Existence of Retinoic Acid-Receptor-Independent Retinoid X-Receptor-Dependent Pathway in Myeloid Cell Function

Takayuki Hida*, Kenji Tai, Naoki Tokuhara, Akira Ishibashi, Kouichi Kikuchi, Shigeki Hibi, Hiroyuki Yoshimura, Mitsuo Nagai, Toshihiko Yamauchi and Seiichi Kobayashi

Tsukuba Research Laboratories for Drug Discovery, Eisai Co., Ltd., 5-1-3 Tokodai, Tsukuba-shi, Ibaraki 300-2635, Japan

Received March 28, 2000 Accepted October 19, 2000

ABSTRACT—We previously reported that ER-27191 (4-[4,5,7,8,9,10-hexahydro-7,7,10,10-tetramethyl-1-(3-pyridylmethyl)anthra[1,2-b]pyrrol-3-yl]benzoic acid) is a potent antagonist of retinoic acid receptor (RAR), and ER-35795 ((2E,4E,6E)-7-[1-(1-methylethyl)-8-chloro-1,2,3,4-tetrahydroquinolin-6-yl]-6-fluoro-3-methyl-2,4,6-nonatrienoic acid) is a novel retinoid X receptor (RXR)-specific agonist. By using these compounds, we investigated whether distinct RAR-dependent and RXR-dependent pathways operate to mediate the diverse activities of retinoids, particularly, the effects of the RXR pathway on cellular function. ER-27191 completely antagonized HL60 cell differentiation induced by all-trans-retinoic acid (atRA). However, the differentiation induced by the ER-35795 was not antagonized at all by the RAR antagonist, but was inhibited by an RXR homodimer antagonist (LGD100754, (2E,4E,6Z)-7-(3-n-propoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)-3-methylocta-2,4,6-trienoic acid). Its agonistic action on RXR/RAR heterodimer, on the other hand, was neutralized by the RAR antagonist. During HL60 cell differentiation, atRA induced RARβ2 mRNA, while the RXR had no effect. Interestingly, a functional RXR-pathway was also seen in lipopolysaccharide-induced inhibition of mouse splenocyte proliferation. These results strongly suggest the existence of a pharmacological RXR-dependent pathway that is activated by a ligand that can bind to RXR.

Keywords: Retinoic acid receptor, Retinoid X receptor, ER-27191, ER-35795, HL60 differentiation

Research to elucidate the molecular mechanisms of transactivation via RAR and RXR, members of the nuclear receptor family, is advancing rapidly. atRA is known as a multifunctional physiological regulator of cell proliferation and differentiation (reviewed in refs. 1 – 3). Because atRA binds only to RARs, the biological activities induced by atRA are thought to be mediated via RARs. On the other hand, 9cRA, an isomer of atRA, was identified as a ligand of RXR (4, 5). Since 9cRA can bind not only to RXR, but also to RAR (4 – 6), the mechanisms of its actions appear to be complicated.

Many nuclear receptors activate transcription by forming heterodimers with RXR in the presence of a specific ligand. During heterodimer activation, RXR was thought to be a silent promiscuous partner. Recent findings showed that RXR ligands can enhance heterodimer formation with a counter receptor, such as RAR or PPAR (7, 8). It has also been demonstrated that the function of the heterodimer was modified by RXR ligands (9) and that 9cRA can activate heterodimer formation of some orphan receptors with RXR (9 – 11). In any case, the cellular pathway induced by RXR ligands as well as the participation of 9cRA in physiological nuclear receptor signaling is still unclear.

To better understand the cellular mechanisms that depend on RXR, many RXR-selective compounds were synthesized. RXR agonists are reported to affect myeloid cell

*Corresponding author. FAX: +81-298-47-2037
E-mail: t-hida@hhc.eisai.co.jp

The abbreviations used are (in alphabetical order): CRBP I, cellular retinol-binding protein I; CRBP II, cellular retinol-binding protein II; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; ER-27191, 4-[4,5,7,8,9,10-hexahydro-7,7,10,10-tetramethyl-1-(3-pyridylmethyl)anthra[1,2-b]pyrrol-3-yl]benzoic acid; ER-35795, (2E,4E,6E)-7-[1-(1-methylethyl)-8-chloro-1,2,3,4-tetrahydroquinolin-6-yl]-6-fluoro-3-methyl-2,4,6-nonatrienoic acid; FBS, fetal bovine serum; LGD100754, (2E,4E,6Z)-7-(3-n-propoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)-3-methylocta-2,4,6-trienoic acid; LPS, lipopolysaccharide; PLAP, placenta-derived secretion-type alkaline phosphatase; PMA, phorbol 12-myristate 13-acetate; PPAR, peroxisome proliferator-activated receptor; 9cRA, 9-cis retinoic acid; atRA, all-trans-retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid-responsive element; RT-PCR, reverse transcription-polymerase chain reaction; RXR, retinoid X receptor.
binding, and IC_{50} values were obtained from logarithmic plots. The selectivities of compounds for each receptor were indicated as relative IC_{50}, obtained by dividing the IC_{50} value for each receptor of the compound by that of the natural ligand (atRA or 9cRA).

