The cytosolic aldehyde dehydrogenase gene (*Aldh1*) is developmentally expressed in Leydig cells

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Abstract Cytosolic aldehyde dehydrogenase, ALDH1, participates in the oxidation of different aldehydes including that of all-*trans* retinal to retinoic acid. The accumulation of mouse *Aldh1* transcripts is characterized by having different patterns in different tissues. This paper reports the greatest expression of *Aldh1* in testis and liver. It was demonstrated that in testis, *Aldh1* is specifically expressed in Leydig cells and is under developmental regulation. In vitro studies of cultured Leydig TM3 cells confirmed these results though such gene expression was found not to be mediated by LH regulation. Previous investigations have associated androgen receptors, and hence the androgen insensitivity syndrome in man, with the presence of ALDH1 in genital skin fibroblasts. However, this relationship was not established in a functional cell type, as is reported here for Leydig cells. These results could suggest a model for a molecular pathway from androgen receptor to retinoic acid biogenesis in Leydig cells via the mediation of ALDH.

Key words: *Aldh1*; Leydig cell; Spermatogenesis; Gene expression

1. Introduction

The aldehyde dehydrogenases (ALDH) (EC 1.2.1.3) are a family of enzymes that catalyze the oxidation of a great number of aldehydes to their corresponding acids. The participation of different ALDH isozymes in the detoxification of acetaldehyde from ethanol ingestion [1], metabolism of corticosteroids [2], biogenic amines [3], neurotransmitters [4] and retinoic acid (RA) [5] has been reported.

ALDH1 from humans [6–8] and rodents [9,10] shows a high capacity to oxidize retinal to RA. RA also stimulates testosterone synthesis in cultures of Leydig cells [11]. Further, it has been reported that RA is able to reinitiate spermatogenesis in vitamin A-deficient rats [12], and that retinoic acid receptor mRNA levels are regulated during spermatogenesis [13].

The androgen insensitivity syndrome is a complex, X-linked disorder in humans, with a wide spectrum of phenotypic expressions ranging from testicular feminization syndrome to subfertility [14]. Some patients with mutations in the X-linked androgen receptor gene show a lack of expression of a 56 kDa protein, characterized as ALDH1 [15], found in normal genital skin fibroblasts. The correlation between androgen insensitivity syndrome and RA synthesis mediated by ALDH1 has been suggested [16,17]. However, how such interactions may occur and in what specific cell type they might take place, is unknown. This paper reports a developmental expression pattern of *Aldh1* in testis [18], initially detected during differential screening analysis of a mouse prepuberal testis subtractive library enriched with early-expressed, testis-specific transcripts [19].

To investigate a possible role for cytosolic Aldh during spermatogenesis, Northern blot analysis and in situ hybridization of mRNA *Aldh1* were performed on testis cells. These approaches revealed a defined expression of the *Aldh1* gene in Leydig cells in a manner which showed a developmental pattern from fetal to adult testis. Expression was also seen in cultured Leydig-type cells. To extend this work, in vitro experiments to investigate *Aldh1* gene regulation by LH as a steroidogenesis mediator were performed.

Evidence is here described of defined Leydig cell-regulated *Aldh1* expression that provides a logical pathway from androgen receptors to retinal oxidation to retinoic acid, mediated by *Aldh1*. RA is involved in testosterone synthesis and interacts with Sertoli cells, leading to spermatogenesis.

2. Materials and methods

2.1. RNA isolation and cell cultures

Tissue samples from brain, kidney, intestine, heart, liver and testis were removed from adult mice. Testis samples were obtained from fetal (days 14, 15, 16, 17, and 18 post coitum) newborn and postnatal mice (days 3, 6, 10, 12, 14, 22 and postpartum). Total RNA was isolated from tissues by the acid guanidinium method [20].

TM3 cells [21] (a Leydig-type cell line), were provided by the American Type Culture Collection. Cultured cells were grown at 37°C in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium containing 1.2 g/l sodium bicarbonate, 15 mM HEPES and 4.5 g/l glucose, horse serum 5%, FBS 2.5% [21]. Total RNA from cells was isolated using RNAzol B (Biotecx). To check the effect of LH on *Aldh1* transcription in TM3 cells, cultures were grown in the described medium. The culture medium was changed from complete medium to serum-free medium and cells cultured for 2 h (time 0) before adding LH (Sigma) at 1 ng/ml or 100 ng/ml. RNA samples were isolated from TM3 cells after 2, 4, 6, 8, 12 and 24 h of LH treatment.

