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 aldol-keto 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 PGE2. Supplementing HL-60 cultures with PGE2 mimicked treatment with AKR1C3-inhibitors by enhancing the differentiation of the cells in response to ATRA. However, PGE2 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 recombinant AKR1C3 when PGD2 was predominately formed. In addition, PGJ2 but not PGE2, recapitulated the potentiation of HL-60 differentiation by PGE2 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 PGF2α and away from PGJ2. In addition, these observations identify AKR1C3 as a plausible target for the non-cyclooxygenase-dependent antineoplastic actions of NSAIDs.

INTRODUCTION

NSAIDs1 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 these 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-
molar doses of drugs that are achieved in vivo (reviewed in Ref. 8). Consequently, the novel target has remained uncertain. However, micromolar concentrations of a broad spectrum of NSAIDs also potently inhibit the aldol-keto reductase AKR1C3 (9–12). We and others have shown previously that myeloid leukemia cell lines express AKR1C3 and that steroidal and NSAID inhibitors of the enzyme promote their sensitivity to the physiological inducers of differentiation ATRA and D3 (13–17).

In this report, we demonstrate that overexpression of AKR1C3 in HL-60 cells generates the reciprocal phenotype, with the cells becoming more resistant to ATRA and D3, thus confirming the enzyme as a novel regulator of nuclear receptor-regulated cell differentiation. AKR1C3 is able to metabolize diverse substrates including 3α- and 17β-hydroxysteroids (9, 12). However, the best substrate in terms of affinity (Km) and rate of turnover [Kcat/Km (specificity constant)] is PG D2 (or PGD2), against which the enzyme exerts 11-ketoreductase activity resulting in the generation of PGF2α (11). We also demonstrate that the provision of excess PGD2, but not steroid substrates of AKR1C3, mimicked AKR1C3 inhibition by potentiating ATRA- and D3-induced HL-60 cell differentiation. PGD2 is unstable and is non-enzymatically converted to PGJ2 and, then, stepwise to 15α-PGJ2, a natural ligand of the PPARγ (reviewed in Ref. 18). Importantly, we also show that the capacity of AKR1C3 inhibitors or PGD2 to potentiate ATRA-induced HL-60 cell differentiation was replicated by PGJ2 and, furthermore, that the PPARγ antagonist GW 9662 reversed the enhancement of cell differentiation conferred by all of these treatments. We, therefore, propose that AKR1C3 functions to regulate cell differentiation by the diversion of PGD2 catabolism from the generation of J-series prostanoids and toward the generation of PGF2α, thereby protecting PPARγ from activation by endogenous ligands. The broader implications of these findings for the possible exploitation of AKR1C3 as a target in human malignancies and on the understanding of the antineoplastic activity of NSAIDs and dietary AKR1C3 inhibitors are also discussed.

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 × 105 cells/ml in 25-cm2 filter-capped cell culture flasks (Nucleon, Nunc Nalge International, Paisley, United Kingdom) or at 5 × 106 cells in 750 μl in 48-well cell culture plates (Nuncleoon), 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 DMSO was stored in the dark at −20°C. Working stocks were prepared for each experiment by diluting...
this stock in culture medium and were stored at 4°C. A stock solution of 4 mM
D3 in isopropanol was provided as a gift by Dr. Lisa Binderup (Leo Pharma-
cueticals, 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 nm 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 PGE2 and PGD2 (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-androstane-
3β-diol-3-one; Sigma) and 5 mM 3α-androstanediol (5α-androstan-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.

Production of AKR1C3 Expression Vectors. The pBluescript SK+ vector
(stratagene, La Jolla, CA) containing human AKR1C3 cDNA (KIAA0119;
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,
Ipsley, United Kingdom). Orientation and nucleotide sequence of the
constructed 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 SalI-PshAI fragment from pcDNA3.1/
Hygro (+)/AKR1C3 was ligated to the 1.3-kbp HpaI-NotI and the 5.3-kbp
NotI-Xhol fragments from MSCV-I-GFP (19).