Transactivation assay
To evaluate transactivation activity, receptor expression and reporter plasmids were transiently co-transfected in COS-1 cells. For antagonist evaluation, only reporter plasmid was transfected into the RAR-expressing stable transformant cells. The cells at 80% confluence in a 60-mm dish were incubated with 6 µg of receptor expression vector, 6 µg of reporter plasmid, and 40 µl of Lipofectamine™ (Gibco-BRL, Rockville, MD, USA) per dish in OPTI-MEM (Gibco-BRL). After 4 h of incubation, the medium was replaced with DMEM supplemented with 10% FBS and incubation continued for 20 h. The cells were suspended in DMEM supplemented with 10% FBS and seeded at 3 × 10^4 per well in 96-well plates. After 6 h of incubation, compounds at various concentrations were added to duplicate wells. The cells were incubated for a further 48 h, and then PLAP activity of the cell supernatants was assayed for transcriptional activity. To inactivate nonspecific activity, the samples were preheated at 65°C for 20 min. Aliquots of 15 µl were mixed with 60 µl of assay buffer (0.28 M Na_2CO_3, pH 10.0, with 8 mM MgSO_4) and reacted with 75 µl of Lumistain™ (Sumitomo, Osaka). After the reaction mixture had incubated for 30 min at 37°C and 30 min at room temperature, chemiluminescence was measured with a micro plate luminometer (23). The selectivities for each receptor were also presented as relative ED_{50}. Because in our investigation, it was found that the response to some RAR-selective compound tended to reach a plateau near 50% of that of atRA. Therefore, ED_{50} values were used to represent the transcriptional activity for the evaluation.

HL60 culture and differentiation measurements
HL60 culture and measurement of CD11b expression (as the marker of differentiation) were performed as previously reported (21). Briefly, HL60 cells (1 × 10^5/ml) were exposed to compounds for 5 days; and for surface antigen analysis, the cells were harvested exposed to FITC-anti CD11b monoclonal antibody (Immunootech, Marseille, France) at 4°C for 1 h, and fixed with 0.2% paraformaldehyde. The proportion of CD11b-positive cells in viable cells was determined with a FACScan™ (Becton Dickinson, San Jose, CA, USA). In some cases, we also measured the magnitude of superoxide anion production from HL60 cells as a functional differentiation marker. For measurement of superoxide production, HL60 cells treated with compounds for 5 days were stimulated with PMA (10 ng/ml) in the presence of cytochrome c (Sigma, St. Louis, MO,
USA) for 30 min at 37°C, the change of absorbance (OD 550 nm) was measured, and the quantity of superoxide anion production per cell was estimated from the millimolar extinction coefficient of reduced cytochrome c as 28.0.

Detection of RARβ mRNA induction

HL60 cells exposed to compounds for 24 h were collected and washed, then total RNA was extracted with the TRIzol™ (Gibco-BRL) according to the manufacturer’s instructions. RT-PCR was performed with an RNA-PCR kit (Perkin Elmer, Branham, CA, USA) from 0.25 μg of total RNA, according to the manufacturer’s instructions. Primers used for the PCR were as follows: RARβ sense (1-22), 5'-ATGTTTGACTGTATGGATGTTC-3'; RARβ antisense (222-245), 5'-AAGCAGGTTGTCACATCG-3'; human β-actin; RT-PCR Amplimer Sets (Clontech, Palo Alto, CA, USA). A reaction cycle consisted of 1 min at 98°C, 1 min at 57°C and 2 min at 73°C, with 22 cycles for β-actin and 35 cycles for RARβ. A 10-μl aliquot of each reaction sample was examined on 1.5% agarose gels stained with ethidium bromide.

Spleen cell culture and proliferation assay

Spleen cells were prepared from 6-week-old male BALB/C mice as reported (24). The spleen cells were suspended at 6 x 10⁷/ml in RPMI1640 medium supplemented with 10% FBS and 50 μM 2-mercaptoethanol; and 0.2-ml aliquots were plated in 96-well plates and then cultured with 5 μg/ml of E. coli LPS (Sigma) in the presence of compound. After 3 days of culture, the cells were pulsed with 0.5 μCi of [³H]thymidine per well (Amersham, Buckinghamshire, UK) for 6 h, then harvested on glass filters, and incorporated radioactivity was counted using a β-plate (Pharmacia, Uppsala, Sweden).