2.2. Northern analysis

Northern blots were performed using total RNA from tissues and TM3 cultured cells. 15 μg of total RNA samples were separated by electrophoresis under denaturing conditions in 1% agarose with 8% formaldehyde. Agarose gels were blotted onto nylon membranes by a vacuum blotter for 4 h and UV cross-linked. Blots were hybridized with an *Aldh1* probe and reprobed with a β-actin probe as a loading control. The *Aldh1* cDNA probe was obtained from a mouse prepuberal testis subtractive library [19,22]. The probes were labeled by random priming with [α-^{32}P]dCTP and hybridized at 42°C for 24 h. Filters were then washed twice in 2×SSC, 0.1% SDS at room temperature for 15 min and three times in 0.1×SSC, 0.1% SDS at 65°C for 15 min each. Films were exposed overnight at −80°C.

2.3. In situ hybridization

Testes were removed from adult mice and immediately frozen in dry

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ice. Sections of 12 μm were obtained in a cryostat, mounted on RNAse-free slides coated with poly-l-lysine (50 μg/ml) and kept frozen at −80°C. Slides were fixed in 4% paraformaldehyde in PBS, dehydrated and acetylated by incubation in 0.25% acetic anhydride in 0.1 M triethanolamine and 0.15 M NaCl (pH 8.0) for 10 min. Preparations were washed in 2×SSC, dehydrated, delipided in chloroform for 5 min and hybridized with an antisense riboprobe corresponding to *Aldh1* cDNA, using the T7 promoter of pBluescript (Stratagene) in which *Aldh1* was cloned. The T3 promoter was used to synthesize the sense riboprobe used as a negative control.

The riboprobe was labeled with [α-^{33}P]UTP and following alkaline hydrolysis was hybridized overnight at 55°C in a humidified chamber. 5×10^5 cpm per slide were used in a buffer containing 50% formamide, 1×Denhardt, 10% dextran sulfate, 0.2 M NaCl, 50 mM Tris-HCl (pH 8.0), 2.5 mM EDTA (pH 8.0) and 0.25 mg/ml tRNA.

After hybridization the slides were washed 4 times in 4×SSC, dehydrated and washed under stringent conditions for 10 min at 60°C in 50% formamide, 40 mM Tris-HCl, 1 mM EDTA (pH 8.0). The slides were then incubated in RNAse A in 0.5 M NaCl, 10 mM Tris- HCl (pH 8.0), 1 mM EDTA at 37°C for 30 min, washed in 2×SSC, 1×SSC and 0.5×SSC for 5 min at room temperature followed by 0.1×SSC at 50°C for 15 min and 5 min at room temperature. The slides were dehydrated and exposed in a cassette with β-max hyperfilm (Amersham) to test the signal intensity. After 4 days of exposure the film was developed and the slides coated with LM emulsion (Amersham). After convenient exposure they were developed, dehydrated and stained with hematoxylin-eosin or Hoechst 33258 and permanently mounted on Entellan (Merck). Slides were visualized and photographed using fluorescence microscopy, bright-field and dark-field illumination.

3. Results

3.1. *Aldh1 is expressed in tissue-specific patterns*

To investigate the differential pattern of expression of *Aldh1* during spermatogenesis, different tissues and developmental stages were analyzed. Northern analysis showed the highest levels of expression of *Aldh1* in testis and liver. No expression was detected in kidney and brain. An intermediate signal was observed in intestine (Fig. 1A). However, in long exposure autoradiographs, a small signal appeared in heart and skeletal muscle samples. The latter showed a longer transcript (2.7 kb) than that usually observed (2.1 kb) (Fig. 1B). In mouse, as in man, a high level of expression was observed in liver, a tissue in which a metabolic role for Aldh1 has been suggested [23].

The high expression in testis suggests an important role for Aldh1 in spermatogenesis. To determine the pattern of gene regulation during testis development, Northern analyses were performed on tissues from newborns to adults. High expression was detected at birth which progressively decreased until day 14 of postnatal (pn.) life. Subsequently a new increase was detected with the maximum level observed in adult testis (Fig. 2).

In mouse, the first cytologically recognized meiotic cells – those at leptotene – can be detected at day 10 pn. Cells at pachytene can be seen at day 14 pn. [24] when *Aldh1* presents the lowest level of expression. In the present study the increase of *Aldh1* was coincident with the appearance of haploid cells. The presence of these differentiated cells generates new cell-cell interactions [25], capable of affecting the metabolism of Leydig cells, such as its steroidogenic activities (reviewed by [26]), by paracrine control [27,28]. However, the change in the level of expression was coincident with the progressive substitution of fetal Leydig cells by adult cells [29]. As observed in comparative Northern analysis (Fig. 3), *Aldh1* was also expressed in the early stages of embryonic development of testis tissue.

