JLB

Article

All-*trans* retinoic acid and a novel synthetic retinoid tamibarotene (Am80) differentially regulate CD38 expression in human leukemia HL-60 cells: possible involvement of protein kinase C-δ

Akira Uruno, Naoya Noguchi,* Ken Matsuda,* Koji Nata,* Takeo Yoshikawa,* Youichiro Chikamatsu,* Hiroyuki Kagechika,† Hideo Harigae,* Sadayoshi Ito,* Hiroshi Okamoto,* and Akira Sugawara*,[1]*

*Department of Advanced Biological Sciences for Regeneration, Tohoku University Graduate School of Medicine, Sendai, Japan; and †School of Biomedical Science, Tokyo Medical and Dental University, Tokyo, Japan

RECEIVED JANUARY 12, 2009; REVISED FEBRUARY 15, 2011; ACCEPTED FEBRUARY 16, 2011. DOI: 10.1189/jlb.0109025

ABSTRACT

ATRA and a synthetic RAR agonist tamibarotene (Am80) induce granulocytic differentiation of human acute leukemia HL-60 cells and have been used in antineoplastic therapy. ATRA induces CD38 antigen during HL-60 cell differentiation, which interacts with CD31 antigen on the vascular EC surface and may induce disadvantages in the therapy. We here examined the mechanisms of the ATRA-mediated CD38 induction and compared the difference between ATRA- and tamibarotene-mediated induction. Tamibarotene-induced HL-60 cell adhesion to ECs was 38% lower than ATRA, and NB4 cell adhesion to ECs by tamibarotene was equivalent to ATRA, which induced CD38 gene transcription biphasically in HL-60 cells, the early-phase induction via DR-RARE containing intron 1, and the delayed-phase induction via RARE lacking the 5′-flanking region. In contrast to ATRA, tamibarotene induced only the early-phase induction, resulting in its lower CD38 induction than ATRA. A PKCδ inhibitor, rottlerin, and siRNA-mediated PKCδ knockdown suppressed the ATRA-induced CD38 promoter activity of the 5′-flanking region, whereas a RAR antagonist, LE540, or RAR knockdown did not affect it. Cycloheximide and rottlerin suppressed the delayed-phase induction of CD38 expression by ATRA but did not affect the early-phase induction. Moreover, ATRA, but not tamibarotene, induced PKCδ expression without affecting its mRNA stability. The diminished effect of tamibarotene on CD38-mediated HL-60 cell adhesion to ECs compared with ATRA is likely a result of the lack of its delayed-phase induction of CD38 expression, which may be advantageous in antineoplastic therapy. *J. Leukoc. Biol.* 90: 235–247; 2011.

Introduction

CD38, a type II single-chain transmembrane glycoprotein, is widely represented on lymphoid and myeloid lineages but absent from most mature resting lymphocytes [1, 2]. CD38 is widely used as a marker to study T and B lymphocyte activation and differentiation [3] and acts as a ligand of the CD31 molecule on vascular ECs [4]. ATRA potently increases CD38 in myeloid HL-60 cells during granulocytic differentiation [5], and the induced CD38 expression increases adhesion of differentiated myeloid cells to vascular ECs because of the protein–protein interaction between CD31 and CD38 molecules [6]. We previously reported the human CD38 promoter sequence and genome structure [7]. The first intron of the human CD38 gene contains a DR-RARE, and its ATRA-mediated transcription has been analyzed [8]. On the other hand, the role of the human CD38 gene 5′-flanking region lacking any DR5-RARE [7] in the ATRA-mediated transcription remains unknown.

Tamibarotene (Am80) is a synthetic RARα/β agonist containing chemical and pharmacological advantages over ATRA, including higher chemical stability, a lower affinity for cellular RA-binding protein, and an absence of affinity to RARγ, which is expressed abundantly in dermal epithelium [9]. Although ATRA induces granulocytic differentiation of HL-60 cells in medium, with or without serum, tamibarotene induces their

Abbreviations: 9cRA=9-*cis* retinoic acid, APL=acute promyelocytic leukemia, ATF=activating transcription factor, CLL=chronic lymphocytic leukemia, DCFH-DA=2′,7′-dichlorodihydrofluorescein diacetate, DR=direct repeat, EBM-2=endothelial basal medium 2, EC=endothelial cell, HMVEC-L=human lung microvascular endothelial cell, MFI=mean fluorescence intensity, RAR=retinoic acid receptor, RARE=retinoic acid response element, RAS=retinoic acid syndrome, RXR=retinoid X receptor, siRNA=small interfering RNA, SLE=systemic lupus erythematosus, SNP=single nucleotide polymorphism

1. Correspondence: Department of Advanced Biological Sciences for Regeneration, Tohoku University Graduate School of Medicine, 2-1, Seiryo-machi, Aoba-ku, Sendai, 980-8575, Japan. E-mail: akiras2i@med.tohoku.ac.jp.

granulocytic differentiation only in medium supplemented with serum [10], indicating their different characteristics in granulocytic differentiation. In the present study, we focused on the differential effects between ATRA and tamibarotene on the adhesion of differentiated HL-60 cells to ECs and the CD38 induction and examined the mechanisms of CD38 up-regulation in the cells. We here demonstrated the decreased adhesion of tamibarotene-differentiated HL-60 cells to ECs compared with that of ATRA-differentiated HL-60 cells, most likely as a result of their difference in CD38 gene transcription regulation.

MATERIALS AND METHODS

Reagents

ATRA and 9cRA, cycloheximide, and PMA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tamibarotene, HX630, LE540, and HX531 were described previously [11, 12]. CD2019 was kindly gifted by Dr. Soichi Kojima (Molecular Cellular Pathology Research Unit, Riken, Wako, Japan). CD437 was purchased from Tocris (Ellisville, MS, USA). Ro-31-8220, rottlerin, Go6976, and DCFH-DA were purchased from Calbiochem (Gibbstown, NJ, USA). Actinomycin D was purchased from Wako Pure Chemical (Osaka, Japan). Calcein-AM was purchased from Dojindo (Kumamoto, Japan).

Cell culture

Cells were cultured in a humidified incubator at 37°C with 5% CO_2. HL-60 and NB4 cells were provided by Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and were grown with RPMI 1640 supplemented with 10% FBS and penicillin-streptomycin-glutamine (Invitrogen, Carlsbad, CA, USA). HMVECs-L were purchased from Takara Bio (Ohtsu, Japan) and were grown with EBM-2 (Takara Bio), supplemented with 2% FBS, 10 ng/mL human epithelial growth factor, 5 ng/mL FGF-2, 1 μg/mL hydrocortisone, 10 μg/mL heparin, 50 μg/mL gentamicin, and 50 ng/mL amphotericin-B. HMVECs-L were used between the third and fourth passages.

Adhesion assay

HMVECs-L were seeded in 24-well plates at 0.5×10^5 cells/well 48 h before assay and were incubated with EBM-2 supplemented with 2% FBS. HL-60 and NB4 cells were treated with or without ATRA or tamibarotene in RPMI 1640 supplemented with 2% resin and charcoal-treated (stripped) FBS for the indicated times. At the end of the incubation period, 5 μg/mL calcein-AM and 1 μg/mL mouse IgG1 [mouse monoclonal anti-CD38 (clone: 0.N.109, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-integrin β2 (clone: 3H1050, Santa Cruz Biotechnology), or negative control mouse IgG1 (Dako, Glostrup, Denmark)] were added to the media. After incubation for 1 h, cells were washed twice with 10 mL PBS. The calcein-AM-labeled cells were suspended with RPMI 1640 supplemented with 2% FBS, and 5×10^5 cells were seeded onto the HMVEC-L monolayers. The cells were cocultured for 4 h. After washing the cells twice with 1 mL PBS, the bound cells were incubated with 0.2 mL PBS containing 5 mmol/L EDTA for 15 min at room temperature, and the detached cells were transferred to 96-multiwell dishes. Their fluorescent intensities were determined using a Fluoroskan Ascent fluorescent multiplate reader (ThermoLab Systems, Mainz, Germany) and were normalized by the fluorescent intensity of 1×10^5 calcein-AM-labeled cells.

Flow cytometry

HL-60 and NB4 cells were treated with or without retinoids in RPMI 1640 supplemented with 2% stripped FBS, and 1×10^6 cells were stained by FITC-conjugated anti-CD38 or CD11b mAb (BD PharMingen, San Diego, CA, USA) in PBS for 30 min on ice (1:20 dilution for CD38 and 1:10 dilution for CD11b). The stained cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), acquiring at least 10,000 events/sample. Data were analyzed using CellQuest software (BD Biosciences) and were expressed as histograms of the fluorescence intensity versus the cell number or as the MFI.

RNA preparation and real-time PCR

HL-60 and NB4 cells were treated with or without retinoids in RPMI 1640 supplemented with 2% stripped FBS for the indicated times. For determination of the mRNA stability, cells treated with retinoids were thereafter, incubated with actinomycin D (5 μg/mL) for an additional 3 h before harvesting. Total RNAs were extracted with the FastPure RNA kit (Takara Bio), according to the manufacturer's instructions. The extracted RNAs were then subjected to RT reaction using the RNA PCR kit (avian myeloblastosis virus), Version 3.0 (Takara Bio), with random 9-mer primer, according to the manufacturer's instructions. Thereafter, the obtained templates (200 ng) were used for real-time PCR (95°C, 3 min for one cycle; 95°C, 15 s; 60°C, 10 s; 72°C, 30 s for 40 cycles) with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) by a DNA engine thermal cycler attached to a Chromo4 detector (Bio-Rad). The following primer sequences were used: CD38 (forward AGAGCCCAACTCTGTCTTGG, reverse CTGAGTTCCCAACTTCATTA; PCR product: 285 bp); GAPDH (forward CCATGGAGAAGGCTGGGG, reverse CAAAGTTGTCATGGATGACC; PCR product: 195 bp); CD11b (forward ACCCCTGGTTCACCTCCTTC, reverse CATGACATAAGGTCAAGGCTG; PCR product: 88 bp); RARα (forward GGACATTGACCTCTGGGACA, reverse AAGGTCATGGTGTCCTGCTC; PCR product: 198 bp); RARβ (forward GGTTTCACTGGCTTGACCAT, reverse TTGGCAAAGGTGAACACAAG; PCR product: 200 bp); RARγ (forward GGCTGTGGGACAAGTTCAGT, reverse GTCGGAGAAGGTCATGGTGT; PCR product: 197 bp); PKCδ (forward ATTATCCCCGCTGGATCAC, reverse CTCTTGGTTGGTTCCCTTTC; PCR product: 73 bp).

Respiratory burst activity

HL-60 and NB4 cells were treated with or without ATRA or tamibarotene in RPMI 1640 supplemented with 2% stripped FBS for the indicated times. DCFH-DA at 10 μmol/L, with or without PMA at 100 ng/mL, was added to the cells, which were then incubated for 3 h at 37°C. The fluorescent intensity of the cells was analyzed with a FACSCalibur flow cytometer acquiring at least 10,000 events/sample. Data were analyzed using CellQuest software (BD Biosciences) and were expressed as histograms of the fluorescence intensity versus the cell number or as the MFI.

