Effects on M5076-Hepatic Metastasis of Retinoic Acid and N-(4-Hydroxyphenyl) Retinamide, Fenretinide Entrapped in SG-Liposomes

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Retinoic acid (RA), a potent inducer of cell differentiation, and N-(4-hydroxyphenyl)retinamide (4-HPR, fenretinide), a potent inducer of apoptosis, are well known as anticancer agents that are administered orally to patients for leukemia, breast and prostate cancer, respectively. However, it has not been studied whether both retinoids are effective on metastatic cancer. In mice implanted with M5076 cells, murine reticulum cell sarcoma survival times were prolonged by i.v. treatment of RA and 4-HPR entrapped in liposomes containing soybean-derived sterylglucoside mixture (SG), which accumulates in liver. In contrast, free RA and 4-HPR were inactive. These results indicate that RA and 4-HPR in SG-liposomes exhibit anticancer efficacy on metastatic cancers, and may have great potential for clinical use in the treatment of various cancers.

Key words retinoic acid; fenretinide; anticancer; M5076; liver metastasis; liposome

Retinoic acid (RA) induces terminal differentiation of the human acute myeloid leukemia cell line HL60 to cells having many of the functional and morphologic characteristics of mature granulocytes.1,2 RA induces the differentiation of cells from acute promyelocytic leukemia patients in vitro3 and, as a sole agent, it induces complete remission of patients with acute promyelocytic leukemia in vivo.4—6 A high percentage of patients in complete remission relapse within a few months,6,7 being resistant to further treatment with RA.6,7

N-(4-Hydroxyphenyl)retinamide (4-HPR, fenretinide), a synthetic amide of RA (Fig. 1) alone is a poor inducer of differentiation of HL60 cells as compared to RA.8 It induces apoptosis in HL60 and NB4 human leukemia cell lines,9 as well as C33A, a human cervical carcinoma cell line,10 and neuroblastoma.11 4-HPR is an effective chemopreventive12 and antiproliferative agent13,14 used against a wide variety of tumor types, including breast, prostate, ovary, and bladder cancers. 4-HPR currently is in clinical trial for treatment of breast,15 bladder,16 and neuroblastoma16 malignancies. In clinical trial, 4-HPR treatment showed side effects, decreasing serum retinol levels and causing night blindness.17

Previous studies have shown the metastatic properties of the murine reticulum sarcoma cell line M5076. When these tumor cells were implanted i.v. injection, metastasis occurred preferentially to liver, ovary, spleen, and kidney.18 Recently, it has been shown that liposomes having glucose residues (SG-liposome) accumulated in liver and in spleen to the extent of approximately 40 and 8% total liposomes respectively at 2 h after i.v. injection. These have been useful as an effective liver targeting drug delivery system.19

In order to evaluate effects on metastatic cancer, we administered RA and 4-HPR encapsulated in SG-liposomes with the intent of sustaining release and targeting the liver. In the present study, we found that RA and 4-HPR entrapped in SG-liposomes, prolonged the survival of mice implanted with M5076 cells and exhibited antitumor efficacy against this metastatic cancer.

MATERIALS AND METHODS

Chemicals and Cells  RA and cholesterol (Ch) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), 4-HPR was provided by Dr. R. C. Moon, University of Illinois, Chicago, IL, U.S.A. Dipalmitoylphosphatidylcholine (DPPC) was purchased from NOF (Tokyo, Japan). A soybean-derived sterylglucoside mixture (SG) containing β-sitosterol 3-β-D-glucoside, campesterol, stigmasterol, and brassicasterol was provided by Ryukakusan Co. (Tokyo, Japan). All other chemicals were of reagent grade. Murine reticulosarcoma (M5076) was obtained from Dr. T. Yamori of the Cancer Chemotherapy Center, Japan Foundation for Cancer Research (Tokyo, Japan).

