Vitamin A is a potent anti-proliferative agent when used against gallbladder cancer cells, which is the most difficult to cure. Therefore, there is a pressing need to establish effective methods of treatment for these cancers (nutrients or therapeutic agents).

In order to evaluate the effects retinol might have on cancer, we measured serum retinol concentrations in xenograft mice with human refractory cancers (pancreatic cancer MiaPaca2 and JHP-1, gallbladder cancer NOZ C-1, and cholangiocarcinoma HuCCT1). In addition, we examined whether the maintenance of serum retinol concentrations could suppress carcinogenesis (prevention) and cancer advancement (therapy), using the NOZ C-1 cell line, which is a refractory cancer having significant drug-resistance.

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MATERIALS AND METHODS

**Chemicals and Cells** RA, retinol, and retinyl palmitate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade. The human pancreatic cancer cell line, JHP-1 and the human gallbladder cancer cell line, NOZ C-1 were kindly supplied by Dr. Tomokazu Matsuura of Jikei University School of Medicine (Tokyo, Japan). The human pancreatic cancer cell line, MIA Paca2 and the human cholangiocarcinoma cell line, HuCCT1 were purchased from Riken Cell Bank (Ibaraki, Japan).

**Cell Culture** JHP-1 and NOZ C-1 cells were grown in William’s Medium E medium (GIBCO, Grand Island, NY, U.S.A.) containing 2 mM l-glutamine (GIBCO) and 10% fetal bovine serum (FBS, GIBCO). MIA Paca2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (low glucose medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% FBS. HuCCT1 cells were grown in RPMI 1640 medium (GIBCO) containing 10% FBS. Cells were subcultured every week, and attached cells were removed with trypsin–ethylenediaminetetraacetic acid (EDTA) (GIBCO). All cells described above 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.).

**Animals and Inoculation of Tumor Xenografts** Specific-pathogen-free (SPF) KSN/Slc nude mice (4 week-old, male) were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). All experiments were carried out inside a SPF room. Animals were allowed free access to water and standard complete rodent chow diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan), and mice were maintained on a 12-h dark-light cycle, with a period of darkness between 8 a.m. to 8 p.m. To generate tumor xenograft mice, male mice (n = 5) were injected subcutaneously in the flank with four human cancer cell lines, MIA Paca2 (8×10⁶ cells), JHP-1 (8×10⁶ cells), HuCCT1 (8×10⁶ cells), or NOZ C-1 (2.5×10⁶ cells), or NOZ C-1 (2.5×10⁶ cells), or NOZ C-1 (2.5×10⁶ cells) were cultured every week, and attached cells were removed with trypsin–ethylenediaminetetraacetic acid (EDTA) (GIBCO). All cells described above 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.).

**Measurement of Retinoid Concentrations** 1) Serum retinol concentrations were measured according to previously reported methods. Supernatant sera (50 µL) prepared as described above, were extracted with ethyl acetate (150 µL) by mixing for 1 min, and then centrifuged (6000×g, 5 min) at 4°C (three times). The ethyl acetate extracts were evaporated by speed vac and residues were dissolved in mobile phase solvent (25 µL) containing 85% MeOH, 15% H₂O, and 10 mM ammonium acetate. Extract (20 µL) was separated by HPLC using a Shimadzu LC-20AD high pressure pump, a Shimadzu CTO-10AS column oven including injector, a Shimadzu SCL-10A VP controller, and a SPD-20A UV spectrophotometric detector (Shimadzu Co., Ltd., Kyoto, Japan). A Luna C₁₈ column (150×4.6 mm, 5 µ particle size) (Phenomenex, Torrance, CA, U.S.A.) was used to separate retinoids. The column was eluted with mobile phase described above at a flow rate of 0.5 mL/min. Retinol was detected at 325 nm. Retention time was 14.7 min for retinol. Quantitation of samples was made using the ratio of peak areas to authentic retinol.

