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The role of purinergic signaling in the liver and in transplantation: effects of extracellular nucleotides on hepatic graft vascular injury, rejection and metabolism

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Abstract

Extracellular nucleotides (e.g. ATP, UTP, ADP) are released by activated endothelium, leukocytes and platelets within the injured vasculature and bind specific cell-surface type-2 purinergic (P2) receptors. This process drives vascular inflammation and thrombosis within grafted organs. Importantly, there are also vascular ectonucleotidases i.e. ectoenzymes that hydrolyze extracellular nucleotides in the blood to generate nucleosides (viz. adenosine). Endothelial cell NTPDase1/CD39 has been shown to critically modulate levels of circulating nucleotides. This process tends to limit the activation of platelet and leukocyte expressed P2 receptors and also generates adenosine to reverse inflammatory events. This vascular protective CD39 activity is rapidly inhibited by oxidative reactions, such as is observed with liver ischemia reperfusion injury. In this review, we chiefly address the impact of these signaling cascades following liver transplantation. Interestingly, the hepatic vasculature, hepatocytes and all non-parenchymal cell types express several components co-ordinating the purinergic signaling response. With hepatic and vascular dysfunction, we note heightened P2- expression and alterations in ectonucleotidase expression and function that may predispose to progression of disease. In addition to documented impacts upon the vasculature during engraftment, extracellular nucleotides also have direct influences upon liver function and bile flow (both under physiological and pathological states). We have recently shown that alterations in purinergic signaling mediated by altered CD39 expression have major impacts

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upon hepatic metabolism, repair mechanisms, regeneration and associated immune responses. Future clinical applications in transplantation might involve new therapeutic modalities using soluble recombinant forms of CD39, altering expression of this ectonucleotidase by drugs and/or using small molecules to inhibit deleterious P2-mediated signaling while augmenting beneficial adenosine-mediated effects within the transplanted liver.

Keywords

Transplantation; Liver; Purinergic Receptors; Bile; CD39; ecto-ATPase; Endothelium; Immunology; Metabolism; NTPDase; Platelet; Vasculature; Review

2. INTRODUCTION

Purinergic signaling comprises release of extracellular nucleotides, stimulation of purinergic receptors and the modulation of nucleotide levels by ectoenzymes. Over the past decade, many advances have been made in the study of purinergic receptors and the regulation of extracellular nucleotide concentrations (1). It is now generally accepted that extracellular nucleotides (e.g. ATP, UTP, ADP), and the derivative nucleosides (e.g. adenosine from ATP), are released in a regulated manner by cells to provide the primary components for purinergic responses (2). Specific nucleotide/nucleoside mediators bind defined purinergic receptors that comprise the second requirement for this intricate signaling network. Almost all cells express cell-surface type-2 purinergic (P2) receptors for nucleotides and adenosine or type-1 purinergic (P1) receptors. There are seven ionotropic (P2X), at least eight metabotropic (P2Y) and four adenosine receptor subtypes that have been identified and characterized to date (3–7). Hepatic parenchymal and non-parenchymal cells express multiple P2X and P2Y receptor subtypes (see later). These receptors operate in both auto- and paracrine loops and are thought to play a complex, important role in the regulation of vascular and immune cell-mediated responses. For example ATP stimulation of endothelium and lymphocytes largely induces pro-inflammatory responses, such as the release of interleukin-1 and interleukin-6. Exposure of dendritic cells (DC) to extracellular ATP induces migration and drives differentiation to promote cellular immune responses (8). Depending upon the receptor subtype, the cell types and signaling pathway involved, these receptors trigger and mediate short-term (acute) processes that affect metabolism, adhesion, activation or migration. Activation of select receptors in a repetitive manner also has a profound impact upon long-term reactions, including cell proliferation, differentiation and apoptosis, as seen in several chronic inflammatory states.

The third, and final, component of purinergic signaling systems comprises ectonucleotidases (9). These ectoenzymes hydrolyze extracellular nucleotides to generate nucleosides that in turn activate adenosine receptors, often with opposing effects as compared to those mediated by P2-receptors. Within the past decade, ectonucleotidases belonging to several enzyme families have been discovered, cloned and further characterized by pharmacological means. Modulated, distinct ectonucleotidase expression may regulate nucleotide-mediated signaling in the vasculature in both temporal and spatial manners. Vascular endothelial and accessory cell activation with associated proliferative responses within grafts may have important consequences for platelet activation, thrombogenesis, angiogenesis, vascular remodeling and the metabolic *milieu* of the vasculature, in response to inflammatory stress and/or immune reactions (9).

This review considers important P2-receptors and major ectonucleotidases of the vascular endothelium and liver, and relates the expression and functions of these to vascular injury,

thromboregulatory issues, rejection patterns, metabolic disturbances and disordered regeneration seen in certain hepatic grafts.

3. OVERVIEW: PRINCIPLE AND PHYSIOLOGY

3.1. Hepatic purinergic signaling

States of biological stress may lead to alteration of release or uptake of nucleotides or may alternatively decrease enzymatic function of ectonucleotidases (9). Besides being implicated in thrombosis, inflammation and vascular injury, purinergic signaling regulates important hepatic processes such as bile secretion, glycogen metabolism and responses to insulin. Hence, the liver is of major importance for purine homeostasis and for the regulation of nucleotide synthesis. This highly metabolically active organ provides the bulk of all purine precursor substrates for the peripheral tissues (10,11). Salvage of nucleotides in bile was recognized over 40 years ago. This process is associated with both systemic nucleotide homeostasis and the potential for local intercellular signaling within the canalicular networks and biliary systems (12).

Within the liver, ATPase activity has been shown to be associated with the vasculature (NTPDase1), periportal fibroblasts (NTPDase2) and bile canaliculus (NTPDase8). Other ATPases such as NTPDase3 and NTPDase5 have been found in hepatic tissue, but their functional relevance is still unclear.

After hepatic ischemia and reperfusion injury, vascular NTPDase activity is lost and deletion of NTPDase1 in mice leads to significantly increased injury and decreased survival. Furthermore, post-transplant biochemical activity of NTPDase1 is lost initially postoperatively and is reestablished within days to weeks later in the surviving, potentially accommodated grafts (13).

In the following sections, we focus on the expression and function of purinergic P2 receptors and ectonucleotidases within hepatic tissue (Table 1) (14). We describe the known implications of purinergic signaling in processes such as vascular injury, modulation of bile and metabolism e.g. of blood glucose and tissue nucleotide levels. We also briefly allude to diseases that are impacted upon by perturbations in nucleotide flux e.g. liver fibrosis and tumor formation.

