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Gene expression profile of quiescent and activated rat hepatic stellate cells implicates Wnt signaling pathway in activation

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***Background/Aims*: Liver fibrosis is characterized by accumulation of extracellular matrix proteins synthesized by activated hepatic stellate cells (HSCs). To understand molecular mechanisms of HSCs activation a comprehensive comparison of gene expression between quiescent and activated HSCs is needed.**

***Methods*: Using DNA microarrays we compared expression of 31,100 genes between quiescent rat HSCs and culture activated rat HSCs. Expression of the components of Wnt signaling was analyzed in HSCs and fibrotic livers by RT-PCR. Activation of β-catenin was analyzed by Western blot.**

***Results*: Nine hundred genes were upregulated more than 4.6-fold and 500 genes were downregulated more than 5.7-fold in activated HSCs. The upregulated genes included Wnt receptor frizzled 2, ligands Wnt4 and Wnt5, which was confirmed in fibrotic livers. Expression of the target genes of Wnt signaling was increased from 5- to 70-fold. Phosphorylation and nuclear translocation of β-catenin were unchanged, indicating activation of the noncanonical Wnt pathway.**

***Conclusions*: Highly upregulated expression of Wnt5a and its receptor frizzled 2 implicates this pathway in differentiation of quiescent HSCs into myofibroblasts. Activation of Wnt signaling pathway in HSCs and in animal models of liver fibrosis has not been described previously, suggesting an important role of Wnt signaling in development of liver fibrosis.**

***Keywords*: Liver fibrosis; Hepatic stellate cells; DNA microarray; Gene expression**

1. Introduction

Liver fibrosis is characterized by progressive accumulation of extracellular matrix proteins (ECM), synthesized by activated HSCs. To discover genes involved in activation of HSCs several studies used DNA microarrays to compare gene expression between normal and cirrhotic livers [1,2]. The differentially expressed genes identified include extracellular matrix proteins, genes involved in liver regeneration, proinflammatory genes and genes encoding serum proteins. Two studies compared gene expression profile between quiescent and culture activated mouse [3] and human HSCs [4]. The upregulated genes identified were the genes encoding ECM, as well as components of protein synthesis machinery, cell cycle and DNA repair genes and apoptotic genes. There was no clearly distinctive class of the downregulated genes.

Wnt signaling is essential for development and implicated in tumorigenesis [5]. Wnt ligands bind to the receptor of frizzled family, which transduce signal to β-catenin causing β-catenin to enter the nucleus and activate the target genes [6–8].

To provide the most comprehensive analysis of genes relevant for activation of rat HSCs published thus far, expression of 31,100 genes was compared between quiescent and culture activated HSCs using DNA

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Abbreviations: HSCs, hepatic stellate cells; ECM, extracellular matrix; Bmp, bone morphogenic protein; FBS, fetal bovine serum.

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microarrays. Our analysis revealed 900 upregulated and 500 downregulated genes, many of which have not been previously described in activation of HSCs. We also report that Wnt signaling pathway [7,8] is stimulated during activation of HSCs. The result was confirmed using an animal model of liver fibrosis. Upregulation of Wnt signaling pathway has not previously been reported in HSCs or liver fibrosis and represents a novel finding in the biology of HSCs.

2. Materials and methods

2.1. Animal model of liver fibrosis

Sprague–Dawley rats were obtained from Charles River. Rats (~200 g) were injected intraperitoneally with CCl_4 (2 μl/g in 50% mineral oil) twice a week for 4 weeks. Control rats received only mineral oil. After 4 weeks of treatment, the livers were harvested for histology and mRNA extraction. All animals received humane care according to the criteria outlined by National Institutes of Health.

2.2. Isolation and culture of rat HSCs

Rat HSCs were isolated from rat livers by perfusion of collagenase and pronase, followed by centrifugation over Nycodenz gradient, as described [9]. After isolation the cells were cultured for one day, trypsinized and cultured for one additional day, when they were harvested as quiescent HSCs. For activated HSCs, HSCs were cultured in uncoated plastic dishes in DMEM supplemented with 10% FBS for total of 8–14 days. Immunofluorescence staining for desmin was performed using anti-desmin antibody (DE-U-10, GeneTex, Inc., San Antonio, TX). Cells were counterstained with Alexa Fluor 594 dye-conjugated goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR). DAPI stain was used to stain the nuclei. Negative controls were performed without primary antibody.