RESULTS

Characterization of compounds used in this study

Relative binding abilities of retinoid compounds are comparably shown in Table 1. Namely the relative binding ability was calculated from the ratio of IC₅₀ of a compound to that of atRA for RAR, or to that of 9cRA for RXR. ER-27191, which we showed previously to be an RAR antagonist (21), could bind to RARα, β and γ with relative IC₅₀ values of 1.3, 1.4 and 3.9, respectively, but was unable to

| Compound         | Structure | Binding (relative IC₅₀ (fold))/Transactivation (relative ED₃₀ (fold)) |
|------------------|-----------|---------------------------------------------------------------|
| atRA             | ![atRA](atRA.png) | RARα: 1, RARβ: 1, RARγ: 43, RXRα: 1, RARβ: 1, RARγ: 1, RXRα: 17 |
| 9cRA             | ![9cRA](9cRA.png) | RARα: 5.4, RARβ: 3.9, RARγ: 7.0, RXRα: 1, RARβ: 1.5, RARγ: 1.1, RXRα: 1 |
| ER-27191         | ![ER-27191](ER-27191.png) | RARα: 1.3, RARβ: 1.4, RARγ: 3.9, RXRα: —, RARβ: —, RARγ: —, RXRα: — |
| ER-35795         | ![ER-35795](ER-35795.png) | RXRα: 6.6, RXRα: 1600, RXRα: 0.067 |
| LGD100754        | ![LGD100754](LGD100754.png) | RXRα: 576, RXRα: 3.2, RXRα: 25, RXRα: 32, RXRα: 110 |

Relative IC₅₀ values and ED₃₀ were calculated as described under Materials and Methods. — indicates that compounds did not inhibit specific binding (less than 25% inhibition of binding) of labeled atRA or 9cRA even at a 500-fold excess or showing less than 30% of maximal activity at 3 μM. All data are shown as the mean of two to four individual experiments.
bind to RXR. In spite of high binding affinities, transactivation activity of ER-27191 was not seen in any RAR subtype. On the other hand, ER-35795, a novel synthetic retinoid, could bind strongly to RXR with a similar affinity to 9cRA and enhanced the transactivation, but did not bind to RARs. Thus, ER-35795 was a selective agonist for RXR. LGD100754, reported as a RXR homodimer antagonist, was described as agonist in the case of RXR/RAR heterodimer (25). In this assay system, LGD100754 actually showed agonistic activity for RAR, but did not induce RXR homodimer transactivation.

Effects of ER-27191 on the transactivation by retinoid compounds via RAR or RXR

The inhibitory profile of ER-27191 on transcriptional activity induced by 10 nM atRA and 9cRA was examined. As shown in Fig. 1, ER-27191 inhibited the transcriptional activities via RARs induced by both atRA and 9cRA in a dose-dependent manner, but failed to inhibit the transcriptional activities via RXR induced by 9cRA or ER-35795, an RXR agonist. In the only RAR/β transformant, ER-27191 itself slightly increased the transactivation, but the transactivation induced by atRA or 9cRA was suppressed to the level by ER-27191. Furthermore, the binding of [3H]9cRA to RARs (RARα, RARβ and RARγ) was also inhibited by ER-27191; however, ER-27191 had no effect on the binding of [3H]9cRA to RXRα (data not shown). These results showed that ER-27191 can completely inhibit the RAR pathway activated not only by atRA, but also by 9cRA. Thus ER-27191 is defined as a pan-antagonist for RAR.

Fig. 1. Antagonistic activity of ER-27191 towards atRA-, 9cRA- or ER-35795-induced transcription. The transformants transfected with the reporter plasmid CRBP I-PLAP (RARα, β, γ) or CRBP II-PLAP (RXRα) in receptor-expressing transformant cells. Cells were cultured in the presence of the indicated concentrations of ER-27191 (●) with 10 nM atRA (■), 10 nM 9cRA (▲), or 10 nM ER-35795 (▲). After 2 days of exposure, the transcriptional activity was determined by measuring the PLAP activity in the supernatant of the cell culture. Three independent experiments were done, and representative data are shown.
HL60 differentiation induced by atRA, 9cRA and RXR agonist

