treated with retinol as compared with control (Figs. 3B, C). These results indicated that retinol treatment did not induce apoptosis in NOZ C-1 cells, and the mechanism of anti-proliferation by retinol on NOZ C-1 cells might not connect with apoptosis induction.

**Effects of Retinol on Phosphorylation of ERK and Akt Proteins in Gallbladder Cancer Cells** The fact that treatment with retinol did not induce apoptosis nor did it promote caspase-3 activity or mRNA expression of Bax and Bcl-2 in NOZ C-1 cells, led us to examine whether signal transduction pathways involving activation of ERK or Akt are affected by retinol treatment. The protein levels of phosphorylated-ERK, ERK, phosphorylated-Akt, and Akt in cells treated without or with 25 \( \mu \text{M} \) retinol, were analyzed by immunoblotting. Retinol did not affect the levels of phosphorylated ERK/ERK (Fig. 4A) or phosphorylated Akt/Akt (Fig. 4B) in NOZ C-1 cells. This indicates that the inhibitory effects on cell growth by retinol were not mediated through mitogen-activated protein extracellular kinase (MEK)/ERK and phosphatidylinositol 3-kinase (PI3K)/Akt pathways.

**Profiles of Genes Involved in Growth Inhibition by Retinol**
The fact that retinol did not induce apoptosis or inhibit either MEK/ERK or PI3K/Akt pathways, led us to examine the gene profile in NOZ C-1 cells following retinol treatment. For this, microarray analysis was performed using RNA extracted from cells incubated without or with retinol for 24h. We found that following treatment with retinol, analysis of 148144 genes showed 367 genes with expression higher than 2-fold of control and 246 genes with lower expression than 0.5-fold of control. Table 1A and B show the top five of up- and down-regulated genes. Growth differentiation factor 15 (GDF15), neutrophil cytosolic factor 2 (NCF2), calbindin 2 (CALB2), heme oxygenase (decycling) 1 (HMOX1), and H19: imprinted maternally expressed transcript (non-protein coding) (H19); microRNA675 (MIR675); ribosomal protein S12 (RPS12), increased 11.12, 10.90, 9.29, 8.45, and 7.72-fold, respectively. In contrast, keratin associated protein 2–3 and 2–4 (KRTAP2–3/KRTAP2–4), synaptotagmin-like 5 (SYTL5), mitogen-activated protein kinase kinase 6
Changes of mRNA Expressions of ER Stress Gene in Gallbladder Cancer Cells Treated with Retinol

ER stress arising from multiple pathways can occur in cells following UV radiation exposure or chemical treatment. When cells are subjected to stress, the unfolded/mis-folded protein respond (UPR) can occur, which induce ER stress in order to maintain homeostasis. Evidence suggests that ER stress can lead to programmed cell death, including apoptosis, necroptosis, and autophagy. Microarray results showed that retinol increases expression of ER stress genes (Table 1). Therefore, we measured mRNA expressions of ER stress markers; HMOX1, CCAAT/enhancer-binding protein homologous protein (CHOP: transcription factor), 78kDa glucose-regulated protein (GRP78: chaperone), and DnaJ (Hsp40) homolog, subfamily B, member 9 (DNAJB9: chaperone) by qPCR. As shown in Fig. 5, mRNA expression of four ER stress markers is induced significantly by retinol treatment. The fold-change for each mRNA expression is approximately 16-times for HMOX1, 4.7-times for CHOP, 1.8-times for GRP78, or 3.5-times for DNAJB9. These results indicate that ER stress occurs in NOZ C-1 cells treated with retinol.

