Original Article

Inhibition of Abcg2 transporter on primitive hematopoietic stem cells by All-trans retinoic acid increases sensitivity to anthracycline

Lawrence Lein¹, Yasuo Nagai¹, Yo Mabuchi¹, Sadafumi Suzuki¹, Satoru Morikawa¹,², and Yumi Matsuzaki¹, *¹

¹Department of Physiology, Keio University School of Medicine, Tokyo, Japan
²Department of Dentistry and Oral Surgery, Keio University School of Medicine, Tokyo, Japan

It is well established that All-trans retinoic acid (RA) regulates the growth and differentiation of a wide variety of tissues and cell types. Here we found that RA has a new role, the breast cancer resistance protein (Abcg2) inhibitor. RA, like the transporter inhibitor reserpine, dose-dependently reduced the efflux of Hoechst dye from HSCs by inhibiting the Abcg2 transporter without requiring new transcription. The effects of RA and reserpine on the SP were reversible upon wash-out. Similar results were obtained in NIH3T3-GFP cells stably expressing Abcg2. RA-treated and Abcg2-deficient HSCs showed increased sensitivity to doxorubicin, a type of anthracycline. Our evidence suggests that, besides playing crucial roles in the survival, growth, and differentiation of HSCs, RA has a novel function as an inhibitor of the Abcg2 transporter.


*Correspondence should be addressed to:
Yumi Matsuzaki, Department of Physiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Fax: +81-3-5363-3566, e-mail: penguin@sc.itc.keio.ac.jp

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Introduction

All-trans retinoic acid (RA) is a derivative of vitamin A and is essential for diverse biological functions, including reproduction, vision, and immune response. The biologic effects of RA are mediated through retinoic acid receptors that are members of the steroid/thyroid hormone superfamily of transcription factors. Transcriptional activation by RAR may trigger differentiation, cell-cycle arrest, and apoptosis, and thus often leads to inhibition of cell proliferation. Here, we found that RA has a novel role in inhibiting the breast cancer resistance protein (BCRP/Abcg2). Abcg2 typically transports chemotherapeutic drugs out of cells, and its inhibition by RA could enhance sensitivity to anthracycline treatment.

Abcg2 is a member of the adenosine triphosphate (ATP) —
binding cassette (ABC) transporter superfamily and is highly expressed in hepatic canicular membranes, renal proximal tubules, and other tissues. It is expressed at high levels in leukemia stem cells and has been shown to cause anthracycline efflux from them. Its overexpression in cell lines confers resistance to a variety of chemotherapeutic drugs, including doxorubicin, supporting the hypothesis that Abcg2 expression in cancer cells contributes to their resistance to chemotherapy.

Primitive HSC cells (CD34−/c-kit+/Sca-1+ lineage-), or hematopoietic precursors (c-kit+Sca-1+ lineage-), are sorted into the “up” side population (SP) by flow cytometry because of their low level of red and blue fluorescence following loading with Hoechst dye. This is caused by the HSCs’ ability to rapidly expel the dye, compared with other cell types, which is conferred by their expression of the Abcg2 transporter. Abcg2 expression is required for the HSCs to appear in the side population (SP) as well as to protect them from doxorubicin toxicity. This idea is supported by the fact that, although the Abcg2-null mouse appears normal, its hematopoietic cells show increased sensitivity to chemotherapeutic reagents like doxorubicin. Here, we found that RA inhibits the Abcg2 transporter, and this inhibition function enhances the sensitivity to anthracycline treatment.

Material and Methods

1) Mice

Adult wild-type mice were purchased for mating from CLEA Japan Inc. (Tokyo, Japan). Bcrp1− (i.e., Abcg2−) knockout mice were kindly provided by Dr. Brian P. Sorrentino (St. Jude Children’s Research Hospital). All experimental procedures were approved by the ethics committee of Keio University and were in accordance with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health).

2) Hoechst and antibody staining for flow cytometry analysis

Bone marrow (BM) preparation, analysis of all SP cells or antibody staining with Lineage− (Ter119, CD45R, CD3e, CD11b, GR-1) and CD34−/c-kit+Sca-1+ phenotype was performed as our previously report. The effects of RA (Sigma R2625) or Reserpine (Sigma Chemical Co.), which was included with the Hoechst staining solutions, on the SP phenomenon were analyzed. Washout studies were performed as outlined in Mogi et al. and Brendel et al.