Plasmids

Newly subcloned chimeric constructs containing the human CD38 genomic DNA and the luciferase cDNA (pGL3-Basic, Promega, Madison, WI, USA) were used for the transient transfection studies: –1731/+75-luc (harboring the CD38 gene 5′-flanking region from –1731 to +75, relative to the transcription start site upstream of the luciferase cDNA in pGL3-Basic); –97/+75-luc; –47/+75-luc; –14/+75-luc; –47/+43-luc; –47/+6-luc; –1731/+75-luc-+309/+1078 (harboring the CD38 gene intron 1 downstream of the luciferase cDNA in –1731/+75-luc). Moreover, seven bases of the CD38 promoter (from –8 to –2 relative to the transcription start site) in –1735/+75-luc and –47/+43-luc were mutated from TTTCAGA to GAGTCCC (–1735/+75-mut-luc and –47/+43-mut-luc) using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). pCMV-β-gal was purchased from Clontech (Palo Alto, CA, USA).

siRNA

HiPerformance-validated siRNAs for RARα (SI00019376), RARβ (SI00019390), RARγ (SI00019425), and negative-control siRNA (sense 5′-UUCUCCGAACGUGUCACGUdTdT-3′ and antisense 5′-ACGUGACACGUUCGGAGAAdTdT-3′) were obtained from Qiagen (Valencia, CA, USA). siRNA for PKCδ (sense 5′-GGCUGAGUUCUGGCUGGACdTdT-3′

and antisense 5′-GUCCAGCCAGAACUCAGCCdTdT-3′) was synthesized and purified by Nihon Gene Research Laboratory (Japan).

Transfection and luciferase assay

HL-60 cells were washed with RPMI 1640, suspended in RPMI 1640 containing 1.25% DMSO at 5×10^7 cells/mL, and incubated for 30 min at room temperature. Cells (2×10^7) were electroporated with 18 μg reporter plasmids and 3 μg pCMV-β-gal using the Gene Pulser (Bio-Rad) at 300 V and a capacitance of 950 μF in a 4-mm electroporation cuvette. In some experiments, siRNAs were cotransfected at 250 nmol/L. The transfected cells were cultured further in RPMI 1640 containing 1.25% DMSO and 10% FBS at 37°C for 24 h or 48 h for siRNA. The media were changed to RPMI 1640 containing 2% stripped FBS, and cells were cultured further with or without retinoids at 37°C for the indicated times. After washing the cells with PBS, cell extracts were prepared using Glo Lysis buffer (Promega) and were analyzed for luciferase and β-gal activities. The transfection efficiency was normalized by the β-gal expression.

Western immunoblot analysis

HL-60 cells were treated with or without ATRA or tamibarotene in RPMI 1640 supplemented with 2% stripped FBS for 48 h. Total cellular extracts were prepared as described previously [13–15]. Samples were separated on a 10% SDS-polyacrylamide gel and transferred onto a PVDF membrane (Bio-Rad). The membranes were then blocked with 5% nonfat dry milk and probed with the primary antibody against PKCδ (Cell Signaling Technology, Danvers, MA, USA; 1:1000 dilution) or β-actin (Sigma-Aldrich; 1:3000 dilution) and thereafter, with HRP-conjugated antibody (GE Healthcare, Waukesha, WI, USA). The membranes were visualized with ECL-plus reagent (GE Healthcare) and a lumino-image analyzer LAS-1000 (Fujifilm, Tokyo, Japan).

Statistical analyses

All data are presented as mean ± SEM. Statistical analyses were performed with ANOVA, followed by Fisher's LSD post hoc test.

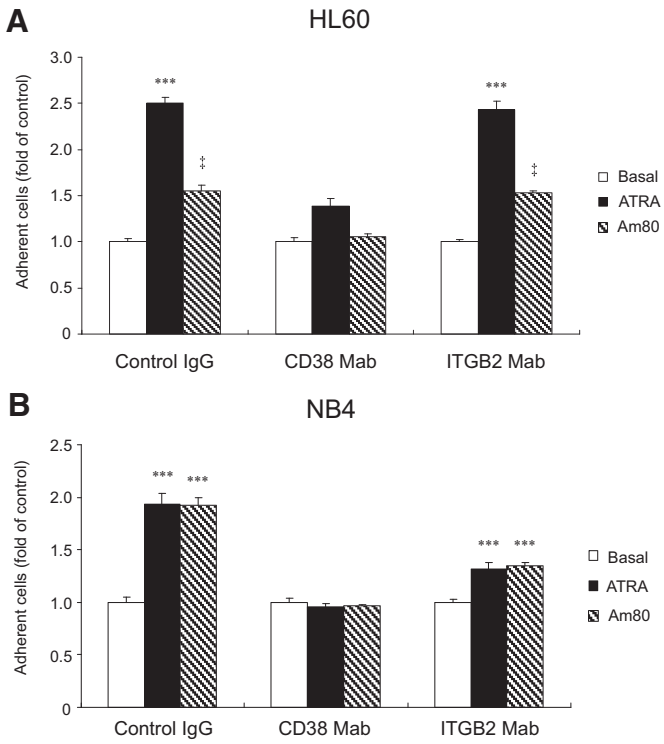

Figure 1. Effects of ATRA and tamibarotene on cell adhesion to ECs. HL-60 (A) or NB4 (B) cells were treated with 0.1% ethanol (Basal), 1 μmol/L ATRA, or 1 μmol/L tamibarotene (Am80) for 72 h. Thereafter, the cells were treated with negative-control mouse IgG1 (Control IgG), mouse anti-CD38, or mouse anti-β2-integrin (ITGB2) mAb for 1 h. Data represent mean ± SEM (n=6); the fluorescent intensity of calcein-AM-labeled HL-60 or NB4 cells bound to the HMVEC-L monolayer (0.1% ethanol with control mouse IgG1 group as 1.0). ***$P <$ 0.001 versus control; ‡$P < 0.001$ versus ATRA.

RESULTS

Differential effects between ATRA and tamibarotene on the CD38-mediated HL-60 cell adhesion to ECs

We performed an adhesion assay of HL-60 and NB4 cells stained with calcein-AM and HMVECs-L. As shown in **Fig. 1A**, incubation with ATRA for 72 h strongly augmented HL-60 cell adhesion to HMVECs-L (2.5-fold increase) in the presence of control IgG, and tamibarotene-induced cell adhesion was significantly (38%) lower than that of ATRA. There was no difference between HL-60 cells grown in RPMI 1640 supplemented with 2% FBS or RPMI 1640 supplemented with 10% FBS for cell growth determined by WST-8 or cell apoptosis determined by Annexin V (data not shown). Separate experiments demonstrated that ~3% of input cells adhered to ECs, in case control (undifferentiated) HL-60 cells were used (data not shown). In contrast, ATRA and tamibatotene only slightly induced HL-60 cell adhesion to ECs to a similar extent at 24 h (data not shown). Although treatment with anti-CD38 mAb almost completely abrogated ATRA- and tamibarotene-induced cell adhesions, treatment with anti-integrin β2 mAb did not affect them. As shown in Fig. 1B, ATRA and tamibarotene augmented NB4 cell adhesion to HMVECs-L (1.9-fold increase) to a similar extent in the presence of control IgG. Anti-CD38 mAb treatment completely suppressed ATRA- and tamibarotene-induced cell adhesions, and anti-integrin β2 mAb partially suppressed their adhesions. These results indicate the diminished effect of tamibarotene on CD38-mediated HL-60 cell adhesion to ECs compared with ATRA at 72 h, but not at 24 h, and ATRA and tamibarotene similarly affected NB4 cell adhesion to ECs.

Differential effects between ATRA and tamibarotene on CD38 induction in HL-60 cells

We next determined the CD38 expression by flow cytometry and quantitative real-time PCR. As shown in **Fig. 2A** and **B**, ATRA and tamibarotene induced CD38 antigen expression on HL-60 and NB4 cells to a similar extent at 24 h. However, CD38 antigen expression induced by tamibarotene on HL-60 cells at 72 h (open histogram) was less than that by ATRA (gray histogram; Fig. 2C), in contrast to NB4 cells, on which ATRA and tamibarotene induced CD38 expression to a similar extent (Fig. 2D). The MFI of HL-60 and NB4 cells using an isotypic IgG1 control (BD PharMingen) was not changed by treatment with ATRA or tamibarotene for 24 h or 72 h (data not shown). Fig. 2E shows the time-course analysis of the CD38 mRNA induction in HL-60 cells. High-dose (1 μmol/L)

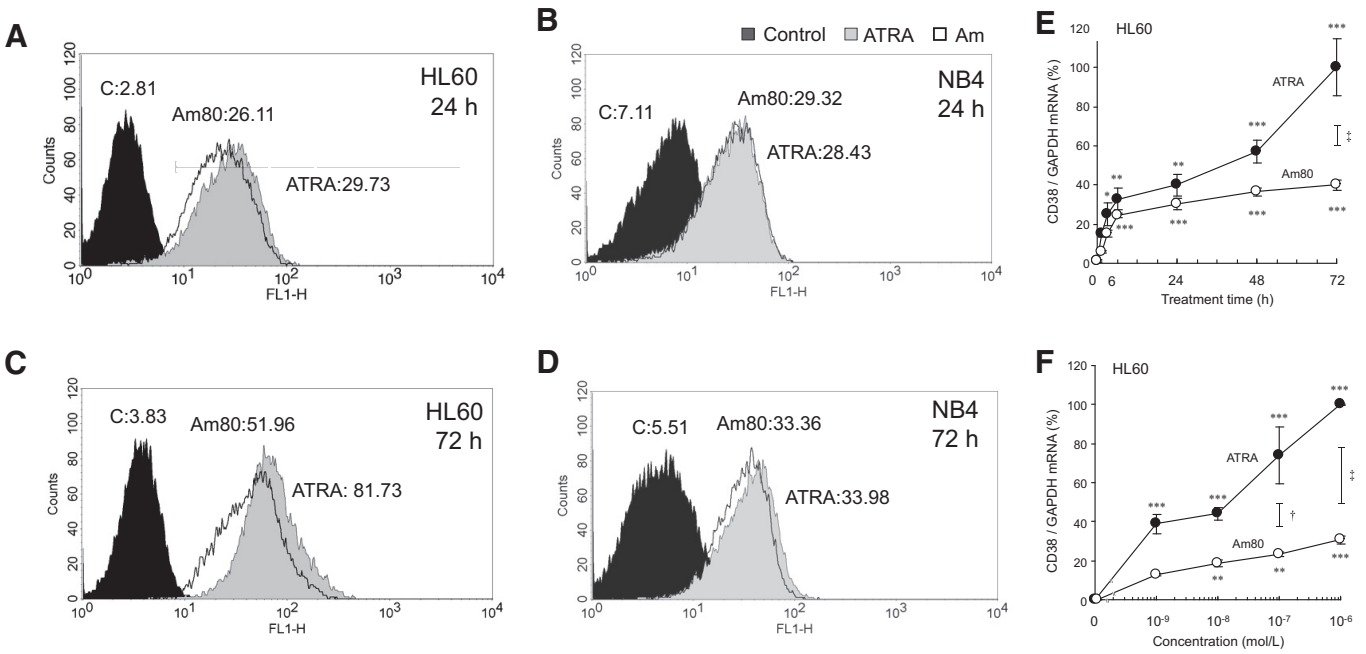

Figure 2. Effects of ATRA and tamibarotene on the CD38 expression. (A–D) Flow cytometry analyses using anti-CD38 mAb. HL-60 (A and C) or NB4 (B and D) cells were treated with 0.1% ethanol (Control, closed histograms), 1 μmol/L ATRA (gray histograms), or 1 μmol/L tamibarotene (Am80, open histograms) for 24 (A and B) or 72 h (C and D). The numbers in each histogram indicate the MFI. FL1-H=Fluorescence 1-height. (E) Time-course analyses of the CD38 mRNA induction. HL-60 cells were treated with ATRA (●) or tamibarotene (Am80; ○) at 1 μmol/L for the indicated times. Data represent mean ± SEM (n=6–9); percentage of the CD38 mRNA expression level normalized by the GAPDH mRNA expression level (ATRA at 72 h group as 100%). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus time 0; $‡P < 0.001$, ATRA versus tamibarotene. (F) Dose-response analyses of the CD38 mRNA induction. HL-60 cells were treated with ATRA (●) or tamibarotene (Am80; ○) at the indicated concentrations for 72 h. Data represent mean ± SEM (n=6–9); percent of the CD38 mRNA levels normalized by GAPDH mRNA levels (ATRA 10^{-6} mol/L group as 100%). $**P < 0.01$, $***P < 0.001$ versus 0 mol/L; $†P < 0.05$, $‡P < 0.001$, ATRA versus tamibarotene.

