The Aldo-Keto Reductase AKR1C3 Is a Novel Suppressor of Cell Differentiation That Provides a Plausible Target for the Non-Cyclooxygenase-dependent Antineoplastic Actions of Nonsteroidal Anti-Inflammatory Drugs

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ABSTRACT

We and others have demonstrated expression of the ald-o-ku reductase AKR1C3 in myeloid leukemia cell lines and that inhibitors of the enzyme, including nonsteroidal anti-inflammatory drugs (NSAIDs), promote HL-60 differentiation in response to all-trans retinoic acid (ATRA) and 1α,25-dihydroxyvitamin D₃ (D₃). Here, we demonstrate that overexpression of AKR1C3 reciprocally desensitizes HL-60 cells to ATRA and D₃, thus confirming the enzyme as a novel regulator of cell differentiation. AKR1C3 possesses marked 11-ketoreductase activity converting prostaglandin (PG) D₂ to PGF₂α. Supplementing HL-60 cultures with PGD₂ mimicked treatment with AKR1C3-inhibitors by enhancing the differentiation of the cells in response to ATRA. However, PGD₂ is chemically unstable, being converted first to PGJ₂ and then stepwise to 15-deoxy-D₁₂,14-prostaglandin J₂ (15α-PGJ₂), a natural ligand for the peroxisome proliferator-activated receptor-γ (PPARγ). Consistent with this, PGD₂ was rapidly converted to PGJ₂ under normal tissue culture conditions but not in the presence of recombiant AKR1C3 when PGD₂ was predominately formed. In addition, PGJ₂ but not PGF₂α, recapitulated the potentiation of HL-60 differentiation by PGD₂ and AKR1C3 inhibitors. Furthermore, the capacity of all of these treatments to potentiate HL-60 cell differentiation was significantly reduced in the presence of the PPARγ antagonist GW 9662. We conclude that AKR1C3 protects HL-60 cells against ATRA and D₃-induced cell differentiation by limiting the production of natural PPARγ ligands via the diversion of PGD₂ toward PGJ₂ and away from PGJ₂. In addition, these observations identify AKR1C3 as plausible target for the non-cyclooxygenase-dependent antineoplastic actions of NSAIDs.

INTRODUCTION

NSAIDs have enjoyed widespread clinical use and have been implicated in chemoprevention of certain cancers. To date, the diverse actions of these compounds have been attributed to their capacity to inhibit COXs (1–7). However, a number of studies have highlighted that drugs may have other important targets. Although a number of non-COX NSAID targets have been described, most require the drugs to be present in millimolar concentrations, exceeding the micro-

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The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; ATRA, all-trans retinoic acid; D₃, 1α,25-dihydroxyvitamin D₃; PG, prostaglandin; PPARγ, peroxisome proliferator-activated receptor-γ; FBS, fetal bovine serum; MPA, medroxyprogesterone acetate; GFP, green fluorescence protein; FACs, fluorescence-activated cell sorting; HSV, herpes simplex virus; VSV-G, vesicular stomatitis virus-G; IRES, internal ribosome entry site; DHT, dihydrotestosterone; 15Δ-PGJ₂, 15-deoxy-D₁₂,14-prostaglandin J₂; AR, androgen receptor; wt, wild type.

MATERIALS AND METHODS

Cells and Cell Culture. HL-60 cells used in this study were from the European Cell and Culture Collection (ECACC; www.ecacc.org; Ref No: 98070106). Stock cultures were maintained in exponential growth in RPMI 1640 + glutamine (Life Technologies, Inc.-Invitrogen Corp., Paisley, United Kingdom) supplemented with 10% fetal heat-inactivated FBS (Life Technologies, Inc.), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). Experimental cultures were seeded at 2.5 × 10⁶ cells/ml in 25-cm² filter-capped cell culture flasks (Nucleon, Nunc Nalge International, Paisley, United Kingdom) or at 5 × 10⁴ cells in 750 μl in 48-well cell culture plates (Nuncleone), and fed appropriately to allow for cellular proliferation. All of the cultures were incubated at 37°C in a fully humidified atmosphere with 5% CO₂.

Modulators of Cellular Proliferation and Differentiation. One mM ATRA (Sigma, Dorset, United Kingdom) prepared in DMEM was stored in the dark at −20°C. Working stocks were prepared for each experiment by diluting...
this stock in culture medium and stored at 4 °C. A stock solution of 4 mM D$_2$ in isopropanol was provided as a gift by Dr. Lisa Binderup (Leo Pharmaceutica, Ballerup, Denmark) and stored in the dark at −20 °C. Working stocks were prepared as required by diluting this stock in culture medium and were stored at 4 °C. Twenty mM indomethacin (Sigma) and 5 mM MPA (Sigma) were prepared in DMSO, stored at 4 °C, and replaced every 4 weeks. The PPARγ antagonist GW 9662 (Cayman Chemical Company, Ann Arbor, MI) was prepared as 1 mM stock in DMSO and used at a final concentration of 1 μM.

