

Identification of a retinoic acid responsive aldoketoreductase expressed in HL60 leukaemic cells

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Abstract Neutrophil and monocyte differentiation can be induced in HL60 leukaemia cells by all-*trans*-retinoic acid (ATRA) and 1 α ,25-dihydroxyvitamin D₃ (D₃), respectively, whose differentiating effects can be enhanced by exposure to ‘anti-inflammatory agents’ and steroids. We have provided evidence that this potentiation is via inhibition of the activity of an enzyme of the aldoketoreductase (AKR) family, but had failed to identify expression of known AKRs in HL60 cells. In this study, we have identified a previously unclassified aldoketoreductase family member (termed HAKR e) that is expressed in HL60 cells. HAKR e is dramatically and transiently up-regulated in HL60 cells within 24 h of exposure to ATRA, further supporting the proposition that a member(s) of this family of enzymes play(s) a role in controlling cell growth and/or differentiation.

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Key words: Aldoketoreductase; HL60; All-*trans*-retinoic acid responsive; Hydroxysteroid dehydrogenase

1. Introduction

The human myeloid leukaemia-derived cell line HL60 has been used extensively for studies of cell differentiation. These cells differentiate towards neutrophils or monocytes in response to all-*trans*-retinoic acid (ATRA) [1] and 1 α ,25-dihydroxyvitamin D₃ (D₃) [2], respectively. In our previous studies, we observed that sensitivity of HL60 cells to the differentiating effects of these two agents is increased by the addition of various agents. Initially, the ‘anti-inflammatory agents’ indomethacin and dexamethasone were observed to potentiate ATRA-induced neutrophilic differentiation and dexamethasone was shown to potentiate D₃-induced monocytic differentiation [3]. We investigated the possibility that indomethacin (a cyclooxygenase inhibitor [4]) and dexamethasone (a phospholipase A₂ inhibitor [5]) were preventing the formation of a differentiation-suppressive prostanoid. Analyses of prostanoids produced by HL60 cells and attempts to reverse indomethacin potentiation of differentiation by adding back selective prostanoids failed to identify an endogenous prostanoid that suppresses differentiation [6].

We identified other agents that potentiate HL60 differentiation, which are aspirin, medroxyprogesterone acetate and 17 β -

estradiol [6]. These agents, together with indomethacin and dexamethasone, are all effective inhibitors of 3 α -hydroxysteroid dehydrogenase (3 α -HSD) [7–10]. We demonstrated that medroxyprogesterone acetate and 17 β -estradiol do not interfere with prostanoid metabolism in HL60 cells. Furthermore, the pharmacological profile for potentiation of differentiation by all the agents we have used matches that for inhibition of 3 α -HSD rather than that for inhibition of cyclooxygenase or PLA₂ [6]. A protein that is recognised by an antiserum to 3 α -HSD is expressed in HL60 cells; this protein is the same size (34 kDa) as rat liver 3 α -HSD and as recombinant 3 α -HSD. From these studies, we concluded that decreased activity of a so far unidentified member of the aldoketoreductase (AKR) family of dehydrogenases leads to increased responsiveness of HL60 cells to differentiating agents [6].

3 α -HSD is a member of a large family of dehydrogenases of similar size and primary sequence. Four human enzymes have been identified that are structurally related to 3 α -HSD; these are expressed at high levels in liver, and are known as human aldoketoreductases a–d (HAKR a–d) [11]. We have used PCR and restriction digest to identify which, if any, of these aldoketoreductases are present within HL60 cells. From this, we identified a further closely related member of the human HAKR family (termed HAKR e). Analysis of levels of mRNA and protein during HL60 neutrophil and monocyte differentiation have revealed that HAKR e expression is ATRA inducible.