By using the compounds characterized above, we next examined the contributions of the RAR and RXR pathways to cell function, especially those triggered by 9cRA and the selective RXR agonist. As HL60, which only expresses RARα and RXRα, differentiate in response to atRA and 9cRA (12, 26–31), we chose it as a model cell for our purpose. First, the HL60 differentiation was analyzed by flow cytometry, and the percentage of CD11b-positive cells was determined. In the presence of 10 nM atRA or 9cRA, HL60 fully expressed CD11b (Fig. 2A). Interestingly, ER-35795, an RXR agonist, which lacks binding ability with RAR and has no inducing activity for transactivation through RARs, also increased the proportion of CD11b-positive cells in a dose-dependent manner. Subsequently, superoxide production from HL60 was measured in terms of functional cell differentiation. Concomitantly with the differentiation of HL60 cells, proliferation was decreased (data not shown). The inhibition of proliferation and increase of CD11b expression were accompanied with significant elevation of superoxide anion production in response to PMA (Fig. 2B). This functional differentiation was induced by not only 9cRA, but also the RXR agonist ER-35795. Finally it was examined whether these differentiation pathways were the same or not by observing RARβ transcription. It is known that the RXR/RAR dimer can up-regulate the RARβ mRNA directly in the presence of ligand for RAR, atRA and 9cRA, because RARE exists upstream of the RARβ gene (32–34). Therefore, induction of RARβ mRNA might show that the RAR pathway would work during HL60 differentiation. In HL60 cells, 1 μM

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

Fig. 2. Differentiation of HL60 induced by RAR and RXR agonists. A: Elevation of CD11b induced by RAR and RXR ligands. HL60 cells were cultured with various concentrations of atRA ( ), 9cRA ( ) and ER-35795 ( ) for 5 days, and the percentage of CD11b-positive cells was measured by flow cytometer. Three separate experiments were done, and representative data are shown. B: Cellular function induced by RAR and RXR agonists. HL60 cells were incubated with the indicated compounds for 5 days. The cells were stimulated by PMA and production of superoxide anion was measured in terms of reduction of cytochrome c. Data are expressed as the mean of three data points ± S.E.M. Statistical analysis was performed by Student’s t-test (*P<0.05 was considered to indicate statistical significance). C: Induction of RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA. HL60 cells were exposed to the compound in the presence or absence of ER-27191 for 24 h. Total RNA was prepared, and RT-PCR was performed. PCR samples were examined on 1.5% agarose gels and stained with ethidium bromide. PCR products were detected at 269 bp for RARβ mRNA.

Three independent experiments were done, and representative data are shown.
atRA could induce the transcription of RARβ mRNA (Fig. 2C: lane 2), and this induction was diminished by cotreatment with 3 µM ER-27191. On the other hand, the induction of RARβ mRNA in the cells treated with 1 µM 9cRA (lane 3) was not so prominent, even though the extent of functional cell differentiation was similar to that with 1 µM atRA. Furthermore, ER-35795, even at the concentration of 10 µM, failed to induce RARβ transcription (lane 4).

**Effect of RAR and RXR antagonists on the differentiation of HL60**

ER-27191, an RAR-selective antagonist, did not itself affect the differentiation of HL60, but suppressed the appearance of CD11b-positive cells induced by exposure to 10 nM atRA or 9cRA, with similar dose dependence in each case (Fig. 3A). On the other hand, when a higher ligand concentration (100 nM) was employed, ER-27191 inhibited the HL60 differentiation induced by atRA, but the inhibitory effect on the differentiation induced by 9cRA was apparently diminished (Fig. 3B). As in the case of 10 or 100 nM, ER-35795 was not inhibited at all by ER-27191 (Fig. 3B). To clarify the pathways activated by each ligand, ER-27191, an antagonist for RAR, and LGD100754, an RXR homodimer antagonist, were used in the HL60 differentiation system. LGD100754 was reported to have antagonist activity in RXR/RXR homodimer transactivation, but it turned into an activator of the RXR/RAR heterodimers (25). We found that LGD100754 partially induced CD11b expression on the HL60 cell surface, and this activity seemed to be mediated by RXR/RAR heterodimer activation, because it was antagonized in the presence of 1 µM ER-27191, an RAR antagonist (Fig. 3C). The RAR antagonist-resistant activities induced by the ER-35795 and by a high dose of 9cRA were both inhibited by the RXR/RXR homodimer antagonist, LGD100754, in the presence of the RAR antagonist (Fig. 3C). Thus, co-treatment of LGD100754 with RAR antagonist extracted the RXR homodimer antagonistic activity of LGD100754 in cellular response. These data strongly suggest that two pathways exist for the induction of HL60 differentiation: 1) an RAR-antagonist-inhibitable RAR-dependent pathway and 2) an RXR-antagonist-inhibitable RXR-dependent pathway, which is RAR-independent.