2) Liver retinol and RP concentrations were measured according to previously reported methods. Liver tissues (0.3 g) were homogenized with an ice-cold solution containing 100 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) buffer (pH 7.4, 1 mL) and methanol (0.5 mL). An ethanolic KOH solution (0.5 M, 7.5 µL), ethanol (99.9%, 22.5 µL) and hexane (600 µL) were added to the liver homogenates (150 µL). The resulting solution was mixed and then centrifuged (6000×g, 10 min) at 4°C. The hexane layer was collected and combined with a second extract [hexane (600 µL) and 6 N HCl (100 µL)] performed under conditions as described for the first extraction. Mixed extracts were evaporated by speed vac and residues were dissolved in mobile phase solvent (100 µL) [acetoniitrile–tetrahydrofuran–water containing 1% ammonium acetate=50:35:15 (v/v/v)]. Extracts (20 µL) were separated by HPLC as described above. A Phenomenex Luna C₁₈-column (250×4.6 mm, 5 µ particle size) was used to separate retinol and RP at a flow rate of 1 mL/min with detection by absorbance at a wavelength of 325 nm. Retention times were 5.2 min for retinol and 48.7 min for RP. Quantitation of samples was made using the ratio of peak areas of authentic retinol and RP. All experiments were performed under yellow light.

**Evaluation of Effects on Cancer Cell Growth (Cancer Advancement) in Xenograft Mice by Post-Administration of RP** KSN/Slc nude mice (4 week of age, male) were separated into three experimental groups (n=8): 1) C, control group administered corn oil only; 2) L, RP group administered a low level of RP (1000 IU/mouse); 3) H, RP group administered a high level of RP (2500 IU/mouse). Mice were injected subcutaneously in the right flank with NOZ C-1 cells (1×10⁶ cells, 0.2 mL) suspended in sterile phosphate-buffered saline (1.5 mM KH₂PO₄, 8.1 mM NaH₂PO₄, 136.9 mM NaCl, pH 7.2) (PBS). After one week, mice were administrated RP orally dissolved in corn oil. The administration was performed daily for three weeks. Control mice were given only corn oil. Tumor size was measured with calipers, and tumor volume was calculated according to the equation: volume=1/2×(width)²×length. After dissection, tumor weight was determined immediately, and retinoid concentrations were quantitated as described above.

**Evaluation of Effects of Cancer Cell Attachment (Carcinogenesis) in Mice by Pre-Administration of RP** KSN/Slc nude mice (4 week of age, male) were separated into three experimental groups (n=8): 1) low-vitamin A diet (VAD); 2) normal diet (VAC); 3) normal diet and oral administration with RP (VAO). After preliminary breeding with a normal diet for one week, the diet of the VAD group was changed to a low-vitamin A diet (CLEA Japan Inc., Tokyo, Japan), and mice in the VAO group were administrated RP dissolved in corn oil (2500 IU/mouse). After 10d, all mice were injected subcutaneously in right flank with NOZ C-1 cells (1×10⁶ cells, 0.2 mL) suspended in sterile PBS, and then cancer growth was observed for the following 10d. Tumor volume was calculated according to the formula: volume=1/2×(width)²×length. After dissection, tumor weight was determined immediately, and retinoid concentrations were quantitated as described above.

**Statistical Analysis** Data were analyzed using Prism version 6. Statistical significance was assessed using one-way
ANOVA followed by Dunnet’s multiple comparison’s test. *p<0.05, **p<0.01, and ***p<0.001 were considered significant.

RESULTS

Serum Retinol Concentrations in Cancer-Bearing Mice
We examined whether the presence of cancer cells in the body affects serum retinol concentrations using xenograft mice. Mice were inoculated with four human refractory cancer cell lines (MIA Paca2, JHP-1, NOZ C-1, and HuCCT1). After 4 weeks, the concentrations of retinol in the serum from mice were measured using HPLC analysis.

Serum retinol concentrations were significantly reduced in mice bearing MIA Paca2 (17%), JHP-1 (47%), NOZ C-1 (40%), and HuCCT1 (27%) cells as compared with control mice (Fig. 1). On the other hand, retinol and RP concentrations in liver tissues did not show significant changes (data not shown). These results indicate that serum retinol concentrations could potentially be an important index in controlling cancer development. This suggests that serum retinol levels may be useful biomarkers for cancer prevention as well as treatment.