Stock solutions of 20 mM PGD$_2$ and PGD$_2$ (both from Affiniti Research Products, near Exeter, United Kingdom) were prepared in ethanol and stored at −20 °C and −80 °C, respectively. Stocks of 5 mM 5α-DHT (5α-androstan-17β-diol-3-one; Sigma) and 5 mM 3α-androstane-diol (3α-androstane-3α,17β-diol; Sigma) were prepared in ethanol and stored at −20 °C.

Assessment of Cell Proliferation and Differentiation. Viable cell counts were performed using a hemocytometer and phase contrast microscopy. CD11b is a marker of both HL-60 neutrophil and monocyte differentiation that is expressed by <5% of cells in cultures not exposed to inducers of differentiation. Phycocyanin-conjugated mouse monoclonal antibody against human CD11b (Leu-15 Becton Dickinson; supplied by Coulter-ImmunoTech, Lonot, United Kingdom) was used to stain cells for 30 min at 4 °C. The cells were washed and fixed with 100 μl of PBS containing 1% (v/v) formalin (Sigma) and 2% (v/v) FBS and were assayed for CD11b expression using a Becton Dickinson FACS-Calibur cell sorter and Becton Dickinson Cell Quest software.

To permit morphological analysis of cell differentiation, 75–100 μl of a cell suspension at a density of 3.5–5 × 10$^6$ cells/ml were spun onto an alcohol-washed microscope slide at 500 rpm for 3 min using a cytocentrifuge (Shandon CytoSpin 2). The slides were then air-dried before being fixed in methanol (Fisher, Lichestershire, United Kingdom) for 5 min at room temperature and were stained using a conventional Jenner Giemsa protocol.

Production of AKR1C3 Expression Vectors. The pBluescript SK$^+$ vector (Strategene, La Jolla, CA) containing human AKR1C3 cDNA (KIAA0919, Ref. 14) kindly donated by Dr. T. Nagase of the Kazusa DNA Research Institute, Chiba, Japan. the cDNA fragment encoding AKR1C3 was removed using BamHI and NotI restriction enzymes (Promega, Southampton, United Kingdom), was gel-purified, and was ligated with similarly digested mamma- lian expression vector, pcDNA3.1/Hygro (+) (Invitrogen Life Technologies, Inc. Paisley, United Kingdom). Orientation and nucleotide sequence of the construct pcDNA3.1-AKR1C3 were confirmed by sequencing.

The MSCV-AKR1C3-I-GFP retroviral vector plasmid was constructed by a three-way ligation in which the 1.2-kbp SstI-PshAI fragment from pcDNA3.1/Hygro (+)/AKR1C3 was ligated to the 1.3-kbp Hpal-NotI and the 5.3-kbp NotI-XhoI fragments from MSCV-I-GFP (19).

The VSV-G-pseudotyped MSCV-AKR1C3-I-GFP retroviral vector containing supernatants were made essentially as described previously (19). 293T cells (Chinese hamster ovary transfected by calcium phosphate coprecipitation of the retroviral vector plasmid MSCV-AKR1C3-I-GFP (15 μg) with the plasmid pEQ-PAM3-E (15 μg), which expresses the retroploid gag and pol (20), and the plasmid pRSa-G (10 μg), which expresses the envelope protein of the vesicular stomatitis virus (21). The following morning, the medium was changed, and 48 h later, the conditioned medium, containing the VSV-G-pseudotyped retroviral vector, was harvested and concentrated by ultracentrifugation, passed through a 0.45 μm filter, snap-frozen on dry ice, and subse- quently stored at −80 °C until needed. The presence of active viral vectors was confirmed by titration onto 3T3 cells as described previously (21).

Liposomal Delivery of AKR1C3. Five μl of DMRIE-C (Invitrogen Life Technologies, Inc., Paisley, United Kingdom) reagent were added to 0.5-ml aliquots of RPMI 1640 supplemented with 1% (v/v) ITS+ (Stratech Scientific, Lonot, United Kingdom) and mixed before adding 4 μg of pcDNA3.1: AKR1C3 in 0.5 ml of RPMI 1640 ITS+ and 0.5 ml of chloroform (Fisher Chemicals) at room temperature (5 °C) and the mixtures then evaporated to dryness at 4 °C under vacuum. The liposomal mixture was then incubated at 37 °C with 5% CO$_2$ for 5 h before the addition of 800 μl of RPMI 1640, 10% FBS, and antibiotics. After 48 h, cells were expressed to 500 μg/ml G418 (Sigma) for 1 week before transfer to methylcellulose (Sigma) in the continued presence of G418 selection antibiotic. Clones, growing through selection after an additional 3 weeks, were isolated and cultured out of methyl cellulose in RPMI culture medium with G418, and their transfection status was confirmed by PCR for the neomycin resistance gene of pcDNA3.1 (data not shown).