ATRA and tamibarotene induced the early-phase increase of CD38 mRNA expression at 1.5–6 h. However, although ATRA induced the delayed-phase increase of CD38 mRNA expression after 24 h, tamibarotene induced little increase after 6 h. As a result, the level of CD38 mRNA expression induced by tamibarotene at 72 h was ~40% of that by ATRA. When low-dose (10 nmol/L) ATRA was used, only monophasic induction of CD38 mRNA was observed (data not shown). Fig. 2F shows the dose-response analyses of the CD38 mRNA induction in HL-60 cells. ATRA significantly increased CD38 mRNA at 10^{-9} mol/L and more strongly so in a dose-dependent manner at 10^{-8}–10^{-6} mol/L. Tamibarotene augmented CD38 mRNA from 10^{-8} to 10^{-6} mol/L at the same level, and the tamibarotene-mediated CD38 mRNA expression was lower than that by ATRA at 10^{-7} and 10^{-6} mol/L. These results indicate that although ATRA, at its high dose, biphasically induces CD38 mRNA expression, tamibarotene monophasically induces it in HL-60 cells. In contrast, no difference between ATRA and tamibarotene on the CD38 induction was observed in NB4 cells.

Effects of ATRA and tamibarotene on myeloid cell differentiation

We next investigated the effects of ATRA and tamibarotene on myeloid cell differentiation to granulocytes. First, the respiratory burst activity was determined using DCFH-DA. ATRA slightly increased the fluorescent intensity of DCFH in the absence of PMA (**Fig. 3B**, closed histogram; MFI: 5.16) compared with the control group (Fig. 3A, closed histogram; MFI: 3.91) in HL-60 cells, and there was no increase by tamibarotene (Fig. 3C, closed histogram; MFI: 3.72). On the other hand, in the presence of PMA, the fluorescent intensity of DCFH was increased by ATRA (Fig. 3B, gray histogram; MFI: 33.38) and tamibarotene (Fig. 3C, gray histogram; MFI: 35.17) to a similar extent compared with the control group (Fig. 3A, gray histogram; MFI: 9.15) in HL-60 cells. In NB4 cells, the fluorescence intensity of DCFH was not altered by ATRA or tamibarotene in the absence of PMA (Fig. 3D–F, closed histograms). However, in the presence of PMA, tamibarotene induced stronger fluorescence intensity (Fig. 3F, gray histogram; MFI: 61.23) than ATRA (Fig. 3E, gray histogram; MFI: 54.63) in NB4 cells. We next examined CD11b expression in HL-60 and NB4 cells. CD11b antigen on HL-60 cells was increased slightly by ATRA (Fig. 3G, red histogram) or tamibarotene (Fig. 3G, blue histogram), and it was increased stronger by tamibarotene (Fig. 3H, blue histogram; MFI: 36.11) than ATRA (Fig. 3H, red histogram; MFI: 21.97) on NB4 cells. However, the MFI of HL-60 and NB4 cells using an isotypic IgG1 control was not changed by treatment with ATRA or tamibarotene (data not shown). ATRA significantly increased the CD11b mRNA expression levels in HL-60 cells, and only a

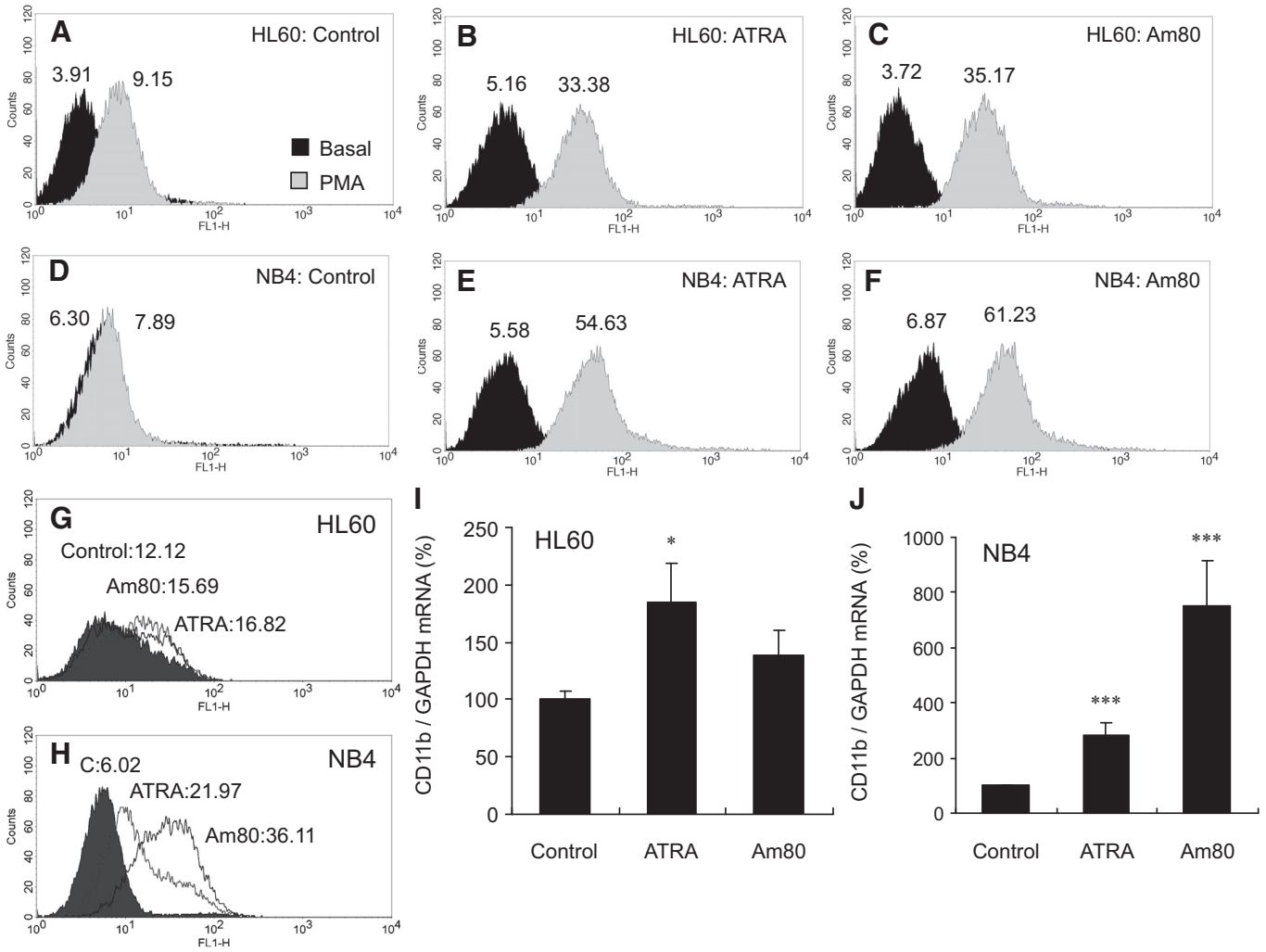

Figure 3. Effects of ATRA and tamibarotene on granulocytic differentiation. (A–F) Respiratory burst activity assessed by NADPH oxidase activity. HL-60 (A–C) or NB4 (D–F) cells were treated with 0.1% ethanol (Control; A and D), 1 μmol/L ATRA (B and E), or 1 μmol/L tamibarotene (Am80; C and F) for 72 h. Thereafter, 0.1% DMSO (Basal; closed histograms) or 100 ng/mL PMA (gray histograms) were added to the media, and the cells were incubated further for 3 h. The numbers in each histogram indicate the MFI of DCFH-DA-mediated fluorescent intensity. (G and H) Flow cytometry analyses using anti-CD11b mAb. HL-60 (G) or NB4 (H) cells were treated with 0.1% ethanol (Control; closed histograms), 1 μmol/L ATRA (gray line histograms), or 1 μmol/L tamibarotene (Am80; black line histograms) for 72 h. The numbers in each histogram indicate the MFI. (I and J) CD11b mRNA expression in HL-60 or NB4 cells. HL-60 (I) or NB4 (J) cells were treated with 0.1% ethanol (Control), 1 μmol/L ATRA, or 1 μmol/L tamibarotene (Am80) for 72 h. Data represent mean ± SEM (n=6); percent of the CD38 mRNA levels normalized by GAPDH mRNA levels (control as 100%). $^*P < 0.05$, $^{***}P < 0.001$ versus control.

weak induction was obtained by tamibarotene (Fig. 3I). On the other hand, the CD11b mRNA expression levels were more strongly induced by tamibarotene than by ATRA in NB4 cells (Fig. 3J). These data suggest that ATRA induces myeloid cell differentiation of HL-60 cells more potently than tamibarotene, and tamibarotene induces myeloid cell differentiation of NB4 cells more potently than ATRA.

ATRA increased CD38 promoter activity in HL-60 cells

To elucidate the mechanism by which ATRA biphasically induces CD38 in HL-60 cells, we next examined its promoter activity. As CD38 gene intron 1 contains a DR5-RARE [8], we constructed reporter vectors including the 5′-flanking region (–1731/+75) and/or intron 1 (+309/+1078) of the CD38 gene. As shown in **Fig. 4A**, ATRA increased the luciferase activity of –1731/+75-luc only at 24 h but not at 6 h. In contrast, the luciferase activity of –1731/+75-luc-+309/+1078 was increased at 6 h and 24 h. As shown in Fig. 4B, tamibarotene did not affect the luciferase activity of –1731/+75-luc at 6 h or 24 h, whereas that of –1731/+75-luc-+309/+1078 was increased by tamibarotene only at 6 h. The time-course analyses (Fig. 4C) revealed that ATRA increased the luciferase activity of –1731/+75-luc after 24 h. The dose-response analyses (Fig. 4D) revealed that ATRA increased the luciferase activity of –1731/+75-luc from 10^{-7} to 10^{-6} mol/L in a dose-dependent