3.2. Nucleotide release

Extracellular nucleotides and nucleosides are released in a regulated manner from cells by a variety of mechanisms. Such pathways include exocytosis of ATP/UTP-containing vesicles, facilitated diffusion via connexin-43 hemichannels, by putative ABC transporters or potentially by poorly understood electrodiffusional movements through ATP/nucleotide channels (2,15–21). Under pathophysiological conditions, the release of nucleotides and the expression of purinergic receptors is increased markedly in injured or stressed cells (17).

Extracellular ATP entering the liver by the portal vein is rapidly metabolized after a single passage through the liver (22). Therefore, metabolic stimulation by extracellular nucleotides might expected to be more pronounced in the periportal region. In addition, ATP can be released by hepatocytes into different extracellular compartments: *via* basolateral, sinusoidal or apical exocrine routes. Secretion of ATP into the bile is mediated by an increase in cholangiocyte cell volume, which stimulates nucleotide release by vesicular exocytosis (23,24). Canalicular nucleotide and nucleoside levels are further regulated and controlled by the presence of a canalicular Na^+-dependent nucleoside transporter that removes adenosine from the bile (25). Salvage of nucleotides in bile may be crucial in the maintenance of

appropriate nucleotide/nucleoside concentrations within hepatocytes or within the entire organism (11).

3.3. Purinergic receptors

Over fifteen P2-receptors of different specificities transmit signals from extracellular nucleotides, triggering and modulating vascular and immune cell activation processes, metabolism, nitric oxide (NO) release, adhesion, migration, proliferation and apoptosis (26,27). Since the original cloning of the P2Y2R subtype, at least eight P2YR and seven P2XR subtypes have been cloned and functionally identified.

There are two main families of nucleotide receptors: P2X are "rapid" ligand-gated ion channels permeable for Na^+, K^+ and also Ca^{2+} (subtypes P2X1-7) (28) and P2Y are the "slow" metabotropic receptors (P2Y1, 2, 4, 6, 11–14). P2Y receptors are 7-transmembrane G_q- or G_i-protein linked and initiate signal transduction coupled to activation of phospholipase C, or to inhibition of adenylate cyclase, respectively (29).

There are also four known P1 cell surface receptors for nucleosides, chiefly adenosine (A1, A2a, A2b and A3) that are classified according to their affinities for adenosine and variant coupling to adenylate cyclase (30,31).

Depending upon the repertoire of receptors and signaling components, P2R influence cellular activation, proliferation and the induction of apoptosis. For example, in the vascular system, extracellular nucleotides and nucleosides can influence platelet activation, thrombosis, inflammatory processes, cardiac function and vasomotor responses, (32–35). ATP and ADP appear to regulate hemostasis through the activation of platelet P2 receptors. ADP is a major platelet recruiting factor originating from platelet dense granules, released upon activation whereas ATP derived from the same sources is considered a competitive antagonist of ADP for platelet P2Y1 receptors (17,36,37). Platelet P2Y12 is perhaps the best known purinergic receptor and several drugs (thienopyridine agents e.g. clopidogrel) are used in clinical practice to target ADP-mediated cardiovascular thrombosis (38).

ATP (and UTP) also stimulate endothelial P2Y receptors to release prostacyclin (PGI2) and nitric oxide (NO); two vasodilators and inhibitors of platelet aggregation (39–43). This latter protective action of ATP may limit the extent of intravascular platelet aggregation and to help localize thrombus formation to areas of vascular damage (41,44,45). The receptors P2Y1 and P2Y2 on vascular endothelial cells, smooth muscle cells and monocytes are also important receptors in the mediation of vascular inflammation (46–48).

In addition, ATP also stimulates P2X receptors to cause plasma membrane permeabilization, induction of apoptosis, organic anion transport, and stimulation of Ca^{2+} mobilization (33,49). The active form of ATP that causes membrane permeabilization is a fully ionized tetrabasic ion (or ATP^4) that interacts with the unique multimeric P2X7 receptors expressed on endothelium and monocyte-macrophages (50). This nucleotide is a relative inhibitor of NTPDases, as these are divalent cation dependent enzymes (51). Such P2X7 receptors are also stimulated by the nonhydrolyzable ATP analog ATP-γ-S but are unresponsive to UTP (35,52). The major effect of P2X7 receptors is the induction of cellular activation and apoptosis (52,53).

Pathways of nucleotide-mediated signaling are further complicated by P2-receptor desensitization phenomena. Thus, adenine nucleotides may also directly limit ADP-mediated reactivity of platelets (33,36,54,55). Like other members of the G protein coupled receptor family, some P2Y receptors readily undergo agonist-induced desensitization (56). This may involve phosphorylation of the receptor by multiple protein kinases (27). For

unclear reasons, P2X- and P2Y-type receptors differ widely with respect to desensitization rates: rapidly desensitizing receptors and/or channels (P2Y1, P2Y2, P2X1 and P2X3) contrast with slow desensitizing receptors such as the P2Y6, P2X2 and P2X7 receptors (the last has proven to be very important in inflammatory reactions); P2X4 is intermediate in this regard (57).

3.4. Ectonucleotidases

Ectonucleotidases consist of families of nucleotide metabolizing enzymes that are expressed on the plasma membrane and have externally orientated active sites (Figure 1). These ectoenzymes operate in concert or consecutively and metabolize nucleotides to the respective nucleoside analogs. They have the potential to decrease extracellular concentrations of nucleotides and to generate nucleosides. The relative contribution of the distinct enzymes to the modulation of purinergic signaling may depend on the availability and preference of substrates and on cell and tissue distribution.

Ectonucleotidases modulate P2-receptor-mediated signaling. Alterations in extracellular nucleotide levels can increase or decrease P2 activity or lead to P2 receptor desensitization (58). Desensitization of P2 receptors is dose dependent. *In vitro* the desensitization of P2-mediated anion secretion responses requires 10-min preincubations with the P2Y2 receptor agonist UTP. Recovery from UTP or ATP-induced desensitization can either be rapid (<10 min) at preincubation concentrations of approximately 2 μM or requires progressively longer time periods at higher concentrations. Furthermore, generation of extracellular adenosine not only abrogates nucleotide-mediated effects but also activates adenosine receptors, often with opposing (patho-) physiological effects.