To detect newly transcribed *Aldh1* RNA, runoff experiments were performed in purified spermatocytes. Negative results were obtained for the expression of *Aldh1* in comparison to the transcription of genes expressed during germ cell differentiation [19] (data not shown).

In order to localize those cells expressing *Aldh1*, in situ hybridization analysis with antisense riboprobes was performed in adult testis sections. A strong signal was detected on the interstitial compartments of the seminiferous tubules. In rodents these are mainly composed of Leydig cells (Fig. 4).

3.2. *Analysis of in vitro expression and LH modulation*

The above experimental observations were confirmed by studies with cultured TM3 cells. This cell strain, with characteristics of normal mouse Leydig cells, expressed *Aldh1* but at lower levels than those observed in total testis cells (Fig. 5). Since the developmental pattern of *Aldh1* expression is parallel to the synthesis of murine testosterone [30,31], and since TM3 cells respond to LH, experiments were performed to asses the use of these cells as a model of LH-mediated *Aldh1* expression. However, TM3 cells cultured in a serum-free medium with different concentrations of LH did not modify *Aldh1* expression when compared to serum-deprived and LH-free cultures. Interestingly, in the absence of serum in the

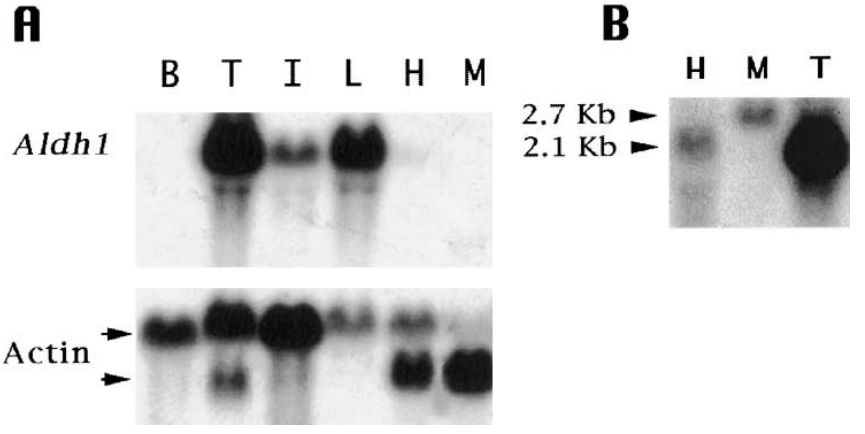

Fig. 1. Northern blot of mouse adult tissues. Total RNA from: B, brain; T, testis; I, intestine; L, liver; H, heart; M, skeletal muscle was extracted and 15 μg run on an agarose gel under denaturing conditions. Blots were hybridized with an *Aldh1* cDNA probe [19]. A: Filter exposed overnight. B: Exposed 72 h. Blots were rehybridized with a β-actin probe as loading control.

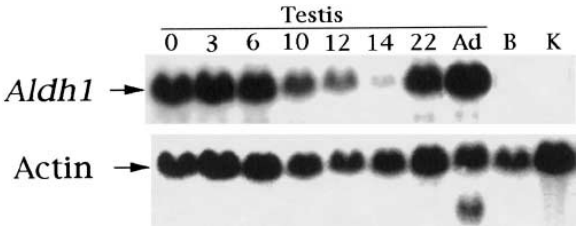

Fig. 2. Northern blots of total RNA from mouse testis over 0–22 days of postnatal development. Blots were hybridized with the *Aldh1* cDNA probe and rehybridized with a β-actin probe. Tracks of testis samples correspond to postnatal days and adult samples (Ad); B, brain; K, kidney.

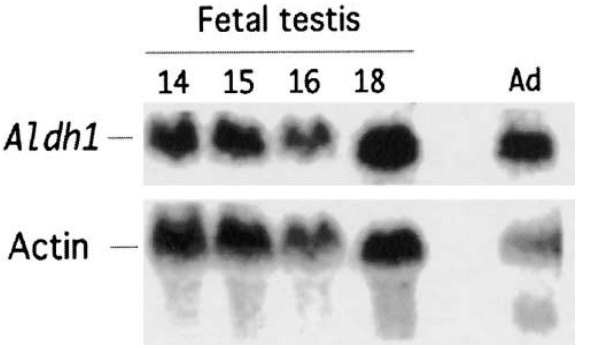

Fig. 3. Time course of expression of *Aldh1* RNA in fetal testis. Northern analysis was performed as in Figs. 1 and 2. Adult testis RNA was run (Ad) in comparison to RNAs from different post coitum days. A β-actin probe was used to rehybridize the same filter and compare the relative expression of the *Aldh1* gene.

culture medium, and independently of the presence of LH, TM3 cells exhibit an increase of *Aldh1* expression after 6 h of culture. This decayed after 24 h of culture (Fig. 5). This suggests that an unidentified element of the serum could modulate *Aldh1* gene expression as a negative regulator.