2. Materials and methods

2.1. Cell culture

Stock cultures of HL60 cells were maintained in exponential growth in serum free conditions containing RPMI 1640 medium (Gibco, Paisley, UK) containing 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco) and supplemented with a serum replacement of 1% ITS (insulin-transferrin-selenium ions) (Stratec, Luton, UK). To induce monocyte differentiation, HL60 cells were treated with a high dose of D₃. For Northern analyses of AKR mRNA levels, we used 5 $\times$ 10^{−7} M D₃, whilst for Western analyses of protein levels we used 1 $\times$ 10^{−7} M D₃. Induction of neutrophil differentiation of serum free-grown HL60 cells requires the presence of a high dose of ATRA (5 $\times$ 10^{−7} M) together with a small amount of D₃ (5 $\times$ 10^{−14} M) [12]. HL60 cells were also treated with a high dose of ATRA alone (5 $\times$ 10^{−7} M), which results in growth arrest but sub-optimal neutrophil differentiation (around 25% mature cells) [12]. For Northern analyses, we also treated HL60 cells with a high dose of ATRA (5 $\times$ 10^{−7} M) and a moderate amount of D₃ (5 $\times$ 10^{−9} M), which result in monocyte differentiation [12]. Western analyses of protein levels were undertaken up to day 5 and cultures were fed with medium containing appropriate agents at day 2.

2.2. RT-PCR and restriction analysis of human AKR products

Cells were washed and RNA was extracted using a RNeasy kit

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(Qiagen). RNA (1 µg) was reverse transcribed for 10 min at 42°C, the reverse transcriptase was inactivated at 99°C for 15 min and finally cooled to 5°C for 1 min. Two µl of the RT reaction were amplified using HAKR3' and HAKR5' primers and the following cycle conditions: 5 cycles of 95°C for 45 s, 50°C for 60 s, 72°C for 60 s; 30 cycles of 95°C for 45 s, 55°C for 60 s, 72°C for 60 s; followed by a soak at 4°C. The primer sequences were: HAKR3' (antisense), 5'-CAT TCA ACC TGG TTG CAC AC-3'; HAKR5' (sense), 5'-GAC ATA TTC TAC ACT TCA AAG C-3'. The 346-bp fragment amplified represented bases 239–585 of HAKR a, 242–588 of HAKR b, 223–569 of HAKR c and 238–584 of HAKR d. PCR products were run on a 1.5% agarose gel, and the resultant fragment size was, as expected, around 350 bp.

PCR fragments were initially digested with *Bsa*I, *Bbv*I or *Ava*II for comparison with the digest patterns for the four HAKRs. A second series of enzymes (*Hinf*I, *Bbv*I, *Nco*I or *Bsa*I) were used to refine identity. The PCR fragment was used to screen a cDNA library from uninduced HL60 cells. Two matches were sequenced and compared to the four published sequences for HAKR a–d. The clone KIAA0119 (a novel HAKR) was a gift from Kazusa DNA Research Institute, Kasuza, Chiba, Japan.

2.3. Analyses of HAKR mRNA and protein levels

RNA (5 µg) from uninduced HL60 cells and from HL60 cells treated with differentiation agents for 1, 2 and 3 days were run in a formamide agarose gel, and Northern blotted. The blot was hybridised overnight with the full length KIAA0119 clone, which is identical in sequence to HAKR e, at 65°C, washed and exposed for 48 h. The filter was also probed with an actin cDNA to confirm equal loading. The filters were scanned and the expression level of HAKR e relative to that of ABL obtained.

HAKR protein levels were measured by immunostaining Western blots with a rabbit polyclonal antiserum raised against rat 3α-HSD. This antiserum (71536) was a gift from Professor T. Penning, University of Pennsylvania. Briefly, extracts from 5×10^6 cells were boiled in sample buffer and loaded onto a 12.5% SDS-polyacrylamide gel. After electrophoresis at 120 V, proteins were transferred onto an Immobilon-P (Millipore) membrane, which was blocked with Tris-buffered saline Tween 20 (0.1%, pH 7.5), 2.5% milk powder for 1 h, then incubated with the rabbit antiserum (at 1:800 in PBS). Antibodies bound to proteins were detected using an ECL chemiluminescence system (Amersham). Gel lanes were loaded with equal amounts of proteins and to ensure even loading the membrane was stained afterwards with Ponceau S. Densitometry was performed on the bands to quantify changes in HAKR protein levels.