Ectonucleotidases also produce the key molecules for purine salvage and consequent replenishment of ATP stores within multiple cell types (59). Indeed, although nucleotides do not appear to be taken up by cells, dephosphorylated nucleoside derivatives interact with several specific transporters to enable intracellular uptake via membrane passage (11,60).

In the liver functional ATPases were shown as early as 1960 to be associated with canalicular plasma membranes in association with alkaline phosphatases (61). This canalicular ecto-ATPase activity was subsequently shown to be modulated by bile duct ligation and to be distinct from the classical Na^+K^+-ATPase-pumps that are preferentially expressed on the basolateral membranes (11). The initially cloned putative ecto-ATPase was not a true ATPase but rather shared significant sequence homology with several canalicular glycoproteins such as C-Cam105 (11).

There are four major families of ectonucleotidases in the liver microenvironment namely CD39/NTPDases (ecto-nucleoside triphosphate diphosphohydrolases), nucleotide pyrophosphatase/phosphodiesterase (NPP)-type ecto-phosphodiesterases, alkaline phosphatases and ecto-5'-nucleotidases/CD73 (62).

3.4.1. Ecto- nucleoside triphosphate diphosphohydrolases (NTPDases of the CD39 family)—It is now known that there are three ectonucleotidases largely responsible for the bulk of ATPase activity in the liver: NTPDase1, which is present on Kupffer and vascular endothelial cells, NTPDase2, which is expressed by portal fibroblasts and activated hepatic stellate cells and NTPDase8 that seems to be the major ATPase of the hepatic canaliculus. Other hepatic NTPDases such as NTPDase3 on stellate cells and NTPDase5 have been described on liver cells, however, functional data is still lacking.

3.4.2. Nucleotide pyrophosphatase/phosphodiesterases (NPP)—NPP hydrolyze pyrophosphate or phosphodiester bonds of nucleotides, metabolizing ATP to AMP and

diphosphates. The family of NPPs consists of seven members (NPP1-7). NPP1 is expressed in hepatocytes and localizes basolateral membrane (63–65). The expression of NPP1 is associated by hepatocellular growth. It is absent during the fetal period and decreased during liver regeneration following 70% partial hepatectomy (66). This observation underscores the relevance of ATP for hepatocellular proliferation (67). NPP3 is the major NPP isoenzyme at the apical membrane of both hepatocytes and cholangiocytes (64,65).

3.4.3. Alkaline phosphatases (ALP)—Alkaline phosphatases are hydrolases responsible for removing phosphate groups in the 5- and 3- positions from many types of molecules, including nucleotides, proteins, and alkaloids. As the name suggests, alkaline phosphatases are most effective in an alkaline environment. Biliary and canalicular expression of ALP may provide a defense mechanism modulating endotoxin toxicity, not addressed here in detail (68))

3.4.4. Ecto-5'-nucleotidase / CD73—Ecto-5'-nucleotidase (CD73; EC 3.1.3.5) terminates the dephosphorylation cascade of nucleotides to adenosine (69). This enzyme has been detected in the canalicular plasma membrane, in the connective tissue of the portal triads and central veins, and HSC (11,70). Ecto-5'-nucleotidase is co-expressed with NTPDase8 on the canalicular membrane, thereby regulating biliary nucleotide and nucleoside levels (71).

3.5. Distribution and functions

3.5.1. Hepatocytes

3.5.1.1. Purinergic receptors: Select P2X and P2Y purinergic receptors are expressed by hepatocytes (Figure 1) (72,73). Of the P2Y receptor family, mRNA species of P2Y1, P2Y2, P2Y4, P2Y6 and P2Y13 have been observed. Of these, only the P2Y1, P2Y2 and P2Y13 receptors appear to be of functional relevance for the hepatocyte (74,75). P2X receptors have been noted in association with the apical membrane as shown below.

Extracellular nucleotides have several effects on isolated hepatocytes such as repetitive changes of transients in cytosolic free calcium concentration ([Ca (2+)]i) directly in response to ATP or UTP, or in response to ADP after conversion to ATP (76–79). On the basis of the pharmacological profile of the nucleotide-induced Ca2+-responses in human hepatocytes, it is believed that extracellular ATP and UTP increase [Ca2+]i by activating P2Y2 and possibly P2Y4 receptors coupled to the Ca^{2+}-phosphatidylinositol signaling cascade (80). The major metabolic effects of ATP include modulation of glycogen metabolism by glycogenolysis and inactivation of glycogen synthesis (81). For example, it has been demonstrated that activation of P2Y1 receptors in rat hepatocytes substantially stimulates glycogen phosphorylase (82).

3.5.1.2. Ectonucleotidases: The major ectonucleotidases activity of hepatocytes lies within the bile canalicular domain and is comparable to what is described for the cholangiocyte, as discussed below.

3.5.2. Cholangiocytes / bile ducts

3.5.2.1. Purinergic receptors: Cholangiocytes express mRNA of distinct P2Y receptor family members P2Y1, P2Y2, P2Y4 and P2Y6 (83). Microperfusion of intrahepatic bile ducts by apyrase and the P2 receptor inhibitor suramin also reveals the presence of functional apical P2 receptors (60). Similar experiments using the perfusion of ATP-γ-S show net alkalization of bile ducts (83).

The apical purinergic receptor agonist preference is consistent with the P2Y2 subtype and this receptor has been shown to be a marker of the apical cilia (60,84). On the basolateral membrane the function of P2Y receptors appears to be more complex. The kinetics of ATP degradation in apical and basolateral compartments are distinct, suggesting that there are domain-specific signaling pathways that contribute to purinergic responses in polarized cholangiocytes (60,83). Reverse transcriptase PCR from cultured normal rat cholangiocytes shows transcripts for P2X receptors P2X2, P2X3, P2X4, and P2X6. In lysates of cholangiocytes, P2X4 protein can be also detected, and immunohistochemical staining of intact rat liver reveals P2X4 protein within intrahepatic bile ducts. Of P2X receptors, P2X4 is chiefly involved in regulation of bile secretion *in vitro* (85). Hepatocytes and bile ductular cells have been shown to interact and communicate *via* ATP release in a paracrine manner and nucleotides may be involved in the regulation of canalicular contraction and bile secretion. ATP/UTP within the cholangiole stimulates anion and fluid secretion. The binding of nucleotides to apical P2Y2-receptors may facilitate the coordination of bile formation as a consequence of the paracrine hepatobiliary coupling. (85)

3.5.2.2. Ectonucleotidases: Functional ATPases were previously shown to be associated with bile canalicular plasma membranes by histochemical techniques (12). The corresponding enzyme was originally identified as c-CAM105, but this turned out to be incorrect (86–88). Recent studies have revealed that the canalicular ecto-ATPase corresponds to NTPDase8 (89).