4. Discussion

Previous studies with human tissues that confined the presence of ALDH1 in testis to genital skin fibroblasts (GSF) [32,15,33] failed to explain the specificity of *ALDH1* expression. However both cell types, GSF cells [34] and Leydig cells [35], have androgen receptors, although the expression of *Aldh1* has not been investigated before in Leydig cells.

Leydig cells have two phases of growth during the mammalian life-span: fetal and adult. Fetal-type cells are responsible for androgen-induced differentiation of the male genitalia. Fetal-type Leydig cells are progressively replaced by adult-type cells during puberty. These persist throughout adult life [36,31]. The pattern of *Aldh1* expression could therefore be analyzed in relation to the proportion of Leydig cells present in testis during development and during the replacement of fetal by adult Leydig cells [37]. A decrease in *Aldh1* accumulation can be observed from birth to day 14 pn. This is coincident with a decrease in the proliferative activity of the Leydig cells and the replacement of fetal cells by adult Leydig cells [29]. Moreover, the subsequent increase in the *Aldh1* expression at the late prepuberal stage is coincident with the appearance and proliferation of adult Leydig cells in the interstitial compartment [29,37]. It could be argued that *Aldh1* mRNA levels were due to the relative number of Leydig cells in testis tissue during development rather than to differential expression. However, the relative number of cells in the interstitial compartment decreases at the end of puberty with respect to the total number of cells in the testis. This is due to the proliferation of germ cells in the seminiferous epithelium. The increase of *Aldh1* expression could, therefore, be attributed to changes in functional patterns in testis tissue. This increase is coincident with the appearance of the mature adult-type Leydig cells [28]. Additionally it has been shown that mouse Leydig cells from day 25 of pn. development require receptor-mediated androgen activity for normal functional development [35]. These data correlate androgen-receptor mediation with *Aldh1* pattern expression.

A structural and functional analysis of the human *ALDH1* promoter has recently been reported [38]. The 5′-region contains two putative androgen responsive elements that could explain its tissue and cell-specific transcriptional regulation. This 5′-region contains two hormone response elements that are 75% homologous to the consensus glucocorticoid response element [16]. Based on this, it has been suggested that *ALDH1* could be transcriptionally activated by an androgen/androgen-receptor complex [8]. However, functional analysis of the pro-

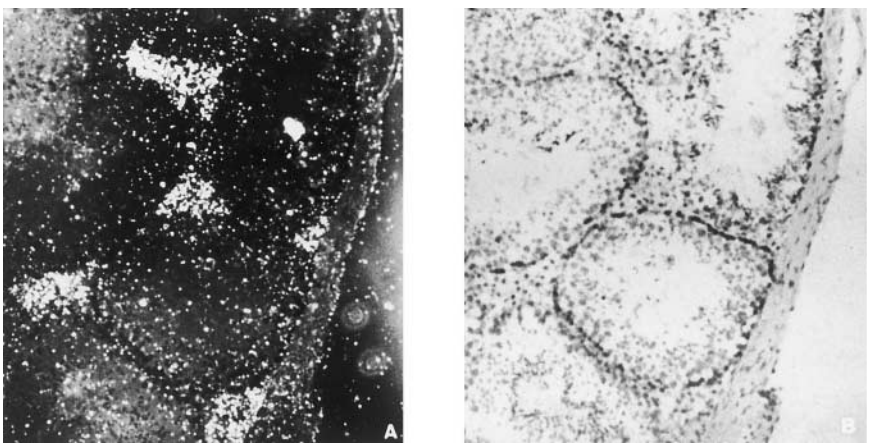

Fig. 4. In situ hybridization in adult testis sections. The *Aldh1* antisense riboprobe was labeled with [α-P^{33}]UTP. A: *Aldh1* transcripts accumulated at the interstitial tissues of seminiferous tubules. Simultaneous dark-field illumination. B: Haematoxylin-eosin staining under bright-field illumination.

