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

Chuan Li,[#] Masahiko Imai,[#] Shinya Hasegawa, Masahiro Yamasaki, and Noriko Takahashi*

Laboratory of Physiological Chemistry, Institute of Medicinal Chemistry, Hoshi University, 2-4-1 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan.

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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 Bcl-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 retinal 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 mucocutaneous formation, growth,²⁾ apoptosis,³⁾ and embryonic development as well as induction of cell differentiation,⁴⁾ and immune regulation.^{5–7)} 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 cholangiocarci-

noma) 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 2 mM L-glutamine (Invitrogen) and 10% fetal bovine serum (FBS, Invitrogen). Cells were sub-cultured every week

[#]These authors contributed equally to this work.

*To whom correspondence should be addressed. e-mail: t-noriko@hoshi.ac.jp

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 (1×10⁴ 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.^{26,27} 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.^{28,29} 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 acetoacetate 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 Gene 2.0 ST Array (Affymetrix, Inc., Santa Clara, CA, U.S.A.) at RIKEN Genesis (Chiba, Japan). Changes in gene expression levels were calculated based on signal_{target}/signal_{control} values of the gene expression level.

Quantitative RT-PCR (qPCR) Total RNA of cultured NOZ C-1 cells was prepared with ISOGEN reagent (Wako Pure Chemical Industries, Ltd.). cDNA was prepared by reverse transcriptase (RT) reactions from 1 μg total RNA using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). The sequences of the PCR primers were as follows: *Bax* (forward 5'-ATGCGTCCACCAAGAAGCTGAG-3', reverse 5'-CCCCAGTTGAAGTTGCCATCAG-3'), *Bcl-2* (forward 5'-TGCACCTGACGCCCTTCAAC-3', reverse 5'-AGACAGCCAGGAGAAATCAAAACAG-3'), heme oxygenase 1

(*HMOX1*) (forward 5'-CCAGCAACAAGTGCAAGATT-3', reverse 5'-CTGCAGGAACTGAGGATGCTG-3'), CCAAT/enhancer-binding protein homologous protein (*CHOP*) (forward 5'-CTCTGGCTTGGCTGACTGA-3', reverse 5'-GCTCTGGAGGTGCTTGT-3'), 78 kDa glucose-regulated protein (*GRP78*, also known as Binding immunoglobulin protein, *BiP*) (forward 5'-GAACATCCTGGTGTGACCC-3', reverse 5'-CCCAGATGAGTATCTCCATT-3'), DnaJ (Hsp40) homolog, subfamily B, member 9 (*DNAJB9*) (forward 5'-CCTGACAAAATAAGAGCCCGG-3', reverse 5'-GATGCCTTTGTCTACTGGAACC-3'), and *β-actin* (forward 5'-CCTTCTGGGCAATGAGTCTCTG-3', reverse 5'-GGAGCAATGATCTTGATCTTTC-3'). Quantitative mRNA expression analysis was performed by a real time PCR system (StepOne™, Applied Biosystems Inc., Foster City, CA, U.S.A.). To estimate the level of transcripts quantitatively, *β-actin* transcript was used as an internal control for each prepared sample.

Caspase Activity Assay For measurement of caspase-3 activity of cellular lysates, the Caspase-3/CPP32 Colorimetric Assay Kit (MBL, Nagoya, Japan) was used according to manufacturer's instructions.

Immunoblotting Analysis Whole cell lysates were prepared with NP40 lysis buffer [25 mM Tris–HCl (pH 8), 150 mM NaCl, 0.5% NP40, 4 mM NaF, 0.1 mM Na₃VO₄, and protease inhibitor cocktail].³⁰ The protein concentration of the whole cell lysates was determined by the Bicinchoninic Acid (BCA) Protein Assay (Pierce, Rockford, IL, U.S.A.). Whole lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and were transferred to Immobilon-P membranes (Millipore, Billerica, MA, U.S.A.). The membranes were probed with polyclonal antibodies against extracellular signal-regulated kinase (ERK), phosphorylated (p)-ERK, Akt, and LC-3 (Cell Signaling, Danvers, MA, U.S.A.), or p-Akt and *β-actin* (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Proteins were visualized using ECL-plus (Invitrogen).