Effect of Serum Retinol Concentrations on Cancer Cell Growth To elucidate the relationship between serum retinol and cancer development, we regulated serum retinol levels following oral administration of RP. We divided our in vivo experiments into two parts to examine cancer development, cancer cell growth and attachment, using the human gallbladder cancer cell line NOZ C-1, which is highly aggressive in both attaching and growing. First, we evaluated whether increases in serum retinol concentrations following RP administration suppress cancer cell growth. Mice were inoculated with NOZ C-1 cells, and after 7 d, RP (0, 1000, and 2500IU) was orally administered (21 d).

The body weights of mice did not show significant differences among the three groups (Fig. 2A). In contrast, tumor volumes were reduced approximately 50% by both doses of RP as compared with control (Fig. 2B). Tumor weight, determined after dissection on day 28, were significantly reduced; approximately 63 and 68% by the administrations of RP 1000IU and RP 2500IU, respectively, as compared with control (Fig. 2C).

Serum retinol concentrations in mice were determined to be 0.57 µM for control group, 0.64 µM (1.12-fold vs. control) for the 1000IU RP group, and 0.75 µM (1.32-fold vs. control) for the 2500IU RP group. Serum retinol levels tended to increase depending on the administered dose of RP (Fig. 3A). In contrast, retinol levels in the liver were 0.041 nmol/mg for the control group, 0.390 nmol/mg (9.5-fold vs. control) for the 1000IU RP group, and 0.352 nmol/mg (8.5-folds vs. control) for the 2500IU RP group. The differences in liver retinol levels between control and 1000 or 2500IU RP groups were significant (Fig. 3B). In addition, liver RP levels increased approximately 2.5-fold (1.59 nmol/mg) and 4.5-fold (2.82 nmol/mg) by 1000 and 2500IU RP administration. These represent significant differences as compared with the control (0.627 nmol/mg) (Fig. 3C).

The data indicate that serum and liver retinol concentrations and liver RP levels were increased by RP administration and that this was accompanied by reduced tumor size and weight. This is consistent with retinol being a critical component that can inhibit solid tumor growth of NOZ C-1 cells.

Effect of Serum Retinol Concentrations on Cancer Cell Attachment Next, we examined the effects of serum retinol concentrations on cancer cell attachment in mice inoculated with NOZ C-1 cells. Mice were fed with low-vitamin A diet (VAD), normal diet (VAC), or normal diet and oral administration with RP (VAO). After 10 d, NOZ C-1 cells were inoculated into all groups of mice. Body weights of mice in the three groups increased slowly without apparent adverse effects, while body weights of mice in the VAO group decreased slightly as compared to VAC mice (Fig. 4A). Tumor volumes increased gradually, beginning from early time points (day 13). Tumor volumes decreased in mice in the VAD and VAO groups, showing significant differences versus the VAC group (Fig. 4B). Tumor volumes in the VAD and VAO mice on day 21, were reducing by approximately 59.8 and 68.2%, respectively as compared with VAC mice (Fig. 5A). In addition, tumor weights in the VAD and VAO groups were suppressed by approximately 61% and 70%, which represent significant differences relative to the VAC group (Fig. 5B). Overall, the reduction of tumor volumes (Fig. 5A) and tumor weights (Fig. 5B) correlated well.

As shown in Fig. 6A, serum retinol concentrations in the VAO group were significantly higher (1.83-fold) than those in the VAC group. Unexpectedly, serum retinol concentrations in the VAD group rather than being lower, were slightly higher (1.14-fold) than the VAC group (Fig. 6A). In addition, liver retinol concentrations were significantly higher in the VAO (7.8-fold) and VAD (5-fold) groups as compared to those in VAC groups (Fig. 6B). In contrast, liver RP concentrations were higher (12.6-fold) in the VAO group and lower in the VAD group (0.36-fold) as compared with the VAC group (Fig. 6C). These results indicate that in VAD mice, liver RP was
transformed into liver retinol, which supplied retinol for blood circulation, thereby providing a source for the inhibition of cancer cell attachment. Retinol appears to be a critical factor in suppressing attachment and growth of cancer cells.