2.3. DNA microarray analysis

Total RNA extracted from quiescent and culture-activated rat HSCs was used for DNA microarray analysis. Two independent isolations and microarray analysis (experiment 1 and experiment 2) were performed using Affimetrix rat 430.A2 chip by the Institute for Fluorescence Analysis, University of Regensburg, Germany, which also performed statistical evaluation of the data.

2.4. RT-PCR analysis of gene expression

Total RNA of quiescent and activated rat HSCs was extracted using an RNA isolation kit (Eppendorf, Westbury, NY). Poly(A)$^+$ RNA of normal livers and CCl_4-induced fibrotic livers was extracted by a direct poly(A)$^+$ RNA isolation kit (Sigma, St. Louis, MO). RT-PCRs were done with 100 ng of total RNA or 20 ng of poly(A)$^+$ RNA using rTth reverse transcriptase (Boca Scientific, Boca Raton, FL) in the presence of 2.5 μCi of [α-^{32}P]dCTP, according to the previously used protocol [10]. PCR products were resolved on a sequencing gel and visualized by autoradiography. The number of cycles was adjusted to be in the linear range of the reaction. The gene specific primers and sizes of the expected PCR products are listed in Table 1. Expression of β-actin and GAPDH was used as an internal control.

2.5. Western blot analysis

Total and nuclear proteins were prepared by standard procedure [11]. Total protein concentration was estimated by Bradford assay with BSA as standard. Western blots were done using 40 μg of protein and anti-β-catenin antibody (Transduction Laboratories, Lexington, KY), anti-phosphorylated β-catenin antibody (ser33/37/thr41, Cell Signaling Technology, Beverly, MA) and anti-tubulin antibody (Zymed Laboratories, San Francisco, CA).

3. Results

3.1. Global changes in gene expression identify gene families upregulated in activated HSCs

A pure population of viable HSCs with quiescent phenotype (Fig. 1A) was obtained by our protocol 2 days after isolation. More than 95% of the quiescent HSCs showed positive desmin staining indicating pure HSCs. To obtain activated HSCs, the cells were cultured for eight days and stained for desmin (Fig. 1C). More than 95% of activated HSCs were desmin positive indicating pure population. The isolation and activation was repeated twice and expression of 31,100 genes was compared between quiescent and activated HSCs using DNA microarrays in two independent experiments. We found 900 genes that were upregulated more than 4.6-fold in activated HSCs compared to quiescent HSCs. Five hundred genes were downregulated more than 5.7-fold in activated HSCs. According to their function, these genes were classified into 14 groups and the fraction of genes in each group is shown in Fig. 2. The genes encoding extracellular matrix proteins, cytoskeleton and components of protein synthesis machinery were preferentially upregulated in activated HSCs. 7% of upregulated genes encoded cytoskeleton proteins, at the same time only 1.6% of downregulated genes were cytoskeletal genes (Fig. 2). The same was for extracellular matrix proteins where upregulated genes represented 7.6% and downregulated genes represented 1% and for genes involved in protein synthesis with 3.1% of upregulated genes compared to 1.7% of downregulated genes. A less pronounced bias was observed for the genes related to cell adhesion, cytokines and growth factors. 5.6% of upregulated genes versus 4% of downregulated genes were cell adhesion molecules; 8.5% of upregulated genes versus 6.9% of downregulated genes were cytokines and growth factors. The best known profibrogenic cytokines were highly upregulated in activated HSCs; in two experiments TGFβ2 was increased 20- and 3-fold, TGFβ3 45- and 26-fold and CTGF 36- and 97-fold. Interestingly, expression of TGFβ1 was unchanged. For the downregulated genes a slight bias was seen for signal transduction molecules and proinflammatory genes. From the global changes in gene expression we concluded that increased synthesis of extracellular matrix proteins, which contribute to the bulk of increase in total protein synthesis in HSCs [10], and the change in cell morphology due to cytoskeletal rearrangements [12] were correctly reflected in our DNA microarray analysis.