Cell Synchronization and Flow Cytometry NOZ C-1 cells were trypsinized and suspended in appropriate medium without FBS. Cells (1×10⁴ 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 μg/mL 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 of Human Refractory Gallbladder Cancer We first examined the effects of retinoids (RA, retinol, and RP) on the growth of the human

Fig. 1. Effects of Retinoid on Cell Growth of Human Gallbladder Cancer

Human gallbladder cancer NOZ C-1 cells (1×10^4 cells/mL) were grown with various concentration of RA (▨), retinol (■) and RP (▤) in medium containing 10% FBS. Cell growth was measured at 72h as described in "MATERIALS AND METHODS." Each bar represents the mean $\pm$ S.D. of each group ($n=3$). * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ compared with control.

refractory gallbladder cancer line NOZ C-1. Cells were treated with various concentrations of retinoids for 72h, 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

Fig. 2. Incorporation of Retinol and RA into Human Gallbladder Cancer Cells

NOZ C-1 cells (1×10^6 cells/mL) were incubated in the presence of retinol (■) and RA (◆) at $10 \mu\text{M}$ concentration in medium containing 10% FBS for the indicated time. Cells were harvested and measurements of cell incorporation were performed using HPLC with a LUNA[®] 5 μ C18 (2) column (150 $\times$ 4.6 mm, Phenomenex) as described in "MATERIALS AND METHODS." Retinol and RA were eluted with 80% MeOH, 20% H₂O, and 10 mM ammonium acetate at a flow rate of 0.5 mL/min. Each point is the mean of three measurements. Results represent the mean $\pm$ S.D. of each group ($n=3$). ** $p<0.01$ and *** $p<0.001$ compared with RA.

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 72h, 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 Reti-

Fig. 3. Effects of Retinol on Caspase-3 Activity and mRNA Expression of Bax and Bcl-2 in Human Gallbladder Cancer Cells

NOZ C-1 cells (1×10^4 cells/mL) were treated with DMSO (Control, Con) and retinol (Rol) at $25 \mu\text{M}$ for 72 h. Cells were harvested. (A) Caspase-3 activity was performed using Caspase-3/CPP32 Colorimetric Assay Kit as described under "MATERIALS AND METHODS." The activity of control was defined as 1.0. (B, C) Total RNA ($5 \mu\text{g}$) was prepared from the cells, and qPCR was performed using specific primers against *Bax* and *Bcl-2* as described under "MATERIALS AND METHODS." The *Bax* and *Bcl-2* expression of control was defined as 1.0. Each bar represents the mean $\pm$ S.D. of each group ($n=3$). n.s., not significance.

Fig. 4. Protein Levels of Phosphorylated-ERK, ERK, Phosphorylated-Akt, and Akt in Gallbladder Cancer Cells

NOZ C-1 cells (1×10^4 cells/mL) were treated with DMSO (Con, control) or retinol (Rol) at $25 \mu\text{M}$ for 24 h. The protein levels of (A) phosphorylated-ERK, ERK, (B) phosphorylated-Akt, and Akt were analyzed by Western blot analysis with specific antibodies against phosphorylated-ERK, ERK, phosphorylated-Akt, Akt, and β -actin as described in "MATERIALS AND METHODS." Intensities of bands were analyzed by scanning densitometry. Each bar represents the mean $\pm$ S.D. of each group ($n=3$). n.s., not significance.

nol in Gallbladder Cancer Cells 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 24 h. 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*), synaptotagmi-like 5 (*SYTL5*), mitogen-activated protein kinase kinase 6

Table 1. Up- or Down-Regulated Genes and Their Functional Classification in Human Gallbladder Cancer Cells Treated with Retinol

