Potential Role of aldo-keto Reductase Family 1, member B10 (AKR1B10) as a Molecular Target in Alcoholic Hepatitis

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Background: Alcoholic Hepatitis (AH) is the most severe form of Alcoholic Liver Disease (ALD) and current therapies are not fully effective. Targeted therapies are urgently needed. *AKR1B10* was recently shown to be overexpressed in patients with AH.

Objectives: To analyze the expression of *AKR1B10,* an aldose reductase, through a translational approach in order to better understand the potential for aldose reductase as a novel target of therapy in AH.

Methods: RNA was extracted from human tissue samples from patients with various liver diseases, animal models of fibrosis and alcoholic liver disease, and cultured Hepatic Stellate Cell (HSC) lines stimulated with proinflammatory, profibrogenic, and AKR1B10 treatments, and the samples were quantified using qPCR analysis.

Results: Human samples showed nearly a 100-fold increase of *AKR1B10* expression in patients with AH. An animal model of liver fibrosis showed a small increase in *Akr1b8* (*AKR1B10* mouse analogue) expression. HSCs did not show any noticeable increase in expression of *AKR1B10* regardless of treatment, and did not show any noticeable increase in expression of proinflammatory or profibrogenic genes when treated with AKR1B10.

Conclusion: The increased expression of *AKR1B10* and its mouse analogue, while present in patients with AH and fibrotic mice, respectively, may not be mediated by HSCs. Further studies are needed to better understand location and nature of overexpression.

INTRODUCTION

Alcoholic Liver Disease (ALD) is a major etiology of end-stage liver disease, accounting for 40% of mortality from cirrhosis[1] and a higher annual mortality than for hepatitis C virus (HCV)[2]. The natural history of ALD includes the development of steatosis in 80-90% of heavy drinkers, followed by development of more severe and symptomatic forms of ALD in 20-40% of patients with steatosis[3]. Alcoholic Hepatitis (AH) is a severe acute-on-chronic disease that is characterized by an inflammation of the liver that results in liver injury, and severe cases have a mortality rate of approximately 30-50%[4]. Unfortunately, the pathogenesis of this disease is not well characterized. The most effective therapy for patients with AH is corticosteroid therapy, which was initially developed over 40 years ago[3-6], and while it has shown to be effective in increasing the survival of some patients with AH, not all patients respond well and the mortality rate remains high[4,7]. The development of new therapeutic strategies has been made difficult due to poor knowledge of involved mechanisms and lack of appropriate animal models[8]. It is thus important to look for new targets of therapy.

Several promising new targets have been identified, including pro-inflammatory cytokines, chemokines and their receptors, anti-inflammatory molecules[8,9], and tumor necrosis factors[10]. Interestingly, a transcriptome analysis of patients with AH, previously performed in our group, yielded a 432-fold overexpression of aldo-keto Reductase Family 1, member B10 (*AKR1B10*)[10], known as aldose reductase, which does not have any known role in AH. AKR1B10 is related to the aldose reductase AKR1B1, which reduces glucose to sorbitol as part of the Polyol pathway and is expressed ubiquitously in human tissues[11]. The Polyol pathway is involved in secondary diabetic complications such as retinopathy[12], neuropathy[13], and nephropathy[14]. *AKR1B10* has a similar function, but is primarily expressed in the small intestine

and colon with little expression in the liver under normal conditions and metabolizes retinals, carbonyls, and several lipid substrates, which may play a crucial role in promoting carcinogenesis[11,15]. Additionally, aldose reductase has been shown to be highly expressed in liver[16], colon[17], and prostate cancer[18], among others[19]. Consequently, the overexpression of *AKR1B10* in AH patients may play an important role in ALD disease progression and the mortality and morbidity associated with AH. It is unknown through which hepatic cell the *AKR1B10* may be playing a role in AH and the nature of the pathway of expression in patients with AH.

In chronic liver injury, Hepatic Stellate Cells (HSCs) differentiate from a fat-storing phenotype to a myofibroblast-like phenotype that secretes fibroblastic cell markers and extracellular matrix that is indicated in fibrosis and cirrhosis[20]. Patients with ALD generally have activated HSCs. Alcohol consumption enhances gut permeability, leading to a translocation of bacterial derived liposaccharide (LPS) which primarily stimulates Kupffer cells (and to a certain extent, HSCs) in the liver, causing inflammation and fibrogenesis[8,21]. Platelet-Derived Growth Factor (PDGF), produced by Kupffer cells and Biliary cells, is the main mitogen for activated HSCs, directly promoting fibrogenesis[22]. Stimulation of HSCs by the proinflammatory LPS and LPS-derived cytokines from Kupffer cells, and profibrogenic PDGF lead to differential gene expression in HSCs for various functions related to fibrosis. It is therefore feasible that an overexpression of *AKR1B10* would be seen in HSCs, one of the primary damage response cells, and that *AKR1B10* expression would be differentially mediated by LPS and PDGF.