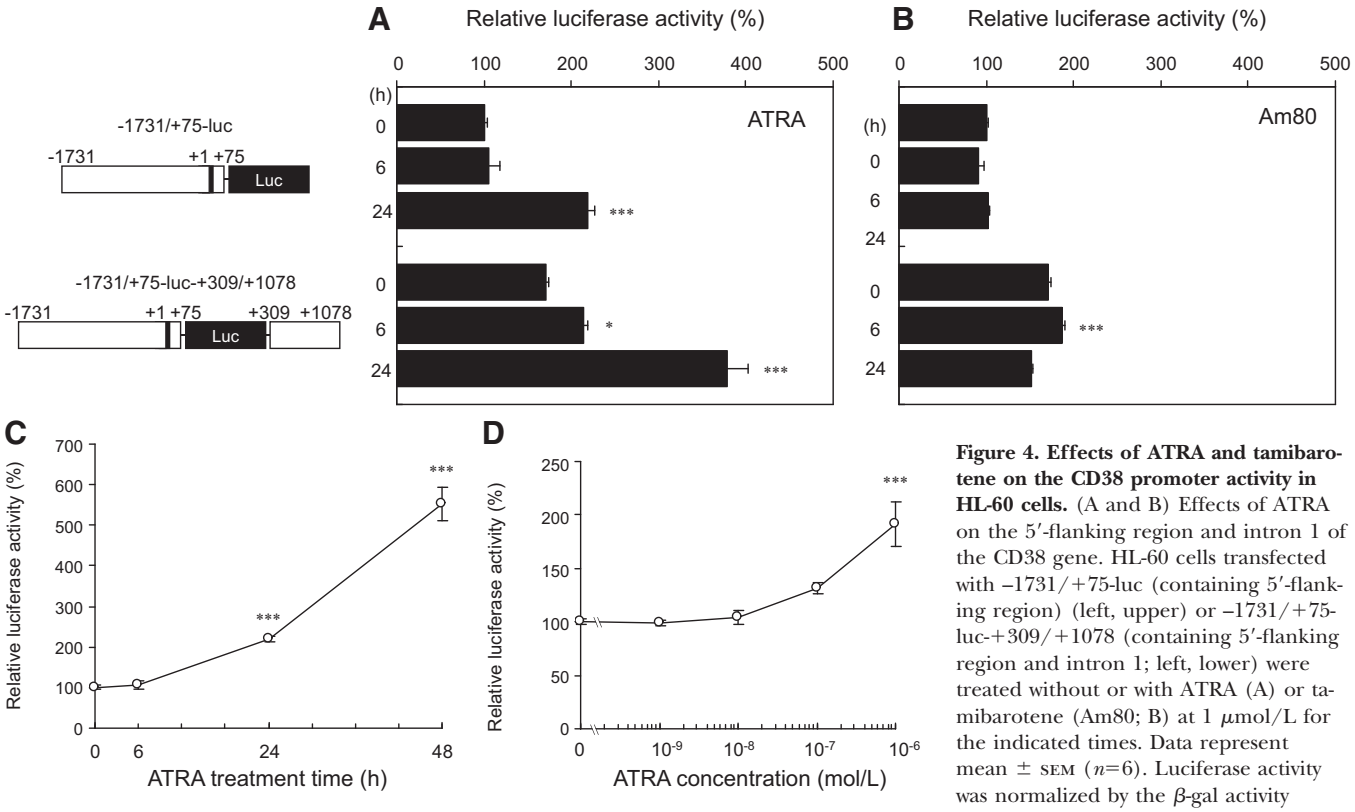

Figure 4. Effects of ATRA and tamibarotene on the CD38 promoter activity in HL-60 cells. (A and B) Effects of ATRA on the 5′-flanking region and intron 1 of the CD38 gene. HL-60 cells transfected with –1731/+75-luc (containing 5′-flanking region) (left, upper) or –1731/+75-luc-+309/+1078 (containing 5′-flanking region and intron 1; left, lower) were treated without or with ATRA (A) or tamibarotene (Am80; B) at 1 μmol/L for the indicated times. Data represent mean ± SEM (n=6). Luciferase activity was normalized by the β-gal activity (–1731/+75-luc at time 0 as 100%). *$P < 0.05$, ***$P < 0.001$ versus time 0. (C) Time-course analyses of the CD38 promoter activity. HL-60 cells transfected with –1731/+75-luc were treated with ATRA (1 μmol/L) for the indicated times. Data represent mean ± SEM (n=6); percentage of luciferase activity (time 0 as 100%). ***$P < 0.001$ versus time 0. (D) Dose-response analyses of the CD38 promoter activity. HL-60 cells transfected with –1731/+75-luc were treated with ATRA for 24 h at the indicated concentrations. Data represent mean ± SEM (n=6); percentage of luciferase activity (0 mol/L as 100%). ***$P < 0.001$ versus 0 mol/L.

manner. These results suggest that intron 1 of the CD38 gene is involved in the early response to ATRA (before 6 h), and the 5′-flanking region is involved in the delayed response (after 24 h).

ATRA increased CD38 promoter activity of the 5′-flanking region independently of RARs in HL-60 cells

As shown in **Fig. 5A**, ATRA and 9cRA increased the CD38 promoter activity of the 5′-flanking region in HL-60 cells, and tamibarotene [Am80 (RARα/β) agonist], CD2019 [(RARβ) agonist], CD437 [(RARγ) agonist], and HX630 [(RXR) agonist], as well as their combinations, did not affect it. Fig. 5B shows the expression of RAR isoforms in HL-60 cells. In the absence of ATRA, RARα and -γ were expressed, and RARβ was not. In the presence of ATRA, expression of all RAR isoforms was induced. The ATRA-induced increase of –1731/+75-luc activity was not affected by RAR antagonist LE540, RXR antagonist HX531, or their combination (Fig. 5C). siRNA-mediated knockdown of each RAR isoform (α/β/γ) as well as all isoforms did not affect the ATRA-induced increase of –1731/+75-luc activity (Fig. 5D). The element responsible for the ATRA-mediated activation of the CD38 gene 5′-flanking region was next localized. As shown in **Fig. 6A**, ATRA increased the luciferase activity of all indicated constructs, suggesting that the ATRA-response element in the 5′-flanking region was between –14 and +6 relative to the transcription start site, which did not contain any DR5-RARE. We next created mutated constructs of the 5′-flanking region and performed transient transfection studies. As shown in Fig. 6B, ATRA increased the luciferase activity in –1731/+75-luc and –47/+43-luc but not in –1731/+75-mut-luc and –47/+43-mut-luc. These results indicate that the ATRA-induced CD38 promoter activation of the 5′-flanking region is independent of RAR.

CD38 promoter activity of the 5′-flanking region increased by ATRA was dependent on PKCδ in HL-60 cells

Nonclassical RA signaling, independent of the conventional RAR/RXR pathway, has been demonstrated in PKCδ-mediated CREB protein activation [16]. Therefore, we next examined the effects of PKCδ on CD38 induction by ATRA. As shown in **Fig. 7A**, the ATRA-induced CD38 protein expression on HL-60 cells (MFI: 57.54) was strongly suppressed by a selective PKCδ inhibitor, rottlerin (MFI: 7.26), but not by a selective PKCα inhibitor, Go6976 (MFI: 52.20). However, the MFI of HL-60 cells using an isotypic IgG1 control was not affected by treatment with ATRA, ATRA/rottlerin, or ATRA/Go6976 (data not shown). As shown in Fig. 7B, the ATRA-induced CD38 mRNA expression was suppressed by rottlerin and a nonselective PKC inhibitor, Ro-31-8220, in a dose-dependent manner, but not by Go6976. The ATRA-induced –1731/+75-luc activity was sup-

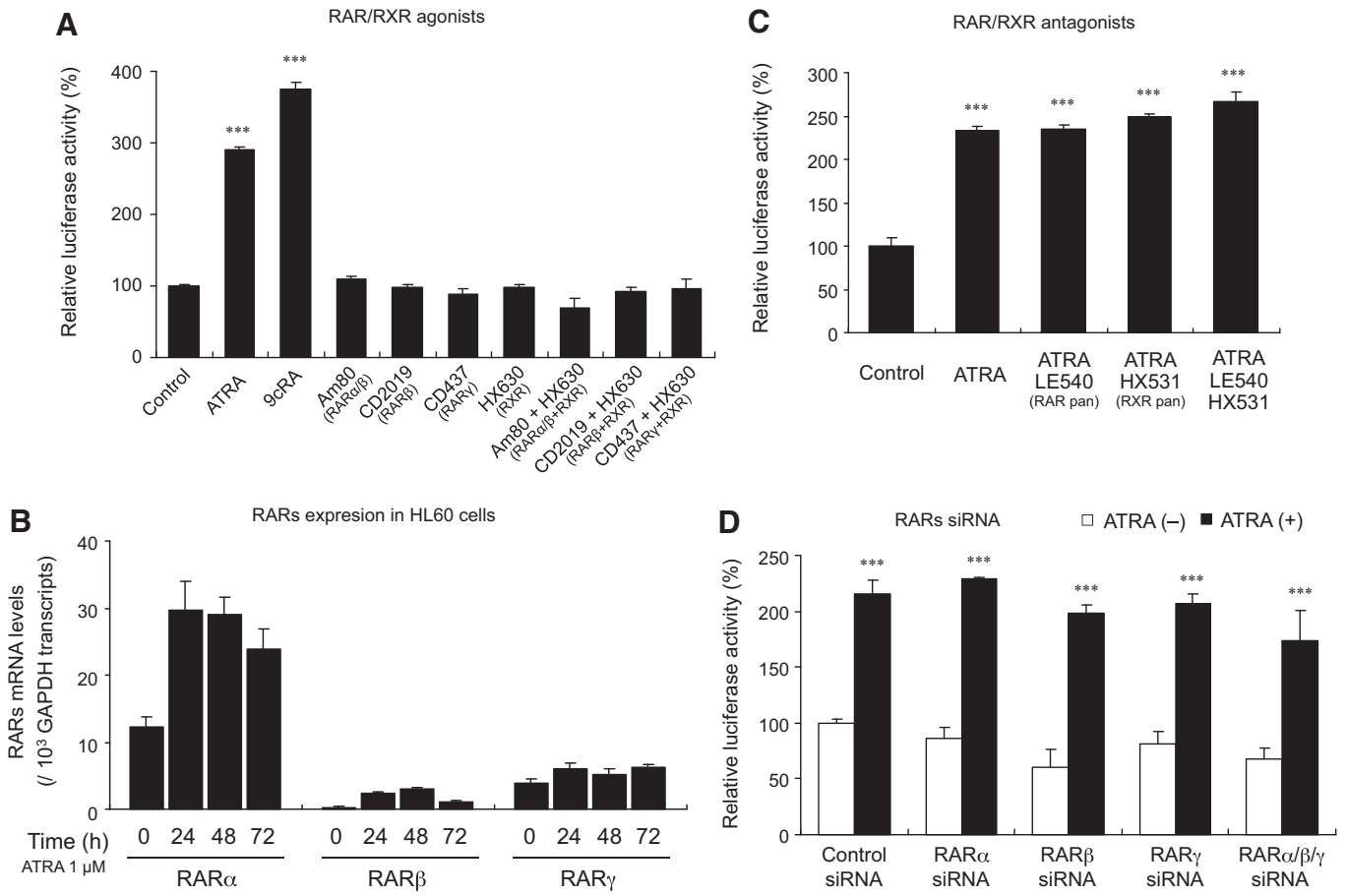

Figure 5. Effects of RAR on the CD38 promoter activity. (A) Effects of RAR/RXR agonists on the CD38 promoter activity of the 5′-flanking region. HL-60 cells transfected with –1731/+75-luc were treated with or without retinoid (1 μmol/L each) for 24 h. Data represent mean ± SEM (n=6); percentage of luciferase activity (control as 100%). ***$P < 0.001$ versus control. Am80 (RARα/β), Tamibarotene. (B) RAR mRNA expression. HL-60 cells were treated with ATRA (1 μmol/L) for the indicated times. Data represent mean ± SEM (n=3); RAR mRNA expression level normalized by the GAPDH mRNA expression level. (C) Effects of RAR/RXR antagonists. HL-60 cells transfected with –1731/+75-luc were treated without or with ATRA at 1 μmol/L in the absence or presence of RAR/RXR antagonists (1 μmol/L each) for 24 h. RAR/RXR antagonists were added to media 1 h prior to ATRA addition. Data represent mean ± SEM (n=6); percentage of luciferase activity (control as 100%). ***$P < 0.001$ versus control. (D) HL-60 cells transfected with RARα, RARβ, or RARγ siRNA, as well as –1731/+75-luc, were treated with (closed bars) or without (open bars) ATRA at 1 μmol/L for 24 h. Data represent mean ± SEM (n=6); percentage of luciferase activity (control siRNA without ATRA as 100%). ***$P < 0.001$ versus ATRA (–).

pressed by Ro-31-8220 and rottlerin in a dose-dependent manner but not by Go6976 (Fig. 7C). Moreover, siRNA-mediated knockdown of PKCδ suppressed the ATRA-induced –1731/+75-luc activity (Fig. 7D). The PKCδ expression in HL-60 cells was demonstrated to be diminished by siRNA (data not shown). These results indicate that the ATRA-induced CD38 promoter activation of the 5′-flanking region is mediated via PKCδ.