Retroviral Delivery of AKR1C3. HL60 cells in exponential growth were seeded at a concentration of 2 × 10$^5$ cells/ml in 500 μl of RPMI 1640 + 10% FCS and 500 μl of concentrated viral suspension with 6 μg/ml Polybrene (Sigma) and were incubated overnight. The multiplicity of infection for the MSCV-AKR1C3 was 13 plaque-forming units/cell and for MSCV-IRES-GFP was 100 plaque-forming units/ml. The following morning, 1 ml of fresh RPMI 1640 + 10% was added to each culture, and the cells were cultured for an additional 48 h. To determine the transduction efficiency, the percentage of GFP-positive cells was determined by FACS (39 and 81% GFP positive for MSCV-AKR1C3 and MSCV-IRES-GFP, respectively), these populations were expanded for an additional 48 h before FACS sorting for populations of GFP-positive cells. After sorting, cells were cultured for an additional 3 days to obtain sufficient numbers of cells for assays and for frozen stocks.

Western Blot Analysis of AKR1C3 Expression. Approximately 1 × 10$^7$ cells were pelleted, washed in PBS, and resuspended in 200 μl of radioimmunoassay precipitation assay (RIPA) buffer containing protease inhibitors (Boehr- ringer Mannheim, Mann., Germany). After 30 min on ice with occasional shaking, samples were microfuged and the supernatant stored at −20 °C. Protein concentrations were determined using the Bio-Rad protein assay kit (2 Bio-Rad, Hertfordshire, United Kingdom) using BSA as a standard. Before separation protein samples were diluted 1 in 2 (v/v) in sample buffer, incubated at 100 °C for 5 min, and microfuged for 10 min at 13,000 × g. Proteins were separated using a 10% polyacrylamide resolving gel and a 4.5% polyacrylamide stacking gel (pH 6.8). 5 μl of prestained high molecular weight rainbow markers (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) were loaded in one lane of each gel. After electrophoresis, the proteins were transferred to an immobilon-P membrane (0.4 μm; Millipore Corp, Bedford, MA) using a trans-blot SD semi-dry transfer cell (Bio-Rad) at 15 V for 2 h. After transfer, the membrane was stained with Ponceau stain (Sigma), to ensure the proteins had resolved, that the transfer had been successful and to confirm equal loading of protein. Membranes were blocked for 1 h with 5% (w/v) nonfat milk powder (Marvel; Premier Brands, Lin- colnhire, United Kingdom) in PBS 0.1% (v/v) Tween (Sigma) and probed overnight with rabbit polyclonal anti-3α-HSD [a kind gift from Professor T. Penning, University of Pennsylvania, Philadelphia, PA, and used in our earlier studies (16)]. After washing, membranes were probed with horseradish per- oxidase-conjugated donkey anti-rabbit immunoglobulin (Amersham Life Sci- ences, Amersham, United Kingdom) and binding was revealed using enhanced chemiluminescence (Amersham Pharmacia Biotech) as instructed by the manufacturer.

Purification of his-Tagged AKR1C3. The generation of his-tagged AKR1C3 will be described in detail elsewhere.4 Recombinant protein was purified using the Novagen (Madison, WI) Ni-NTAHisBind Resin kits and the manufacturer’s protocols. Eluates were subsequently analyzed using the Bio-Rad protein assay kit (2 Bio-Rad) and by SDS-PAGE. Protein aliquots were stored at −80 °C until use. Recombinant AKR1C3 activity assays contained 100 mM potassium phosphate buffer (Sigma; pH 7.0) along with either 2.3 mM NADP for reduction reactions or 2.3 mM NAD for oxidation reactions (both Sigma). One μl of [3H]-labeled substrate (5α-DHT; Amersham Pharmacia Biotech), 3α-androstane-3β-ol (NEN Life Science, Boston, MA) or PGD$_2$ (Amersham Pharmacia Biotech), and 2–6 μg of recombinant protein were added in a final reaction volume of 100 μl. The reactions were performed in stopped borosilicate glass culture tubes (13 × 100 mm; Corning, New York) and incubated at 37 °C for 1 or 2 h. Reactions were quenched by the addition of 400 μl ethyl acetate (Aldrich, Dorset, United Kingdom), and the mixtures then evaporated to dryness under a stream of air at room temperature.

Cell Line Activity Assays. To measure AKR1C3 activity in intact cells, 1 μl of [3H]3α-androstenediol was added to 4 ml cultures set at 1 × 10$^6$ cells/ml, and the cells were cultured overnight. To extract steroid metabolites, cultures were split into two 2-ml aliquots and placed in borosilicate glass culture tubes (Corning); 4 ml of chloroform (Fisher Chemicals) were added, and the mixtures were vortexed for 4 min. The reaction mixtures were centrifuged at 2500 rpm for 10 min, and the aqueous and proteinaceous layers were removed by aspiration. The steroid-containing chloroform fraction was

then evaporated to dryness under a stream of air at 35°C. The sterols were redissolved in 40 μl of methanol and spotted onto Silica gel TLC plates (Fluka; Sigma).

For extraction of prostanoids from culture medium, the medium was split into four 1-ml aliquots, placed in borosilicate glass culture tubes (Corning), and 2 ml of methanol (Fisher Chemicals) and 10 μl 10% (v/v) formic acid (Sigma) were added to each aliquot. After vortexing for 2 min, 3.9 ml of chloroform (Sigma) and 200 μl of 4.2% (v/v) KCl (Sigma) were also added, and the samples were vortexed for an additional 4 min. The reaction mixtures were then centrifuged at 2500 rpm for 10 min and the resulting aqueous and proteinaceous layers removed by aspiration. The prostanoid-containing organic fractions were then pooled and evaporated to dryness under a stream of air at room temperature. The prostanoids were redissolved in 40 μl of chloroform and applied to Silica gel TLC plates (Fluka).