3) Doxorubicin fluorescence assay

To determine the doxorubicin (DOX) efflux fluorescence effect of the doxorubicin (Adriamycin, Kyowa Hakko Kogyo Co., Ltd.) alone or in combination with RA or reserpine, cells were incubated with 2 μM doxorubicin in DMEM for 1.5 hours. After 1.5 hours, the cells were stained with KSL antibodies (described above). The cells were analyzed in a flow cytometer at 488 nm and emission integrated above 530 nm.

4) Actinomycin D

The cells were incubated with HBSS+ with 2 μg/mL actinomycin D (Sigma), 3 hours prior to Hoechst staining. Actinomycin D-treated BM cells were then co-incubated with various concentrations of RA during Hoechst staining and analyzed as described above.

5) Cell Culture And Cell Viability Assay

The colony-forming unit in culture (CFU-C) assay was performed according to the manufacturer’s recommendations (MethoCult, Stem Cell Technologies Inc.). RA, Reserpine or doxorubicin, if used, was added to the methylcellulose medium with the CD34-KSL sorted cells. After 14 days, the colonies were analyzed. NIH3T3-Abcg2-GFP cells were prepared as outlined in Zhou et al.

Results

1) RA inhibits the efflux of Hoechst dye from SP cells

To examine the effect of RA on the SP cells, we performed flow cytometry analysis. The BM cells were incubated with different concentrations of RA (1,10, or 50 μM) during Hoechst 33342 staining. As a positive control, we showed that the SP fraction completely disappeared, indicating there was no efflux of Hoechst in the presence of 7 μM of reserpine, a known inhibitor of Abc transporters. In the presence of RA, the SP fraction significantly decreased in a dose-dependent manner, especially in the Hoechst-low region of the SP (Figure A and B). These data suggested two possibilities. Either the RA inhibited the Hoechst efflux from SP cells or the RA killed the SP cells. To learn if RA killed the HSC-rich SP cells, the effect of RA on the KSL population was examined. Total BM cells were incubated with Hoechst and different concentrations of RA for 1 hour, and the KSL cells were analyzed by flow cytometry. At the highest concentration of RA (50 μM), the KSL cells were detected with the same frequency as at lower concentrations (Figure C and D). To examine whether the KSL cells were intact and functional, we sorted the total BM cells into 5 groups, untreated, RA treated (1,10, or 50 μM RA), or reserpine (7 μM)-treated, and performed a colony-forming unit in culture (CFU-C) assay for each group. After two weeks, the number of colonies in the five
groups was similar (Figure 1E and F). These results indicated that at the moderate concentration of 50 μM, RA did not destroy the SP cells, but inhibited the efflux of Hoechst.

2) Abcg2 reduces or prevents the intracellular accumulation of doxorubicin

Since Abcg2 activity decreases the intracellular level of doxorubicin (DOX) through a direct efflux mechanism, and since doxorubicin is fluorescent and detectable, we used flow cytometry to study the intracellular doxorubicin efflux activity of KSL cells in vitro. Flow cytometry analysis showed that doxorubicin did not accumulate in most KSL cells, but it did accumulate intracellularly in Lineage (Ter119, CD45R, CD3e, CD11b, Gr-1) positive cells, which do not express the Abcg2 transporter. Neither RA-only nor reserpine-only affected the doxorubicin fluorescence in these cells (Figure 2A). We also found that KSL cells treated with the combination of doxorubicin and RA (50 μM) or with reserpine (7.5 μM) showed stronger doxorubicin fluorescence than KSL cells treated with doxorubicin alone (Figure 2B). All of these data can be explained if RA inhibited the efflux of doxorubicin from KSL cells by blocking the function of the Abcg2 transporter.

3) HSCs in which Abcg2 is inhibited or deleted show increased sensitivity to doxorubicin

In previous reports, Abcg2-deficient cells were shown to develop normally, but to be more sensitive to doxorubicin than control cells. Therefore, we tested whether HSCs became susceptible to doxorubicin when RA was used to inhibit the Abcg2. We assayed 100 highly purified HSCs (CD34-KSL cells) sorted
Fig. 2  Efflux of doxorubicin by the Abcg2 transporter, and HSCs are sensitive to doxorubicin when Abcg2 is blocked or knocked out