**DISCUSSION**

In our current study, we examined retinol concentrations in xenograft mice with four human cancer cells and measured the effects of RP administration on *in vivo* proliferation and attachment of human gallbladder cancer NOZ C-1 cells using a xenograft model mice. As compared to control mice, serum retinol concentrations were found to be significantly lower in xenograft mice bearing four refractory cancers (Fig. 1). The post-administration of RP into xenograft mice inoculated with NOZ C-1 cells, resulted in increasing concentrations of serum retinol, liver retinol, and liver RP in dose-dependent fashion (Fig. 3). This suppressed tumor growth (Fig. 2). In addition, in xenograft mice inoculated with NOZ C-1 cells after pre-administrated with RP (VAO) or in mice fed with low-
vitamin A diet (VAD), retinol concentrations in serum and liver increased (Fig. 6), and both tumor volume and weight were dramatically suppressed (Figs. 4, 5). Based on this, we conclude that retinol produced from RP reduces tumor size by inhibiting cell growth and attachment.

Cancer is considered to be a lifestyle disease, which is closely linked to differences in working, studying, smoking, drinking, and eating (nutrient conditions). We assume that improving lifestyles could potentially lead to a lower incidence of cancer. In the current study, we focused on an essential nutrient, vitamin A, and evaluated the relationship between vitamin A and cancer development using in vivo xenograft mouse models that allow us to investigate under the same conditions, cancers with different primary lesions. We found that serum retinol concentrations decreased in xenograft mice having four refractory cancers, MIA Paca2, JHP-1, NOZ C-1, and HuCCT1 (Fig. 1). These results suggest that cancers with distinct primary lesions may influence endogenous retinol concentrations.

Previous clinical studies have shown changes in serum retinol concentrations in patients with pancreatic, gallbladder, prostate, breast, and head/neck cancers. However, the findings among these studies have been inconsistent. This is because serum retinol levels in patients can be affected by cancer stage grade, chemotherapy, radiotherapy, age, sex, body weight, body mass index (BMI), and race etc. Previously, it has been reported that serum retinol levels are low in gallbladder and biliary duct (cholangiocarcinoma) cancer patients as compared with healthy volunteers. These data are in agreement with our results, where serum retinol concentrations were significantly lower in xenograft mice having human gallbladder cancer NOZ C-1 cells and human cholangiocarcinoma HuCCT1 cells, as compared with control mice (Fig. 1).

It is possible that circulating retinol is taken up into cancer tissues and that this could suppress cancer cell growth and adhesion. Unfortunately, it would be difficult to examine under the same conditions serum retinol levels in cancer patients having different primary lesions. Therefore, we used xenograft mice implanted with NOZ C-1 cells as an in vivo cancer model. We found that increases in serum retinol levels by post- and pre-administration of RP, which did not affect NOZ C-1 cell growth, suppressed the growth of tumors and their subsequent weights (Figs. 2–6). This indicated that retinol, and not RP, inhibited growth and weight of NOZ C-1 tumor. It would be interesting to determine retinol levels proximal to or within solid tumors.

Over 75% of retinoids in circulating blood exist in the form of retinol bound to retinol binding protein (RBP or RBP4). Recently, stimulated by retinoic acid 6 (STRA6) has been discovered, which is a RBP receptor located on cell membranes. STRA6 recognizes the binding of RBP to retinol in the circulation, and the uptake of retinol into cells. Thus, STRA6 mediates cellular uptake of retinol, and this is the only mechanism currently known to facilitate the cellular uptake retinoids. Cellular RA is a transformation product obtained from retinol via retinol by an oxidative process. RA binds to receptors (RAR and RXR), and in so doing, regulates gene expression. Previous studies have shown that retinol inhibits the growth and the invasion of RA-resistant colon cancer cells in vitro and in vivo. Retinol reduces invasiveness by decreasing the activity and mRNA expression of matrix
metalloprotease and phosphatidylinositol 3-kinase (PI3K) activity, and not through RAR/RXR pathways. In addition, we have shown that retinol may act in refractory cancer cells through mechanisms that are distinct from RAR/RXR. This is because retinol is more potent than RA and RP on inhibiting growth and adhesion of human refractory cancers, including gallbladder cancer NOZ C-1 cells in vitro,\(^2\) and that retinol reduces tumor size by inhibiting growth and attachment of NOZ C-1 cells in vivo (Figs. 2, 4, 5). Protein modification by retinol\(^3\) or mitochondrial energy homeostasis,\(^3\) could be one mechanism employed by retinol to affect cancer cells. Currently, elucidation of the mechanisms of retinol’s actions, which are distinct and depend on cell type or experimental methods (activity, mRNA or protein expression etc.) are under investigation.

