Expression of mediators of purinergic signaling in human liver cell lines

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Abstract Purinergic signaling regulates a diverse and biologically relevant group of processes in the liver. However, progress of research into functions regulated by purinergic signals in the liver has been hampered by the complexity of systems probed. Specifically, there are multiple liver cell subpopulations relevant to hepatic functions, and many of these have been effectively modeled in human cell lines. Furthermore, there are more than 20 genes relevant to purinergic signaling, each of which has distinct functions. Hence, we felt the need to categorize genes relevant to purinergic signaling in the best characterized human cell line models of liver cell subpopulations. Therefore, we investigated the expression of adenosine receptor, P2X receptor, P2Y receptor, and ecto-nucleotidase genes via RT-PCR in the following cell lines: LX-2, hTERT, FH11, HepG2, Huh7, H69, and MzChA-1. We believe that our findings will provide an excellent resource to investigators seeking to define functions of purinergic signals in liver physiology and liver disease pathogenesis.

Keywords Liver cell line · Purinergic signaling · Hepatic stellate cell · Hepatocyte · Cholangiocyte

Introduction

One of the fundamental aspects of liver physiology and disease pathogenesis is the diversity of resident cell types

involved. Liver cell subpopulations include such distinct specialized cell types as parenchymal cells or hepatocytes [1] and non-parenchymal cells including, cholangiocytes [2], portal fibroblasts [3], hepatic stellate cells (HSC) [4], mesothelial cells [5], sinusoidal endothelial cells [6], and immune cells [7, 8]. For most of these cell types, the establishment of various immortalized cell lines in human and rodent species represents a major experimental advance, providing in vitro models for the study of liver functions in cell-specific manner. Most of these cell lines have been effectively characterized and are increasingly used in fundamental experiments probing the basic biology of liver under normal and pathological conditions.

The diverse roles of purinergic signals in the liver have been increasingly appreciated in recent years [9–11]. For instance, purinergic signals regulate proliferation [12–14] and glucose release [15] in hepatocytes; secretion [16, 17], proliferation [18], and mechanosensation [19] in cholangiocytes; and fibrogenic activity [20, 21] and properties in HSC [22, 23]. Of note, the purinergic signaling system is quite diverse, including multiple subtypes of G protein-coupled receptors for adenosine P1 receptors [24] and nucleotide P2Y receptors [25], nucleotide P2X ligand-gated ion channels [26], and a variety of plasma membrane-bound ecto-enzymes that regulate extracellular nucleotide/nucleoside levels, namely ecto-nucleoside triphosphate diphosphohydrolases (ENTPDases) [27, 28], CD73/ecto-5'-nucleotidase [29, 30], and tissue-non-specific alkaline phosphatase (TNAP) [31]. Thus, there is tremendous, almost overwhelming, complexity both in the relevant cell types and purinergic signaling systems relevant to liver physiology and disease [32].

To help remedy this concern, we set out to categorize the specific genes relevant to purinergic signaling in well-characterized human liver cell lines. We propose that the data collection created from this work will be of value to all

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investigators interested in the role of purinergic signaling in the liver.

Materials and methods

Materials/reagents Dulbecco's Modified Eagles medium (DMEM), DMEM/F12 medium, fetal bovine serum (FBS), penicillin-streptomycin (10,000 U/mL) antibiotic solution, human recombinant insulin, and zinc solution were purchased from Gibco (Life Technologies, Grand Island, NY). Adenine, epinephrine, triiodothyronine-transferrin, epidermal growth factor, and hydrocortisone reagents used for cell culture medium were purchased from Sigma-Aldrich (St. Louis, MO). Deoxyribonuclease 1 (DNase 1) and nuclease-free water were purchased from Ambion (Life Technologies, Grand

Island, NY). Qubit RNA Assay Kit was purchased from Invitrogen (Life Technologies), and iScript RT-Superscript kit was purchased from Bio-Rad (Hercules, CA). RNeasy Plus total RNA extraction Kit, Qias shredder homogenizer spin columns, and TopTaq polymerase chain reaction Mastermix Kit were purchased from Qiagen (Valencia, CA). Human Liver Poly A+RNA was purchased from Clontech (Mountain View, CA).