(A) BM-derived KSL cells were incubated with doxorubicin (DOX), and/or reserpine (7 μM) and/or RA (50 μM), or without any drugs, and analyzed for doxorubicin fluorescence. Upper row, BM KSL cells expressing Abcg2 were incubated for 1.5 hours without any drugs (control) or with doxorubicin (2 μg/mL), RA, or reserpine. Lower row, BM Lineage+ cells lacking Abcg2 expression, were incubated with doxorubicin (2 μg/mL) for 1.5 hours. Reserpine- and RA-treated cells did not show doxorubicin fluorescence. (B) BM KSL cells were co-incubated with doxorubicin (2 μg/mL) and RA (left) or doxorubicin and reserpine (right). (C) CD34-KSL cells from WT and Abcg2−/− mice grown in methylcellulose culture at various concentrations of doxorubicin. Four treatment conditions were tested: untreated wild-type (WT) CD34-KSL cells, WT CD34-KSL cells treated with RA, WT CD34-KSL cells treated with reserpine, and Abcg2−/− CD34-KSL cells. All four groups were challenged with increasing concentrations of doxorubicin. CFU-C survival expressed as the percentage of colonies relative to the number of colonies in the untreated culture. Values are ± SD; n = 3.

4) RA does not inhibit Abcg2 transporters at the transcriptional level

The inhibitory role of RA could be explained by direct inhibition of the transporter or by the inhibition of Abcg2 transcription. To address this, we investigated the effect of Actinomycin D, an RNA synthesis inhibitor, on the SP population. BM cells were pretreated with 2 μM Actinomycin D 3 hours prior to Hoechst staining. RA was added at different concentrations (1, 10, and 50 μM) during the Hoechst-staining step. In the presence of RA, the SP fraction significantly decreased, dose-dependently, as shown in Figure 1. Despite the inhibition of transcription by Actinomycin D, there was no difference in SP cell frequency compared with the control group (Figure 3A,B). These data show that the RA-induced inhibition of doxorubicin efflux does not require new transcription.

To verify that our protocol for treating the cells with Actino-
Fig. 3 Inhibition of the Abcg2 transporter by RA does not require transcription
(A) Murine BM cells incubated with 5 μg/ml Hoechst33342 in the presence or absence of 7 μM reserpine or 1, 10, or 50 μM RA. Prior to the drug treatment, the BM cells were incubated with 2 μg of Actinomycin D per ml of culture medium. Representative data are shown. (B) Percentages of side population cells treated as in (A), compared with control [Actinomycin D(-)]. Values are ± SD; n = 3. (C) RT-PCR results showing that Actinomycin D reduced the transcription of RA target early genes egr-1 and G0S2 in BM cells. β-actin served as an internal control. (D) Quantitative RT-PCR analysis of Abcg2 mRNA isolated from SP cells showing that RA treatment had no effect on the Abcg2 expression levels, compared with the Abcg2 mRNA levels in untreated SP cells (defined as 1.0). Abcg2 expression was normalized to that of β-actin.

5) RA abrogates Abcg2-mediated Hoechst 33342 transport and the SP phenotype in HSCs and Abcg2-expressing NIH 3T3 cells
As a final investigation into the role of transcription in RA’s inhibition of Abcg2, wash-out studies were performed with BM cells11,12. To examine the effect of RA on the dye efflux activity of SP cells, RA or reserpine was added during the Hoechst staining step. In the presence of RA or reserpine, the SP fraction decreased, but after the RA or reserpine was washed out, the SP cells were again visible in the SP gate (Figure 4A,B).

Next, we examined whether the cells that shifted from the SP region to the non-SP region were the same cells that appeared following the wash-out. A previous study showed that both the SP and the CD34-KSL cells express Abcg2 mRNA and that CD34-KSL cells are sorted into the SP fraction9. We found that the CD34-KSL cells dose-dependently shifted to the non-SP fraction after treatment with either RA or reserpine. Once the RA or reserpine had been washed out, these CD34-KSL cells reappeared in the SP fraction (Figure 4C).

To test whether RA inhibited Abcg2 directly, we performed similar experiments with NIH3T3-GFP cells that had been stably transfected with Abcg2. We found that, although NIH3T3-GFP cells did not show an SP fraction, NIH3T3-GFP-Abcg2 cells did appear in the SP (Figure 5C). Treatment with RA or reserpine dose-dependently reduced the percentage of NIH3T3-GFP-Abcg2 cells in the SP fraction. Then, 30 min after the wash-
Fig. 4 RA-induced inhibition of the Abcg2 transporter is reversible

(A) Murine BM cells incubated with Hoechst in the presence or absence of 50 μM RA or 7 μM reserpine for 1 hour as control (upper panels, Non-wash-out) or (lower panels) incubated for an additional hour in Hoechst- and inhibitor-free medium (Wash-out). The area enclosed by black lines indicates the side population cells. (B) Percentage of BM cells in the side population was assessed in three independent experiments. Values are ± SD; n = 3. (C) CD34-KSL cells, denoted by black dots, and whole bone marrow cells, denoted by gray area, stained with Hoechst in the presence or absence of RA or 7 μM Reserpine. Upper panels show the results of non- wash-out as control. Lower panels show the results of a wash-out experiment performed as in (A).