Human and rat NTPDase8 cDNA has been cloned and the genes are located on chromosome loci 9q34 and 3p13, respectively (71,89). NTPDase8 resembles the chicken ecto-ATPase cloned from oviduct and liver (90,91). Major ATPase and ADPase activity was detected in the bile canaliculi by immunohistochemistry after incubation with nucleotides. It has been proposed that NTPDase8 is the canalicular ecto-ATPase and that it is responsible for a major proportion of canalicular NTPDase activity (71).

3.5.3. Vasculature

3.5.3.1.Purinergic receptors: Functional expression profiles of P2Y1 and P2Y2 receptors have been described for the hepatic artery and portal vein (92,93). These endothelial cells of the hepatic artery and portal vein mediate nitric oxide (NO) release by P2Y mediated mechanisms (92–94)., We have detected mRNA for P2Y1, P2Y2, P2Y6, P2X4 and P2X7 receptors in sinusoidal endothelial cells (Figure 2). In response to nucleotides, sinusoidal endothelial cells secrete Prostaglandin E2 (PGE2) in a P2Y dependent manner (95).

On vascular smooth muscle cells, P2Y1, P2Y2, P2Y6, P2X1, P2X3, P2X4, P2X5 and P2X7 have been described (96–101). Vasoconstriction is the major effect on arterial smooth muscle cells in response to ATP and UDP and is mediated by P2X and P2Y6 receptors (96,97). In the portal vein, vascular responses seem to be mediated by P2X and P2Y2 receptors (98,99,102).

3.5.3.2. Ectonucleotidases: In the liver NTPDase1 can be detected immunohistochemically on endothelial cells of muscularized vessels and Kupffer cells (Figure 3) (103). The NTPDase/CD39 family includes genes that share considerable sequence homology inclusive of five conserved regions (102). CD39 was originally characterized as a cell activation marker, identified on B cells, subsets of activated NK-cells and T-lymphocytes (104–106). We and others have since demonstrated that CD39 is an NTPDase (EC 3.6.1.5) that hydrolyzes both extracellular ATP and ADP to AMP (50,105,107). Members of this family of ectoenzymes are all membrane-attached *ab initio*, with external-oriented active sites. Select members such as NTPDase5 and NTPDase6 are solubilized after proteolytic degradation. Quiescent sinusoidal endothelial cells do not express CD39. However, under

specific conditions of activation e.g. proliferation after partial hepatectomy, expression of CD39 on sinusoidal endothelial cells is notably upregulated and is of substantial functional relevance (Beldi et al, manuscript submitted). In all human and murine specimens examined to date, neither canalicular nor biliary expression of NTPDase1 has been observed, whereas strong expression on Kupffer cells and endothelial cells of muscularized vessels is found (Figure 3) (108).

Interactions between hepatic NTPDases and P2 may exist, as NTPDases may modulate hepatocyte or bile duct epithelial P2-receptor activity. However, there is no current data to support this hypothesis. Other evidence suggests that certain P2-receptors expressed in the gastrointestinal tract can undergo desensitization in the *Cd39*-null mouse (58).

3.5.4. Stellate cells / portal fibroblasts

3.5.4.1. Purinergic receptors: Quiescent hepatic stellate cells (HSC) can function as pericytes in the liver and express the P2Y subtypes P2Y2 and P2Y4 that are activated by ATP and UTP. Activated HSC in turn express the P2Y subtype P2Y6 (108). Early reports have shown secretion of prostaglandins F2 and D2 after stimulation with ATP (109). Recent studies revealed that P2Y receptors link extracellular ATP to inositol triphosphate-mediated cytosolic calcium signals resulting in localized calcium signaling and cell contraction (110,111).

Portal fibroblasts show functional P2Y activity and express NTPDase2. NTPDase2 (or CD39L1/Cd39L1) was originally cloned from rat brain and chicken smooth muscle and was characterized later from human/mouse sources (112–114). This protein shares 40% sequence homology with rat NTPDase1 but has a 32-fold preference for hydrolysis of ATP over ADP (leading to sustained accumulation of ADP). Functional and molecular expression of NTPDase2 has been shown in portal fibroblasts, near the basolateral membranes of bile duct epithelia (115). This distribution may represent a previously unrecognized mechanism for regulation of nucleotide signaling in bile ducts and other epithelia. Under basal conditions portal fibroblasts hydrolyze ATP to AMP and thereby inhibit activation of basolateral P2Y receptor expression by bile duct epithelia. After injury, such as following bile duct ligation, portal fibroblasts transform into portal myofibroblasts. Loss of NTPDase2 might predispose to stimulation of proliferation of bile duct epithelia (116). Decreased levels of NTPDase2 expression in human biliary cirrhosis, as well as in models of bile duct ligation in the rat, have been observed. Expression of NTPDase 2 also shifts from the portal area to the areas with bridging fibrous bands in cirrhosis with hepatitis C (117).

3.5.4.2. Ectonucleotidases: Hepatic stellate cells express CD39 mRNA both under quiescent and activated conditions (108). Upon activation HSC also express NTPDase2 (108,115). The functional relevance of NTPDase2 on HSC remains unclear. NTPDase 2 is also expressed on portal fibroblasts as discussed below. *In vivo*, NTPDase2 is not expressed on hepatocytes (118). However, certain wild-type mouse hepatoma cells also contain both constitutive and inducible ecto-ATPase activities with unclear cellular localization (118). NTPDase2 was also found to be expressed on mouse hepatocytes *in vitro* after dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin) treatment, but this remains controversial (119).

3.5.5. Kupffer cells

3.5.5.1.Purinergic receptors: There are limited data concerning the expression and function of P2 receptors on Kupffer cells. Peritoneal macrophages typically express mRNA for P2Y1, P2Y2, P2Y6 and possibly P2Y4 as well as P2X1, P2X4 and P2X7 receptors (120–122). P2X7 activation is associated with Interleukin-1 secretion and killing of Mycobacteria, Chlamydia and fungi in monocyte-macrophages (52,123–128). Polymorphisms of P2X7

have been associated with decreased killing of Mycobacteria (129–131). Furthermore secretion of interleukin-6 possibly by stimulation of P2X4 receptors activates innate immunity (132). In one report, liver injury by ATP was attenuated by administration of gadolinium chloride, which depletes Kupffer cells; Suramin a P2 receptor antagonist has comparable effect. (133). Prostaglandin E2 is released after P2Y receptor activation in non-parenchymal cells (95).