Effects of Retinol on Cell Cycle in NOZ C-1 Cells

NOZ C-1 cells (1 × 10⁴ cells/mL) were synchronized, and then grown in the absence or presence of retinol for 24 h. Cells were harvested, and RNA was prepared from the cells and microarray analysis was performed as described under "MATERIALS AND METHODS." A: The top 5 up-regulated genes having expression at least 2-fold higher than control, B: The top 5 down-regulated genes having expression lower than 0.5-fold of control, C: The top 5 genes based on an ontology analysis for up-regulated genes having expression at least 2-fold higher than control, D: The top 5 genes based on an ontology analysis for down-regulated genes having expression lower than 0.5-fold of control. *Unique sorted transcript cluster ID, **H19|MIR675|RPS12; H19, imprinted maternally expressed transcript (non-protein coding), MIR675 (microRNA675), and RPS12 (ribosomal protein S12).
The percentage of cells in the G₀/G₁ phase increased significantly by treatment with 25 \( \mu \text{M} \) retinol (Fig. 6), while the percentage of cells in S phase decreased. In contrast, there were no changes in the cell cycle in NOZ C-1 cells without synchronization (data not shown). These results indicated that retinol treatment delayed and slowed cell division.

Alteration of Autophagy-Related Protein LC3 Expression in Gallbladder Cancer Cells Treated without and with Retinol

Enhancement of ER stress by retinol treatment in NOZ C-1 cells, led us to examine the effects of retinol on autophagy pathways involving LC3. The conversion from LC3-I to LC3-II is associated with the formation of autophagosomes, a marker of autophagy. The protein levels of LC3 (LC3-I and LC3-II) and \( \beta \)-actin in synchronized NOZ C-1 cells treated without and with \( 25 \mu \text{M} \) retinol, were analyzed by immunoblotting. Retinol increased the levels of the LC3-II approximately 6-fold in NOZ C-1 cells as compared with control (Fig. 7). In contrast, there were no significant changes in LC3-II levels in NOZ C-1 cells without the synchronization (data not shown). These results indicate that retinol induces autophagy in NOZ C-1 cells and that anti-proliferative effects of retinol might be due to autophagy through ER stress.

DISCUSSION

In our current work, we have shown greater inhibitory effects of retinol against the growth of human gallbladder cancer (NOZ C-1) cells than is achieved with RA (Fig. 1). The cellular incorporation of retinol was less than RA (Fig. 2). Retinol neither induced apoptosis nor suppressed MEK/ERK and PI3K/Akt pathways (Figs. 3, 4). Retinol treatment significantly increased mRNA expression of HMOX1, CHOP, GRP78, and DNAJB9, which are genes related to ER stress (Table 1, Fig. 5). The cell cycle was delayed and expression of LC3-II protein was increased in synchronized NOZ C-1 cells treated with retinol, as compared with controls lacking treatment (Figs. 6, 7). These results suggest that retinol suppresses the growth of NOZ C-1 cells along with ER stress signaling, autophagy, and cell cycle delay.

It is unlikely that retinol acts through RA-mediated nuclear receptors that bind specifically to RA and directly activate
transcription of target genes by binding to specific DNA sequences. Retinol and RA may act on cancer cells through distinct mechanisms, since retinol and RA inhibit cell growth of different cancer cell lines, and retinol is more potent than RA against pancreatic (MIA PaCa2, JHP-1), gallbladder (NOZ C-1), and prostate (PC-3) cancer cells and cholangiocarcinoma (HuCCT1), but not breast cancer (MCF-7) cells. In the current study, the low incorporation of retinol into NOZ C-1 cells seemed to contradict its greater cell growth inhibition, as compared with RA (Figs. 1, 2). Therefore, the inhibitory effects of retinol on NOZ C-1 cell growth, are not mediated via RAR/RXR pathways. In addition, a previous study has shown that cell growth inhibition in a RA-resistant human colon cancer by retinol, was RA, RAR, and RARE independent. Elucidation of the complete mechanism of retinol action may give important insights that could facilitate the creation of new effective anti-cancer drugs for cancer patients.