In the present study, we explored the potential role of AKR1B10 as a therapeutic target for patients with AH. We investigated the expression of *AKR1B10* in human samples, an animal

model, and cell culture of HSC cell lines, and we sought to better characterize the location and pathway of the overexpression of *AKR1B10* in order to lead to novel therapies for AH.

METHODS

Patients with Alcoholic Hepatitis (AH) and selection of normal control livers.

The data that was collected was derived from the following methods performed by the laboratory. Patients admitted to the Liver Unit, Hospital Clínic of Barcelona with clinical, analytical and histological features of AH from 2007 to 2010 were prospectively included in the study[7,23,24]. The inclusion criteria have been described in a similar study[10]. All patients had histological diagnosis of AH (n = 34). Patients with hepatocellular carcinoma or any other potential cause of liver disease were excluded from the study. Liver biopsy was obtained using a transjugular approach. As controls, fragments of normal liver tissue (n = 6) were obtained from optimal cadaveric liver donors (n = 3) or resection of liver metastases (n = 3) as described in detail in a similar study.[10] All liver specimens were analyzed by an expert pathologist and a part of the biopsy was submerged into a RNA stabilization solution (RNAlater, Ambion, Austin, Texas, USA). The protocol was approved by the Ethics Committee of the Hospital Clinic and all patients gave informed consent.

Mouse models

The data that was collected was derived from the following methods performed by the laboratory. Hepatic fibrosis was induced in male C57BL/6J mice (Bar Harbor, ME, USA) following administration of carbon tetrachloride (CCl_4) (Sigma-Aldrich, St Louis, MO, USA) injected intraperitoneally at a dose of 0.5 ml/kg, 12.5% diluted in olive oil, twice a week for four weeks, CCl_4 (Sigma-Aldrich). Control mice were given olive oil at the same dose.

Alcoholic liver disease was induced in male male C57BL/6J mice by administering a high-fat liquid diet (HFD) containing ethanol. The dose of alcohol started at 5 g/kg/day and the concentration of alcohol gradually increased until the total dose of alcohol administered reached up to 20 g/kg/day. Control mice were given high-fat liquid diet without ethanol. Each group included at least 3 mice. Mice were housed in temperature and humidity-controlled rooms and kept on a 12-hour light/dark cycle. Mice were sacrificed and collection of liver and blood samples was performed. All experimental procedures were reviewed and approved by The University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Cell Cultures

To study AKR1B10 production and biological effects, LX-2 cells (from Scott L. Friedman, Mount Sinai Hospital, New York, NY, USA), a human HSC line[25], were serum starved for 24 h and then incubated with LPS 1 µg/mL (Sigma-Aldrich), PDGF 20 ng/mL (Sigma-Aldrich), and recombinant AKR1B10 10 ng/mL, 100 ng/mL, and 1 µg/mL (Novus Biologicals, Littleton, CO, USA) for 20 h. Fetal Bovine Serum (FBS) 20%/mL (Sigma-Aldrich) was used as a control.

RNA Isolation and PCR Analysis

RNA was isolated from liver tissues, animal models, and cells using Trizol and the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Two thousand nanograms of RNA were retrotranscribed using a high-capacity complementary DNA reverse-transcription kit (Applied Biosystems, Foster City, CA, USA) and complementary DNA (cDNA) were then amplified using Taqman Technology (Applied Biosystems) in a final PCR volume of 10 µL using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) and various Assay-on-Demand probes and primers were used (Applied Biosystems). For human and animal model

samples, primer-probe pairs for AKR1B10 and AKR1B8 were utilized, respectively. For cell culture samples, primer-probe pairs for COL1A1, ICAM1, AKR1B10, TIMP1, IL6, CCL2, MMP2, ACTA2, and IL8 were used. However, only the primer-probe pairs for COL1A1, ICAM1, AKR1B10, and TIMP1 were analyzed due to sample integrity problems with other assessed target genes. Results were normalized to 18s rRNA expression (housekeeping gene). The gene expression values were calculated based on the ΔΔCt method and the results were expressed as $2^{-\Delta\Delta Ct}$ referred to as fold increase compared with the mean expression quantified on normal livers.