Cells Murine reticulosarcoma cell line (M5076) was grown in RPMI 1640 medium (GIBCO, Grand Island, NY, U.S.A.) supplemented with 10 μm 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), pH 7.3 and 15% fetal bovine serum (FBS) as described previously.20 Human hepatoma cell line, HepG2 was obtained from RIKEN cell bank (Tokyo, Japan). HepG2 cells were grown in RPMI 1640 medium containing 10% FBS and subcultured every week. Attached cells were removed from the tissue culture flask surface with trypsin–EDTA (GIBCO).

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) and viability was determined by trypan blue dye exclusion.

Cell Growth M5076 cells (2×10⁵/ml) were grown in RPMI 1640 medium containing 15% FBS and various concentrations of retinoids. M5076 cells were harvested by trypsinizing to dislodge cells into medium. Cell number and viability were measured. The percentage of net growth is shown with values adjusted by subtracting the initial cell concentration (cells/ml) of experimental cultures from the initial concentrations of control cultures which were defined as 100% (Fig. 3). Values for percent net growth were calculated with the following formula: [(cell concentration of experimental culture)−(initial cell concentration)/(cell concentration of control culture)−(initial cell concentration)]×100.

Fig. 1. Chemical Structures of RA and 4-HPR

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Preparation of Liposome-Entrapped Retinoids  Liposome-entrapped retinoids were prepared from 60 μmol DPPC, 10 μmol SG, 30 μmol Ch and 3 μmol retinoid (DPPC:SG:Ch:retinoid=6:1:3:0.3 molar ratio) according to the reverse-phase evaporation method described previously. Liposomes were extruded through a polycarbonate membrane (Nuclepore, U.S.A.) with a pore size of 200 nm at 50—60 °C. Entrapped retinoids were separated from free retinoids through a Sephadex G-50 column (Pharmacia, Sweden) with the 1/10 diluted phosphate-buffered saline. Aliquots of liposomes were analyzed for the measurement of retinoids as described below. The particle size distribution of liposome was determined using a dynamic laser light scattering instrument (Model ELS-800, Ostuka Electronics Co., Ltd., Osaka) and lipid concentration of liposomes were determined using a Wako phospholipid B test (Wako Pure Chemical Ind., Ltd., Osaka). Liposomes were stored at 4 °C in the absence of light.

Quantitation of Retinoids by High Pressure Liquid Chromatography (HPLC)  HepG2 cells (2 × 10⁶ cells/ml) were incubated at 37 °C for 0—1 h with free or SG-liposome entrapped retinoids at the concentration of 360 nM. Retinoids in mixtures were extracted with equivalent volumes of methyl acetate containing 1% acetic acid under argon gas. Mixtures were centrifuged at 10000 × g for 5 min, then retinoids in organic layers were analyzed by HPLC using a LC-10As pump, SIL-10A injector, and RF-10AXL detector (Shimadzu Co., Kyoto, Japan). A ZORBAX ODS (C₁₈ reverse-phase column, 4.6 mm ID×25 cm, 5 μm, HEWLETT PACKARD, Palo Alto, CA, U.S.A.) was used to separate RA and 4-HPR. The column was eluted with 10 mM NH₄OAc in a methanol/water gradient (30—70% (0—10 min), 70—90% (10—32 min), 90—100% (32—36 min) and then, 100% (36—40 min)) at a flow rate of 1.5 ml/min with UV monitor at wave lengths of maximum absorbance for each retinoid; 340 nm for RA and 4-HPR. Elution time was 26 min for RA and 31 min for 4-HPR. Measurements were made using authentic RA and 4-HPR as internal standards.