3.5.5.1. Ectonucleotidases: NTPDase1/CD39 is expressed on Kupffer cells, however its function remains unclear. In theory, NTPDase1 would modulate P2 receptor function on Kupffer cells and mimic effects on macrophage function with respect to cytokine production (132,134).

3.5.6. Liver associated lymphocytes

3.5.6.1.Purinergic receptors: Natural killer (NK) and natural killer T (NKT) cells as well as B cells express a variety of P2 receptors. Lymphocytes express P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12 and P2Y14 as well as P2X1, P2X2, P2X4 and P2X7 (135–143). In the liver activation of P2X7 by released nucleotides has been shown to mediate, at least in part, the injury noted in Concanavalin A dependent autoimmune hepatitis (144).

3.5.6.2. Ectonucleotidases: NTPDase1/CD39 is expressed on subsets of liver lymphocytes such as NK, NKT and B cells. We have recently shown, that NTPDase1 is expressed on regulatory T cells (145).

4. IMPACT OF PURINERGIC SIGNALING

4.1.Hepatic homeostasis in health

4.1.1. Bile flow—Bile acid secretion is disturbed after liver transplantation, thereby possibly exacerbating hepatic ischemia and reperfusion injury (146,147). Extracellular nucleotides are released into the canaliculus and modulate bile secretion. The biliary epithelium and hepatocytes constitutively release ATP into the bile (11). In biliary epithelium ATP is stored in vesicles and is released in response to cell swelling (23); concentrations of canalicular adenine nucleotides in bile samples are estimated to be 5±0.9μM (148,149). Extracellular nucleotides entering the bile ducts are potent stimuli for secretion of bile fluid including anions, and canalicular contraction, which is in part explained by interaction and communication between hepatocytes and bile duct cells via local ATP release (25,149–151). These effects are mediated and coordinated by apical P2Y2 receptors and NTPDase8 (71,83,89).

4.1.2. Nucleotide synthesis and salvage—Canalicular localization of NTPDase8 in tandem with ecto-5'-ectonucleotidase suggests involvement in the regulation of nucleoside salvage and consequently nucleotide homeostasis and purine salvage (71). Ultimately, the generation of extracellular adenosine from dephosphorylated ATP not only activates adenosine receptors but also produces the key molecule for purine salvage and consequent replenishment of ATP within many cell types (11,59). In the canaliculus Na^+ dependent nucleoside transporters might further aid in concentrating nucleotides within the bile (152). Nucleoside transporters are of major importance to organs and cells incapable of *de novo* nucleotide synthesis such as the brain, muscle, intestinal mucosa and bone marrow (10,25).

Erythrocytes also have these nucleoside transporters and may facilitate the transport of purines between hepatocytes and dependent tissues (153). As the liver appears to be a major source of purines for these tissues, curtailment of nucleotide loss into the bile may be important to maintain appropriate nucleotide/nucleoside concentrations within hepatocytes

(11). Thus, dephosphorylation of nucleotides by ectonucleotidases may be critical for appropriate systemic purine homeostasis (25).

4.1.3. Metabolism (glucose/fatty acids)—Extracellular nucleotides are involved in the regulation of glycogen, fatty acid metabolism and insulin resistance. Extracellular nucleotides activate glycogen storage and are glycogenolytic (81). Overall glycogen phosphorylase and the generation of inositol triphosphate (IP3) are stimulated. Glycogen synthase is inhibited, adenosine 3': 5'-cyclic monophosphate levels are lowered and activation of phospholipase D is decreased (81,154–156). In addition, administered ATP appears to facilitate up-regulation of the gluconeogenic enzyme, phosphoenolpyruvate carboxykinase, and down-regulation of the glycolytic enzyme, pyruvate kinase; hence promoting glucose output by the liver (157,158). Of the P2Y receptors that mainly control metabolism and proliferation, P2Y2 seems to primarily control both glycogen metabolism and proliferation-associated responses such as increased [Ca ($^{2+}$)](c) and modulation of mitogen-activated protein kinase cascades (82).

Effects of extracellular ATP on fatty acid metabolism indicate inhibition of acetyl-CoA carboxylase activity and fatty acid synthesis with a concomitant decrease of intracellular malonyl-CoA concentrations (159).

Purinergic signaling also impacts upon insulin signaling. Disordered regulation of extracellular nucleotides induces insulin resistance. Cd39 knockout mice show impaired glucose tolerance and sensitivity to insulin. Furthermore, plasma insulin levels are significantly higher when compared to wild-type mice. Hyperinsulinemic euglycemic clamp studies have revealed altered glucose metabolism in the livers of mice null for CD39/NTPDase1. The impaired sensitivity was accompanied by increased susceptibility to activation of c-jun N-terminal kinase /SAP by extracellular ATP in CD39 null mice liver, (Enjyoji at al, submitted).

4.2. Liver disease

4.2.1. Vascular injury—Many processes of vascular injury result in the release of adenine nucleotides that exert a variety of inflammatory effects on endothelial cells, platelets and leukocytes (reviewed in (9,33,160–162)). Perturbation of nucleotide signaling in the vasculature can influence vasomotor responses, platelet activation, inflammatory processes and also cardiac function (32).

In the setting of organ transplantation NTPDase1 activity is lost with reperfusion or rejection and up-regulation is seen with graft survival (13). In a model of total hepatic ischemia and reperfusion injury we have shown a significantly decreased survival associated with increased biochemical and structural liver injury in Cd39-null animals as compared to wild-type controls (163). Furthermore, ADP is a major platelet recruiting factor originating from platelet dense granules during activation (17,36,37). ATP also stimulates endothelial cell production of prostacyclin (PGI2) and NO; two vasodilators and inhibitors of platelet aggregation (39,40).

Administration of soluble NTPDases in the blood stream or up regulation of CD39 post-adenoviral infection has been shown to prolong transplanted graft survival and abrogate platelet sequestration within the vasculature. Finally, mutant mice deficient in *Cd39* have evidence for a vascular-based thrombotic state, paradoxically associated with marked prolongation of experimental bleeding times and acquired platelet hypofunction (164).