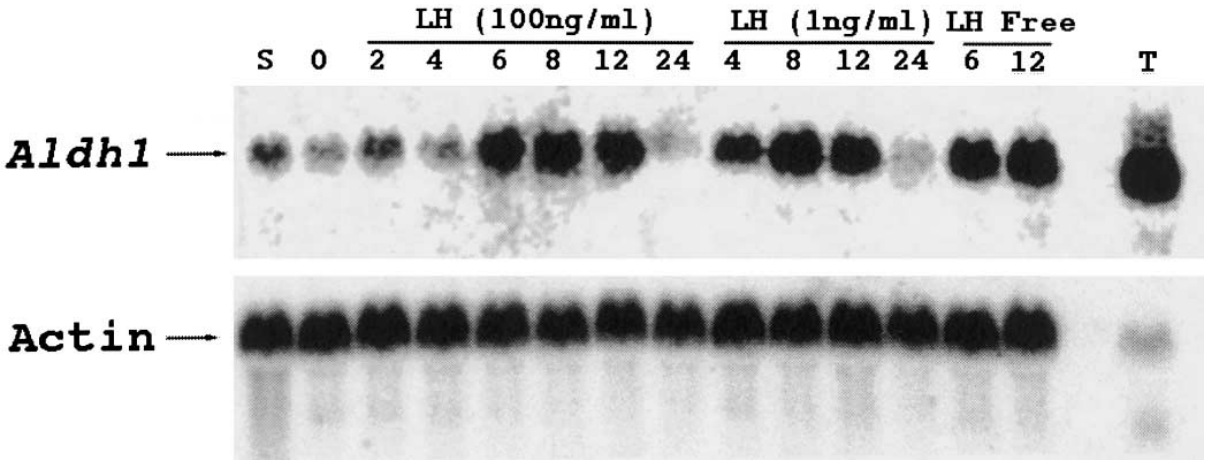

Fig. 5. Pattern of *Aldh1* transcript accumulation in TM3 cells cultured in the presence of LH. Track S, RNA from cells grown with serum in standard conditions. Track 0, 2 h after change to fresh medium without serum before adding LH. Two concentrations of LH in serum-free medium were used: 100 ng and 1 ng/ml. Cultured cells were killed at different times (2, 4, 6, 8, 10, 12, 24 h) after addition of LH and Northern blots were performed using total RNA. An apparent increase of *Aldh1* expression was detected after 6 h of culture without serum (LH-free tracks), and similar to that observed in different LH concentration treatments. As a positive control of *Aldh1* expression, total RNA from adult testis was included in the blot (T). This track was underloaded to compensate for the higher expression detected in adult testis. As before, a β-actin probe was used as a loading control after rehybridization.

moter region has not yet fully elucidated the molecular mechanism of the supposed androgen-receptor mediated expression of *ALDH1*. This could be facilitated by the use of mouse Leydig cells that present a developmental regulation as shown in these results.

The in vivo observations of the present study were confirmed by studies using cultured TM3 cells. However, TM3 cells cultured in serum-free medium with different concentrations of LH did not modify *Aldh1* expression. These experiments could suggest that, in vivo, there is no physiological response of *Aldh1* transcription to LH. Further, studies in experimental animal models may define the possible influence of gonadotrophins on Aldh1-mediated steroidogenesis.

The presence of retinoic acid in testis at concentrations sufficient to activate nuclear retinoic acid receptors has recently been reported [39]. Moreover, the expression of class I alcohol dehydrogenase has been also detected in mouse Leydig cells [39]. Class I Adh could participate in the oxidation of retinol to retinal and the oxidation of this to retinoic acid by Aldh1.

In conclusion, the characterization of specific *Aldh1* expression in Leydig cells and the understanding of its developmental regulation, may give rise to a new way of elucidating the molecular pathogenesis of androgen insensitivity and testicular feminization syndrome. Androgen regulation of the *Aldh1* gene could be mediated by androgen receptors in Leydig cells via the hormone response elements. Translated *Aldh1* could participate in the oxidation of retinal (from oxidized retinol [39]) to RA. Retinoic acid could then act as an autocrine signal to regulate testosterone synthesis. It could also act in a paracrine interaction with Sertoli cells and early meiotic cells, via retinoid receptors [40], to drive germ cell differentiation. This complex interaction, which includes *Aldh1* expression and regulation, could be elucidated by generation of mutant mice disrupted at different steps in the pathway. Similar approaches have recently demonstrated degeneration of the seminiferous epithelium caused by mutations of selectively expressed retinoid receptors in seminiferous epithelium cells, such as RARα [41] or RXRβ [42].

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