A: Up-regulated gene (Top 5)			
Cluster ID*	Gene symbol	Gene description	Fold change
16859795	GDF15	Growth differentiation factor 15	11.12
16697095	NCF2	Neutrophil cytosolic factor 2	10.90
16820849	CALB2	Calbindin 2	9.29
16929562	HMOX1	Heme oxygenase (decycling) 1	8.45
16734355	H19 MIR675 RPS12**		7.72
B: Down-regulated gene (Top 5)			
Cluster ID*	Gene symbol	Gene description	Fold change
16844585	KRTAP2-3	Keratinassociatedprotein 2–3	0.08
	KRTAP2-4	Keratinassociatedprotein 2–4	
17102566	SYTL5	Synaptotagmin-like 5	0.18
16837348	MAP2K6	Mitogen-activated protein kinase kinase 6	0.19
16799315	THBS1	Thrombospondin 1	0.19
16859205	UCA1	Urothelial cancer associated 1 (non-protein coding)	0.21
C: Up-regulated gene ontology analysis (Top 5)			
GO accession	GO term		p-Value
GO:0044699	Single-organism process		7.16E-10
GO:0044763	Single-organism cellular process		1.87E-09
GO:0034976	Response to endoplasmic reticulum stress		2.65E-09
GO:0030968	Endoplasmic reticulum unfolded protein response		7.35E-09
GO:0034620	Cellular response to unfolded protein		8.05E-09
D: Down-regulated gene ontology analysis (Top 5)			
GO accession	GO term		p-Value
GO:0001977	Renal system process involved in regulation of blood volume		8.11E-07
GO:0001965	G-Protein alpha-subunit binding		5.97E-07
GO:0003071	Renal system process involved in regulation of systemic arterial blood pressure		3.47E-06
GO:0050921	Positive regulation of chemotaxis		7.76E-06
GO:0002684	Positive regulation of immune system process		1.01E-05

NOZ C-1 cells (1×10^4 cells/mL) were treated with DMSO (Control) and retinol at $25 \mu\text{M}$ concentration 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).

(*MAP2K6*), thrombospondin 1 (*THBS1*), and urothelial cancer associated 1 (non-protein coding) (*UCA1*), decreased 0.08, 0.18, 0.19, 0.19, and 0.21-fold, respectively.

HMOX1 was up-regulated 8.45-fold by retinol treatment (Table 1A). HMOX1 is a cytoprotective protein that catalyzes the degradation of heme to biliverdin, iron, and carbon monoxide and is stimulated by ER stress. Results of ontology analysis showed several items related to ER stress (Table 1C). In contrast, down-regulated genes in the ontology results were related to renal and immune system (Table 1D). These results indicate that ER stress might be significant, and that it might be related to the anti-proliferative mechanism of retinol in NOZ C-1 cells.

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 main-

tain homeostasis. Evidence suggests that ER stress can lead to programmed cell death, including apoptosis, necroptosis, and autophagy.^{31,32)} 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 were synchronized, and then grown in the absence or presence of retinol for 24 h. The cell cycle has previously been analyzed under these conditions to elucidate retinol's

Fig. 5. Effects of Retinol on mRNA Expression of ER Stress Markers HMOX1, CHOP, GRP78, and DNAJB9 in Human Gallbladder Cancer Cells

NOZ C-1 cells (1×10^4 cells/mL) were treated with DMSO (Control, Con) and retinol (Rol) at $25 \mu\text{M}$ concentration for 24 h. Cells were harvested, and RNA was prepared. qPCR was performed on the RNA ($5 \mu\text{g}$) using specific primers against *HMOX1*, *CHOP*, *GRP78*, and *DNAJB9* as described under "MATERIALS AND METHODS." Expression of control (Con) was defined as 1.0. Each bar represents the mean $\pm$ S.D. of each group ($n=3$). * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ compared with control.

mechanism of action.¹⁸⁾ The percentage of cells in the G_0/G_1 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 β -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.^{33,34} 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.²¹ 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.¹⁸ 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 had 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*,^{18–20} and that retinol does not induce apoptosis. However, retinol does affect cell cycle progression in synchronized colon cancer cells.¹⁸ 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.³⁵ 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,³⁶ which cause ER stress.³⁷ 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 (1×10^4 cells/mL) were synchronized, and then treated with DMSO (Control, □) or $25 \mu\text{M}$ retinol (■) 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 $\pm$ 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 (1×10^4 cells/mL) were synchronized, and then treated with DMSO (Con) or $25 \mu\text{M}$ 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 $\pm$ 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/mis-folded 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 through RAR-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.