3. Results

3.1. HL60 cells express a novel HAKR

To identify which, if any, of the four known HAKRs is present in HL60 cells, RNA was reverse transcribed and amplified by PCR using primers that flank a region of high homology (98%). This region, between 242 and 588 bp (in HAKR b), was also chosen because sequence differences between HAKRs can be identified by simple restriction fragment analyses of PCR fragments (Table 1A). Restriction analysis suggested that the PCR fragment amplified from HL60 cells consisted of either HAKR b (288) or HAKR c (290). Further restriction analyses, with a second series of enzymes, indicated that the vast majority of the PCR product was or closely resembled HAKR b (280) (Table 1B).

The HAKR PCR fragment was used to probe an uninduced HL60 cDNA library. Two positive clones were identified. Sequence analysis of these clones indicated a very close homology with HAKR b (98.5%), however, both clones had 100% homology with a cDNA (D17793, KIAA0119) isolated from a library constructed from the human myeloid cell line KG1 [13]. We suggest that KIAA0119 should be classified as HAKR e. This HAKR has only a 1.1% divergence from HAKR b, and, in the analyses undertaken, shows the same restriction enzyme digestion pattern as HAKR b.

3.2. Expression of HAKR e is ATRA inducible

Treatment of serum free-grown HL60 cells with 5×10^{-7} M ATRA together with 5×10^{-14} M D₃ resulted in maximum differentiation to neutrophils. After such treatment and at day 5, 72% of cells phagocytosed yeast and <0.5% of cells were stained for monocyte specific esterase (ANAE), using α-naphthyl-acetate as substrate. At day 1, there was an initial dramatic increase in expression of mRNA followed by a gradual down-regulation of the mRNA (Fig. 1). A similar pattern of expression was observed when HL60 cells were treated with 5×10^{-7} ATRA alone. The use of ATRA alone, as reported

Table 1
Restriction digest analysis of the HAKR expressed in HL60 cells

A	Accession no.	<i>Bsa</i> I		<i>Bbv</i> I		<i>Ava</i> II	
		No. of bands	Fragment lengths	No. of bands	Fragment lengths	No. of bands	Fragment lengths
HAKR a	S68287	2	166, 181	2	46, 301	2	70, 277
HAKR b	S68288	1	346	2	46, 301	3	25, 25, 297
HAKR c	S68290	1	346	2	70, 277	2	50, 297
HAKR d	S68330	1	346	–	346	2	50, 297
PCR fragment		1	~ 350	1+	~ 50, ~ 300	1+	~ 300

B	Accession no.	<i>Hinf</i> I		<i>Bbv</i> I		<i>Nco</i> I		<i>Bsa</i> I	
		No. of bands	Fragment lengths	No. of bands	Fragment lengths	No. of bands	Fragment lengths	No. of bands	Fragment lengths
HAKR b	S68288	2	37, 310	2	46, 301	2	130, 217	1	346
HAKR d	S68330	1	346	2	70, 277	1	346	1	346
PCR fragment		1+	~ 310	1+	~ 300	2	~ 130, ~ 220	1	~ 350

PCR fragment, approximate size 350 bp, amplified using HAKR5' and HAKR3' primers were digested initially with *Bsa*I, *Bbv*I and *Ava*II individually (A). These digests suggested that the PCR fragment was either HAKR b or HAKR d. Further digests (B) indicated that the vast majority of the PCR product was HAKR b. The 1+ indicates that only 1 fragment was observed by ethidium bromide staining, but was smaller than the undigested PCR fragment, therefore the smaller digest fragments were presumable present, but undetected.

previously [13], resulted in sub-optimal neutrophil differentiation (25% mature cells). HAKR e mRNA levels were unaffected when HL60 cells were treated with 5×10^{-7} M D_3 to induce monocyte differentiation. Following this induction regimen, and at day 5, 77% of cells were observed to phagocytose yeast and 66% of cells expressed ANAE. An immediate up-regulation in the level of expression of HAKR e mRNA was observed when HL60 cells were treated with a high dose of ATRA (5×10^{-7} M) together with a moderate dose of D_3 (5×10^{-9} M). This combination of a high dose of ATRA and a moderate amount of D_3 , as reported previously [13], induced HL60 cells to differentiate to monocytes. Levels of mature monocytes were comparable to those observed in cultures treated with the high dose of D_3 alone. Thus, an immediate up-regulation of HAKR e mRNA expression was observed post-ATRA treatment per se and irrespective of whether HL60 cells had undergone neutrophil differentiation or monocyte differentiation or even maximum differentiation.