The VSV-G-pseudotyped MSCV-AKR1C3-I-GFP retroviral vector contain-
ing supernatants were made essentially as described previously (19). 293T
cells (2×106) were transfected by calcium phosphate precipitation of the
retroviral vector plasmid MSCV-AKR1C3-I-GFP (15 μg) with the plasmid
pEQ-PAM3-E (15 μg), which expresses the retroviral 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 ultracentrif-
ugation, 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+ (Strategich Scientific,
Luton, United Kingdom) and mixed before adding 4 μl of pcDNA3.1-
AKR1C3 in 0.5 ml of 1× PBS. The cells were transfected by calcium phosphate precipitation of the
retroviral vector plasmid for 45 min. After incubation, 2×105 cells in 200 μl of RPMI
1640, 10% FBS, and antibiotics. After 48 h, cells were exposed to 500 μg/ml
G418 (Sigma) for 1 week before transfer to methycellulose (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 neo37-resistance gene of pcDNA3.1 (data not shown).

Retroviral Delivery of AKR1C3. HL60 cells in exponential growth were
seeded at a concentration of 2×105 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 used 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 1×107
cells were pelleted, washed in PBS, and resuspended in 200 μl of radioimmu-
no precipitation assay (RIPA) buffer containing protease inhibitors (Boehr-
gringer 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 (pH 8.8) 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-
colnshire, 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 antirabbit 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-NTA HisBind 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 on ice at 4°C or at −80°C. Recombinant AKR1C3 activity assays contained 100 mM potassium phosphate
buffer (Sigma; pH 7.0) along with either 2.3 mM NADPH for reduction
reactions or 2.3 mM NADP for oxidation reactions (both Sigma). One μl of
[3H]-labeled substrate (5α-DHT; Amersham Pharmacia Biotech), 3α-andro-
stanediol (NEN Life Science, Boston, MA) or PGD2 (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-androstanediol was added to 4 ml cultures set at 2×105 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

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(AKR1C3): crystal structures of binary and ternary complexes, manuscript in preparation.

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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 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/culture (×10^5) 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 culture 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 culture 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 culture 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 (7), 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 5α-DHT (△) or 5 μM PGD2 (●), 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-HL-60AKR1C3 cells (P = 0.002). Analysis of cell morphology also confirmed reduced differentiation of MSCV-HL-60AKR1C3 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 prostanoid 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 PGD$_2$ (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 PGD$_2$ showed features characteristic of HL-60-derived neutrophils (Fig. 3c). Furthermore, prolonged culture of HL-60 cells in 5 μM PGD$_2$ 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 PGD$_2$ may be in itself a weak stimulator of differentiation. However, increasing PGD$_2$ 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 PGD$_2$ to induce apoptosis in other cell models (reviewed in Ref. 18). In contrast to PGD$_2$, its AKR1C3 metabolite PGF$_{2α}$, 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$^3$] compared with parallel control cultures [(45 ± 0.1) × 10$^3$; P = 0.05].

**AKR1C3 Activity Protects against the Chemical Conversion of PGD$_2$ to PGJ$_2$.** Although biologically active in diverse systems, PGD$_2$ is chemically unstable and is rapidly converted, nonenzymatically, to PGJ$_2$ and, subsequently, to Δ$^{15}$-PGJ$_2$, and then to the PPARγ ligand 15Δ-PGJ$_2$. Thus, many of the biological activities ascribed to PGD$_2$ are likely to be mediated by J-series PGs. We observed that, after overnight incubation of PGD$_2$ in tissue culture conditions, only 23.9 ± 3.4% remained as PGD$_2$, and the majority had converted to a metabolite that comigrated with PGJ$_2$ in TLC assays (see also Fig. 4a). Furthermore, PGJ$_2$ treatment of HL-60 cells resulted in an enhanced HL-60 response to ATRA that was similar to that observed in response to PGD$_2$ (Fig. 4b). Together these observations indicate that the actions of PGD$_2$ in enhancing HL-60 cell differentiation are mediated downstream of its chemical conversion to PGJ$_2$.

The chemical instability of PGD$_2$ in the extracellular environ of HL-60 cultures precluded us from demonstrating the PGD$_2$ 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 PGD$_2$ solution to PGF$_{2α}$ in just 2 h (~5.4 pmol/μg of AKR1C3/min). Thus, at least in vitro, AKR1C3 is able to inhibit the formation of PGJ$_2$ from PGD$_2$ by the efficient catalysis of its conversion to PGF$_{2α}$. In a previous study, we demonstrated the constitutive generation of PGD$_2$ by HL-60 cells (16). The data we have shown here now suggest that, if not tightly regulated, such a pool of PGD$_2$ would lead to the endogenous generation of J-series PGs, resulting in the promotion of differentiation.