**Effects of retinoids on the proliferation of splenocytes**

Since atRA and 9cRA inhibit B cell proliferation induced by LPS (24, 35), we investigated whether the putative RAR-dependent and RXR-dependent pathways are functional in normal cells. As shown in Fig. 4A, atRA and 9cRA both inhibited the proliferation of spleen cells. ER-27191, an RAR antagonist, alone did not show any inhibition up to 100 nM. Interestingly, ER-35795, an RXR agonist, could also inhibit the splenocyte proliferation at a concentration of 10 nM or higher. The inhibition of B cell growth induced by 10 nM atRA or 9cRA was completely reversed by 100 nM ER-27191 (data not shown). In order to know which pathway is contributing to the inhibition of ER-35795, we examined the effect of LGD100754. Consistent with previous findings, LGD100754 partially induced CD11b expression on the HL60 cell surface, and this activity was antagonized by RAR antagonist ER-27191 (Fig. 3C). The RAR antagonist-resistant activities induced by the ER-35795 and by a high dose of 9cRA were both inhibited by the RXR/RXR homodimer antagonist, LGD100754, in the presence of the RAR antagonist (Fig. 3C). Thus, co-treatment of LGD100754 with RAR antagonist extracted the RXR homodimer antagonistic activity of LGD100754 in cellular response. These data strongly suggest that two pathways exist for the induction of HL60 differentiation: 1) an RAR-antagonist-inhibitable RAR-dependent pathway and 2) an RXR-antagonist-inhibitable RXR-dependent pathway, which is RAR-independent.

**Fig. 3.** Inhibitory effects of ER-27191 and LGD100754 on HL60 differentiation induced by atRA or 9cRA and RXR agonist. A and B: HL60 cells were incubated with the indicated concentrations of ER-27191 (□); in the presence of 10 nM atRA (■), 9cRA (●) or ER-35795 (▲) (A); in the presence of 100 nM atRA (□), 9cRA (●) or ER-35795 (▲) (B). C: HL60 cells were cultured with various concentrations of LGD100754 (●) in the presence of 1 µM ER-27191 (○), 100 nM 9cRA (●), 100 nM 9cRA and 1 µM ER-27191 (●), or 30 nM ER-35795 and 1 µM ER-27191 (▲). The extent of cell differentiation was measured in terms of the percentage of CD11b-positive cells measured by flow cytometry. Three separate experiments were done, and representative data are shown.
with the results of the HL60 assay, LGD100754 also inhibited LPS-induced B cell proliferation, but it was reversed by ER-27191, an RAR antagonist (Fig. 4B), although inhibition of the response by 100 nM ER35795, an RXR agonist, was not reversed by only ER-27191 and LGD100754, but it was reversed by both antagonists in combination (Fig. 4B).

DISCUSSION

ER-27191 was found to be an antagonist of atRA-induced HL60 cell differentiation (21, 26). In this study, ER-27191 exhibited strong binding affinity for all RAR-subtypes, while it failed to bind to RXR. Furthermore, ER-27191 antagonized the transactivation of RARα, β and γ transformants induced by not only atRA, but also 9cRA, although it can not inhibit the RXRα transactivation induced by 9cRA (Fig. 1). This antagonistic activity of ER-27191 correlated with the ability to displace [3H]atRA (Table 1) or [3H]9cRA bound to RAR (data not shown), suggesting that ER-27191 binds to the common region of RARs recognized by atRA and 9cRA.