Cell lines and culture conditions LX-2 [33] (kindly provided by Dr. Scott Friedman, Mont Sinai School of Medicine, New York, New York), hTERT [34] (kindly provided by Dr. Tatiana Kisseleva, University of California at San Diego, San Diego, CA), FH11 [35] (kindly provided by Dr. Meena Bansal, Mont Sinai School of Medicine, New York, New York), Mz-Cha-1 [36], HepG2 [37], and Huh7 [37] (kindly

Table 1 Sequences of PCR primers used for expression analysis of human purinergic genes

Targeted gene symbol	Forward primer sequence	Reverse primer sequence	Product size (bp)	Gene accession number	Positive Control
Adenosine receptors					
ADORA ₁	CCTCCATCTCAGCTTCCAG	AGTAGGTCTGTGGCCCAATG	222	NM_000674	Brain
ADORA _{2a}	AACCTGCAGAACGTCACCAA	GTCACCAAGCCATTGTACCG	245	NM_000675	Brain
ADORA _{2b}	GAGACACAGGACGCGTGTACG	CGGGTCCCCGTGACCAAACT	353	NM_000676.2	Brain
ADORA ₃ v1, v3	GACACAGGGAACCAGCTCAT	TGCAGCTTCTGGTTTTGTG	199	NM_020683	Brain
ADORA ₃ v2	TGTTTGCTGGAACATGAAA	ATAGATGGCGCACATGACAA	155	NM_000677	Brain
P2X receptors					
P2RX ₁	GCTACGTGGTGCAAGAGTCA	GTAGTTGGTCCCGTTCTCCA	215	NM_002558	Brain
P2RX ₂	GCTCCTTTCATCTCACTGG	GGAAGTGAGCAGCCCTGTAG	237	NM_170682.3	Brain
P2RX ₃	ACAGCCAGGGACATGAAGAC	AGCCGGGTGAAGGAGTATTT	186	NM_002559	Liver
P2RX ₄	GAGATTCCAGATGCGACC	GACTTGAGGTAAAGTAGTGG	296	NM_002560	Brain
P2RX ₅	CTGGTCGTATGGGTGTTCTT	CTGGGCTGGAATGACGTAGT	159	NM_002561	Brain
P2RX ₆	ACTCTGTGTGGAGGGAGCTG	GGCAAGTGGGTGTCAGAACT	151	NM_005446.3	Brain
P2RX ₇	AAGCTGTACCAGCGAAAGA	GCTCTGGCCTTCTGTTTGT	202	NM_002562	Brain
P2Y receptors					
P2RY ₁	AAAAGTAGCCCCCTGCAACT	GATCTGATGCCGATGAAGT	153	NM_002563	Brain
P2RY ₂	CCACCTGCCTTCTCACTAGC	TGGGAAATCTCAAGGACTGG	163	NM_176072	Liver
P2RY ₄	CGTCTTCTCGCCTCCGCTCTCT	GCCCTGCACTCATCCCCTTTTCT	411	NM_002565	Liver
P2RY ₆	AGCTGGGCATGGAGTTAAGA	GCTGACTGGGACCTCTCAAG	139	NM_176797	Liver
P2RY ₁₁	CCTCTACGCCAGCTCCTATG	CACTGCGGCCATGTAGAGTA	211	NM_002566	Brain
P2RY ₁₂	TTTGCCCGAATTCCTTACAC	ATTGGGGCACTTCAGCATAC	192	NM_022788	Brain
P2RY ₁₃	CCCCTGGTACACTTGAAGA	TACAGAGGAGGGGGTGATTG	125	NM_176894.2	Liver
P2RY ₁₄	TCTTTGGGCTCATCAGCTTT	TCCGTCCCAGTTCACTTTTC	213	NM_014879	Brain
Ecto-nucleotidases					
ENTPD1	CAGAACAAAGCATTGCCAGA	CCACATCCAGAACCCTGTCT	340	NM_001776.4	Brain
ENTPD2	TCAATCCAGCTCCTTGAACC	TCCCCAGTACAGACCCAGAC	167	NM_203468.1	Brain
ENTPD3	TTGACCTCAGGGCTCAGTTT	TGAGGGGGTTCAGTGCTTAC	159	NM_001248.2	Brain
ENTPD8	ACTGGGCTACATGCTGAACC	GCACCATGAACACCACTTTG	107	NM_198585.2	Liver
NT5E	TGGAACACGCTATCCATGTG	ATGCTCAAAGGCCCTTCTTCA	171	NM_002526	Brain
ALPL	CTCTCCAAGACGTACAACACCAA	ATGGTGCCCGTGGTCAAT	735	NM_000478.4	Brain