REFERENCES

- 1) Patek AJ Jr, Haig C. The occurrence of abnormal dark adaptation and its relation to vitamin A metabolism in patients with cirrhosis of the liver. *J. Clin. Invest.*, **18**, 609–616 (1939).
- 2) Lin E, Chen MC, Huang CY, Hsu SL, Huang WJ, Lin MS, Wu JC, Lin H. All-*trans* retinoic acid induces DU145 cell cycle arrest through Cdk5 activation. *Cell. Physiol. Biochem.*, **33**, 1620–1630 (2014).
- 3) Sha J, Pan J, Ping P, Xuan H, Li D, Bo J, Liu D, Huang Y. Synergistic effect and mechanism of vitamin A and vitamin D on inducing apoptosis of prostate cancer cells. *Mol. Biol. Rep.*, **40**, 2763–2768 (2013).
- 4) Breitman TR, Collins SJ, Keene BR. Terminal differentiation of human promyelocytic leukemic cells in primary culture in response to retinoic acid. *Blood*, **57**, 1000–1004 (1981).
- 5) Beijer MR, Molenaar R, Goverse G, Mebius RE, Kraal G, den Haan JM. A crucial role for retinoic acid in the development of Notch-dependent murine splenic CD8⁺ CD4⁺ and CD4⁺ dendritic cells. *Eur. J. Immunol.*, **43**, 1608–1616 (2013).
- 6) Duriancik DM, Hoag KA. Vitamin A deficiency alters splenic dendritic cell subsets and increases CD8(+)Gr-1(+) memory T lymphocytes in C57BL/6J mice. *Cell. Immunol.*, **265**, 156–163 (2010).
- 7) Wolbach SB, Howe PR. Epithelial repair in recovery from vitamin A deficiency: an experimental study. *J. Exp. Med.*, **57**, 511–526 (1933).
- 8) Huang ME, Ye YC, Chen SR, Chai JR, Lu JX, Zhao L, Gu LJ, Wang ZY. Use of all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia. *Blood*, **72**, 567–572 (1988).
- 9) Warrell RP Jr, Frankel SR, Miller WH Jr, Scheinberg DA, Itri LM, Hittelman WN, Vyas R, Andreeff M, Tafuri A, Jakubowski A, Gabrilove J, Gordon MS, Dmitrovsky E. Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-*trans*-retinoic acid). *N. Engl. J. Med.*, **324**, 1385–1393 (1991).
- 10) Castaigne S, Chomienne C, Daniel MT, Ballerini P, Berger R, Fenaux P, Degos L. All-*trans* retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results. *Blood*, **76**, 1704–1709 (1990).
- 11) Meyskens FL Jr, Salmon SE. Inhibition of human melanoma colony formation by retinoids. *Cancer Res.*, **39**, 4055–4057 (1979).
- 12) Mondul AM, Watters JL, Mannisto S, Weinstein SJ, Snyder K, Virtamo J, Albanes D. Serum retinol and risk of prostate cancer. *Am. J. Epidemiol.*, **173**, 813–821 (2011).
- 13) Toma S, Raffo P, Isnardi L, Palumbo R. Retinoids in lung cancer chemoprevention and treatment. *Ann. Oncol.*, **10** (Suppl. 5), S95–S102 (1999).
- 14) Fritz H, Kennedy D, Fergusson D, Fernandes R, Doucette S, Cooley K, Seely A, Sagar S, Wong R, Seely D. Vitamin A and retinoid derivatives for lung cancer: a systematic review and meta analysis. *PLoS ONE*, **6**, e21107 (2011).
- 15) Donato LJ, Noy N. Suppression of mammary carcinoma growth by retinoic acid: proapoptotic genes are targets for retinoic acid receptor and cellular retinoic acid-binding protein II signaling. *Cancer Res.*, **65**, 8193–8199 (2005).
- 16) Wu K, Kim HT, Rodriguez JL, Munoz-Medellin D, Mohsin SK, Hilsenbeck SG, Lamph WW, Gottardis MM, Shirley MA, Kuhn JG, Green JE, Brown PH. 9-*cis*-Retinoic acid suppresses mammary tumorigenesis in C3(1)-simian virus 40T antigen-transgenic mice. *Clin. Cancer Res.*, **6**, 3696–3704 (2000).
- 17) Endo Y, Deonauth K, Prahalad P, Hoxter B, Zhu Y, Byers SW. Role of Sox-9, ER81 and VE-cadherin in retinoic acid-mediated *trans*-differentiation of breast cancer cells. *PLoS ONE*, **3**, e2714 (2008).
- 18) Park EY, Dillard A, Williams EA, Wilder ET, Pepper MR, Lane MA. Retinol inhibits the growth of all-*trans*-retinoic acid-sensitive and all-*trans*-retinoic acid-resistant colon cancer cells through a retinoic acid receptor-independent mechanism. *Cancer Res.*, **65**, 9923–9933 (2005).
- 19) Park EY, Wilder ET, Lane MA. Retinol inhibits the invasion of retinoic acid-resistant colon cancer cells *in vitro* and decreases matrix metalloproteinase mRNA, protein, and activity levels. *Nutr. Cancer*, **57**, 66–77 (2007).
- 20) Park EY, Pinali D, Lindley K, Lane MA. Hepatic vitamin A preloading reduces colorectal cancer metastatic multiplicity in a mouse xenograft model. *Nutr. Cancer*, **64**, 732–740 (2012).
- 21) Li C, Imai M, Matsuura T, Hasegawa S, Yamasaki M, Takahashi N. Inhibitory effects of retinol are greater than retinoic acid on the growth and adhesion of human refractory cancer cells. *Biol. Pharm. Bull.*, **39**, 636–640 (2016).
- 22) Chambon P. A decade of molecular biology of retinoic acid receptors. *FASEB J.*, **10**, 940–954 (1996).
- 23) Repa JJ, Hanson KK, Clagett-Dame M. All-*trans*-retinol is a ligand for the retinoic acid receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 7293–7297 (1993).
- 24) Chen Y, Reese DH. Corexit-EC9527A disrupts retinol signaling and neuronal differentiation in P19 embryonal pluripotent cells. *PLoS ONE*, **11**, e0163724 (2016).
- 25) Shibamoto Y, Takagi I. Cytotoxic effects of new antineoplastic