4.2.2. Rejection—Purinergic receptors and ectonucleotidases are expressed on a variety of immune cells. However, data concerning hepatic graft survival are lacking. NTPDase1

deficiency appears to have two opposing impacts on nucleotide-mediated signaling, that is, inhibition via desensitization of some but not all P2 receptors and augmentation of certain other responses via impaired hydrolysis of their ligands (134). Consequently, in Cd39 null mice, defects in dendrititc cell function, antigen presentation, T cell responses to haptens (type IV hypersensitivity reactions) and delayed cellular rejection responses are observed (8,131,134). We have noted membrane expression of CD39 by classic T-regulatory (Treg) cells that appears to be of crucial importance in the immunomodulation of skin allograft rejection (145). These T cell subsets have recently been shown to directly influence chronic vascular injury (165).

4.2.3. Regeneration—In the area of split liver and living donor related liver transplantation liver regeneration has gained additional interest in order to maintain and increase regenerative capacities of remaining and transplanted liver lobes. ATP activates proliferation of hepatocytes *in vitro* and *in vivo* and modulates growth factor responses possibly via P2Y2 receptor activation (67). The response of hepatocytes is comparable to early signaling events associated with liver regeneration such as transient activation of c-jun N-terminal kinase and immediate early genes. VEGF receptor 2 signaling, which is of importance during liver regeneration, is modulated by the P2Y2 receptor (48). In mice null for CD39 liver regeneration is significantly decreased. Stimulation of cultured liver sinusoidal endothelial cells (LSEC) with UTP and VEGF leads to enhanced VEGF receptor 2 phosphorylation that is abrogated in cultures with Cd39 −/− LSEC (Beldi G, Wu Y, Sun X Imai M, Enjoji K, Zimmermann H, Candinas D, Robson SC, The vascular ectonucleotidase CD39 is permissive for sinusoidal endothelial cell proliferation during liver regeneration Hepatology 44 (4): 190A-191A 8 Suppl. 1 2006).

4.2.4. Tumors—Recurrence of hepatocellular carcinoma is an important problem post-transplantation, especially in patients with larger tumors exceeding standard selection criteria for liver transplantation (166,167). As shown above, deletion of Cd39 and perturbations in extracellular nucleotides influence hepatocellular proliferation. Curiously, mice null for Cd39 also exhibit an increased incidence of spontaneous and diethyl nitrosamine induced hepatocellular carcinoma (Wu Y, Sun X, Imai M, Sultan B, Csizmadia E, Enjyoji K, Jackson S, Usheva A, Robson SC. Modulation of RAS/ERK signaling by CD39/ENTPD1 during liver regeneration. Hepatology 2005 42, 4, Suppl.1, Abst.1138, page 643A). Although the mechanism is unclear, mitogenic effects of extracellular nucleotides may predispose to the development of malignancy in these livers. There are also documented associations between CD39 and RanBPM, a membrane scaffolding protein that may be responsible for heightened levels of hepatocyte Ras activation observed following genetic deletion of CD39. This, in turn, may predispose to hepatocarcinogenesis (168).

4.2.5. Fibrogenesis and cirrhosis—Liver fibrosis and cirrhosis due to an array of underlying etiologies are the leading cause world wide of end stage liver disease. In patients post liver transplantation with hepatitis B and C, fibrosis has additionally been seen in conditions such as fibrosing cholestatic syndrome (169,170). The main cellular components that cause hepatic fibrosis, namely portal fibroblasts and HSC, contain a variety of P2 receptors and express NTPDase2. In this system, closely regulated extracellular nucleotides can activate HSC leading to transcription of procollagen-1 (108). The proliferation of bile duct epithelium which is important for fibrogenesis is inhibited by NTPDase2 on portal fibroblasts leading to blockade of P2Y activation (116).

4.2.6. Cachexia—Individuals with liver disease may develop metabolic imbalances with cholestasis, consequent bone marrow suppression, wasting or severe neurological manifestations (including cerebral edema) that may progress to coma and death over days to

weeks. Hypothetically, uncompensated failure of hepatic nucleotide synthetic pathways and disordered salvage may contribute, at least in part, to these complications of acute or chronic liver failure.

The cachexia seen in untreated AIDS patients also has a component that may be associated with purine salvage defects and adenosine mediated toxicity. Extracellular adenosine deaminase that degrades extracellular adenosine, has crucial functional and structural associations with CD26. This interaction results in co-stimulatory signals in T cells and thereby modulation of cell-to-cell contacts in the immune response. Interestingly, adenosine deaminase is also displaced from CD26 by HIV envelope proteins and the consequent perturbations in purine metabolism might be linked to or associated with T cell death and/or AIDS wasting (146).

5. CONCLUSIONS AND PERSPECTIVE

We have reviewed the basic principles of purinergic signaling in some depth and have also shown substantial experimental data implicating extracellular nucleotides, signaling by purinergic receptors and modulation by NTPDases of the CD39 family in the regulation of hepatic blood flow, bile production, homeostasis and metabolism. These pathways impact upon vascular and immune responses to transplantation. In addition to graft injury, disordered bile flow and ongoing graft ischemia secondary to vascular rejection may impact upon the outcome of organ transplantation.

Additionally, purinergic receptors and CD39 are not only expressed on endothelial cells and hepatic sinusoidal cells but also at high levels on Treg (145,171,172) and NKT cell populations (Beldi et al, submitted). It is particularly important that hepatic metabolic changes can be caused by extracellular nucleotides thought to serve primarily as inflammatory mediators. These new data provide evidence for integration of nucleotide-mediated vascular and immune responses and associated metabolic disturbances e.g. insulin resistance that are so crucial to the clinical outcomes following hepatic allografting.

Our expectation is that therapeutic avenues will develop using soluble CD39/NTPDase1 and/or small molecules to enhance NTPDase ecto-enzymatic functions that may be used together with selective adenosine receptor agonists. Other approaches to inhibit NTPDase activity may be considered to slow progression of fibrosis e.g. in chronic pancreatitis. It is of interest that certain drugs already in clinical practice influence CD39 function and expression. Commonly prescribed lipid lowering therapies that have salutary effects on the vasculature (e.g. HMG-CoA reductase inhibitors) and membrane phospholipids have in parallel important positive effects on endothelial cell NTPDase activity (173).

Studies are planned to address the efficacy of CD39-based therapeutics in the setting of hepatic reperfusion injury and in the appropriate immune modulation of hepatic allograft rejection.