Steroids were separated using chloroform and ethyl acetate (4:1 v/v) and prostanoids using chloroform/methanol/glacial acetic acid (Sigma)/distilled H₂O (90:8:1:0.8 v/v/v/v). The solvents were added to the running tank and allowed to equilibrate for 2 h before use. The plates were developed at room temperature for ~2 h. After chromatography, the plates were removed from the running tank and allowed to dry at room temperature. The location of the radiolabelled steroids and prostanoids was then identified using a Bioscan plate reader (Bioscan Inc., Washington, DC). Sterols and prostanoids were identified by their comigration with standards the positions of which were identified under an UV lamp.

RESULTS

Overexpression of AKR1C3 by HL-60 Cells Results in Diminished Sensitivity to ATRA. We used two approaches to overexpress AKR1C3 in HL-60 cells. The first used liposomal delivery in the mammalian expression vector pcDNA3.1 and the second, retroviral delivery using MSCV pseudotyped with VSV-G. pcDNA3.1 transfected cells were selected using 500 μg/ml G418- and MSCV-transduced cells by GFP expression driven independently of AKR1C3 expression, by virtue of an interstitial IRES. Although generated using different vectors and selection protocols, all AKR1C3 overexpressing HL-60 cells (HL-60AKR1C3) displayed increased resistance to ATRA (Fig. 1). For example, 5 days exposure of pcDNA3.1:HL-60AKR1C3 to 50 nM ATRA resulted in 30.8 ± 3.4% of cells expressing the differentiation marker CD11b as compared with 72.5 ± 5.7% of wt-HL-60 cells (P = 0.00035). Similarly 50 nM ATRA induced differentiation in 37.8 ± 5.0% of MSCV:HL-60AKR1C3 cells compared with 76.3 ± 2.1% of MSCV:HL-60 vector control cells (P = 0.00019). Studies of cell morphology confirmed that the reduced numbers of CD11b-positive cells in ATRA-treated HL-60AKR1C3 cultures reflected fewer cells undergoing terminal cell differentiation (Fig. 1b).

Elevated AKR1C3 expression was also associated with resistance to the antiproliferative action of ATRA. For example, 5-day 50-nM ATRA-treated MSCV:HL-60AKR1C3 cultures contained 7.2 ± 0.3 × 10^5 cells as compared with 5.2 ± 0.6 × 10^5 in parallel MSCV:HL-60 vector control cultures (P = 0.033). Consequently, it was possible that similar actual numbers of maturing cells were produced in HL-60AKR1C3 and control cultures. In this scenario, the action of AKR1C3 overexpression may have been to promote the proliferation (and/or survival) of HL-60 cells that failed to respond to ATRA, thereby increasing the cell number in ATRA-treated cultures and diminishing the proportion of cells expressing CD11b. However, as shown in Fig. 1a, combining the number of cells produced with the proportion of mature cells at day 5 clearly demonstrated that fewer cells were differentiating in HL-60AKR1C3 cultures than in controls.

Cell viability in all of the cultures was recorded to be 95–98%. Thus, elevated AKR1C3 did not alter cell survival, and, therefore, its overexpression most likely resulted in reduced differentiation by altering the proportion of cells entering terminal differentiation.

Loss of AKR1C3 trans-Gene Expression by HL-60 Cells Correlated with Loss of Resistance to ATRA. trans-Gene expression by both pcDNA3.1:HL-60AKR1C3 and MSCV:HL-60AKR1C3 diminished on prolonged passage of the cells. At the time of their isolation, overexpression of AKR1C3 by pcDNA3.1:HL-60AKR1C3 was readily detected by both Western blot analyses and by their elevated 17β-HSD activity against 3α-androstenediol (Fig. 2a). However, after 4-weeks passage, AKR1C3 overexpression was no longer detectable by either parameter and, concomitantly, the transfectants lost their increased resistance to ATRA (Fig. 2a). In the case of MSCV:HL-60AKR1C3 cells, the progressive loss of trans-gene expression was most readily monitored by the loss of GFP expression and was again closely associated with a progressive diminution of the cellular resistance to ATRA (Fig. 2b). However, regular resorting of GFP-bright MSCV:HL-60AKR1C3 cells sustained the AKR1C3-associated phenotype (data originally shown in Fig. 1).