- agent decetaxel, which affects microtubules/tubulin system, on human gallbladder carcinoma cell line, NOZ C-1. *J. Jpn. Biliary Assoc.*, **14**, 42–50 (2000).
- 26) Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, Boyd MR. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.*, **48**, 589–601 (1988).
- 27) Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.*, **47**, 936–942 (1987).
- 28) Ohba T, Yamauch T, Higashiyama K, Takahashi N. Potent anticancer activities of novel aminophenol analogues against various cancer cell lines. *Bioorg. Med. Chem.*, **15**, 847–853 (2007).
- 29) Imai M, Takahashi N. Growth inhibition and mechanism of action of *p*-dodecylaminophenol against refractory human pancreatic cancer and cholangiocarcinoma. *Bioorg. Med. Chem.*, **20**, 2520–2526 (2012).
- 30) Sagara C, Takahashi K, Kagechika H, Takahashi N. Molecular mechanism of 9-*cis*-retinoic acid inhibition of adipogenesis in 3T3-L1 cells. *Biochem. Biophys. Res. Commun.*, **433**, 102–107 (2013).
- 31) Su Z, Yang Z, Xu Y, Chen Y, Yu Q. MicroRNAs in apoptosis, autophagy and necroptosis. *Oncotarget*, **6**, 8474–8490 (2015).
- 32) Sano R, Reed JC. ER stress-induced cell death mechanisms. *Biochim. Biophys. Acta*, **1833**, 3460–3470 (2013).
- 33) Giguere V, Ong ES, Segui P, Evans RM. Identification of a receptor for the morphogen retinoic acid. *Nature*, **330**, 624–629 (1987).
- 34) Yim CY, Mao P, Spinella MJ. Headway and hurdles in the clinical development of dietary phytochemicals for cancer therapy and prevention: lessons learned from vitamin A derivatives. *AAPS J.*, **16**, 281–288 (2014).
- 35) Park EY, Wilder ET, Chipuk JE, Lane MA. Retinol decreases phosphatidylinositol 3-kinase activity in colon cancer cells. *Mol. Carcinog.*, **47**, 264–274 (2008).
- 36) Cerignoli F, Guo X, Cardinali B, Rinaldi C, Casaletto J, Frati L, Screpanti I, Gudas LJ, Gulino A, Thiele CJ, Giannini G. retSDR1, a short-chain retinol dehydrogenase/reductase, is retinoic acid-inducible and frequently deleted in human neuroblastoma cell lines. *Cancer Res.*, **62**, 1196–1204 (2002).
- 37) Deisenroth C, Itahana Y, Tollini L, Jin A, Zhang Y. p53-Inducible DHRS3 is an endoplasmic reticulum protein associated with lipid droplet accumulation. *J. Biol. Chem.*, **286**, 28343–28356 (2011).
- 38) Myhre AM, Takahashi N, Blomhoff R, Breitman TR, Norum KR. Retinoylation of proteins in rat liver, kidney, and lung *in vivo*. *J. Lipid Res.*, **37**, 1971–1977 (1996).