De novo protein synthesis and PKCδ were required for the delayed-phase increase of the ATRA-induced CD38 mRNA expression

We next examined whether de novo protein synthesis and PKCδ were necessary for the ATRA-induced CD38 mRNA expression. The ATRA-mediated CD38 mRNA increase at 6 h was not affected by cycloheximide (**Fig. 8A**). As shown in Fig. 8B, ATRA gradually increased CD38 mRNA from 48 h to 72 h in the absence of cycloheximide (open bars); however, 12 h treatment with cycloheximide (from 6 h to 18 h during ATRA treatment) completely abrogated the ATRA-mediated CD38 mRNA increase from 24 h to 72 h. As shown in Fig. 8C, although the ATRA-mediated CD38 mRNA induction was only weakly decreased by rottlerin at 6 h, it was suppressed significantly by rottlerin at 72 h. These results therefore indicate that de novo protein synthesis and PKCδ are involved in the delayed-phase increase of the ATRA-induced CD38 mRNA expression but not in its early-phase increase.

ATRA, but not tamibarotene, induced PKCδ expression in HL-60 cells

We next examined the effects of ATRA and tamibarotene on PKCδ expression in HL-60 cells. As shown in **Fig. 9A** and **B**, ATRA induced PKCδ protein and mRNA expression in HL-60

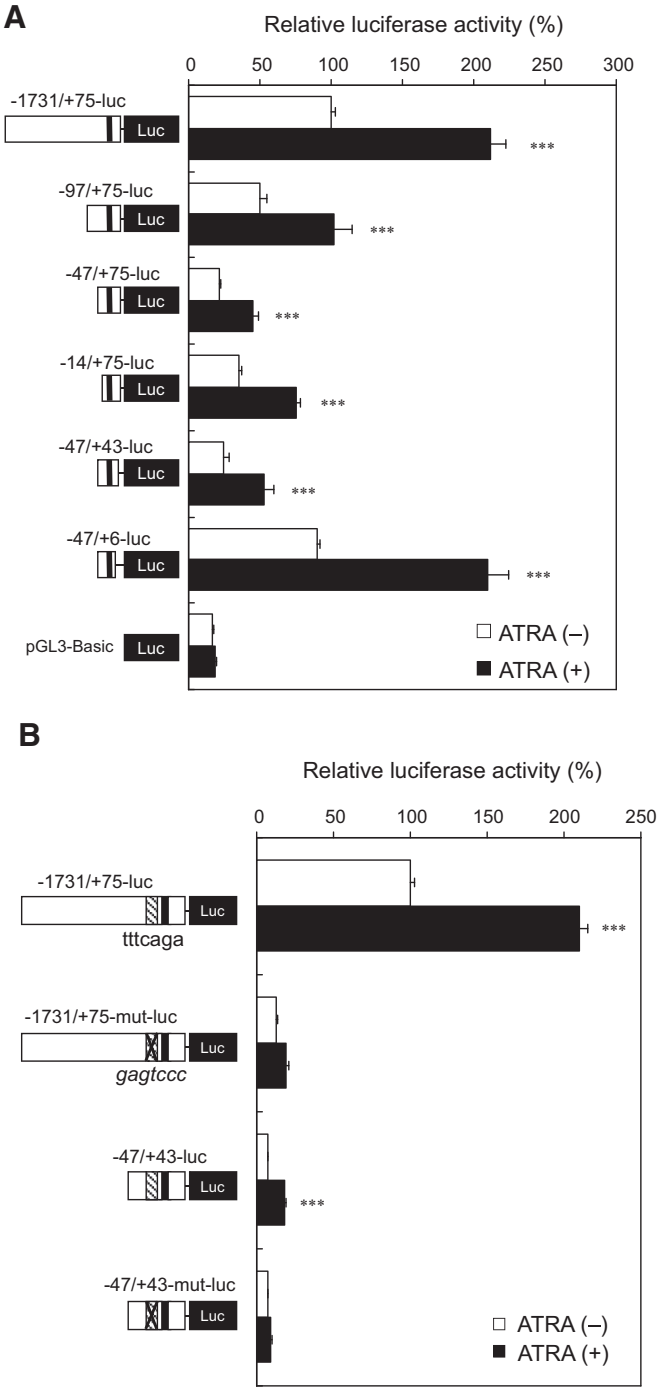

Figure 6. ATRA-response element in the CD38 gene 5′-flanking region. (A) Deletion mutant analyses of the CD38 gene 5′-flanking region. HL-60 cells transfected with reporter plasmids were treated with (closed bars) or without (open bars) ATRA at 1 μmol/L for 24 h. Data represent mean ± SEM (n=6); percentage of luciferase activity (–1731/+75-luc without ATRA as 100%). ***$P < 0.001$ versus ATRA (–). (B) Mutant analyses of the CD38 gene 5′-flanking region. HL-60 cells transfected with reporter plasmids were treated with (closed bars) or without (open bars) ATRA at 1 μmol/L for 24 h. Data represent mean ± SEM (n=6); percent of luciferase activity (–1731/+75-luc without ATRA as 100%). ***$P < 0.001$ versus ATRA (–).

cells, whereas tamibarotene did not. Time-course analyses revealed that ATRA increased PKCδ mRNA expression after 24 h (Fig. 9C). Dose-response analyses revealed that ATRA increased PKCδ mRNA expression significantly at 10^{-6} mol/L (Fig. 9D). The RNA stability of PKCδ evaluated by actinomycin D treatment was not altered by ATRA or tamibarotene (Fig. 9E). These results indicate that ATRA induces PKCδ expression without affecting its stability in HL-60 cells.

DISCUSSION

Retinoids, including ATRA and tamibarotene, both of which are known to induce granulocytic differentiation of HL-60 cells, have been used recently in the treatment of neoplasms. During HL-60 cell differentiation, ATRA induces CD38 antigen, which mediates HL-60 cell adhesion to ECs. The occurrence of granulocyte-EC adhesion during antineoplastic therapy is most likely deleterious for patients. Therefore, it is important to clarify the differential effects between ATRA and tamibarotene on the cell adhesion/CD38 expression for the future innovation of antineoplastic retinoid therapy. We demonstrated that tamibarotene-induced HL-60 cell adhesion to ECs was significantly lower than that by ATRA (Fig. 1). Although ATRA and tamibarotene acutely induced CD38 mRNA expression in HL-60 cells at 1.5–6 h (the early-phase induction), the delayed-phase induction after 24 h was only observed with ATRA (Fig. 2E). ATRA and tamibarotene induced the early response via stimulation of the CD38 gene intron 1 in HL-60 cells, and only ATRA, especially at its high concentration (1 μmol/L), induced the delayed response via the stimulation of the CD38 gene 5′-flanking region (Fig. 4A and D). Although a natural retinoid 9cRA also induced the CD38 promoter activity of the 5′-flanking region, synthetic RAR/RXR agonists, including tamibarotene, did not (Fig. 5A). Interestingly, the ATRA-induced CD38 promoter activity of the 5′-flanking region was entirely dependent on the activation of PKCδ in HL-60 cells (Fig. 7), whose expression was up-regulated by ATRA but not by tamibarotene (Fig. 9). ATRA could therefore cause a strong induction of the CD38 transcription by activating intron 1 containing a DR5-RARE in the early response and the 5′-flanking region through PKCδ induction in the delayed response, resulting in a strong induction of the CD38-mediated HL-60 cell adhesion to ECs. On the other hand, tamibarotene failed to induce PKCδ expression as well as the CD38 promoter activity of the 5′-flanking region and thus, caused only a weak induction of CD38 transcription by the activation of intron 1, resulting in a weak induction of the CD38-mediated HL-60 cell adhesion to ECs.

The early response of CD38 to ATRA and tamibarotene was mediated via intron 1 (Fig. 4A and B). These results were partially consistent with those of a previous report describing the involvement of a DR5-RARE in intron 1 of the CD38 gene in the ATRA-mediated CD38 induction and in vitro binding of the RAR/RXR heterodimer to the element [8]. That study therefore suggested that the ATRA-induced CD38 transcription via intron 1 was mediated thorough RAR. On the other hand, the delayed response of CD38 to ATRA was completely suppressed by cycloheximide or rottlerin (Fig. 8B and C).