Analyses of changes in HAKR e proteins levels during HL60 differentiation were undertaken by immunostaining blots with an antibody to 3 α -HSD (Fig. 2). In these experiments, analyses were extended up to day 5. At day 5, percentages of mature cells (phagocytic) in cultures that had differentiated to neutrophils (5×10^{-7} M ATRA/ 5×10^{-14} M D_3) and monocytes (1×10^{-7} M D_3) were 78% in both instances. Sub-optimal neutrophil differentiation was observed in cultures treated with 5×10^{-7} M ATRA alone (28% phagocytic cells). Between days 0 to 3, data obtained for protein levels

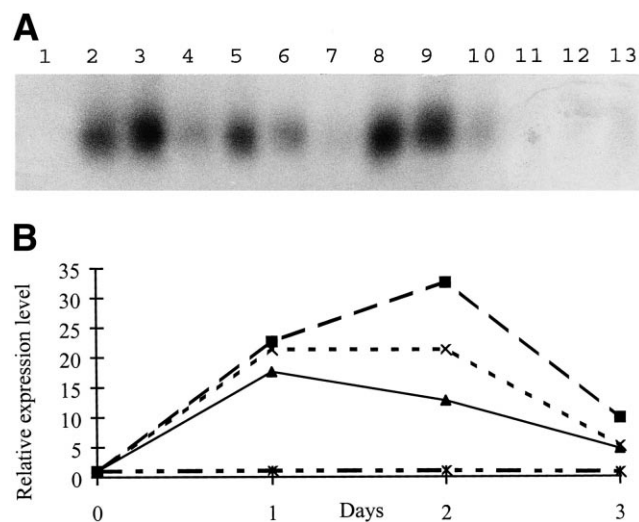


Fig. 1. HAKR e is ATRA inducible. A: Northern blot of RNA extracted from uninduced HL60 cells (lanes 1), or cells exposed to 5×10^{-7} M ATRA (limited neutrophil differentiation) (lanes 2–4), 5×10^{-7} M ATRA/ 5×10^{-9} M D_3 (monocyte) (lanes 5–7), 5×10^{-7} M ATRA/ 5×10^{-14} M D_3 (neutrophil) (lanes 8–10) or 5×10^{-7} M D_3 (monocyte) (lanes 11–13) over a 3-day period of exposure. Lanes 2, 5, 8 and 11 represent day 1, lanes 3, 6, 9 and 12 are day 2, and lanes 4, 7, 10 and 13 are day 3. The filter was hybridised with the full length KIAA0119 cDNA. B: Relative expression of KIAA0119 on each of the days of the ATRA/ D_3 treatments standardised for loading and calculated relative to expression in uninduced HL60 cells. (—■—) Limited neutrophil differentiation (5×10^{-7} M ATRA only); (—▲—) neutrophil (5×10^{-7} M ATRA, 5×10^{-9} M D_3); (—×—) monocyte (5×10^{-7} M ATRA, 5×10^{-14} M D_3); (—*—) monocyte (5×10^{-7} M D_3 only).

