Inhibitory Effects of Retinol Are Greater than Retinoic Acid on the Growth and Adhesion of Human Refractory Cancer Cells

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Received October 15, 2015; accepted December 25, 2015; advance publication released online January 26, 2016

Vitamin A constitutes include retinal, which plays a role in vision, and retinoic acid (RA), which has been used in the therapy of human acute promyelocytic leukemia. However, the effects on cancer of retinol (Rol) and its ester, retinyl palmitate (RP) are not known well. In the current study, we examined the effects of these agents on proliferation and adhesion of various cancer cells. Rol exhibited dose-dependent inhibition of the proliferation of human refractory and prostate cancer cells, while RA and RP showed little or no effect. In contrast, RA inhibited the growth of human breast cancer cells to a greater extent than Rol at low concentrations, but not at high concentrations. Rol suppressed adhesion of refractory and prostate cancer cells to a greater extent than RA, while it suppressed adhesion of breast cancer cells as well as RA and of JHP-1 cells less effectively than RA. These results indicate that Rol is a potent suppressor of cancer cell growth and adhesion, which are both linked to metastasis and tumor progression. Rol might be useful for the clinical treatment of cancer.

Key words vitamin A; retinol; retinoic acid; retinyl palmitate; anti-cancer

MATERIALS AND METHODS

Chemicals and Cells RA (all-trans), Rol, RP, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). JHP-1 and NOZ C-1 cells were supplied by Dr. Tomokazu Matsuura, Jikei University School of Medicine (Tokyo, Japan). MIA Paca2 and HuCCT1 cells were purchased from Riken Cell Bank (Ibaraki, Japan). PC-3 cells were a gift of Dr. Yoshie Maitani, Hoshi University (Tokyo, Japan). MCF-7 cells were purchased from American Type Culture Collection (Rockville, MD, U.S.A.).

Cell Culture JHP-1 and NOZ C-1 cells were grown in William’s Medium E containing 2 mM L-glutamine and 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, U.S.A.). MIA Paca2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (low glucose) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% FBS. HuCCT1 cells were grown in RPMI 1640 medium (Invitrogen) containing 10% FBS. PC-3 and MCF-7 cells were grown in RPMI 1640 medium containing 10% FBS and 1 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) (Invitrogen). 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 Growth Cells (1×10⁴ cells/mL) were incubated at 37°C for 1 d, and then various concentrations of Rol, RA, and RP were added to the cultures. Control cells were treated with 0.1% DMSO or ethanol. Cells were incubated for 72 h, and then viable cell numbers were estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously. Values for percent net cell growth were calculated using the following formula: [(absorbance of experimental cell density)−(absorbance of initial cell density)]/[(absorbance of control cell density)−(absorbance of initial cell density)]×100.

Cell Adhesion Cells (3.8×10⁴ cells/well) were transferred onto 24-well plates either having a hydrophilic surface that allows attachment factors to adhere (HPS-plates; Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) or to plates coated with collagen IV as described previously (COL-plates), which lacked this hydrophilic character. The cells were then treated with Rol or RA at 40 µM concentration or with 0.1% DMSO (control). Cells were incubated for 0.5–2 h, and then viable cell
numbers were estimated using MTT.\textsuperscript{5)}

**Statistical Analysis** Results represent the mean±standard deviation (S.D.) of each group (n=4). Data were analyzed using Prism version 6. Statistical significance was assessed using one-way ANOVA followed by Dunnet's or Bonferroni's multiple comparison's test. *p<0.05, **p<0.01, and ***p<0.001 vs. control. &p<0.05, &&p<0.01, and &&&p<0.001 vs. RA.

**RESULTS AND DISCUSSION**

We first examined the effects of retinoids on the growth of cancer cells (Fig. 1). The growth of MIA Paca2, JHP-1, HuCCT1, and PC-3 cells were suppressed 60, 78, 97, and 79% with 10\(\mu\)M Rol, respectively. NOZ C-1 cell growth was inhibited 78% by 20\(\mu\)M Rol. On the other hand, RA and RP had little or no effect on cancer cell growth. In contrast, treatment with even 1\(\mu\)M RA inhibited MCF-7 cell growth 50%, while 20\(\mu\)M RA reduced cell proliferation 57%. Rol inhibited MCF-7 cell growth in dose-dependent manner, achieving 87% inhibition at 15\(\mu\)M concentration. The IC\textsubscript{50} values of the retinoids (Table 1), show that Rol is an effective anti-proliferative agent, being more potent than RA against the cancer cells examined, with the exception of MCF-7 cells.