**The Ability of PGD$_2$, PGJ$_2$, 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 PGD$_2$ or PGJ$_2$ 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 steroidal AKR1C3-inhibitor MPA (Fig. 5b). In addition to reversing the potentiation of HL-60 anti-proliferative responses by PGD$_2$, PGJ$_2$, 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 PGD$_2$ increased this to 53 ± 12% (P = 0.006). However, in the presence of 1.56 nM ATRA, 5 μM PGD$_2$, 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 PGD$_2$ without GW 9662, and P = 0.9 when compared with 1.56 nM ATRA alone). Thus, GW 9662 completely abrogated the ability of PGD$_2$ 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 ± 2.1% 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 D$_3$ is mediated by the prevention of PPARγ activation via the directed metabolism of PGD$_2$ toward PGF$_{2α}$ and the consequent depletion of J-series PGs. The phenomenon of “receptor signaling” by ligand metabolizing enzymes is well established for many nuclear receptors, including thyroid hormone and steroid, retinoid, and D$_3$ 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 PG$_E_2$ pro-

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**Fig. 4.** PGD$_2$ mediates its action upon HL-60 cell differentiation downstream of its chemical conversion to PGJ$_2$. a. AKR1C3 activity prevents PGD$_2$ degradation to PGJ$_2$: Typical TLC traces for PGD$_2$ (top panel), PGD$_2$ incubated for 2 h at 37°C in the presence of human recombinant AKR1C3 (middle panel), and PGD$_2$ incubated overnight at 37°C in naked tissue culture medium (bottom panel). PGJ$_2$ and PGF$_{2α}$ were identified by comigration of “cold” standards. b. PGJ$_2$ promotes ATRA-induced HL-60 cell differentiation. HL-60 cells were treated with the doses of ATRA shown either alone (○) or in combination with 5 μM PGJ$_2$ (□) and the resultant differentiation assayed by FACS analysis of CD11b expression. Data are the mean ± SE of three experiments.
AKR1C3 inhibitors and PPAR
combination with the shown concentrations of PGD₂ or PGJ₂ and in either the presence
H9253 in inhibitors indomethacin and MPA is dependent on PPAR
f, 3.125 (E), and 6.25 (Œ) n M ATRA in for 4 days in the presence of 0 (F/H17004), 1.56 (E), shown alone (ƒ), or in the presence of
performed in triplicate.

PGD₂ was not ablated by this treatment, but, in contrast, its
duction (a widely used assay for COX inhibition). Importantly, however, PGD₂ was not ablated by this treatment, but, in contrast, its AKR1C3-derived product, PGF₂α, was (16). Thus, at the doses that potentiate HL-60 differentiation, indomethacin acts as both a COX and a AKR1C3 inhibitor. It is, therefore, not possible to exclude that the potentiation of differentiation by NSAIDs is dependent on both activities. However, the data that we have described here are the first to clearly demonstrate the role of AKR1C3 in these processes. Furthermore, in our previous study, we observed that a spectrum of AKR1C3 inhibitors, including steroidal inhibitors that do not inhibit COX-inhibitory actions, all potentiated HL-60 cell differentiation (16).

AKR1C3 has a broad tissue distribution and, at least in vitro, recognizes diverse substrates. This raises the possibility that the enzyme has separate roles in different tissues. In the case of the prostate, it has been suggested 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 PGD₂ as the key AKR1C3 substrate and that the enzyme diverts its metabolism toward PGF₂α, and away from J-series prostanoids. These findings are supported by those of others demonstrating that PGD₂ and PGJ₂ 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 PGD₂ and PGJ₂ 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 PGD₂-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 PGD₂ that are mediated downstream of its chemical conversion to PGJ₂. Although abundant in PGD₂, the extracellular environment is not rich in free PGJ₂, 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 PGJ₂ from which myeloid progenitor cells have to protect themselves. Our study now indicates that the ability of AKR1C3 to divert PGD₂ toward PGF₂α and away from PGJ₂ 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, PGF₂α, 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 PGF₂α 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.

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5 Unpublished observations.
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 (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 in vegetable content have been demonstrated to protect against both diseases (48–50). As yet it is uncertain exactly how this protection occurs. However, a recent study has identified more than 20 dietary plant constituents that act as inhibitors of AKR1C3 (51), and therefore, it is now possible to provide a plausible rationale for unifying the protective antineoplastic activities of NSAIDs and diet in these diseases.

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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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