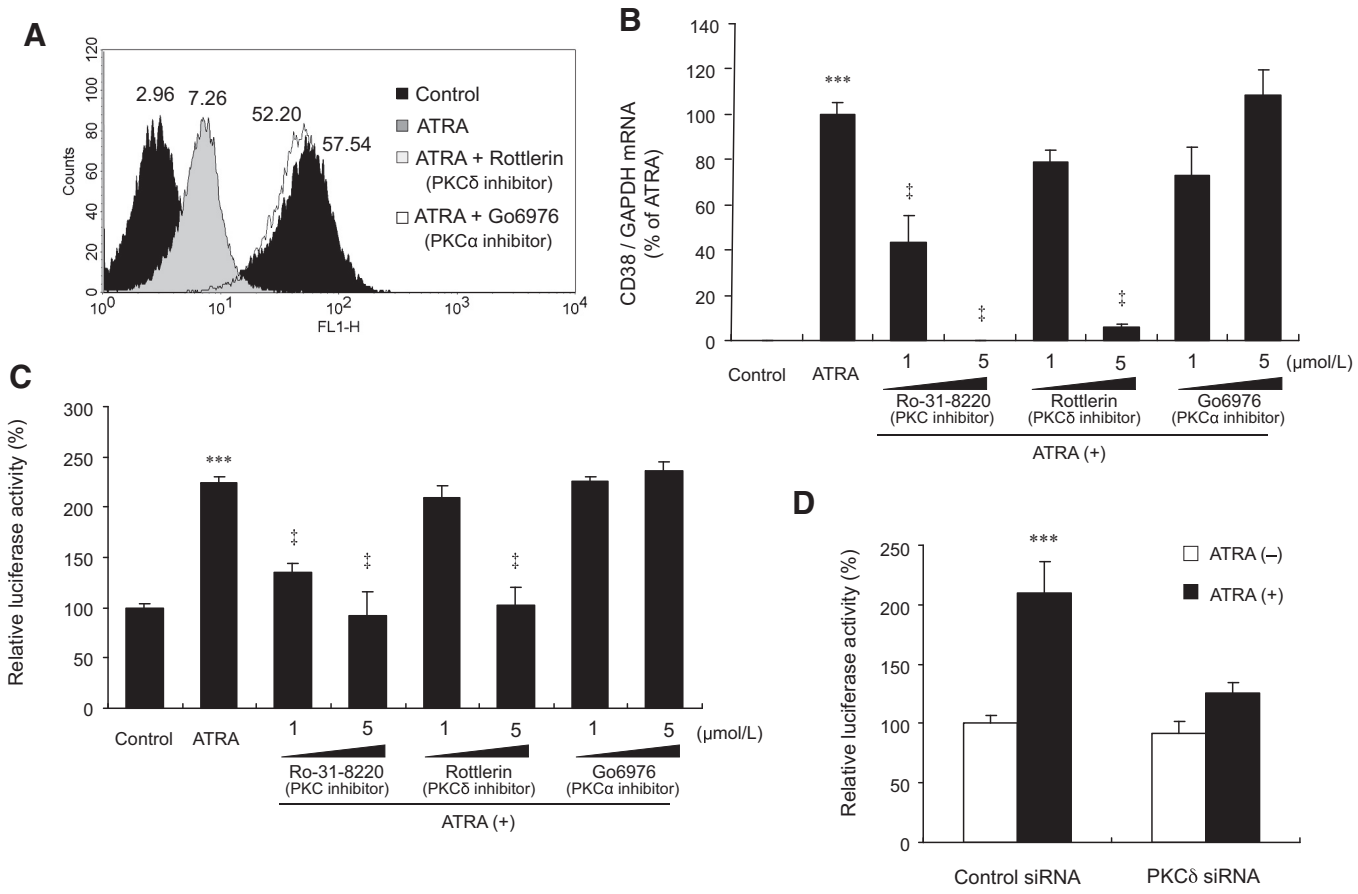

Figure 7. Involvement of PKCδ in the ATRA-induced CD38 expression. (A) Flow cytometry analyses using anti-CD38 mAb. HL-60 cells were treated with vehicle control (closed histograms), 1 μmol/L ATRA (dark-gray histograms), ATRA plus rottlerin (light-gray histograms), or ATRA plus Go6976 (open histograms) for 72 h. Rottlerin and Go6976 were added to media 30 min prior to ATRA addition at 5 μmol/L. The numbers in each histogram indicate the MFI. (B) Effects of PKC inhibitors on the CD38 mRNA expression. HL-60 cells were treated without or with ATRA at 1 μmol/L for 72 h in the absence or presence of PKC inhibitors. Ro-31-8220, rottlerin, and Go6976 were added to media at the indicated concentrations, 30 min prior to ATRA addition. Data represent mean ± SEM (n=6) of the CD38 mRNA expression level normalized by the GAPDH mRNA expression level; percentage of ATRA. ***$P < 0.001$ versus control; ‡$P < 0.001$ versus ATRA. (C) Effects of PKC inhibitors on the CD38 promoter activity of the 5′-flanking region. HL-60 cells transfected with –1731/+75-luc were treated without or with ATRA at 1 μmol/L in the absence or presence of PKC inhibitors for 24 h. Ro-31-8220, rottlerin, and Go6976 were added to media 30 min prior to ATRA addition. Data represent mean ± SEM (n=6); percentage of luciferase activity control as 100%). ***$P < 0.001$ versus control; ‡$P < 0.001$ versus ATRA. (D) HL-60 cells transfected with PKCδ siRNA as well as –1731/+75-luc were treated with (closed bars) or without (open bars) ATRA at 1 μmol/L for 24 h. Data represent mean ± SEM (n=7); percentage of luciferase activity (control siRNA without ATRA as 100%). ***$P < 0.01$ versus ATRA (–).

Moreover, ATRA was observed to increase the PKCδ protein expression (Fig. 9A). Although direct interaction between ATRA and PKC has been reported previously [17], our data suggest that the ATRA-induced CD38 promoter activation may be mediated via the de novo protein synthesis of PKCδ. RA-response genes can be classified into two groups, early RA-response genes, most of which contain one or more RAREs in their regulatory region, and delayed RA-response genes, most of which lack RAREs and require de novo protein synthesis for the activation of gene expression [18]. The CD38 gene therefore contains the characteristics of the early and delayed RA-response genes.

Tamibarotene partially induced the differentiation of HL-60 cells (AML M2 cell line; Fig. 3A–C, G, and I) [19] but less potently than by ATRA and with weaker cell adhesion to ECs than that by ATRA (Fig. 1A). On the other hand, tamibarotene induced the differentiation of NB4 cells (APL, AML M3 cell line) more potently than ATRA (Fig. 3D–F, H, and J) without increasing the cell adhesion to ECs compared with ATRA (Fig. 1B). In patients with APL, an increase in CD38 expression was observed in vivo following a single oral dose administration of ATRA [20]. CD38 probably plays an important role in the development of RAS [6], observed during differentiation-inducing therapy for APL by ATRA, with extensive infiltration of myeloid cells into organs, including lungs, skin, kidneys, liver, and LNs [21–23]. RAS was also observed during differentiation therapy by tamibarotene [24]. Based on data of AML M3 NB4 cells in this study, it is predicted that the incidence rate of RAS between ATRA and tamibarotene may not be different. However, if cell types other than M3 cells are in-

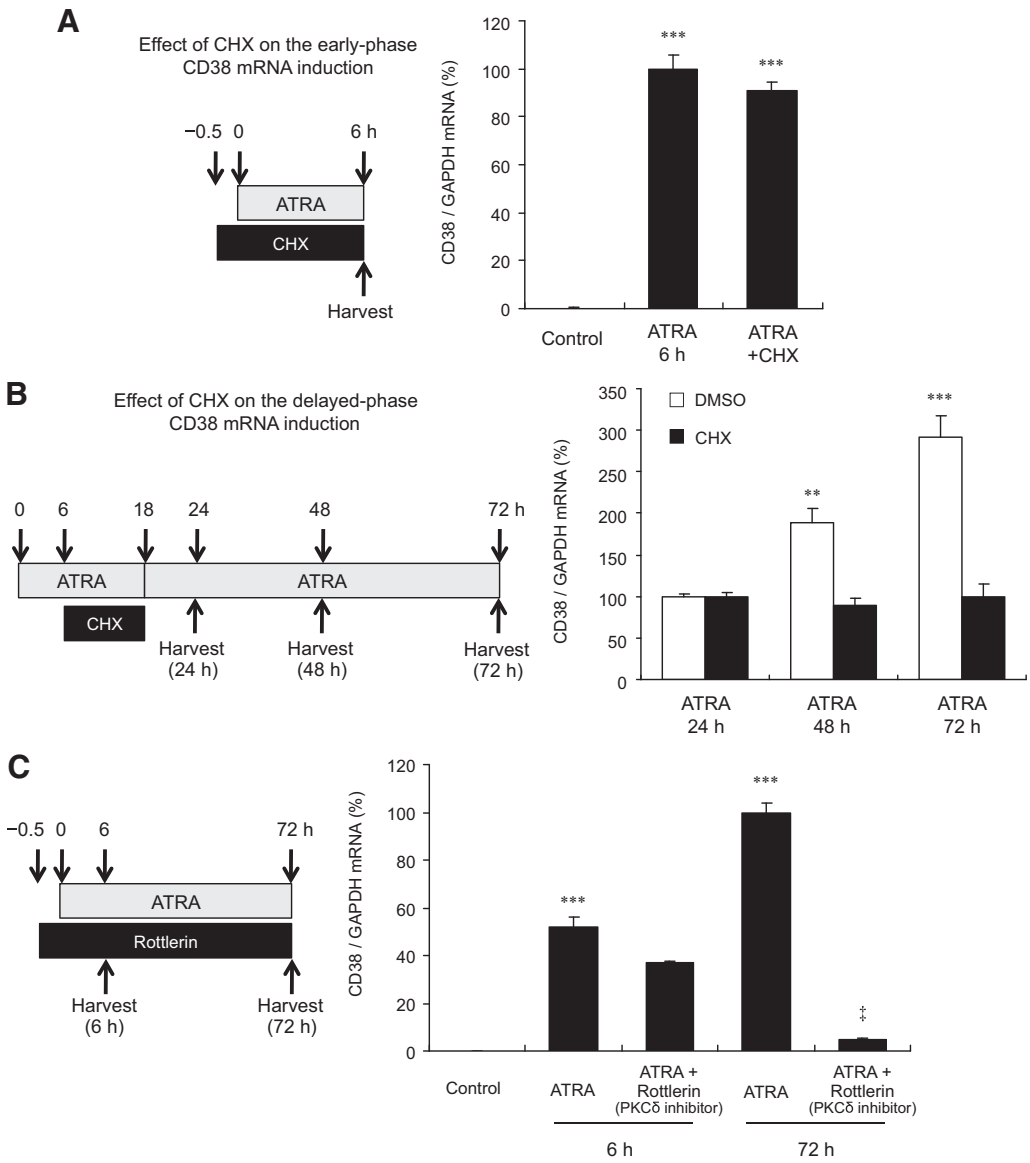

Figure 8. Involvement of PKCδ and de novo protein synthesis in the delayed-phase increase of the ATRA-induced CD38 mRNA expression. (A) Effects of cycloheximide (CHX) on the early-phase CD38 mRNA induction. HL-60 cells were treated without or with ATRA at 1 μmol/L in the absence or presence of cycloheximide (1 μg/mL) for 6 h. Cycloheximide was added to media 30 min prior to ATRA addition. Data represent mean ± SEM (n=6); percentage of the CD38 mRNA expression level normalized by the GAPDH mRNA expression level (ATRA as 100%). ***$P < 0.001$ versus control. (B) Effects of cycloheximide on the delayed-phase CD38 mRNA induction. HL-60 cells were incubated with 1 μmol/L ATRA for 18 h, and the cells were additionally treated with cycloheximide (1 μg/mL; closed bars) or DMSO (as vehicle control, 0.1%; open bars) during the last 12 h of the incubation period. Thereafter, media were freshly changed, and the cells were incubated further with ATRA for indicated times (total time from the first ATRA addition). Data represent mean ± SEM (n=6); percentage of the CD38 mRNA expression level normalized by the GAPDH mRNA expression level (ATRA at 24 h as 100%). *$P < 0.01$, ***$P < 0.001$ versus control. (C) Effects of rottlerin on the early- and delayed-phase CD38 mRNA induction. HL-60 cells were treated without or with ATRA at 1 μmol/L in the absence or presence of rottlerin (5 μmol/L) for the indicated times. Rottlerin was added to media 30 min prior to ATRA addition. Data represent mean ± SEM (n=6); percentage of the CD38 mRNA expression level normalized by the GAPDH mRNA expression level (ATRA at 72 h as 100%). ***$P < 0.001$ versus control; ‡$P < 0.001$ versus ATRA at 72 h.

volved in cell infiltration during RAS, tamibarotene is expected to improve its symptoms compared with ATRA. In addition to APL, retinoids have therapeutic potentials against solid tumors, including glioblastoma [25–27], neck cancer [28], prostate cancer [29], and adrenocorticotropic hormone-secreting pituitary adenoma [30, 31]. The incidence of RAS by retinoids in these solid tumors is uncertain. However, it is possible that retinoids induce CD38-positive granulocytes, which may adhere to ECs and infiltrate into organs, resulting in a RAS-like phenomenon. Our observation regarding HL-60 cells therefore indicates the advantage of tamibarotene for their treatment.

The ATRA-induced CD38 promoter activation of the 5′-flanking region was independent of RAR in HL-60 cells (Fig. 5), whose mechanism closely resembled that of ATRA-mediated CREB activation, independent of RAR [16]. In this study, ATRA increased PKCδ mRNA expression in HL-60 cells without affecting its stability, and tamibarotene failed to increase it (Fig. 9). Additionally, the PKCδ mRNA induction (Fig. 9D) and the stimulation of the CD38 promoter activity of the 5′-flanking region (Fig. 4D) were significant only at high concentration(s) of ATRA, further confirming the possible involvement of the induced PKCδ in the ATRA-mediated CD38 promoter activation. The induction of PKCδ expression has been demonstrated previously in several nuclear receptor ligands, including androgen, which activates the PKCδ gene promoter via the androgen receptor [32]. Our data, in contrast, suggest that ATRA may increase PKCδ mRNA expression via transcriptional regulation independently of RAR, as observed in the 1,25-dihydroxyvitamin D_3-mediated PKCδ expression increase