v1, v2, v3 distinct genetic variants

Fig. 1 Adenosine receptor genes expression in human liver cell lines. RT-PCR analysis was performed on LX-2, hTERT, FH11, HEPG2, HUH7, H69, and MZCHA1 cell cDNA samples. Human brain or liver cDNA was used as positive control (see Table 1). Negative control was obtained by replacing cDNA by deionized water. RT-PCR products were visualized on a UV transilluminator with ethidium bromide staining

provided by Dr. Michael Nathanson, Yale University School of Medicine, New Haven, CT) cells were cultured in DMEM containing 10 % fetal bovine serum and 1 % antibiotic. H69 cells [38] (kindly provided by Dr. Doug Jefferson, Tufts University School of Medicine, Boston, MA) were cultured, as previously described [38], in a mixture (1:1, volume/volume) of DMEM and DMEM/F12 media containing 10 % FBS, 1 % antibiotics, 1 % adenine, 0.125 % insulin, 0.1 % epinephrine, 0.1 % triiodothyronine-transferrin, 0.33 % epidermal growth factor, and 0.267 % hydrocortisone. All cell cultures were maintained at 37 °C with a 5.0 % CO₂, humidified environment, and passaged every 2–3 days.

Total RNA extraction and RT-PCR analysis Total RNA was isolated from human liver cell lines (LX-2, hTERT, FH11, HEPG2, HUH7, H69, and Mz-Cha-1) in culture using RNeasy Plus Kit following homogenization with supplementary Qiashredder spin columns according to the manufacturer's instructions. Total RNA sample concentration was quantified using the Qubit RNA Assay Kit with a Qubit 2.0 Fluorometer (Life Technologies). In order to remove any genomic DNA contamination from isolated RNA samples, 1 µg of total RNA was digested with DNase 1 according to the manufacturer's instructions before performing the reverse transcription reaction using the iScript RT Supermix according to the manufacturer's instructions. The product of the RT reaction was diluted 1:10 with nuclease-free water; 1 µl of that diluted RT reaction

Fig. 2 P2X receptor genes expression in human liver cell lines. RT-PCR analysis was performed as described in Fig. 1

Fig. 3 P2Y receptor genes expression in human liver cell lines. RT-PCR analysis was performed as described in Fig. 1

sample was further used for PCR amplification using the TopTaq Master Mix Kit as DNA polymerase. PCR amplification was performed with the following protocol for the PCR reactions: Initialization at 94 °C for 2 min followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 60 °C (except for all adenosine receptors at 62 °C and nucleotide P2Y₄ receptor at 54 °C), and 30 s elongation at 72 °C; the amplification was then completed with a 10-min final elongation at 72 °C, using an S1000 Thermo Cycler (Bio-Rad). The primer sequences used for the semi-quantitative RT-PCR reactions are listed in Table 1. Amplification products were visualized on 3 % agarose gels via ethidium bromide staining.