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Abbreviations

P1	type 1 purinergic (adenosine) receptor
P2	type 2 purinergic receptor
ATPase	ATP diphosphohydrolase
DC	dendritic cell
EC	endothelial cell
NTPDase	ecto-nucleoside triphosphate diphosphohydrolase
ALP	alkaline phosphatases
IL	interleukin
NPP	nucleotide pyrophosphatase/phosphodiesterase
HSC	hepatic stellate cell
NK	natural killer cell
NKT	natural killer T cell
Treg	T regulatory cells
VEGF	Vascular endothelial growth factor
LSEC	liver sinusoidal endothelial cell

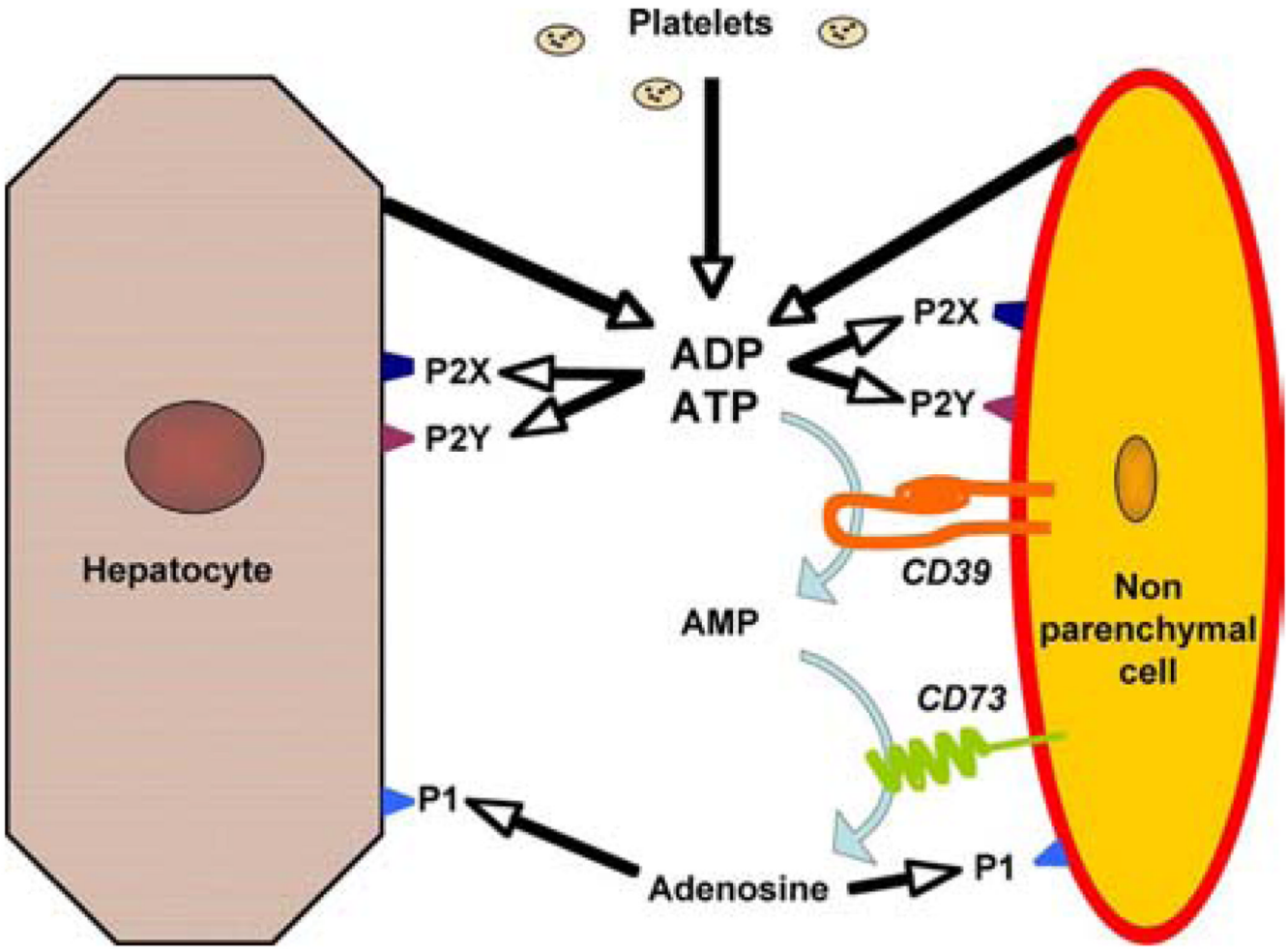

Figure 1.
Principles of purinergic signaling in the hepatic microenvironment. Nucleotides are released from parenchymal cells, non-parenchymal cells and platelets. These nucleotides then activate P2 receptors or are hydrolyzed by NTPDases and 5'nucleotidase to adenosine, which activates P1 receptors.