Animals and Tumor Models  Specific-pathogen-free female C57BL/6 mice (19—20 g, 7 weeks old) were purchased from Tokyo Laboratory Animal Science Company Co. Ltd. (Tokyo, Japan). M5076 cells were kept in vivo as subcutaneous solid tumors in C57BL/6 mice by transplantation every two weeks into the right axillary subcutaneous tissue. To obtain a suspension of tumor cells for transplantation, every two weeks into the right axillary subcutaneous tissue. Aliquots of liposomes were analyzed for the measurement of retinoids as described below. The particle size distribution of liposome was determined using a dynamic laser light scattering instrument (Model ELS-800, Ostuka Electronics Co., Ltd., Osaka) and lipid concentration of liposomes were determined using a Wako phospholipid B test (Wako Pure Chemical Ind., Ltd., Osaka). Liposomes were stored at 4 °C in the absence of light.

Evaluation of Antitumor Activity  In therapeutic experiments of liver metastatic cancer, a group of nine C57BL/6 mice were inoculated intravenously on day 0 with M5076 cells (6 × 10⁶ cells, 0.2 ml) per mouse. On Day 8 after implantation of tumor cells, mice were given as single i.v. doses of free or liposomal retinoids (0.585 mg/kg for RA; 0.762 mg/kg for 4-HPR). The control group was given sterile saline instead of retinoid on Day 8. Mice were weighed everyday. Survival times were recorded in days following tumor administration. Antitumor activity was evaluated by comparing mean survival times of treated animals (T) with those of controls (C), and by calculation of increased life-span (ILS), (T/C—1) 100 (%).

Statistical Analysis  The statistical significance of the results was assessed by the nonparametric test of Kruskal-Wallis and Student’s t-test for the survival experiment.

RESULTS

Characterization of SG-Liposome Entrapping of RA and 4-HPR  Characterization of SG-liposome entrapping of RA and 4-HPR (Fig. 1) is summarized in Table 1. Properties of SG-liposome containing RA and 4-HPR did not differ significantly, e.g. drug and lipid concentrations, drug/lipid (drug and lipid ratio), and size, even though their structures were distinct (Fig. 1).

Survival Times of M5076 Bearing-Mice Treated with RA and 4-HPR  Previous studies have shown that with the exception of metastatic cancer RA and 4-HPR exhibit potent anticancer activities against various cancers, in vitro and in vivo.8,10,11,22—24 These results led us to investigate the antitumor efficacy of these retinoids entrapped in SG-liposome in a liver metastatic cancer model with M5076 cells in vivo.

M5076 cells were injected into mice via tail vein on Day 0. On Day 8 retinoids encapsulated in liposomes and liposomes without retinoid as control were administrated by i.v. Body weights of mice in all groups did not change noticeably. Liver, heart, lung, kidney, and spleen of mice 14 d after i.v. injection of M5076 cells were approximately two-fold larger than those in normal mice.23 Livers of M5076 bearing mice exhibited white spots, which were identified as liver tumors.8,23

Figure 2 shows effects of RA and 4-HPR on survival times of M5076-implanted mice. All mice in the control group died at approximately 14 d after tumor implantation. In contrast, the life of mice treated with RA and 4-HPR at a con-

| Drug concentration (μg) | 0.935±0.146 | 0.931±0.159 |
| Lipid concentration (μg) | 14.908±4.475 | 15.863±6.048 |
| Size (nm) | 251.4±67.0 | 287.8±67.9 |
| Drug/lipid | 0.063±0.021 | 0.059±0.014 |

Table 1. Characterization of SG-Liposome Entrapping Retinoids
HepG2 cells, the concentration of RA decreased markedly to RA and 31 min for 4-HPR. After incubation of free RA with and quantified by HPLC. Retention times were 26 min for liposome-entrapped retinoids were incubated at 37 °C with Free and SG-

suppressed by a high concentration of 4-HPR, but not by RA. 3). These results indicate that growth of M5076 cells was not inhibited by 1 μM 4-HPR (Fig. 3). In contrast, cell growth was inhibited M5076 cell growth to the extent of approximately 40% (Fig. 3). In contrast, cell growth was not inhibited by 1 μM or 10 μM RA or 1 μM 4-HPR (Fig. 3). These results indicate that growth of M5076 cells was suppressed by a high concentration of 4-HPR, but not by RA.