The strong ATRA resistance of HL-60AKR1C3 cells, irrespective of the vector used, and the clear association of the phenotype with the level of trans-gene expression, combines with our earlier inhibitor studies to clearly demonstrate that AKR1C3 functions as a key regulator of ATRA-induced differentiation. Furthermore, as predicted by

Fig. 1. AKR1C3 overexpression results in increased resistance to ATRA-induced cell differentiation. a, analyses of CD11b expression. Top panel, wt-HL-60 (○) and pcDNA3.1:HL-60AKR1C3 cells (■) were treated for 5 days with the doses of ATRA shown and were analyzed for expression of the differentiation-associated surface marker CD11b using FACS. Data are the mean ± SE of three experiments. Middle panel, MSCV:HL-60 (○) and MSCV:HL-60AKR1C3 cells (■) were treated for 5 days with the doses of ATRA shown and were analyzed for expression of the differentiation-associated surface marker CD11b using FACS. Data are the mean ± SE of six experiments. Bottom panel, MSCV:HL-60 (○) and MSCV:HL-60AKR1C3 cells (■) were treated for 5 days with the doses of ATRA shown, and the actual numbers of cells expressing CD11b were determined from the data from the middle panel and the mean total numbers of cells per culture (×10^4) for the same six experiments. b, analyses of cell morphology. Jenner-Giemsa-stained cytospin preparations of MSCV:HL-60 and MSCV:HL-60AKR1C3 cells, treated in parallel for 5 days with the doses of ATRA shown. HL-60 neutrophil maturation is characterized by the development of pleomorphic and, occasionally, fully lobed nuclei. The images show greater differentiation by ATRA-treated MSCV:HL-60 cells compared with MSCV:HL-60AKR1C3 cells.
INHIBITION OF DIFFERENTIATION BY AKR1C3

Fig. 2. Resistance to ATRA-induced differentiation is correlated with the level of transgene expression. a, pcDNA3.1-transfected cells. Top panel, Western blot analyses of AKR1C3 expression relative to wt-HL-60 cells in three clones of pcDNA3.1.HL-60AKR1C3 cells before and after 4 weeks continual passage in the absence of G418. Middle panel, analysis of the relative 17β-oxidation of 3α-androstanediol (an activity of AKR1C3 but not AKR1C4) by wt-HL-60 cells and by pcDNA3.1.HL-60AKR1C3 cells before and after 4 weeks continual passage in the absence of G418. Data are the mean ± SE of three experiments. Bottom panel, analysis of the relative sensitivity of wt-HL-60 cells and of pcDNA3.1.HL-60AKR1C3 cells to differentiation induced by 5 days of exposure to 50 nM ATRA, assessed by FACS analysis of CD11b staining, before and after 4 weeks continual passage in the absence of G418. Data are the mean ± SE of three experiments. b, MSCV-transduced cells. Top panels, FACS analyses of GFP expression by MSCV:HL60AKR1C3 cells before and after 4 weeks continual culture. Bottom panel, changes in the relative sensitivity of MSCV:HL60AKR1C3 cells to ATRA-induced differentiation as measured by CD11b expression at 5 days. Cells were treated with the shown ATRA doses at one (), two (), three (), and 4 () weeks after FACS sorting for GFP-positive cells. MSCV:HL60 cells (●), sorted in parallel and in the same window of GFP expression, were also treated with the same doses of ATRA 1 week after sorting.

Fig. 3. a, the AKR1C3 inhibitor indomethacin and PGD2 equally sensitize HL-60 cells to the antiproliferative actions of ATRA. HL-60 cells were treated for 5 days with the doses of ATRA shown, either alone (○) or in combination with 20 μM indomethacin (□) or 5 μM PGD2 (●) and the number of cells generated by each culture determined as described in “Materials and Methods.” b, excess of the AKR1C3 substrate PGD2, but not the steroid substrates 5α-DHT or 3α-androstanediol promotes HL-60 cell differentiation. HL-60 cells were treated with the doses of ATRA shown, either alone (○) or in combination with 5 μM 3α-androstanediol (△), 5 μM 3α-DHT (□) or 5 μM PGD2 (●). c, and the resultant differentiation assayed by FACS analysis of CD11b expression. c, escalation of PGD2 concentration results in cell death. The number of viable cells present in 5-day HL-60 cultures treated with the shown doses of PGD2. Data in a and c are the mean ± SE (×10⁵) of three experiments. Data in b are the mean ± SE of three experiments. d, morphology of PGD2-treated HL-60 cells. Jenner-Giemsa-stained cytospin preparations of HL-60 cells treated as shown.

former inhibitor studies (15, 16), AKR1C3 overexpression similarly bestowed HL-60 cells with increased resistance to D3. For example MSCV:HL60 cultures contained 41.2 ± 2.2% CD11b-expressing cells after 5-days exposure to 4 nM D3 as compared with 25.3 ± 1.6% in MSCV:HL60AKR1C3 cells (P = 0.002). Analysis of cell morphology also confirmed reduced differentiation of MSCV:HL60AKR1C3 cells in response to D3 (not shown). Thus, the capacity of AKR1C3 to suppress cell differentiation is not limited to a single cell lineage nor restricted to the blockade of ATRA-associated differentiation.