Statistical Analysis

Results of quantitative variables are expressed as mean plus standard error unless otherwise specified. The differences between groups were analyzed using non-parametric tests (Mann-Whitney U test) for continuous variables. Statistical analysis was performed using SPSS version 22 (SPSS Inc.,Chicago, IL, USA).

RESULTS

Patients with AH show increased AKR1B10 hepatic expression

AKR1B10 was previously identified as the most upregulated hepatic gene in patients with AH[10]. To confirm this previous result, we analyzed previous laboratory data on hepatic *AKR1B10* expression in a cohort of patients with AH from a transjugular biopsy by real time qPCR analysis. The results confirmed marked upregulation of *AKR1B10* in patients with AH compared with normal liver and other liver diseases with a 99-fold overexpression of *AKR1B10* in patients with AH (Figure 1).

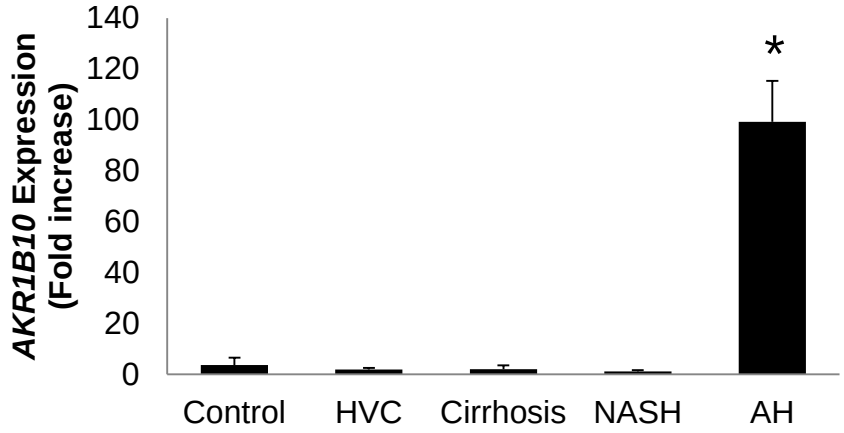

Figure 1. AKR1B10 gene expression in patients with various liver diseases. (*p<0.001 compared to control and all other studied diseases.)

AKR1B10 is overexpressed in fibrosis animal models

There are no animal models that fully characterize the pathogenesis of AH in human patients[8]. However, we looked at previously derived laboratory data from CCl_4-induced fibrotic liver injury in mice as well as an alcohol animal model. We investigated the known mouse analogue for *AKR1B10*, *Akr1b8*[26]. While the high fat diet plus alcohol administration animal model did not show a significant increase in *Akr1b8* expression, the CCl_4-induced fibrosis animal model

showed a significant expression of *Akr1b8* over the control (Figure 2). Because HSCs are the primary cells involved in fibrosis development[20] and *Akr1b8* is overexpressed in a fibrotic animal model, HSCs could be a likely cell source of hepatic AKR1B10 seen in the Transcriptome analysis.

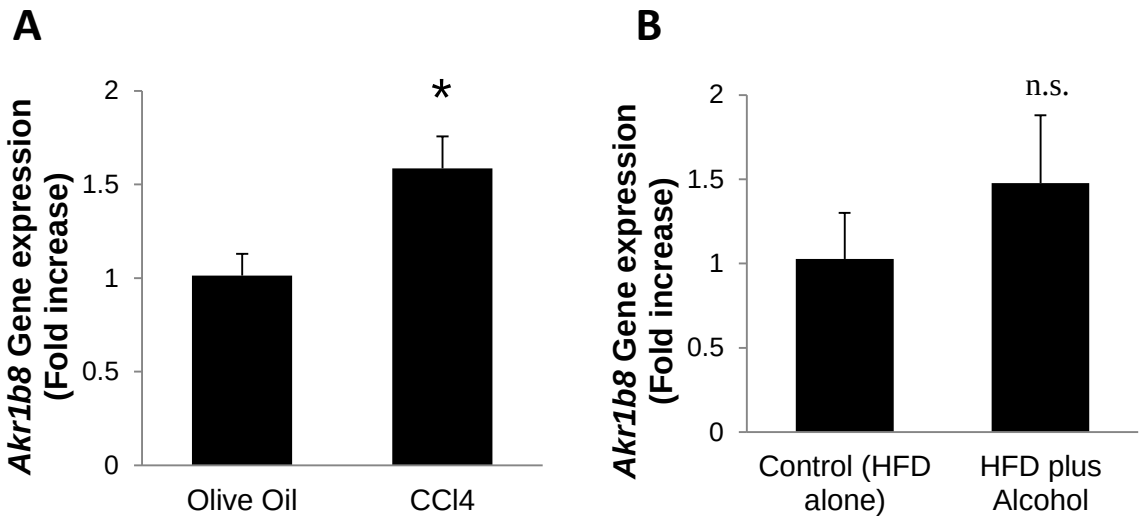

Figure 2. *Akr1b8* expression in animal models of liver injury. (A) Hepatic *Akr1b8* gene expression in mice treated with CCl_4 (n=3). (*$p<0.05$ compared with olive oil control). (B) Hepatic *Akr1b8* gene expression in HFD mice treated with alcohol (n=4). Abbreviation n.s. = not significant.