It is well known that atRA induces differentiation of HL60 cells to granulocytes and subsequently causes cell death by apoptosis mediated by RAR. On the other hand, the role of RXR ligand during HL60 differentiation was thought to be a synergistic enhancer of RAR activity (12). However, the existence of an RAR-independent RXR pathway was recently reported; eg., antagonist-resistant differentiation and apoptosis induced by 9cRA (26), apoptosis in HL60 cells induced by an RXR agonist (27), and RAR-independent NB4 (acute promyelocytic leukemia cell) maturation (13). In this report, we demonstrate that RXR ligand can induce HL60 differentiation and that this may occur through an RXR pathway. This is supported by the following findings. First, the RXR agonist, ER-35795, could fully induce CD11b expression (Fig. 2A) and cell function (Fig. 2B). This differentiation was not inhibited by ER-27191, an RARs-selective antagonist (Fig. 3: A and B). In addition, in the presence of the RAR antagonist, the differentiation induced by the RXR agonist was antagonized by LGD100754, an RXR-homodimer antagonist, in a dose-dependent manner (Fig. 3C). The purpose of combining LGD100754 with RAR antagonist was to eliminate the agonistic action of LGD100754 via RXR/RAR heterodimer, as it has been reported that LGD100754 itself has RXR/RAR and RXR/PPAR agonistic activities (25). In fact, we observed the agonistic activity of the LGD100754 in HL60 differentiation, and found that this effect was diminished in the presence of the RAR antagonist (Fig. 3C). Thus, in the presence of an RAR antagonist, LGD100754 works as a pure RXR antagonist with no HL60 differentiation. This is the first time that LGD100754 manifests its RXR homodimer antagonistic activity in cellular response combined with an RAR antagonist. Second, RAR/β mRNA is
known to be induced by atRA itself through RARE remaining in the promoter region of the RARβ gene (32, 33). During the differentiation of HL60, it was found that atRA induced the transcription of RARβ mRNA, and this induction was weakened in the presence of the RAR antagonist (Fig. 2C: lanes 2 and 6). In contrast to atRA, the RXR agonist failed to induce transcription of RARβ mRNA (lane 4), even though it induced the differentiation of HL60, again suggesting that ER-35795, an RXR agonist, induced HL60 differentiation not through an RAR-dependent, RARE-mediated pathway involving induction of RARβ mRNA, but through another pathway that is not accompanied with induction of RARβ mRNA. As reported by Apfel et al. (12), HL60 differentiation was induced not only by atRA or 9cRA, but also by the RXR agonist. The report described that the RXR agonists enhanced the RXR/RAR heterodimer, even though RXR/RXR homodimer was also formed by RXR ligand on retinoid X responsive element. In many cases, the role of RXR compound was explained by enhancement of heterodimer with RAR, PPAR and the like. However, the results described here suggest the possibility that high concentrations of RXR agonists induce the RXR/RXR homodimer pathway. Recently the possibility was also reported that an RAR-independent RXR signal exists in atRA-sensitive and -resistant NB4 for cell maturation (13). Taken together, it is strongly suggested that a functional RXR pathway exists for myeloid cell differentiation mediated by RXR homodimer. 9cRA is also known to induce HL60 differentiation (26, 31). From the results shown here, 9cRA bound to RXR and activated RXR-dependent transcription, supporting the idea that 9cRA is a natural ligand for RXR (36). However, 9cRA causes granulocytic differentiation of HL60 predominantly not through the RXR pathway, but rather through the RAR pathway at a dose of 10 nM or less, because the differentiation was almost completely blocked by ER-27191, an RARs-selective antagonist. On the other hand, the HL60 differentiation induced by a high concentration (such as 100 nM) of 9cRA, was not antagonized by ER-27191 (Fig. 3C). It was reported that not only atRA but also 9cRA could induce RARβ transcription (33, 34). In HL60 cells, 10 nM of 9cRA could induce RARβ mRNA, but 1 μM of 9cRA was less than that by the same concentration of atRA (Fig. 2C). These results suggest that such a high concentration of 9cRA might change the differentiation-pathway from the RAR-dependent pathway to an RXR pathway that is not functionally antagonized by ER-27191. In the presence of more than 100 nM 9cRA, it was also reported that RXR homodimer formation was induced (12). From kinetic analysis, it was reported that RXR exists as a tetramer in the absence of ligand (37, 38). RXR tetramer dissociates to RXR homodimer in the presence of 9cRA, and subsequently RAR and cognate ligand induce RXR/RAR heterodimer. Since a significant fraction of the dimer remains as a RXR homodimer, it is likely that high concentration of 9cRA preferably activates RXR homodimer. In this study, as the inhibitory profile of RAR antagonist on the differentiation of HL60 induced by 10 nM or 100 nM 9cRA was also quite different, it appears that the activation pathway is dramatically switched from RAR to RXR depending on the concentration of 9cRA employed. To elucidate possible involvement of an RXR pathway, we considered a similar study with splenocytes, as it was known that B cell proliferation induced by mitogens such as LPS is inhibited by atRA, mediating RARα (24). It was also shown that physiological concentrations of atRA and 9cRA inhibit the growth of normal human and murine B cells (35). As shown in Fig. 4, the proliferative response of LPS-stimulated splenocytes was suppressed by the RXR agonist. Thus, functional activation through the RXR pathway has been demonstrated herein by an effect on HL60 cell differentiation and the proliferative response of LPS-stimulated splenocyte. In conclusion, the RXR-selective agonist induced not only RXR transactivation, but also granulocytic differentiation and inhibition of splenocyte proliferation. These cellular functions were not antagonized by an RAR-selective antagonist, but were inhibited by an RXR-homodimer antagonist, while its agonistic action on RAR was neutralized by an RAR antagonist. Thus, we conclude that at least some types of cells retain both an RAR-dependent pathway and an RXR-dependent pathway, both of which are functionally active. 9cRA can utilize both pathways, depending on its concentration. Although RXR had been thought to be a silent receptor, we showed here that RXR homodimer can be activated by RXR ligands. Further studies combining the selective activation pathway induced by RXR homodimer should provide new insights and lead to novel drug discovery.

Acknowledgments

We thank Seiko Higashi for expert technical assistance and Dr. Makoto Asada for his support. We also thank Dr. Akira Kakizuka (Osaka Bioscience Institute) for helpful discussions.