PGD2 but not the AKR1C3 Substrates 5α-DHT or 3α-Androstanediol Potentiates ATRA-induced HL-60 Cell Differentiation. We next determined which activity of AKR1C3 might be responsible for the actions on cell differentiation. We first treated HL-60 cells with an excess (5 μM) of the preferred AKR1C3 3α-hydroxysteroid and 17β-hydroxysteroid substrates 5α-DHT and 3α-androstanediol, respectively (9) and the prostaglandin substrate PGD2 (11). The striking finding was that, whereas 5α-DHT and 3α-androstanediol had no effect on the ATRA response of HL-60 cells, both the antiproliferative and the differentiation responses were markedly enhanced by PGD2 (Fig. 3a and b, and negative data not shown). For example, cultures treated for 5 days with either 5 μM PGD2, or 1.56 nM ATRA alone, generated near identical cell numbers (19.8 ± 1.8 × 10⁵ and 19.8 ± 1.6 × 10⁵, respectively) that were not statistically different from those generated in control cultures (20.8 ± 1.3 × 10⁵). In stark contrast, cultures treated with 1.56 nM ATRA, together with 5 μM PGD2, generated significantly fewer cells (11.4 ± 0.34 × 10⁵; P = 0.001 with respect to control cells). Furthermore, cultures treated with 6.25 nM ATRA and 5 μM PGD2 generated just 4.1 ± 0.5 × 10⁵ cells (~20% of controls) which was similar to the maximal response to ATRA alone (100 nM ATRA, 3.2 ± 0.37 × 10⁵ cells; ~15% of controls). Most noticeable was the observation that the enhanced antiproliferative effect of combined ATRA and 5 μM PGD2 paralleled that observed when ATRA was combined with the NSAID-AKR1C3 inhibitor indomethacin (Fig. 3a). Therefore, as predicted by our model, the provision of an excess of the appropriate AKR1C3 substrate mimicked the effect of inhibiting the enzyme.

PGD2 also greatly enhanced ATRA-induced differentiation, whereas 5α-DHT and 3α-androstanediol did not (Fig. 3b). After
5 days exposure to 1.56 nM ATRA alone, only 3.9 ± 1.8% of the cells expressed CD11b as compared with 53 ± 12% in cultures also treated with 5 μM PGD2 (P = 0.006). Cultures treated with 1.56 nM ATRA alone also lacked features consistent with ATRA-induced differentiation, whereas the majority of cells also treated with 5 μM PGD2 showed features characteristic of HL-60-derived neutrophils (Fig. 3c). Furthermore, prolonged culture of HL-60 cells in 5 μM PGD2 alone led to the cells acquiring features of partial differentiation and the development of weak CD11b staining by up to 25% of the cells (Fig. 3d). Thus PGD2 may be in itself a weak stimulator of differentiation. However, increasing PGD2 doses did not enhance cell differentiation but rather resulted in cell death with features of apoptosis (Fig. 3, c and d), an observation consistent with the ability of PGD2 to induce apoptosis in other cell models (reviewed in Ref. 18). In contrast to PGD2, its AKR1C3 metabolite PGF2α, displayed no capacity to induce either HL-60 cell differentiation or apoptosis. Instead, 20 μM produced a small but significant increase in cell numbers generated over 5 days [(50 ± 0.2) × 10^5] compared with parallel control cultures [(45 ± 0.1) × 10^5]; P = 0.05.

**AKR1C3 Activity Protects against the Chemical Conversion of PGD2 to PGJ2**

Although biologically active in diverse systems, PGD2 is chemically unstable and is rapidly converted, nonenzymatically, to PGJ2 and, subsequently, to Δ12-PGJ2, and then to the PPARγ ligand 15Δ-PGJ2. Thus, many of the biological activities ascribed to PGD2 are likely to be mediated by J-series PGs. We observed that, after overnight incubation of PGD2 in tissue culture conditions, only 23.9 ± 3.4% remained as PGD2, and the majority had converted to a metabolite that comigrated with PGJ2 in TLC assays (see also Fig. 4a). Furthermore, PGJ2 treatment of HL-60 cells resulted in an enhanced HL-60 response to ATRA that was similar to that observed in response to PGD2 (Fig. 4b). Together these observations indicate that the actions of PGD2 in enhancing HL-60 cell differentiation are mediated downstream of its chemical conversion to PGJ2.

The chemical instability of PGD2 in the extracellular environ of HL-60 cultures precluded us from demonstrating the PGD2 11-ketoreductase activity of AKR1C3 within HL-60 cells. However, as shown in Fig. 4a, 6 μg of human recombinant-AKR1C3 in 1 ml of tissue culture medium (~0.2 nM) was able to convert ~70% of a 5-nM radiolabelled PGD2 solution to PGF2α in just 2 h (~5.4 pmol/μg of AKR1C3/min). Thus, at least in vitro, AKR1C3 is able to inhibit the formation of PGJ2 from PGD2 by the efficient catalysis of its conversion to PGF2α. In a previous study, we demonstrated the constitutive generation of PGD2 by HL-60 cells (16). The data we have shown here now suggest that, if not tightly regulated, such a pool of PGD2 would lead to the endogenous generation of J-series PGs, resulting in the promotion of differentiation.