Results

Human LX-2, hTERT, and FH11 hepatic stellate cell lines LX-2 [33] and hTERT cells [34] are immortalized activated human HSCs cell lines, while FH11 cells [35] are primary activated human HSC isolated from liver explants. The expression of adenosine receptors on HSC cell lines varied considerably (as seen in Fig. 1). The LX-2, hTERT, and FH11 cells expressed all four adenosine receptors (A1, A2a, A2b, and A3). The expression of P2X receptors by HSC cell lines was consistent (Fig. 2) with a few exceptions. It is interesting to note that the hTERT cell line exhibited no

Fig. 4 Ecto-nucleotidase genes expression in human liver cell lines. RT-PCR analysis was performed as described in Fig. 1

Table 2 mRNA expression of purinergic genes in human liver cell lines

Targeted gene symbol	Cell line name						
	LX-2	hTERT	FH11	HEPG2	HUH7	H69	MZCHA1
Adenosine receptors							
ADORA ₁	+	+	+	+	–	+	+
ADORA _{2a}	+	+	+	+	+	+	+
ADORA _{2b}	+	+	+	+	+	+	+
ADORA ₃	+	+	+	+	+	+	+
P2X receptors							
P2RX1	+	+	+	+	–	–	–
P2RX2	+	–	+	+	–	+	+
P2RX3	+	–	–	+	+	–	+
P2RX4	+	+	+	+	+	+	+
P2RX5	+	+	+	+	+	+	+
P2RX6	+	+	+	+	+	+	+
P2RX7	+	+	+	–	–	+	–
P2Y receptors							
P2RY ₁	–	+	–	–	+	–	+
P2RY ₂	+	+	+	+	+	+	+
P2RY ₄	–	–	–	+	–	–	+
P2RY ₆	+	+	+	+	+	–	+
P2RY ₁₁	+	+	+	+	+	+	+
P2RY ₁₂	+	–	+	+	–	–	–
P2RY ₁₃	–	–	–	–	–	–	–
P2Y ₁₄	+	–	+	+	–	–	–
Ecto-nucleotidases							
ENTPD1	–	–	+	+	–	–	–
ENTPD2	+	–	–	+	+	+	+
ENTPD3	+	–	+	+	–	–	–
ENTPD8	+	–	+	+	+	–	+
NT5E	+	+	+	+	+	+	+
ALPL	–	+	+	+	+	+	–

expression of P2X2 and P2X3 and that P2X3 was expressed only by LX2 cells. In comparison to the expression of adenosine and P2X receptors, HSC cell lines did not express a wide variety of P2Y receptors (Fig. 3). Neither LX-2, hTERT, nor FH11 cells expressed P2Y₄ or P2Y₁₃ genes. In contrast, the P2Y₂, P2Y₆, and P2Y₁₁ receptors were detected in all three HSC cell lines. P2Y₁ expression was detected solely in hTERT cells. The expression of ecto-nucleotidases in HSC cell lines is presented in Fig. 4. hTERT cells showed no evidence of ENTPDase expression. CD73 was expressed by all three cell lines, while TNAP was expressed by FH11 cells and hTERT cells but was absent in LX-2 cells.

Human HepG2 and Huh7 hepatocyte-like cells HepG2 [37] and Huh7 [39] cell lines are derived from excised hepatocellular carcinomas. HepG2 cells expressed all four adenosine

receptors. Expression of adenosine receptors by Huh7 cells was nearly identical to HepG2, although no expression of the A1 receptor was detected (Fig. 1). HepG2 cells expressed all P2X receptors excluding P2X7, yet Huh7 cells expressed only P2X3, P2X4, P2X5, and P2X6 (Fig. 2). HepG2 cells expressed all P2Y receptors with the exception of P2Y₁ and P2Y₁₃, while Huh7 cells showed expression of P2Y₁, P2Y₂, P2Y₆, and P2Y₁₁, and no expression of P2Y₄, P2Y₁₂, P2Y₁₃, or P2Y₁₄ (Fig. 3). Lastly, HepG2 cells expressed all ecto-nucleotidases examined. On the other hand, Huh7 cells expressed all ecto-nucleotidases tested aside from ENTPDase1 and ENTPDase3.