REFERENCES

1 Sporn MB, Roberts AB and Goodman DS: The Retinoids. Biology, Chemistry, and Medicine, 2nd Ed, Academic Press, Orlando, FL (1992)
2 Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P and Evans RM: The nuclear receptor super family. The second decade. Cell 83, 835 – 839 (1995)
3 Bollag W: The retinoid revolution. FASEB J 10, 938 – 939 (1996)
4 Levin AA, Sturzenbecker LJ, Kazmer S, Bosakowski T,
Huselton C, Allenby G, Speck J, Kratzeisen C, Rosenberger M, Lovey A and Grippo JF: 9-cis Retinoic acid stereoisomer binds and activates the nuclear receptor RXRα. Nature 355, 359 – 361 (1992)
5 Heyman RA, Mangelsdorf DJ, Dick JA, Stein RB, Eichele G, Evans RM and Thaller C: 9-cis Retinoic acid is a high affinity ligand for the retinoid X receptor. Cell 68, 397 – 406 (1992)
6 Allenby G, Bocquel MT, Sanders M, Kazmer S, Speck J, Rosenberger M, Lovey A, Kastner P, Grippo JF, Chambon P and Levin AA: Retinoid X receptors and retinoid X receptors interactions with endogenous retinoid acids. Proc Natl Acad Sci USA 90, 30 – 34 (1993)
7 Mukherjee R, Davies PJ, Crombie DL, Bischoff ED, Cesario JM, Ow L, Hamann LG, Boehm MF, Mondon CE, Nadzan AM, Paterniti JRJ and Heyman RA: Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. Nature 386, 407 – 410 (1997)
8 Westin S, Kurokawa N, Noike RT, Wisely GB, McInerney EM, Rose DW, Milburn MV, Rosenfeld MG and Glass CK: Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators. Nature 395, 199 – 202 (1998)
9 Forman BM, Umesono K, Chen J and Evans RM: Unique response pathways are established by allosteric interactions among nuclear hormone receptors. Cell 81, 541 – 550 (1995)
10 Leblanc BP and Stunnenberg G: 9-cis Retinoic acid signaling changing partners causes some excitement. Genes Dev 9, 1811 – 1816 (1995)
11 Soontjens CD, Rafter JJ and Gustafsson JA: Ligands for orphan receptors? J Endocrinol 150, S241 – S257 (1995)
12 Apfel CM, Kamber M, Klau M, Mohr P, Keidel S and LeMotte PK: Enhancement of HL-60 differentiation by a new class of retinoids with selective activity on retinoid X receptor. J Biol Chem 270, 30765 – 30772 (1995)
13 Benoit G, Altucci L, Flexor M, Ruchaud S, Lillehaug J, Raffelsberger W, Gronemeyer H and Lenotte M: RAR-independent RXR signalling induced t(15;17) leukemia cell maturation. EMBO J 18, 7011 – 7018 (1999)
14 Rosati R, Rammnath N, Adil MR, Ou X, Ali MA, Heyman RA and Kalemkerian GP: Activity of 9-cis-retinoic acid and receptor-selective retinoids in small cell lung cancer cell lines. Anti-cancer Res 18, 4071 – 4075 (1998)
15 Na SY, Kang BY, Chung SW, Han SJ, Ma X, Trinchieri G, Im SY, Lee JW and Kim TS: Retinoids inhibit interleukin-12 production in macrophages through physical associations of retinoid X receptor and NFκB. J Biol Chem 272, 47674 – 47680 (1999)
16 Lenhard JM, Lancaster ME, Paulik MA, Weiel JE, Binz JG, Sundseth SS, Gaskill BA, Lightfoot RM and Brown HR: The RXR agonist LG100268 causes hepatomegaly, improves glycemic control and decreases cardiovascular risk and cachexia in diabetic mice suffering from pancreatic beta-cell dysfunction. Diabetologia 42, 545 – 554 (1999)
17 Solomin L, Johansson CB, Zetterstrom RH, Bissonnette RP, Heyman RA, Olson L, Lundahl U, Frisen J and Perlmann T: Retinoid-X receptor signalling in the developing spinal cord. Nature 395, 398 – 402 (1998)
18 Kastner P, Mark M and Chambon P: Nonsteroid nuclear receptors: What are genetic studies telling us about their role in real life. Cell 83, 859 – 869 (1995)
19 Sucov HM, Dyson E, Gumeringer CL, Price J, Chien KR and Evans, RM: RXR alpha mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. Genes Dev 8, 1007 – 1018 (1995)
20 Kastner P, Mark M, Leid M, Gansmuller A, Chin W, Grondona JM, Decimo D, Krezel W, Dierich A and Chambon P: Abnormal spermato genesis in RXR beta mutant mice. Genes Dev 10, 80 – 82 (1996)