**The Ability of PGD2, PGJ2, and Steroidal and Nonsteroidal Inhibitors of AKR1C3 to Promote HL-60 Cell Differentiation Is Antagonized by the PPARγ Antagonist GW 9662.** In other in vitro models of cell differentiation J-series PGs have been shown to promote differentiation via activation of PPARγ (reviewed in 18). To test whether this was also the case in HL-60 cells, we used the PPARγ antagonist GW 9662, which has been shown by others to inhibit the PPARγ-dependent differentiation of osteoclasts (22). As shown in Fig. 5a, GW 9662 clearly diminished the capacity of either PGD2 or PGJ2 to promote the antiproliferative effects of low-dose ATRA. Similarly, GW 9662 also severely restricted the enhanced antiproliferative actions of ATRA mediated by either the NSAID AKR1C3-inhibitor indomethacin or the steroid AKR1C3-inhibitor MPA (Fig. 5b). In addition to reversing the potentiation of HL-60 antiproliferative responses by PGD2, PGJ2, and AKR1C3 inhibitors, GW 9226 also diminished the enhanced HL-60-cell differentiation associated with these treatments. For example, as stated above, when treated with 1.56 nM ATRA alone, only 3.9 ± 1.8% of the cells expressed CD11b, and treatment with combined 1.56 nM ATRA and 5 μM PGD2 increased this to 53 ± 12% (P = 0.006). However, in the presence of 1.56 nM ATRA, 5 μM PGD2, and GW 9662, only 4.1 ± 0.3% of cells became CD11b positive (P = 0.016 when compared with 1.56 nM ATRA and 5 μM PGD2 without GW 9662, and P = 0.9 when compared with 1.56 nM ATRA alone). Thus, GW 9662 completely abrogated the ability of PGD2 to potentiate the differentiation of HL-60 cells in response to this low dose of ATRA. Similarly, although indomethacin elevated differentiation in response to 1.56 nM ATRA (23 ± 21% CD11b positive), this did not occur in the additional presence of GW 9662 (7.0 ± 1.4% CD11b positive; P = 0.009 when compared with 1.56 nM ATRA and indomethacin without GW 9662; P = 0.1 when compared with 1.56 nM ATRA alone).

We, therefore, conclude that the constitutive action of AKR1C3 in reducing the sensitivity of HL-60 cells to physiological ATRA and D3 is mediated by the prevention of PPARγ activation via the directed metabolism of PGD2 toward PGF2α, and the consequent depletion of J-series PGs. The phenomenon of “prereceptor signaling” by ligand metabolizing enzymes is well established for many nuclear receptors, including thyroid hormone and steroid, retinoid, and D3 receptors. However, ours is the first to identify an enzyme that controls PPARγ-mediated cell activities.

**DISCUSSION**

AKRs are expressed throughout the evolutionary spectrum from prokaryotes to flowering plants and higher mammals (23). This extensive conservation strongly suggests that these enzymes have important ancestral roles in fundamental aspects of cellular behavior. Consistent with this, our study has highlighted AKR1C3, as a novel and potentially important regulator of myeloid cell proliferation and differentiation.

Other workers have demonstrated the expression of both COX-1 and COX-2 by HL-60 cells, and in an earlier study, we demonstrated the generation of multiple PGs by HL-60 cells (16, 24–26). We further showed that indomethacin near totally diminished PGF2 pro-
AKR1C3 inhibitors and PPAR/H9253 combination with the shown concentrations of PGD 2 or PGJ 2 and in either the presence or the absence of the PPAR inhibitors indomethacin and MPA is dependent on PPAR/H9253 AKR1C3-derived product, PGF 2/H17004 for 4 days in the presence of 0 ( ), 1.56 ( ), 3.125 ( ), and 6.25 ( ) μM ATRA in combination with the shown concentrations of PGD, or PGJ, and in the presence or the absence of the PPAR antagonist GW 9662. Data are the mean of two experiments performed in triplicate.

b. HL-60 cells were cultured for 4 days in the doses of ATRA shown alone ( ), in the presence of AKR1C3 inhibitors ( ), or in the presence of AKR1C3 inhibitors and PPARγ antagonist GW 9662 ( ). Data are the mean of two experiments performed in triplicate.

Fig. 5. The potentiation of HL-60 differentiation by PGD 2, PGJ 2, and the AKR1C3 inhibitors indomethacin and MPA is dependent on PPARγ. a. HL-60 cells were cultured for 4 days in the presence of 0 ( ), 1.56 ( ), 3.125 ( ), and 6.25 ( ) nM ATRA in combination with the shown concentrations of PGD, or PGJ, and in either the presence or the absence of the PPAR antagonist GW 9662. Data are the mean of two experiments performed in triplicate. b. HL-60 cells were cultured for 4 days in the doses of ATRA shown alone ( ), in the presence of AKR1C3 inhibitors ( ), or in the presence of AKR1C3 inhibitors and PPARγ antagonist GW 9662 ( ). Data are the mean of two experiments performed in triplicate.

A potentiation of differentiation by NSAIDs was not observed in the absence of ATRA, but was clearly observed in the presence of ATRA, indicating that AKR1C3 mediates the differentiation effect of ATRA. However, the role of AKR1C3 in regulating PPARγ activity is not yet clear. In some tumors, NSAIDs have not been reported to be beneficial in acute myeloid leukemia. However, the lack of activity of AKR1C3 in regulating PPARγ activity in cells that are capable of differentiating in response to ATRA suggests that the enzyme protects the AR by sequential 3α-reduction and 17β-oxidation of the potent androgen 5α-dihydrotestosterone to form the inactive androgen androsterone (9, 17). However, we and others have failed to detect AR expression by HL-60 cells, and thus, it appears likely that the capacity of AKR1C3 to regulate myeloid cell differentiation is independent of androgen action (Ref. 27 and unpublished observations).