Fig. 5 Inhibition of the Hoechst 33342 transport in NIH3T3-Abcg2 cells is dose-dependent and reversible

(A) After transduction with Abcg2, the SP phenotype was confirmed with 80.2 percent of SP. RA or reserpine (Res) inhibited the efflux of Hoechst from the NIH3T3-Abcg2-GFP cells. From left to right, panels show Hoechst-stained NIH3T3-Abcg2-GFP cells incubated with the indicated amounts of RA or reserpine. The six panels were generated after a wash-out step. The area enclosed by purple lines represents the SP region. (B) Percentage of NIH3T3-Abcg2-GFP cells in the side population was assessed in three independent experiments. Values are ± SD; n = 3. (C) Parental NIH3T3 cells showing that none were in side population (low-fluorescence) cells. After transduction with Abcg2, 99.9% of the cells were positive for Abcg2-GFP and express SP as shown in (A).
out step, the Abcg2-GFP cells shifted back to the SP fraction (Figure 5A,B). These data showed that the inhibition of efflux activity by RA was reversible in less than 1 hour, further supporting our hypothesis that RA and reserpine inhibit efflux directly through the Abcg2 transporter. In addition, we found that different types of cells (i.e., NIH3T3 cells and BM cells) responded identically to RA and reserpine as long as they expressed Abcg2.

Discussion
In this study, we found that RA has a new role, the breast cancer resistance protein (BCRP/Abcg2) inhibitor. The effects of RA on Abcg2 were not cell-type specific, because the ability of Abcg2-tranduced NIH3T3 cells to export Hoechst was also hindered by RA treatment. We also evaluated whether RA inhibited the efflux of SP cells via the transcription of target genes. By blocking transcription with Actinomycin D, we found that Actinomycin D had no effect on the RA-induced efflux inhibition (Figure 3A,B). This observation was again supported by our real-time RT-PCR results showing that the Abcg2 transcript level was the same with and without RA treatment (Figure 3D). Wash-out studies further corroborated this interpretation: the RA-induced efflux inhibition of both murine BM cells (Figure 4A, B), and CD34-KSL hematopoietic cells (Figure 4C) was rapidly reversible, shifting back from the non-SP fraction to the tip-SP fraction.

Furthermore, using the stably transfected NIH3T3-Abcg2-GFP cells, we found that the GFP+ cells moved from the tip-SP fraction to the non-SP fraction, and that the size of the effect depended on the dose of RA or reserpine (Figure 5). After the drugs were washed out, the Abcg2-GFP cells reappeared as SP cells. Taken together, our data suggested that RA inhibited the SP efflux activity by reacting with Abcg2 transporters directly, rather than through a transcription-dependent mediator. Although there are possibilities for SP disappearance such as RA causes Abcg2 protein turn over, this possibility is unlikely because the both HSCs and NIH3T3-Abcg2-GFP cells shifted from SP cells to non-SP area only by one hour of RA treatment, and shifted back to SP region after short period of RA wash out, merely 30 minutes. Since it is known that SP phenotype of HSCs is also related to their cell cycle status, there is a possibility that disappearance of SP fraction by RA may reflect recruitment of dormant HSCs into active cell cycle, rather than inhibiting Abcg2. To exam this possibility, we utilized NIH3T3 cells, which are in MP region after staining, and NIH3T3-Abcg2-GFP cells, which are in SP region after staining. It is unlikely that all NIH3T3 cells changed from active cell cycle to dormant cell cycle after transfection by Abcg2, and transfected NIH3T3-Abcg2-GFP cells changed from dormant cell cycle to active cell cycle after adding RA for 1 hour.

From the Abcg2-transduced NIH3T3 experiment in Figure 5, we showed that RA or reserpine is able to inhibit Abcg2 transporter. However, we believe RA or reserpine is able to inhibit not only Abcg2 transporter. In figure 2C, our data showed that reserpine-treated or RA-treated HSC are more sensitive to doxorubicin than Abcg2-/- HSCs. This phenomenon could be explained by the fact that RA or reserpine has the ability to inhibit some other unknown transporters, which also pump out doxorubicin. These unknown transporters are currently under investigation. Overall, we found that RA inhibits the Abcg2 transporter, and this inhibition enhances the sensitivity to anthracycline. This new discovery will provide a means by which researchers can study the other potential effects of RA.

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