Human H69 and Mz-ChA-1 cholangiocyte-derived cells H69 cell line [38] was developed through retroviral SV40 transformation of human cholangiocytes, while Mz-ChA-1 cell line [36] is derived from mechanically dissociated gallbladder

Table 3 Summary of known expression of purinergic signaling mediators

Targeted Gene Symbol	Hepatic Stellate Cells	Hepatocytes	Cholangiocytes
Adenosine Receptors			
ADORA ₁	+		
	Mouse (1)		
ADORA _{2a}	+		
	Mouse (1)		
ADORA _{2b}	+		
	Mouse (1)		
ADORA ₃	+		
	Mouse (1)		
P2X Receptors			
P2RX1		+	+/-
		Rat (5)	Rat (7)
		-	-
		Rat (6)	Rat (10)
P2RX2		+	+/-
		Rat (5)	Rat (7)
		-	-
		Rat (6)	Rat (10)
P2RX3		+	+
		Rat (5)	Rat (7)
		-	-
		Rat (6)	Rat (10)
P2RX4		+	+
		Rat (5)	Rat (7)
		+	+
		Rat (6)	Rat (10)
P2RX5		-	-
		Rat (5)	Rat (7)
		-	-
		Rat (6)	Rat (10)
P2RX6		Rat (5)	Rat (7)
		+	+/-
		Rat (6)	Rat (10)
P2RX7		+	-
		Rat (5)	Rat (7)
		+	-
		Rat (6)	Rat (10)
P2Y Receptors			
P2RY ₁	+	+	+
	Rat (2)	Rat (8)	Rat (10)
P2RY ₂		+	+
		Rat (8)	Rat (10)
P2RY ₄		+	+
		Human (4)	Rat (10)
		+	-
		Rat (8)	-
P2RY ₆	+	+	+
	Rat (2)	Rat (8)	Rat (10)
P2RY ₁₁			
P2RY ₁₂		+	+
		Rat (9)	Rat (9)
P2RY ₁₃		+	+
		Rat (9)	Rat (9)
P2RY ₁₄			
Ecto-Nucleotidases			
ENTPD1	+		
	Rat (2)		
ENTPD2	+		
	Rat (2)		
ENTPD3	+		
	Mouse (3)		
ENTPD8			
NT5E			
ALPL			

adenocarcinoma metastases. All adenosine receptors were shown to be expressed by both H69 and MzChA-1 cell lines (Fig. 1). Neither H69 nor MzChA-1 showed expression of P2X₁; however, both cell lines showed expression of P2X₂, P2X₄, P2X₅, and P2X₆ (Fig. 2). The only P2Y receptors detected in H69 cells were P2Y₂ and P2Y₁₁, yet Mz-ChA-1 cells expressed P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ (Fig. 3). H69 cell ecto-nucleotidase expression was limited to expression of CD73, NTPDase2, and TNAP, while Mz-ChA-1 cells demonstrated NTPDase2, NTPDase8, and CD73 expression (Fig. 4).

Discussion

Purinergic signaling in the liver is biologically relevant. Specifically, purinergic signals contribute to the maintenance of normal liver physiology via regulation of secretory [40, 41] and metabolic [15] functions, as well as disease pathogenesis, via modulation of activation mechanisms in multiple hepatic cell types [21, 42, 43] and of wound healing and inflammatory responses [10, 32, 44]. Furthermore, the ecto-enzymes that control balance of extracellular nucleotides and nucleosides in the liver are equally important in pathological processes such

as regeneration [45], injury [46, 47], ischemia/reperfusion [48], and fibrosis [10]. Thus, it is scientifically worthwhile to clarify the cell-specific distribution of the various purinergic signaling components expressed in the liver tissue.

A distinct aspect of the liver that makes its study difficult is the multitude of subcellular populations expressed. Myofibroblasts derived from HSC are the primary effector cells in liver fibrosis [49–51], whereas hepatocytes and cholangiocytes work in coordinated fashion to secrete bile [52] and are primary targets of a variety of liver diseases [53, 54]. Isolation of HSC, hepatocytes, and cholangiocytes is now technically straightforward [55], yet there are good reasons to use cell culture models of each of these cell types. In particular, cultured cells allow greater cell purity, have longer life span, and maintain a more stable phenotype (even upon passaging) than isolated cells. Thus, characterization of the adenosine receptors, nucleotide receptors, and regulatory ecto-enzymes in immortalized cells is worthwhile. Unfortunately, because the purinergic gene array investigated in our study has never been characterized in a systematic fashion in liver cell culture models, we proposed that doing so represents a useful resource to the liver research community.