Proinflammatory and profibrogenic effects on *AKR1B10* expression in HSCs

As expression of *AKR1B10* in the liver was found to be significantly higher in AH patients, and as HSCs play a key role in the development of liver fibrosis in the during liver injury[8], we next investigated the potential of HSCs to synthesize AKR1B10 and its biological effects on these cells. We used several mediators known to play a role in ALD and typically present in the AH microenvironment, and investigated whether they induced *AKR1B10* expression in human HSC cell lines. However, incubation of HSCs (LX-2) with LPS and PDGF did not show a significant increase in *AKR1B10* levels (Figure 2A). In addition, to investigate the biological effects of

AKR1B10 on HSCs, cells were incubated with recombinant AKR1B10, and this similarly did not induce the expression of proinflammatory or profibrogenic genes in HSC (Figure 2B).

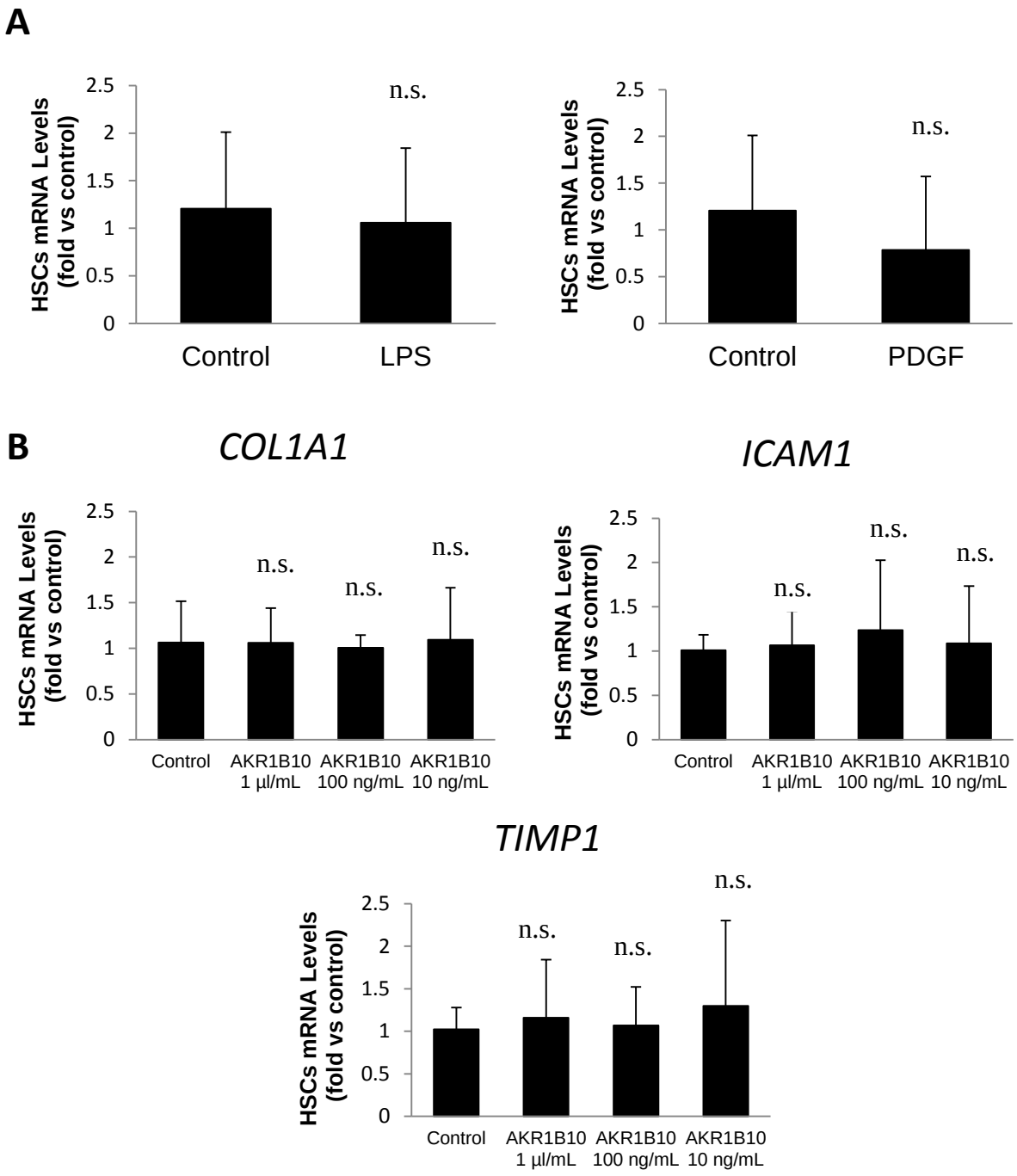

Figure 3. Expression of various genes in human HSC cell lines. (A) Expression of *AKR1B10* in cultured cells treated with PDGF and LPS. (B) Expression of *COL1A1* (profibrogenic), *ICAM1* (proinflammatory), and *TIMP1* (profibrogenic) in cultured cells treated with various concentrations of recombinant AKR1B10. Abbreviation n.s. = not significant.

DISCUSSION

AH is an acute-on-chronic liver disease that is responsible for much of the morbidity and mortality seen in patients with ALD, and is characterized by hepatic inflammation, fibrosis, and damage. It is therefore of great importance to identify the key pathways and molecules of the disease in order to produce novel and effective therapies. In the present study, we investigated the potential role of AKR1B10 as a target for therapies in patients with AH. We performed qPCR analyses of human liver biopsies of patients with various liver diseases and of animal models of fibrotic and alcohol-supplemented mice. Furthermore, we conducted and analyzed tissue culture experiments of HSC cell lines to understand the location and pathway of expression of *AKR1B10*. While the results of the human and animal model samples agreed with transcriptome analysis referenced above and suggested the implication of HSCs, the tissue culture experiments dismissed this hypothesis, and the mechanism of overexpression could not be determined.

The results suggest that overexpression of *AKR1B10* in patients with AH may not be linked to HSCs directly and may be present in other cells of the liver. While there was a 99-fold overexpression of *AKR1B10* in patients with AH and the animal models suggested a fibrosis-producing cell such as HSCs, an increased expression of *AKR1B10* could not be reproduced in HSC cell lines.