Effects of Retinoids on Growth of M5076 Cells
Murine reticulosarcoma M5076 cells, were grown in culture containing various concentrations of retinoids and 15% FBS, which was required for exponential cell growth. 4-HPR at a concentration of 10 μM inhibited M5076 cell growth to the extent of approximately 40% (Fig. 3). In contrast, cell growth was not inhibited by 1 μM or 10 μM RA or 1 μM 4-HPR (Fig. 3). These results indicate that growth of M5076 cells was suppressed by a high concentration of 4-HPR, but not by RA.

Stability of Free and SG-Liposome-Entrapped Retinoids in the Presence of HepG2 Cells Free and SG-liposome-entrapped retinoids were incubated at 37 °C with HepG2 cells, and then remaining retinoids were extracted and quantified by HPLC. Retention times were 26 min for RA and 31 min for 4-HPR. After incubation of free RA with HepG2 cells, the concentration of RA decreased markedly to concentration of 50 μM were prolonged and survival lines were shifted to the right in Fig. 2. The mean survival times of RA and 4-HPR were 16.9 and 18.9 d (Table 2). The differences between control and retinoids were significant (p < 0.01 for RA and p < 0.001 for 4-HPR). Free RA and 4-HPR did not prolong survival times (data not shown). These results indicated that the retinoids encapsuled in SG-liposomes showed great antitumor activity. Percentage increase in life span (ILS) are 16.8% for RA and 30.1% for 4-HPR (Table 1). 4-HPR exhibited more potent antitumor efficacy than RA.

DISCUSSION
The current study shows that SG-liposomes containing RA or 4-HPR (Fig. 1) could be prepared in a stable form that prevented their metabolism (Fig. 4). Survival times of mice implanted with M5076 metastatic cancer, following administration of RA or 4-HPR entrapped in SG-liposomes, were prolonged as compared with control mice (Fig. 2, Table 2). In contrast, free RA and 4-HPR were inactive. In addition, while RA did not inhibit M5076 cell growth, 4-HPR at a concentration of 10 μM was inhibitory (Fig. 3). These results indicated that SG-liposomal RA or 4-HPR may have potential for clinical use in the treatment of various cancers.

One reason for using liposomes was to enhance solubility of RA and 4-HPR, which are highly hydrophobic. Additional reason was to extend blood residence time in order to allow optimal delivery to target tissues. The intent was to stabilize components in liposomes so as to prolong circulation time in blood.25) Previous studies of drug tumor targeting have shown that liposomes consisting of the ganglioside GM126) or amphipathic poly(ethylene glycol)26,27) prevent drug uptake into the reticuloendothelial system. Additionally, liposomes carrying galactose residues tend to target hepatocyte,28) while liposomes having glucose residues (DPPC : SG : Ch = 6 : 1 : 3, molar ratio) accumulate in the liver following i.v. administration, thereby making these useful for effec-

Table 2. Antitumor Efficacy of SG-Liposome-Retinoids against Reticulum Cell Sarcoma (M5076)

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Dose (μM)</th>
<th>RA</th>
<th>4-HPR</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>50</td>
<td>14.5</td>
<td>0.5</td>
<td>14.6</td>
</tr>
<tr>
<td>4-HPR</td>
<td>50</td>
<td>16.9</td>
<td>2.6*</td>
<td>17.0</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>16.8</td>
<td>0.0</td>
<td>16.8</td>
</tr>
</tbody>
</table>

* Percentage increase in life span (ILS), [(T/C − 1) × 100 (%)], where T and C represent the median survival time (d) of the treated and control animals, respectively. ** p < 0.01, *** p < 0.001, significantly different from control. Antitumor activity was evaluated by comparing the mean survival time of the treated animals with that of the controls.

Fig. 3. Growth of M5076 Cells in the Presence of Retinoids
Cells were grown without or with RA and 4-HPR at the concentration of 1 μM and 10 μM in medium containing 15% FBS. Growth was measured at 96 h as described under Materials and Methods. The means of at least four measurements are shown.