The expression patterns of purinergic signaling genes in the liver cell cultures examined are interesting and occasionally unanticipated. When data are categorized by cell type rather than target gene (Table 2), two important findings become utterly apparent. First, there are distinct differences between HSC cell lines, HepG2 cells (hepatocyte-like), and H69 cells (cholangiocyte-like). Specifically, HSC cell lines (LX-2, hTERT, and FH11) have wide expression of adenosine receptors, but more limited and varied expression of P2X and P2Y receptors. HSC cell line expression of ecto-nucleotidases is more consistent. In contrast, HepG2 cells express all adenosine receptors, all P2X receptors besides P2X₇, all P2Y receptors besides P2Y₁ and P2Y₁₂, and all ecto-nucleotidases, and Huh7 cells express all adenosine receptors besides A₁—a more limited number of P2X and P2Y receptors—and all ecto-nucleotidases besides NTPDase1 and NTPDase3. H69 cells primarily express the A_{2b} receptor, P2X₄ and P2X₅ receptors, P2Y₂ and P2Y₁₁ receptors, and CD73. In contrast, Mz-ChA-1 cells express all adenosinergic receptors, all “traditional” Gq-coupled P2Y receptors plus P2Y₁₁ and NTPDase2, NTPDase8, and CD73; the one consistent area is the complement of P2X receptors expressed by H69 cells and Mz-ChA-1 cells, which is nearly identical. These wide differences in gene expression may be explained in part by the origins of these cells. Precisely, HepG2 cells and Huh7 were derived from distinct tumors may explain, while Mz-ChA-1 cells derive from cholangiocarcinoma cells rather than primary cholangiocytes (as is the case for H69).

The second finding of particular interest is the dissimilarity in expression pattern among the three HSC cell lines. While some consistent patterns are apparent—expression of A_{2B}, P2X₄,

P2Y₁₁, and CD73—there are notable differences. To our knowledge, no study has directly compared gene profiles of LX2 cells and hTERT cells, so it is reasonable to expect differences in purinergic gene expression. Since FH11 cells are outgrowths from primary resected cirrhotic livers, it might be expected that they are highly differentiated, with more limited gene expression patterns. Interestingly, FH11 cells express more purinergic genes than hTERT cells. One quite unexpected but consistent observation is that all but LX-2 cells were negative for NTPDase2, which has been reported as a marker for activated HSC [56]; this may be explained by species differences or cell activation state. Also interesting is the consistent expression of P2Y₁₁, which has not been studied previously in activated HSC, and the absence of consistent expression of P2Y₁ and P2Y₆, which are expressed in activated rat HSC (Table 3). Here, cataloguing purinergic gene expression patterns in HSC cell lines may be of particular importance since it will let investigators choose the proper cell lines for study for specific projects.

Lastly, there are some quirks worthy of comment. NTPDase8 has been identified as a hepatocyte canalicular marker [57]; however, expression was detected in HSC and Mz-ChA-1 cells as well. In contrast, CD73 is expressed in hepatocytes in normal liver; however, expression is dramatically redistributed to HSC [58] in the cirrhotic liver. Thus, there may be a disconnection between mRNA expression and protein expression in the cell lines we have examined. This possibility underlines the necessity to validate all of the mRNA expression patterns with assays of protein expression or biochemical function whenever possible when using the cell lines examined for physiological studies.

In summary, we have provided, here, useful data to liver investigators with an interest in purinergic signaling. This is not hypothesis-driven research; rather, it is an attempt at creating a tool or guide for our scientific colleagues. We are optimistic that similar time-saving approaches will be taken in other organ systems with comparable complexity as the field progresses. Recent developments in liver purinergic signaling are exciting, and we wish to see the field advance more rapidly.

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