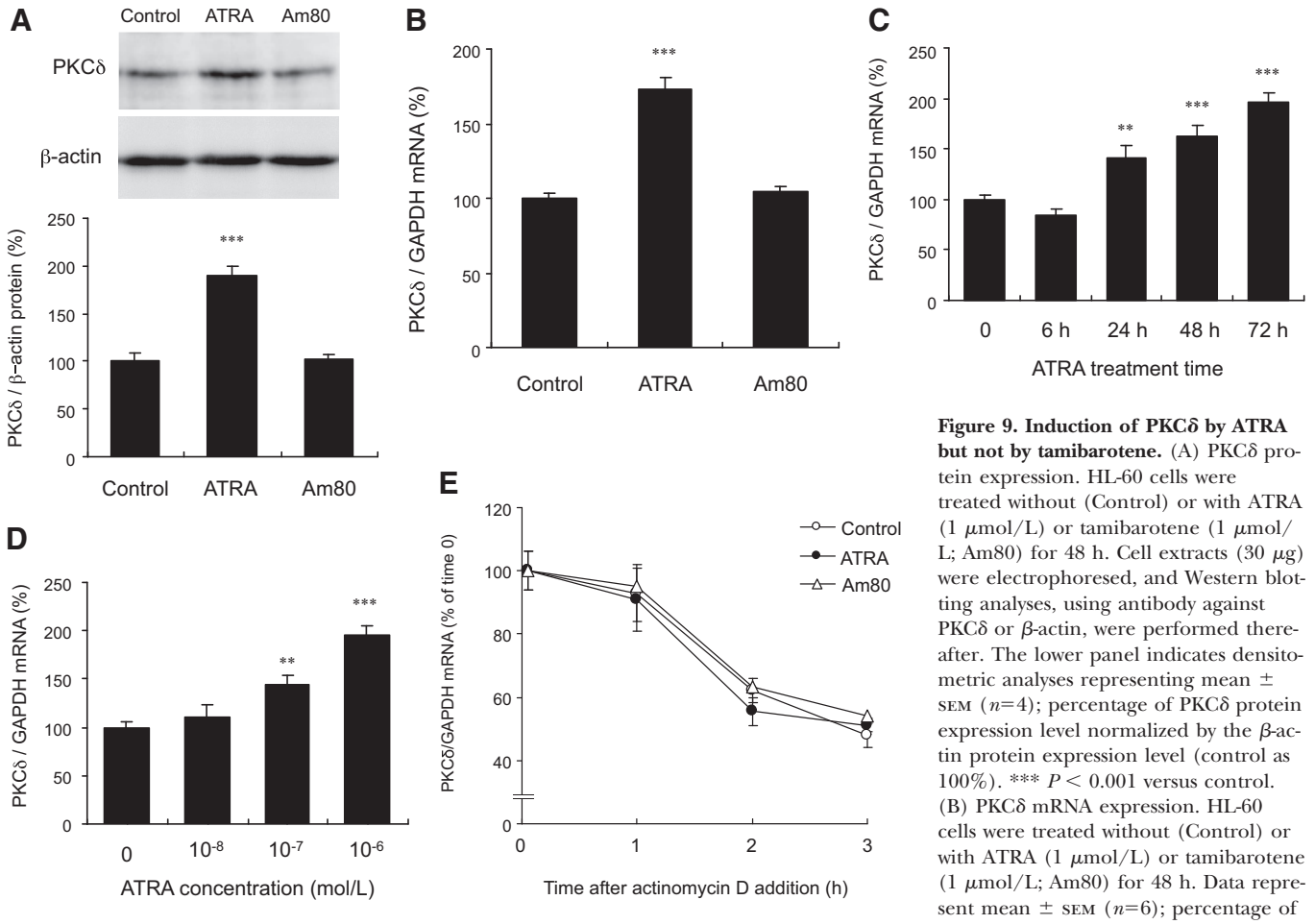

Figure 9. Induction of PKCδ by ATRA but not by tamibarotene. (A) PKCδ protein expression. HL-60 cells were treated without (Control) or with ATRA (1 μmol/L) or tamibarotene (1 μmol/L; Am80) for 48 h. Cell extracts (30 μg) were electrophoresed, and Western blotting analyses, using antibody against PKCδ or β-actin, were performed thereafter. The lower panel indicates densitometric analyses representing mean ± SEM (n=4); percentage of PKCδ protein expression level normalized by the β-actin protein expression level (control as 100%). *** $P < 0.001$ versus control. (B) PKCδ mRNA expression. HL-60 cells were treated without (Control) or with ATRA (1 μmol/L) or tamibarotene (1 μmol/L; Am80) for 48 h. Data represent mean ± SEM (n=6); percentage of the CD38 mRNA expression level normalized by the GAPDH mRNA expression level (control as 100%). *** $P < 0.001$ versus control. (C) Time-course analyses of the PKCδ mRNA expression. HL-60 cells were treated with ATRA (1 μmol/L) for the indicated times. Data represent mean ± SEM (n=6); percentage of the CD38 mRNA expression level normalized by the GAPDH mRNA expression level (time 0 as 100%). **$P < 0.01$, ***$P < 0.001$ versus time 0. (D) Dose-response analyses of the PKCδ mRNA expression. HL-60 cells were treated with ATRA for 72 h at the indicated concentrations. Data represent mean ± SEM (n=6); percentage of the CD38 mRNA expression level normalized by the GAPDH mRNA expression level (0 mol/L as 100%). **$P < 0.01$, ***$P < 0.001$ versus 0 mol/L. (E) CD38 mRNA stability examined by real-time PCR. HL-60 cells were incubated without (Control) or with ATRA (1 μmol/L) or tamibarotene (1 μmol/L; Am80) for 48 h and were incubated further with actinomycin D (5 μg/mL) for an additional 1, 2, or 3 h. Data represent mean ± SEM (n=3); percentage of the CD38 mRNA expression level normalized by the GAPDH mRNA expression level (time 0 as 100%).

that was independent of the vitamin D receptor in NB4 cells [33]. The PKCδ promoter activity is regulated by various transcription factors, including NF-κB [34]. ATRA has been reported to increase NF-κB p65 in HL-60 cells [35], which may possibly be involved in the ATRA-mediated PKCδ induction.

PKCδ is a serine/threonine kinase, which mediates important intracellular signaling pathways and is involved in the ATRA-mediated differentiation of myeloid cells [36, 37]. PKC is involved in diabetes mellitus complications [38]. PKCδ is activated by high glucose in glomeruli [39] and mesangial cells [40], suggesting its involvement in diabetic nephropathy. As tamibarotene did not up-regulate PKCδ expression (Fig. 9A and B), it may be more advantageous for diabetic patients than ATRA.

The response element for the PKCδ-mediated CD38 promoter activation by ATRA in its 5′-flanking region was from −8 to −2, relative to the transcription start site, which contains the putative C/EBPβ response sequence but not DR5-RAREs (Fig. 6A and B). As ATRA has been shown to increase C/EBPβ expression in HL-60 cells [35, 41], the ATRA-induced CD38 promoter activation of the 5′-flanking region may be mediated via C/EBPβ. PKCδ has been reported to interact with various transcription factors, including STAT1 [42], NF-E2-related factor 2 [43], ATF1 [44], ATF2, and NF-κB [45]. PKCδ has also been shown to be involved in the ATRA-mediated activation of RAREs via the formation of a PKCδ-containing, RARE-binding complex in ATRA-treated myeloid cells [36]. As the mechanism(s) by which PKCδ mediates the putative C/EBPβ-response element in the CD38 gene 5′-flanking region remain unclear, further studies are needed.

CD38 is also known to be involved in social behavior [46], and its decreased expression level has been reported in autism patients [47]. Interestingly, SNPs have been observed in the CD38 gene, including intron 1 in patients with autism [48].

The expression level of the CD38 gene may therefore be related to its SNPs. Another SNP in the CD38 gene locates within a restriction endonuclease *PvuII* site in intron 1 and is related to CLL [49], SLE [50], and osteoporosis [51]. Interestingly, the CD38 gene DR5-RARE in intron 1 locates from 670 to 686 bp downstream of the translation start site, which is close to the SNP related to CLL/SLE/osteoporosis, located at 416 bp downstream of the translation start site. Further studies are needed to clarify the relation between the SNP and CD38 expression level.

In summary, the diminished effect of tamibarotene on the CD38-mediated HL-60 cell adhesion to ECs compared with that of ATRA may possibly be a result of the lack of PKCδ induction and the delayed response of CD38 expression, which may be advantageous in antineoplastic therapy. These characteristic differences between tamibarotene and ATRA may affect their differential pharmacotherapeutic effects.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid (no. 17790545 for A.U., no. 16590898 for A.S., and no. 17390245 for S.I.), grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan (for A.S.), Takeda Science Foundation (for A.S.), and the 21st Century Centers of Excellence (COE) Program special research grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan. We thank Dr. S. Kojima (Molecular Cellular Pathology Research Unit, Riken, Wako, Japan) for providing us CD2019.