The mechanisms behind the inhibitory effects of retinol on cancer cell growth are not known well, since it has been considered that retinol acts through RA. Park et al. have shown that retinol inhibits the growth and the invasion of colon cancer cells in vitro, and that retinol does not induce apoptosis. However, retinol does affect cell cycle progression in synchronized colon cancer cells. In addition, based on the fact that retinol inhibits PI3K activity measured in vitro using cell extracts prepared from colon cancer cells, the inhibitory effects of colon cancer cell invasion may be due to the decrease in PI3K activity. In the current study, our finding that retinol does not induce apoptosis in gallbladder cancer cells (Fig. 3), is in agreement with these earlier findings. However, retinol did not suppress the phosphorylation of ERK and Akt in NOZ C-1 cells (Fig. 4). These results suggest that retinol does not inhibit PI3K/Akt and MEK/ERK pathways in human gallbladder cancer cells. This differs from earlier results. It should be noted that the observed effects may depend on cell type or experimental methods and conditions.

Vitamin A (retinol) is a fat-soluble vitamin, and its metabolism is similar to the metabolism of alcohol and lipids. After retinol is incorporated into cells, retinol can be oxidized to retinal, and further oxidized to RA, which is subsequently metabolized by cytochrome P-450s including CYP26A1. Alternatively, it can be converted to RE for storage. Among retinol metabolic enzymes, dehydrogenase/reductase (SDR family) member 3 (DHRS3) catalyzes the reduction of retinal to retinol. A previous study has shown that over-expression of DHRS3 in cells results in the accumulation of RE as oil drops, which cause ER stress. In the current study, we found that retinol treatment of NOZ C-1 cells resulted in ER stress. In cells incubated with retinol for 24 h, up-regulation of the DHRS3 gene was the 7th greatest change, while in cells incubated with retinol for 72 h it showed the greatest change.

Fig. 6. Effect of Retinol on Cell Cycle Distribution in Human Gallbladder Cancer Cells

NOZ C-1 cells (1×10⁶ cells/mL) were synchronized, and then treated with DMSO (Control) or 25 μM retinol (Rol) for 24 h, as described under “MATERIALS AND METHODS.” Cells were collected and the cell cycle was analyzed by flow cytometry after propidium iodide staining. Results represent the mean±S.D. (n=3). **p<0.01 compared with control.

Fig. 7. Protein Levels of LC-3 in Gallbladder Cancer Cells

NOZ C-1 cells (1×10⁶ cells/mL) were synchronized, and then treated with DMSO (Con) or 25 μM retinol (Rol) for 24 h, as described under “MATERIALS AND METHODS.” The protein levels of LC-3 and β-actin were analyzed by Western blot analysis with specific antibodies against LC-3 and β-actin as described in “MATERIALS AND METHODS.” Intensities of bands were analyzed by scanning densitometry. Each bar represents the mean±S.D. of each group (n=3). **p<0.01 compared with control.
among all up-regulated genes (defined as 2-fold higher expression than control) (data not shown).

ER stress involving multiple pathways occurs in cells following UV radiation exposure or chemical treatment. When cells are stimulated by some forms of stress, the unfolded/misfolded protein response (UPR) can occur, which induces ER stress to maintain homeostasis. However, evidence is accumulating that ER stress may lead to programmed cell death, including apoptosis, necroptosis, and autophagy.\(^{31,32}\) GRP78 and DNAJB9 are ER stress markers, which play roles in molecular chaperone and facilitate retrograde transport across the ER membrane of aberrant proteins destined for degradation by the proteasome. The synthesis of GRP78 and DNAJB9 is markedly induced under conditions that lead to the accumulation of unfolded proteins in the ER. It is possible that retinol treatment might increase aberrant levels of proteins that play a role in cell growth. Protein modification by retinol has been reported by Myhre et al.\(^{38}\) to be involved in production of the unfolded/misfolded protein response in cancer cells. Evaluating the relationships between growth inhibition and unfolded protein in NOZ C-1 cells treated with retinol are currently under investigation.

This study shows that retinol acts throughRAR-independent mechanisms to inhibit cell growth of drug-resistant human gallbladder cancers by affecting UPR/ER stress/autophagy pathways and cell cycle progression. Retinol is a potent anti-proliferative agent when used against drug-resistant human refractory cancers, including human gallbladder cancer.\(^{21}\) RA-resistance may limit the effective use of RA derivatives as chemotherapies. Retinol or its derivative may prove to be effective therapies to treat refractory cancers.

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Conflict of Interest The authors declare no conflict of interest.

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