21 Yoshimura H, Nagai M, Hibi S, Kikuchi K, Abe S, Hida T, Higashi S, Hishinuma I and Yamanaka T: A novel type of retinoic acid receptor antagonist synthesis and structure-activity relationships of heterocyclic ring-containing benzoic acid derivatives. J Med Chem 38, 3163 – 3173 (1995)
22 Hibi S, Kikuchi K, Yoshimura H, Nagai M, Tai K and Hida T: Syntheses and structure-activity relationships of novel retinoid X receptor agonists. J Med Chem 41, 3245 – 3252 (1998)
23 Goto M, Yamada K, Katayama K and Tanaka I: Inhibitory effect of E3330, a novel quinone derivative able to suppress tumor necrosis factor-α generation, on activation of nuclear factor-κB. Mol Pharmacol 49, 860 – 873 (1996)
24 Apfel C, Bauer F, Cretzaz M, Forni L, Kamber M, Kaufmann F, LeMotte P, Pirson W and Klaus M: A retinoic acid receptor antagonist selectively counteracts retinoic acid effects. Proc Natl Acad Sci USA 89, 7129 – 7133 (1992)
25 Lala DS, Mukherjee R, Schulman IG, Canon Koch SS, Dardashiti LJ, Nadzan AM, Croston GE, Evans RM and Heyman RA: Activation of specific RXR heterodimers by an antagonist of RXR homodimers. Nature 383, 450 – 453 (1996)
26 Ueno H, Kizaki M, Matsushita H, Muto A, Yamato K, Nishihara T, Hida T, Yoshimura H, Koefler HP and Ikeda Y: A novel retinoic acid receptor (RAR)-selective antagonist inhibits differentiation and apoptosis of HL-60 cells: implications of RARα-mediated signals in myeloid leukemic cells. Leukemia Res 22, 517 – 525 (1998)
27 Nagy L, Thomazy VA, Shipley GL, Fusus WK, Lamph W, Heyman RA, Chandraartana RA and Davies PA: Activation of retinoid X receptors induces apoptosis in HL-60 cell lines. Mol Cell Biol 15, 3540 – 3551 (1995)
28 Nervi C, Grippo JF, Sherman MI, George MD and Jetten AM: Identification and characterization of nuclear retinoic acid-binding activity in human myeloblastic leukemia HL-60 cells. Proc Natl Acad Sci USA 86, 5854 – 5858 (1989)
29 Breitman TR, Solenick SE and Collins SJ: Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoid acid. Proc Natl Acad Sci USA 77, 2936 – 2940 (1980)
30 Park JR, Robertson K, Hickten DD, Tsai S, Hockenbery DM and Collins J: Dysregulated bcl-2 expression inhibits apoptosis but not differentiation of retinoic acid-induced HL-60 granulocytes. Blood 84, 440 – 445 (1994)
31 Kizaki M, Ikeda Y, Tanosaki R, Nakajima H, Morikawa M, Sakashita A and Koefler H: Effects of novel retinoid acid compound, 9-cis-retinoic acid, on proliferation, differentiation, and expression of retinoic acid receptor-alpha and retinoid X receptor-alpha RNA by HL-60 cells. Blood 82, 3592 – 3599 (1993)
32 Sucov HM, Murakami KK and Evans RM: Characterization of an autoregulated response element in the mouse retinoic acid receptor type beta gene. Proc Natl Acad Sci USA 87, 5392 – 5396 (1990)
33 de The H, Vivanco-Ruiz dMM, Tiolais P, Stunnenberg H and
Dejean A: Identification of retinoic acid responsive element in the retinoic acid receptor β gene. Nature 343, 177 – 180 (1990)

Redfern PFC, Lovat PE, Malcolm AJ and Pearson ADJ: Differential effects of 9-cis and all-trans retinoic acid on the induction of retinoic acid receptor-β and cellular retinoic acid-binding protein II in human neuroblastoma cells. Biochem J 304, 147 – 154 (1994)

Fahlman C, Jakobsen SE, Smeland EB, Lomo J, Naess CE, Funderud S and Blomhoff HK: All-trans- and 9-cis-retinoic acid inhibit growth of normal human and murine B cell precursors.

J Immunol 155, 58 – 65 (1995)

Mangelsdorf DJ and Evans RM: The RXR heterodimers and orphan receptors. Cell 83, 841 – 850 (1995)

Kersten S, Dong D, Lee Wy, Reczek PR and Noy N: Auto-silencing by the retinoic X receptor. J Mol Biol 284, 21 – 32 (1998)

Dong D and Noy N: Heterodimer formation by retinoid X receptor: regulation by ligands and by the receptor’s self-association properties. Biochemistry 37, 10691 – 10700 (1998)