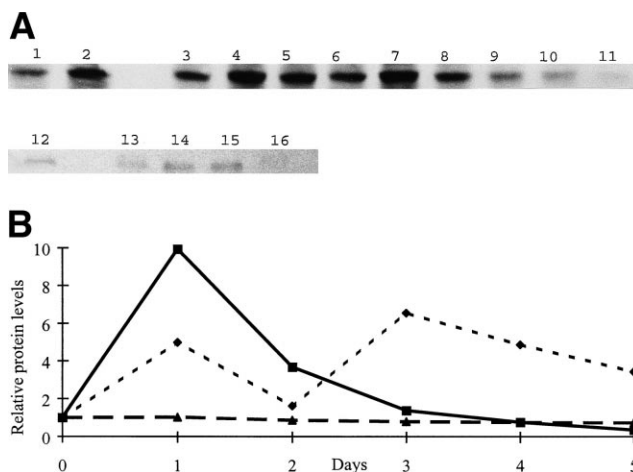


Fig. 2. Changes in HAKR e proteins levels during HL60 neutrophil and monocyte differentiation. A: A representative Western blot of protein extracts from uninduced HL60 cells (lanes 1 and 12) and HL60 cells undergoing growth arrest and limited neutrophil differentiation (5×10^{-7} M ATRA) (lanes 2–6 represent days 1–5 of induction), neutrophil differentiation (5×10^{-7} M ATRA/ 5×10^{-14} M D_3) (lanes 7–11 representing days 1–5 of induction) or monocyte differentiation (1×10^{-7} M D_3) (lanes 13–16 represent days 1–4 of induction). B: Changes in protein levels relative to the amount present in uninduced HL60 cells. HL60 cells were induced to undergo neutrophil differentiation (—■—), monocyte differentiation (—▲—) or growth arrest and limited neutrophil differentiation (—♦—), using the above conditions.

(see Fig. 2) parallel the observations from Northern blots for mRNA levels. During neutrophil differentiation, induced by treatment with 5×10^{-7} M ATRA/ 3.9×10^{-14} M D_3 , there was an immediate up-regulation of expression of HAKR e protein followed by a return to the level observed in uninduced HL60 cells. An immediate up-regulation, at day 1, was also observed when HL60 cells were treated with 5×10^{-7} ATRA alone. There was then a reduction in the level of protein, at day 2, followed by a further increase post-feeding the cells, at day 2, with ATRA. HAKR e protein levels did not change between days 0 to 3 in HL60 cells treated with 1×10^{-7} M D_3 to induce monocyte differentiation. In terminally differentiated neutrophils (5 days post treatment with 5×10^{-7} M ATRA/ 3.9×10^{-14} M D_3) and monocytes (5 days post treatment with 1×10^{-7} M D_3) levels of HAKR e expression were lower than that observed in uninduced HL60 cells. This down-regulation of expression was not observed in cells from cultures which had been treated with 5×10^{-7} ATRA alone and which had showed sub-optimal neutrophil differentiation.

We examined the effect of indomethacin (an inhibitor of 3 α -HSD) on HAKR e expression in HL60 cells deriving a stock of cells (serum free) that has been maintained, for >6 months, in 20 μ M indomethacin. These cells grow slower than the parental cells (doubling time of 36 h vs. 24 h) and have increased sensitivity to vitamin D_3 . Concentrations of D_3 required to give 50% maturation (phagocytic cells) of indomethacin grown cells, cells co-treated with 20 μ M indomethacin and D_3 alone were 1.4 nM, 4 nM and 42 nM, respectively ($n=3$). Repeated determinations of the levels of HAKR e in indomethacin-grown HL60 cells vs. parental cells has shown that indomethacin has no effect on the level of expression of HAKR e (data not shown).

4. Discussion

Proposition of the involvement of an aldoketoreductase in the control of cell differentiation arose from studies which showed that potentiators of HL60 differentiation, such as indomethacin, dexamethasone, aspirin, medroxyprogesterone acetate and 17β -estradiol, are all effective inhibitors of 3α -HSD [7–10]. A 3α -HSD-like protein is expressed in HL60 cells. Four human aldoketoreductases have been identified, with close homology to each other and to 3α -HSD [11]. The high degree of restriction fragment length polymorphisms suggested that these four proteins form the basis of a larger family of human AKRs.

Based on the evidence presented above, we wanted to identify which, if any, of the human AKRs is expressed in HL60 cells. The PCR/restriction digest screening protocol we developed indicated that HAKR b is the major gene expressed in HL60 cells. However, further cloning and sequencing revealed that although the HL60 HAKR has close homology to HAKR b, it is an additional HAKR family member. This gene had been previously isolated from a cDNA library produced from the KG1 myeloblastic cell line [13]. The clone (KIAA0119) was reported to have a region of homology with another member of the AKR family of genes, chlordecone reductase [13]. In addition, Lin and co-workers have recently characterised a 3α -HSD cDNA isolated from a human prostate cDNA library (termed type 2 3α -HSD) [14]. Comparison of the sequence of clone KIAA0119 with the nucleotide sequence of the human prostate type 2 3α -HSD clone revealed a single base difference, which does not affect the predicted amino acid sequence. Thus, the HAKRs cloned from HL60 cells, KG1 cells and prostate cells are identical.