In contrast, the experiments that we have described here strongly implicate PGD 2 as the key AKR1C3 substrate and that the enzyme diverts its metabolism toward PGF 2α and away from J-series prostanoids. These findings are supported by those of others demonstrating that PGD 2 and PGJ 2 inhibited the clonogenic growth not only of the HL-60 cells but also of the K562, KG1, U937, and THP1 myeloid cell lines and, importantly, normal myeloid progenitor cells CFU-GM (28). Thus PGD 2 and PGJ 2 are powerful modulators of both normal and malignant myeloid cell activities. Our findings are also supported by the study of Asou et al. (29) that demonstrated the expression of PPARγ across a panel of myeloid leukemia cell lines and the capacity of its synthetic ligand troglitazone to potentiate the antiproliferative actions of ATRA.

Normal bone marrow is one of the most PGD 2-rich tissues in the body (30). Intuitively, therefore, it appears that myeloid progenitor cells must transiently protect themselves from the antiproliferative and differentiative actions of PGD 2 that are mediated downstream of its chemical conversion to PGJ 2. Although abundant in PGD 2, the extracellular environment is not rich in free PGJ 2, the levels of which appear to be tightly controlled by diverse mechanisms that are as yet poorly defined (31–35). Consequently, it is endogenously generated PGJ 2 from which myeloid progenitor cells have to protect themselves. Our study now indicates that the ability of AKR1C3 to divert PGD 2 toward PGF 2α and away from PGJ 2 represents a major component in this mechanism. However, the role of AKR1C3 in regulating PPARγ activity may be more complex than mere regulation of ligand availability, because in the adipocyte model, PGF 2α, reciprocally, negatively regulates PPARγ activation via induction of its mitogen-activated protein kinase (MAPK)-dependent phosphorylation (36). Therefore, the level of AKR1C3 activity in cells may determine the relative activity of PPARγ by controlling the relative abundance of PGF 2α and J-series prostanoids.

Present therapies for acute myeloid leukemia (AML) rely on combined chemotherapy. However, overall response rates and disease-free survival remain poor. This is, in part, because the majority of patients are not able to tolerate the most intensive treatments. Adjuvant therapies that increase patient responses but which are themselves associated with low toxicities are therefore required. A well-established paradigm for this principle is the success of combined cytotoxic therapy and ATRA in acute promyelocytic leukemia (APL). The data presented here, together with those of our previous studies and those of other workers, now indicate that inhibitors of AKR1C3 may be similarly exploited. In this regard, it is important to note that, unlike in some tumors, NSAIDs have not been reported to be beneficial in acute myeloid leukemia. However, the lack of in vivo evidence from leukemia patients is, in part, compounded by the gut-irritant actions of NSAIDs. Given the fact that reduced platelet counts are associated with leukemia, it is rare that these patients receive NSAIDs. Nonetheless a number of studies have shown that NSAIDs promote hemopoiesis in rodents (37–40). Thus, in the particular case of leukemia, attempts to exploit AKR1C3 inhibition therapeutically will require the use of steroidal inhibitors or indeed the development of novel inhibitors.

5 Unpublished observations.
INHIBITION OF DIFFERENTIATION BY AKR1C3

However, the above issues are less important in the context of other tumors, it is significant that many studies have demonstrated that AKR1C3 has a broad tissue distribution and that PGD2 and PGJ2 promote the in vitro differentiation and apoptosis of diverse cells (reviewed in Refs. 18 and 41). Thus, it remains possible that AKR1C3 activity may influence the progression of diverse malignancies and that the capacity of NSAIDs to protect against certain tumors may in part be mediated by its inhibition. In this regard, it has been demonstrated that natural and synthetic PPARγ ligands are antiproliferative against in vitro and in vivo models of colon and prostate carcinoma, and the synthetic PPARγ ligand troglitazone has been shown to inhibit chemically induced colitis and aberrant crypt formation in rats and mice (42–46). Similarly, in a study of human prostate carcinoma, treatment with troglitazone resulted in an increased incidence of prolonged stabilization of prostate-specific antigen (PSA) levels (47). It is important to note that attempts to better exploit the antineoplastic activities of NSAIDs has centered on the derivation of drugs that selectively inhibit COX-2 over COX-1 (1–4). In future studies, it will be important to determine whether the clinical efficacy of these drugs correlates with their ability to also inhibit AKR1C3. Finally, both gut and prostate tumors share a common etiology in as much as diets high and prostate tumors share a common etiology in as much as diets high

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The Aldo-Keto Reductase AKR1C3 Is a Novel Suppressor of Cell Differentiation That Provides a Plausible Target for the Non-Cyclooxygenase-dependent Antineoplastic Actions of Nonsteroidal Anti-Inflammatory Drugs

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