Previous studies have shown that serum concentrations of retinol and RA are approximately 2.7 (2.6–2.7) μM and 5.7 nM in humans,\(^4\) and approximately 0.8–1.2 μM and 2.7–5.4 nM in mice,\(^5\) respectively. In the current study, the serum retinol concentration in xenograft mice without RP administration was approximately 0.57 μM (Fig. 3A). Post-administration of 1000 and 2500 IU RP, increased serum retinol concentrations to approximately 0.64 (1.12-fold vs. control) and 0.75 μM (1.32-fold vs. control) (Fig. 3A). Because administering 1000 IU RP significantly reduced tumor weight by day 28 to an extent similar to 2500 IU RP administration (Fig. 2C), administering 1000 IU RP might be sufficient to suppress tumor growth under these conditions. These results suggested that in cancer patients maintaining serum retinol concentrations is more significant than increasing it.

Both tumor volume and weight were significantly reduced in VAD and VAO xenograft mice (by approximately 60–70%) (Fig. 5). We had not expected that serum retinol levels in VAD mice fed with a low vitamin A diet would be higher than VAC mice fed with a normal diet (Fig. 6A). However, liver retinol levels in VAD mice were 5-fold higher than in

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**Fig. 4. Effects of a Low-Vitamin A Diet and RP Administration on Body Weight and Tumor Volume in Xenograft Mice**

Male nude mice were 1) fed with a low-vitamin A diet (VAD, △), 2) fed with a normal diet (VAC, ○), or 3) fed with a normal diet and orally administrated with RP (2500 IU/d/mouse)(VAO, ●). After 10d, mice were injected s.c. in the flank with NOZ C-1 cells (1×10⁶ cells/mouse). Body weights (A) and tumor volumes (B) were measured daily as described in “MATERIALS AND METHODS.” Results represent the mean±S.E. of each group (n=8). *p<0.05, **p<0.01, and ***p<0.001 compared with VAC group.
VAC mice, and liver RP levels in VAD mice were reduced by approximately 33% as compared to VAC mice (Figs. 6B, C). These results indicate that under the VAD conditions, RP is actively converted to retinol in the liver, and that retinol is released from liver into the blood circulation and taken up into target cells. Thus, the VAD environment may activate vitamin A metabolic enzymes and maintain serum retinol levels. Under both VAD and VAO conditions, maintaining and increasing serum retinol concentrations resulted in reduced tumor volumes and weights. Accordingly, retinol may directly affect tumor growth and attachment. Maintaining serum retinol by this VAD-activation strategy may be useful for cancer prevention and therapy. Evaluating the relationships between tumor development and vitamin A metabolism in VAD-xenograft mice are currently under investigation.

Cancer is related to smoking and alcohol drinking. Recently, it has been reported that serum retinol concentrations are lower in alcoholics and smokers. The decrease in serum retinol concentrations may be one reason why the risk of carcinogenesis is high in people who often smoke and drink. These results suggested that regulation of vitamin A intake to maintain vitamin A levels, could promote human health by keeping normal metabolism of endogenous substances with suitable balance, even in inappropriate lifestyles.

We have shown that maintaining serum retinol levels might be important for preventing cancer development. Monitoring serum retinol levels and ingesting retinol could be clinically useful for cancer prevention and therapy. Conditions or agents that maintain serum retinol concentrations could be beneficial for human health.

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Conflict of Interest The authors declare no conflict of interest.

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