REFERENCES

1. Stevenson, G. T. (2006) CD38 as a therapeutic target. *Mol. Med.* **12,** 345–346.
2. Malavasi, F., Deaglio, S., Funaro, A., Ferrero, E., Horenstein, A. L., Ortolan, E., Vaisitti, T., Aydin, S. (2008) Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiol. Rev.* **88,** 841–886.
3. Jackson, D. G., Bell, J. I. (1990) Isolation of a cDNA encoding the human CD38 (T10) molecule, a cell surface glycoprotein with an unusual discontinuous pattern of expression during lymphocyte differentiation. *J. Immunol.* **144,** 2811–2815.
4. Deaglio, S., Morra, M., Mallone, R., Ausiello, C. M., Prager, E., Garbarino, G., Dianzani, U., Stockinger, H., Malavasi, F. (1998) Human CD38 (ADP-ribosyl cyclase) is a counter-receptor of CD31, an Ig superfamily member. *J. Immunol.* **160,** 395–402.
5. Drach, J., McQueen, T., Engel, H., Andreeff, M., Robertson, K. A., Collins, S. J., Malavasi, F., Mehta, K. (1994) Retinoic acid-induced expression of CD38 antigen in myeloid cells is mediated through retinoic acid receptor-α. *Cancer Res.* **54,** 1746–1752.
6. Gao, Y., Camacho, L. H., Mehta, K. (2007) Retinoic acid-induced CD38 antigen promotes leukemia cells attachment and interferon-γ/interleukin-1β-dependent apoptosis of endothelial cells: implications in the etiology of retinoic acid syndrome. *Leuk. Res.* **31,** 455–463.
7. Nata, K., Takamura, T., Karasawa, T., Kumagai, T., Hashioka, W., Tohgo, A., Yonekura, H., Takasawa, S., Nakamura, S., Okamoto, H. (1997) Human gene encoding CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase): organization, nucleotide sequence and alternative splicing. *Gene* **186,** 285–292.
8. Kishimoto, H., Hoshino, S., Ohori, M., Kontani, K., Nishina, H., Suzawa, M., Kato, S., Katada, T. (1998) Molecular mechanism of human CD38 gene expression by retinoic acid. Identification of retinoic acid response element in the first intron. *J. Biol. Chem.* **273,** 15429–15434.
9. Miwako, I., Kagechika, H. (2007) Tamibarotene. *Drugs Today (Barc)* **43,** 563–568.
10. Takahashi, N., Breitman, T. R. (1994) Induction of differentiation and covalent binding to proteins by the synthetic retinoids Ch55 and Am80. *Arch. Biochem. Biophys.* **314,** 82–89.
11. Uruno, A., Sugawara, A., Kudo, M., Sato, M., Sato, K., Ito, S., Takeuchi, K. (2003) Transcription suppression of thromboxane receptor gene expression by retinoids in vascular smooth muscle cells. *Hypertens. Res.* **26,** 815–821.
12. Saito, A., Sugawara, A., Uruno, A., Kudo, M., Kagechika, H., Sato, Y., Owada, Y., Kondo, H., Sato, M., Kurabayashi, M., Imaizumi, M., Tsuchiya, S., Ito, S. (2007) All-*trans* retinoic acid induces *in vitro* angiogenesis via retinoic acid receptor: possible involvement of paracrine effects of endogenous vascular endothelial growth factor signaling. *Endocrinology* **148,** 1412–1423.
13. Uruno, A., Sugawara, A., Kanatsuka, H., Arima, S., Taniyama, Y., Kudo, M., Takeuchi, K., Ito, S. (2004) Hepatocyte growth factor stimulates nitric oxide production through endothelial nitric oxide synthase activation by the phosphoinositide 3-kinase/Akt pathway and possibly by mitogen-activated protein kinase kinase in vascular endothelial cells. *Hypertens. Res.* **27,** 887–895.
14. Uruno, A., Sugawara, A., Kanatsuka, H., Kagechika, H., Saito, A., Sato, K., Kudo, M., Takeuchi, K., Ito, S. (2005) Upregulation of nitric oxide production in vascular endothelial cells by all-*trans* retinoic acid through the phosphoinositide 3-kinase/Akt pathway. *Circulation* **112,** 727–736.
15. Uruno, A., Sugawara, A., Kudo, M., Satoh, F., Saito, A., Ito, S. (2008) Stimulatory effects of low-dose 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitor fluvastatin on hepatocyte growth factor-induced angiogenesis: involvement of p38 mitogen-activated protein kinase. *Hypertens. Res.* **31,** 2085–2096.
16. Aggarwal, S., Kim, S. W., Cheon, K., Tabassam, F. H., Yoon, J. H., Koo, J. S. (2006) Nonclassical action of retinoic acid on the activation of the cAMP response element-binding protein in normal human bronchial epithelial cells. *Mol. Biol. Cell* **17,** 566–575.
17. Radominska-Pandya, A., Chen, G., Czernik, P. J., Little, J. M., Samokyszyn, V. M., Carter, C. A., Nowak, G. (2000) Direct interaction of all-*trans*-retinoic acid with protein kinase C (PKC). Implications for PKC signaling and cancer therapy. *J. Biol. Chem.* **275,** 22324–22330.
18. Miano, J. M., Berk, B. C. (2000) Retinoids: versatile biological response modifiers of vascular smooth muscle phenotype. *Circ. Res.* **87,** 355–362.
19. Dalton Jr., W. T., Ahearn, M. J., McCredie, K. B., Freireich, E. J., Stass, S. A., Trujillo, J. M. (1988) HL-60 cell line was derived from a patient with FAB-M2 and not FAB-M3. *Blood* **71,** 242–247.
20. Drach, J., Zhao, S., Malavasi, F., Mehta, K. (1993) Rapid induction of CD38 antigen on myeloid leukemia cells by all trans-retinoic acid. *Biochem. Biophys. Res. Commun.* **195,** 545–550.
21. Frankel, S. R., Eardley, A., Lauwers, G., Weiss, M., Warrell Jr., R. P. (1992) The "retinoic acid syndrome" in acute promyelocytic leukemia. *Ann. Intern. Med.* **117,** 292–296.
22. Patatanian, E., Thompson, D. F. (2008) Retinoic acid syndrome: a review. *J. Clin. Pharm. Ther.* **33,** 331–338.
23. Tallman, M. S., Andersen, J. W., Schiffer, C. A., Appelbaum, F. R., Feusner, J. H., Ogden, A., Shepherd, L., Rowe, J. M., François, C., Larson, R. S., Wiernik, P. H. (2000) Clinical description of 44 patients with acute promyelocytic leukemia who developed the retinoic acid syndrome. *Blood* **95,** 90–95.
24. Tobita, T., Takeshita, A., Kitamura, K., Ohnishi, K., Yanagi, M., Hiraoka, A., Karasuno, T., Takeuchi, M., Miyawaki, S., Ueda, R., Naoe, T., Ohno, R. (1997) Treatment with a new synthetic retinoid, Am80, of acute promyelocytic leukemia relapsed from complete remission induced by all-trans retinoic acid. *Blood* **90,** 967–973.
25. Yung, W. K., Kyritsis, A. P., Gleason, M. J., Levin, V. A. (1996) Treatment of recurrent malignant gliomas with high-dose 13-cis-retinoic acid. *Clin. Cancer Res.* **2,** 1931–1935.
26. Jaeckle, K. A., Hess, K. R., Yung, W. K., Greenberg, H., Fine, H., Schiff, D., Pollack, I. F., Kuhn, J., Fink, K., Mehta, M., Cloughesy, T., Nicholas, M. K., Chang, S., Prados, M., North American Brain Tumor Consortium (2003) Phase II evaluation of temozolomide and 13-cis-retinoic acid for the treatment of recurrent and progressive malignant glioma: a North American Brain Tumor Consortium study. *J. Clin. Oncol.* **21,** 2305–2311.
27. See, S. J., Levin, V. A., Yung, W. K., Hess, K. R., Groves, M. D. (2004) 13-*cis*-Retinoic acid in the treatment of recurrent glioblastoma multiforme. *Neuro-oncol.* **6,** 253–258.
28. Lotan, R. (1996) Retinoids and their receptors in modulation of differentiation, development, and prevention of head and neck cancers. *Anticancer Res.* **16,** 2415–2419.
29. Pasquali, D., Rossi, V., Bellastella, G., Bellastella, A., Sinisi, A. A. (2006) Natural and synthetic retinoids in prostate cancer. *Curr. Pharm. Des.* **12,** 1923–1929.
30. Páez-Pereda, M., Kovalovsky, D., Hopfner, U., Theodoropoulou, M., Pagotto, U., Uhl, E., Losa, M., Stalla, J., Grübler, Y., Missale, C., Arzt, E., Stalla, G. K. (2001) Retinoic acid prevents experimental Cushing syndrome. *J. Clin. Invest.* **108,** 1123–1131.
31. Castillo, V., Giacomini, D., Páez-Pereda, M., Stalla, J., Labeur, M., Theodoropoulou, M., Holsboer, F., Grossman, A. B., Stalla, G. K., Arzt, E. (2006) Retinoic acid as a novel medical therapy for Cushing's disease in dogs. *Endocrinology* **147,** 4438–4444.
32. Gavrielides, M. V., Gonzalez-Guerrico, A. M., Riobo, N. A., Kazanietz, M. G. (2006) Androgens regulate protein kinase Cδ transcription and modulate its apoptotic function in prostate cancer cells. *Cancer Res.* **66,** 11792–11801.

33. Berry, D. M., Antochi, R., Bhatia, M., Meckling-Gill, K. A. (1996) 1,25-Dihydroxyvitamin D3 stimulates expression and translocation of protein kinase Cα and Cδ via a nongenomic mechanism and rapidly induces phosphorylation of a 33-kDa protein in acute promyelocytic NB4 cells. *J. Biol. Chem.* **271,** 16090–16096.
34. Suh, K. S., Tatunchak, T. T., Crutchley, J. M., Edwards, L. E., Marin, K. G., Yuspa, S. H. (2003) Genomic structure and promoter analysis of PKC-δ. *Genomics* **82,** 57–67.
35. Navakauskiene, R., Kulyte, A., Treigyte, G., Gineitis, A., Magnusson, K. E. (2003) Translocation of transcription regulators into the nucleus during granulocyte commitment of HL-60 cells. *Biochem. Cell Biol.* **81,** 285–295.
36. Kambhampati, S., Li, Y., Verma, A., Sassano, A., Majchrzak, B., Deb, D. K., Parmar, S., Giafis, N., Kalvakolanu, D. V., Rahman, A., Uddin, S., Minucci, S., Tallman, M. S., Fish, E. N., Platanias, L. C. (2003) Activation of protein kinase C δ by all-*trans*-retinoic acid. *J. Biol. Chem.* **278,** 32544–32551.
37. Zhao, K. W., Li, X., Zhao, Q., Huang, Y., Li, D., Peng, Z. G., Shen, W. Z., Zhao, J., Zhou, Q., Chen, Z., Sims, P. J., Wiedmer, T., Chen, G. Q. (2004) Protein kinase Cδ mediates retinoic acid and phorbol myristate acetate-induced phospholipid scramblase 1 gene expression: its role in leukemic cell differentiation. *Blood* **104,** 3731–3738.
38. Koya, D., King, G. L. (1998) Protein kinase C activation and the development of diabetic complications. *Diabetes* **47,** 859–866.
39. Babazono, T., Kapor-Drezgic, J., Dlugosz, J. A., Whiteside, C. (1998) Altered expression and subcellular localization of diacylglycerol-sensitive protein kinase C isoforms in diabetic rat glomerular cells. *Diabetes* **47,** 668–676.
40. Kapor-Drezgic, J., Zhou, X., Babazono, T., Dlugosz, J. A., Hohman, T., Whiteside, C. (1999) Effect of high glucose on mesangial cell protein kinase C-δ and -ε is polyol pathway-dependent. *J. Am. Soc. Nephrol.* **10,** 1193–1203.
41. Duprez, E., Wagner, K., Koch, H., Tenen, D. G. (2003) C/EBPβ: a major PML-RARA-responsive gene in retinoic acid-induced differentiation of APL cells. *EMBO J.* **22,** 5806–5816.
42. Zhao, K. W., Li, D., Zhao, Q., Huang, Y., Silverman, R. H., Sims, P. J., Chen, G. Q. (2005) Interferon-α-induced expression of phospholipid scramblase 1 through STAT1 requires the sequential activation of protein kinase Cδ and JNK. *J. Biol. Chem.* **280,** 42707–42714.
43. Zhang, H., Forman, H. J. (2008) Acrolein induces heme oxygenase-1 through PKC-δ and PI3K in human bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **38,** 483–490.
44. Fan, C. Y., Katsuyama, M., Yabe-Nishimura, C. (2005) PKCδ mediates upregulation of NOX1, a catalytic subunit of NADPH oxidase, via transactivation of the EGF receptor: possible involvement of PKCδ in vascular hypertrophy. *Biochem. J.* **390,** 761–767.
45. Page, K., Li, J., Corbit, K. C., Rumilla, K. M., Soh, J. W., Weinstein, I. B., Albanese, C., Pestell, R. G., Rosner, M. R., Hershenson, M. B. (2002) Regulation of airway smooth muscle cyclin D1 transcription by protein kinase C-δ. *Am. J. Respir. Cell Mol. Biol.* **27,** 204–213.
46. Jin, D., Liu, H. X., Hirai, H., Torashima, T., Nagai, T., Lopatina, O., Shnayder, N. A., Yamada, K., Noda, M., Seike, T., et al. (2007) CD38 is critical for social behavior by regulating oxytocin secretion. *Nature* **446,** 41–45.
47. Lerer, E., Levi, S., Israel, S., Yaari, M., Nemanov, L., Mankuta, D., Nurit, Y., Ebstein, R. P. (2010) Low CD38 expression in lymphoblastoid cells and haplotypes are both associated with autism in a family-based study. *Autism Res.* **3,** 293–302.
48. Munesue, T., Yokoyama, S., Nakamura, K., Anitha, A., Yamada, K., Hayashi, K., Asaka, T., Liu, H. X., Jin, D., Koizumi, K., et al. (2010) Two genetic variants of CD38 in subjects with autism spectrum disorder and controls. *Neurosci. Res.* **67,** 181–191.
49. Aydin, S., Rossi, D., Bergui, L., D'Arena, G., Ferrero, E., Bonello, L., Omedé, P., Novero, D., Morabito, F., Carbone, A., Gaidano, G., Malavasi, F., Deaglio, S. (2008) CD38 gene polymorphism and chronic lymphocytic leukemia: a role in transformation to Richter syndrome? *Blood* **111,** 5646–5653.
50. González-Escribano, M. F., Aguilar, F., Torres, B., Sánchez-Román, J., Núñez-Roldán, A. (2004) CD38 polymorphisms in Spanish patients with systemic lupus erythematosus. *Hum. Immunol.* **65,** 660–664.
51. Drummond, F. J., Mackrill, J. J., O'sullivan, K., Daly, M., Shanahan, F., Molloy, M. G. (2006) CD38 is associated with premenopausal and postmenopausal bone mineral density and postmenopausal bone loss. *J. Bone Miner. Metab.* **24,** 28–35.

KEY WORDS:
retinoic acid receptor · endothelial cells · adhesion