The mechanism for the inhibitory effects of Rol on cancer cell growth is not well known. However, it is unlikely that Rol acts through RA-mediated nuclear receptors, which bind specifically to RA and directly activate transcription of target genes by binding to specific DNA sequences.\textsuperscript{1)} Rol and RA most probably act on cancer cells through distinct mechanisms. This is because Rol and RA differentially inhibit the growth of cancer cell lines, and Rol is more potent than RA against refractory cancer and PC-3 cells (Fig. 1). We con-

### Table 1. IC\textsubscript{50} Values of Retinoids against Cancer Cell Growth

<table>
<thead>
<tr>
<th>Cell</th>
<th>IC\textsubscript{50} ((\mu)M)</th>
<th>Rol</th>
<th>RA</th>
<th>RP</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIA Paca2</td>
<td>8</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>JHP-1</td>
<td>6.5</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>HuCCT1</td>
<td>5</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>NOZ C-1</td>
<td>14.2</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>PC-3</td>
<td>7.2</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>MCF-7</td>
<td>6</td>
<td>1</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

IC\textsubscript{50} values are based on data shown in Fig. 1.
firmed that in NOZ C-1 cells the content of Rol taken up is less than that of RA, and that Rol did not affect caspase 3 activity, gene expression of bax and bcl-2, or the cell cycle (data not shown). Protein modification by Rol has been reported by Myhre et al.,7) and this could be one mechanism by which Rol acts in cancer cells.

Previous studies have shown that serum concentrations of Rol and RA are 2.7 µM and 5.7 nm in humans8,9) and 0.8–1.2 µM and 2.7–5.4 nm in the mouse, respectively.10,11) Rol significantly reduces cancer cell growth when given within a 1–20 µM concentration range (Fig. 1). This is 0.4–7-fold of the endogenous Rol concentration in humans (2.7 µM). In contrast, RA decreases MCF-7 cell growth when given in concentrations as low as 1 µM (Fig. 1E), which is 175-fold higher than endogenous RA concentration in humans (5.7 nm). It is known that the serum RA concentration in acute promyelocytic leukemia patients administered with RA is approximately 1 µM.2) Therefore, effective Rol concentrations are low or moderate as compared to endogenous Rol levels. We confirmed that a high concentration of Rol (approximately 2-fold as compared with endogenous levels) did not affect normal cells, tissues or organs in mice in vivo (data not shown). Accordingly, it is possible that regulating Rol levels in blood could be used as a method for treating and preventing refractory cancer.

Invasion and metastasis are the primary causes of death due to cancer. Cell adhesion is the first step in these processes. Among the six cell lines, MIA Paca2 cells were distinguished by not adhering to COL-plates, while MIA Paca2 cells did adhere to HPS-plates (Fig. 2A). When using HPS-plates, Rol inhibited the extent of adhesion of MIA Paca2 (65%), JHP-1 (62%), HuCCT1 (35%), NOZ C-1 (20%), PC-3 (79%), and MCF-7 (58%) cells, as compared with control (Fig. 2B). Suppression of cell adhesion by Rol was greater than by RA, with the exception of JHP-1 cells. When using COL-plates, RA and Rol inhibited adhesion by 23 and 12% for JHP-1 cells, 0.5 and 18% for HuCCT1 cells, 4 and 16% for NOZ C-1 cells, respectively.

![Fig. 2. Effects of Retinoids on Cancer Cell Adhesion](image_url)
cells, and 13 and 23% for PC-3 cells as compared with control (Fig. 3). In contrast, RA and Rol suppressed adhesion 37 and 32% for MCF-7 cells. Rol suppressed adhesion to a greater extent than RA against HuCCT1, NOZ C-1, and PC-3 cells in COL-plates (Fig. 3), and MIA Paca2 and NOZ C-1 cells in HPS-plates (Fig. 2B). Thus, the effects of Rol on cancer cell adhesion were greater than or equal to RA, while it was a more effective anti-adhesive agent than RA in NOZ C-1 cells grown under both conditions. In contrast, RA was more effective against JHP-1 cell adhesion than Rol in both conditions (Figs. 2B, 3). The anti-adhesive activities of RA and Rol in HPS-plates showed good correlation with results obtained in COL-plates.

While numerous reports have noted the biological activities of retinoids, the focus has typically been on RA rather than other forms of retinoids. This is because RA is considered to be the most active metabolite. Our data suggest that Rol is a potent inhibitor of cell growth and adhesion when examined against several cancer cell lines, including refractory cancers, and that its potency is equivalent to or greater than RA.

Distinct differences on cell growth were shown by RA, Rol, and RP (Fig. 1) and on adhesion by RA and Rol (Fig. 3). These results indicate that the sensitivity of cancer cells toward different forms of retinoids varies, and that when treating cancer as well as other diseases, it might be advantageous to employ different forms of retinoids, depending on the primary lesion tissues. The reasons are unclear why retinoid sensitivities vary among different kinds of cancer cells. The evaluation of mechanisms related to uptake efficiency and metabolism rates among RA, Rol, and RP are under investigation.

Clinical studies have shown that serum Rol concentrations are lower in breast cancer patients than in healthy people. In contrast, Rol levels in breast adipose tissue are high in breast cancer patients. It is possible that circulating Rol is taken up into cancer environments, which could suppress cancer cell growth and adhesion. Maintaining serum Rol levels could be important for preventing cancer development. Administration of Rol might be useful for the clinical treatment and prevention of cancer.

Acknowledgments We thank Dr. Terrence Burke, Jr. for helpful comments. This investigation was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan and JSPS Core-to-Core Program, A. Advanced Research Networks of Japan.

Conflict of Interest The authors declare no conflict of interest.
REFERENCES