Interestingly, HAKR e has been cloned from KG1 and HL60 cells, which are myeloid leukaemic cells with minimal morphological maturation. We have postulated that the enzyme activity suppresses differentiation, which could occur as a consequence of promotion of proliferation. In other words, the enzyme product favours proliferation over differentiation. Lin and co-workers have studied levels of HAKR e mRNA in primary cultures of epithelial and stromal cells derived from normal tissues, benign prostatic hyperplasia and prostate carcinoma tissues [14]. Elevated mRNA levels were observed in epithelial cells derived from benign prostatic hyperplasia and prostate carcinoma. Characterisation of this enzyme, by Lin and co-workers [14], has revealed bifunctional $3\alpha/17\beta$ -HSD activity. Hence, these investigators have postulated that increased HAKR activity in diseased prostate may either interfere with the effects of androgens on the abnormal growth of prostate carcinoma cells or reflect androgen-independent growth of the gland. In HL60 and KG1 leukaemia cells and prostate carcinoma cells, activity of HAKR e may be 'pro-proliferation' and, as such, reduce the capacity of cells to respond to differentiating agents.

Analyses of HAKR e mRNA and protein levels in HL60 cells treated with amounts of ATRA and D_3 , alone and in combination, have revealed that this enzyme is ATRA inducible. Immediate up-regulation of enzyme expression occurred post-treatment with ATRA per se regardless of whether HL60 cells had undergone neutrophil differentiation (high dose ATRA/low amount of D_3), monocyte differentiation (high dose of ATRA and moderate amount of D_3) or growth arrest with limited differentiation (high dose of ATRA alone). In our

original study, we used a priming dose of ATRA (10 nM) and observed a very small up-regulation of HAKRe (at day 1), followed by down-regulation, to a level of 20–30% of that in uninduced HL60 cells [6]. The pattern of change observed post-ATRA treatment in the experiments presented in this paper is very similar. The difference is that we observed an initial substantial up-regulation of the level of HAKRe, followed by down-regulation to a level of 35% of that present in uninduced cells. The now clearly defined initial rise in HAKR e is presumably attributable to the higher dose of ATRA (100 nM) used in the present study. Whether the initial rise, subsequent lowering of levels of HAKR e or both events affect HL60 differentiation is, at present, unclear. Changes in the level of expression were not a feature of growth arrest per se since they were not observed when HL60 cells were induced to differentiate to monocytes by a high dose of D_3 alone. Prostate carcinoma cells are also sensitive to the anti-proliferative effects of ATRA, and ATRA mediated regulation of expression of HAKR e may be important in this context. We do not know the substrate and product of HAKR e in HL60 cells and, therefore, it is difficult to speculate why HAKR e is ATRA responsive. The substrate is likely to be endogenously produced and steroid-like since potentiation experiments have been undertaken using HL60 cells that had been grown long term in serum free conditions. In this regard, immediate up-regulation of HAKR-e post ATRA-induced differentiation would be predicted to delay differentiation and promote cell expansion (see above). Indeed, in cultures induced to undergo neutrophil differentiation (by 5×10^{-7} M ATRA and 5×10^{-14} M D_3) mature cells appear predominantly between days 3 to 5, and post the day 1 peak of HAKR e expression. We have, also, to reexamine whether potentiation of differentiation, by indomethacin and dexamethasone, is via inhibition of HAKR e activity. These agents are known to inhibit 3α -HSD activity, but we do not know their effects on activity of the novel AKR cloned from HL60.

HAKR e has now been cloned from two undifferentiated leukaemic cell lines and is highly expressed in prostate carcinoma cells. HAKR b, which has close homology with HAKR e, is expressed in placenta; this tissue can be classified as a 'differentiating' tissue. HAKR e is also ATRA responsive in HL60 cells. These findings strengthen the argument, from studies which revealed that potentiators of HL60 differentiation are inhibitors of AKR activity, that a member(s) of the AKR enzyme family may be a key component(s) of an intracellular mechanism that controls the capacity of cells to grow and/or differentiate.

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