There were several limitations to the present study. The major limitation to the animal studies is the lack of a proper animal model that can reproduce key pathophysiological features of AH[8]. We used data from a study in the laboratory regarding the use of the hepatotoxic CCl_4 which is known to adequately reproduce liver fibrosis and cirrhosis in mice[27].While we observed a 1.5-fold increase in expression of *Akr1b8* ($p < 0.05$) in this model, this result may not be fully

indicative of the expression of *AKR1B10* in AH patients, and the sample size (n = 3) was very small. Additionally, the study was limited to gene expression analysis and did not include protein analysis that could confirm the results of the qPCR gene expression analysis. While supernatant was collected from the cell culture studies, time and resource constraints prevented the analysis of the samples.

Future studies could examine other liver cells that may be the location of the overexpression of *AKR1B10* in patients with AH. Potentially, AKR1B10 could be upregulated in hepatocytes instead of HSCs. Several pathways have been studied regarding the potential fibrosis resulting from improper hepatocyte functioning, such as the activation of HSCs by signals derived from hepatocyte apoptosis[28]. The HepG2 human hepatocellular carcinoma cell line could be employed to explore potential *AKR1B10* overexpression in hepatocytes[29]. Additionally, Liver Progenitor Cells (LPCs) have been shown to correlate well with fibrotic liver damage and are strong prognostic markers for short-term mortality in patients with AH[30,31]. Future studies could assess potential *AKR1B10* overexpression in LPCs and hepatocytes by using a similar approach as the present work. Finally, further work to assess sorbitol accumulation in human samples could help elucidate the molecular function of aldose reductase in the liver during AH pathogenesis.[32]

A better understanding of the role of various factors in the development and progression of AH is of the utmost importance in order to develop novel therapies. The overexpression of aldose reductase in patients with AH is poorly understood and could play an important role in the disease. Our present study confirms the overexpression of *AKR1B10* in AH and suggests that it may be linked to cells other than HSCs. Future studies will be needed to explore the role of AKR1B10 in AH.

CONCLUSION

We confirmed *AKR1B10* is overexpressed in patients with AH and observed an analogous overexpression in a fibrotic animal model but could not reproduce findings in HSCs. We suggest that the expression of the gene is not linked to HSCs and may be found in other cells such as Hepatocytes or LPCs.

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