Fig. 4. Stability of Free or SG-Liposomal Retinoids in the Presence of HepG2 Cells
HepG2 cells (2×10^6 cells/ml) were incubated at 37 °C for 0—1 h with free or SG-liposome entrapped RA (A) or 4-HPR (B) at a concentration of 360 μM. Retinoids were extracted and analyzed by HPLC as described under Materials and Methods. The means of at least three measurements are shown. The S.E. of each data point was ±8% of the mean.

90% at 30 min and to 32% at 1 h as compared with the initial RA concentration, which was defined as 100% (Fig. 4A). On the other hand, the concentration of 4-HPR decreased to 93% after 30 min and to 87% after 1 h incubation (Fig. 4B). However, the levels of RA and 4-HPR in SG-liposomes did not change during the incubation (Fig. 4A, B). These results indicate that free RA and free 4-HPR may be more unstable in the presence of HepG2 cells as compared with SG-liposome-entrapped RA and 4-HPR, and that SG-liposome preparation may protect retinoids against metabolizing enzymes. This may facilitate sustained release, thereby maintaining high concentrations of these retinoids.
tive liver targeting. On the other hand, M5076 murine reticulum cell sarcoma preferentially metastasizes to liver, ovary, spleen, and kidney regardless of method of tumor injection. In the current study, RA and 4-HPR encapsulated in liposomes containing SG, prolonged survival of M5076 cell-implanted mice (Fig. 2, Table 2). This is the first report that RA and 4-HPR entrapped in SG-liposomes exhibit anti-tumor efficacy in an animal M5076 tumor metastasis model. RA and 4-HPR are well known clinical anti-cancer agents that are administrated orally to leukemia patients in a capsule form. In the case of RA, an inducer of cell differentiation, patients with acute promyelocytic leukemia (APL) initially respond to RA-therapy with complete remission. However, frequently relapses occur in association with leukemia cells that are resistant to differentiation by RA. This suggests that processes or steps are affected, e.g. cell growth suppression of M5076 cells, while 4-HPR was active in a metastasis model (Fig. 2, Table 2). However, in assays measuring tumor growth suppression of M5076 cells, while 4-HPR was active in a metastasis model (Fig. 2, Table 2). However, in assays measuring tumor growth suppression of M5076 cells, while 4-HPR was active in vivo using a variety of cell lines. These include HL60 leukemia cells; HL60R cells which are a subclone of HL60 cells that are resistant to RA; MCF-7 breast cancer cells, that have estradiol receptors (ER-positive); MCF-7/AdrR cells which are a subclone of MCF-7 cells that are resistant to RA (ER-negative); HepG2 hepatoma cells and DU-145 prostate cancer cells. RA inhibited cell growth of HL60 and MCF-7 cells with high efficacy, 4-HPR suppressed growth of various human cancer cells (all described above) with a broad spectrum and high efficacy, even against cells that were resistant to RA (e.g. HL60R and MCF-7/AdrR cells). Also, RA and 4-HPR were effective against M5076-implanted mice in vivo, which represents a metastatic cancer model (Fig. 2, Table 2). However, in assays measuring growth suppression of M5076 cells, while 4-HPR was active at a concentration of 10 μM, RA was inactive (Fig. 3). It is possible that both RA and 4-HPR may affect metastasis or growth of metastatic cells. Further studies are needed to ascertain which processes or steps are affected, e.g. cell growth of M5076 (primary neoplasm), metastasis, or cell growth of metastatic cancer etc.

RA and 4-HPR are effective anticancer drugs in M5076 tumor models. The results reported herein suggest potential clinical anticancer applications of RA and 4-HPR encapsulated in SG-liposomes. Studies are presently underway to measure the effectiveness of RA and 4-HPR encapsulated in SG-liposome against other animal tumor models.

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