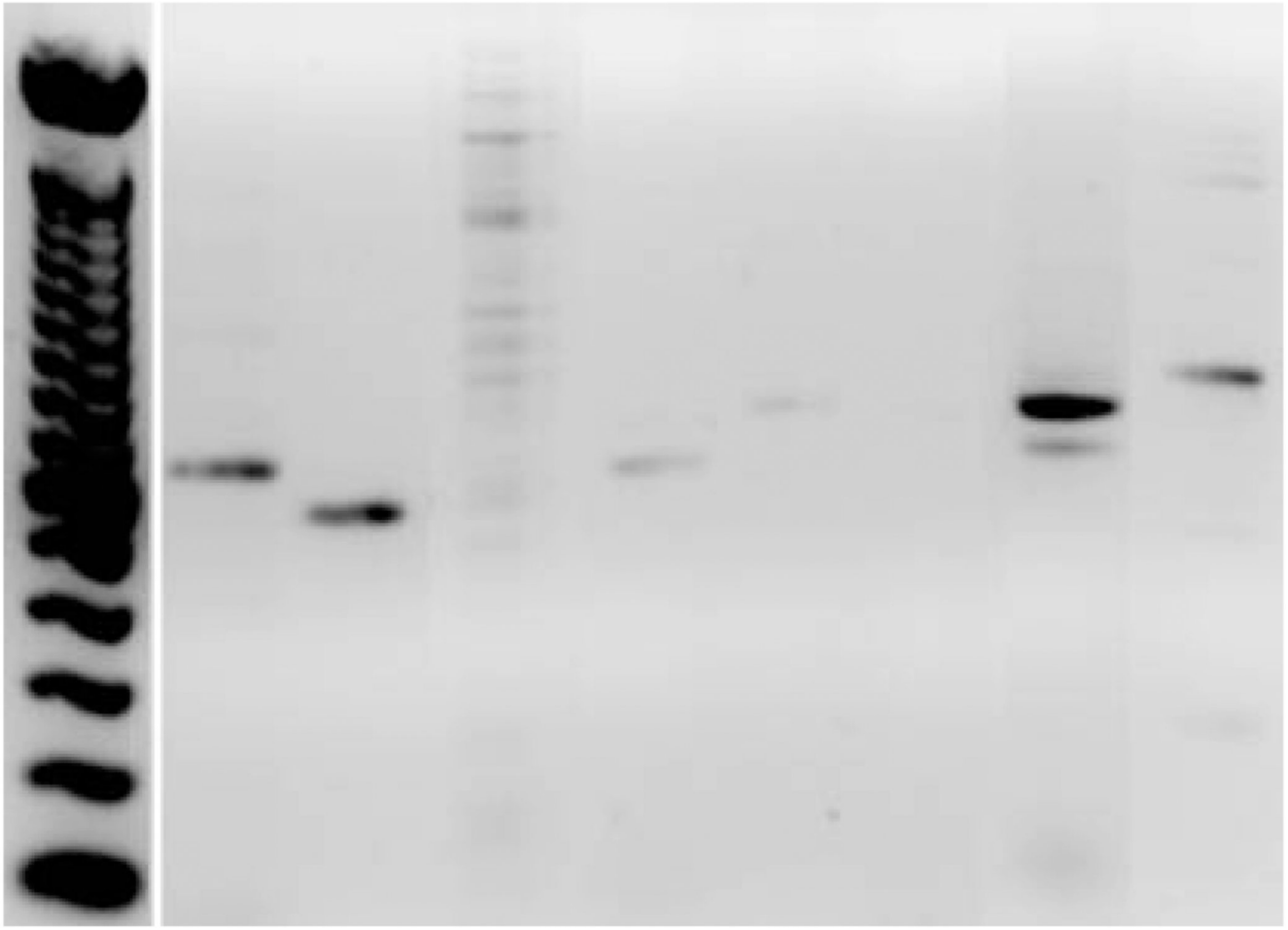

Figure 2.
P2 receptor expression by hepatic sinusoidal endothelial cells. High levels of mRNA for P2Y1, P2Y2, P2X4 and P2X7 are noted. The expression of P2Y6 is weak and no signals for P2Y4, P2X1 and P2X2 are noted.

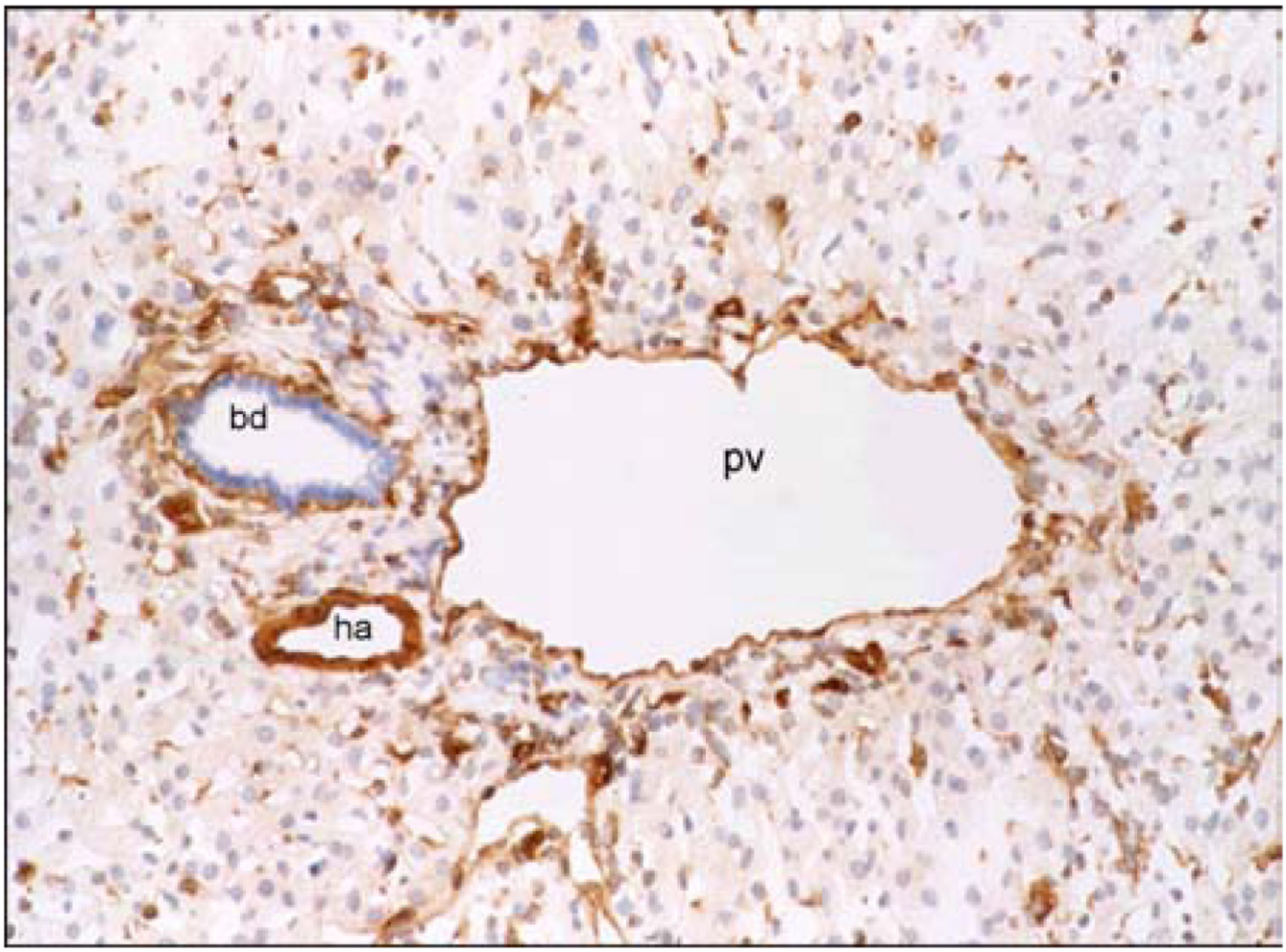

Figure 3.
Immunohistochemical patterns of expression of CD39 in donor human liver. Endothelial cells of the portal vein (pv), hepatic artery (ha) and Kupffer cells strongly express CD39. Quiescent sinusoidal endothelial cells and bile duct (bd) epithelia do not stain for CD39.

Table 1

Expression and function of P2-receptors and ectonucleotidases by cellular components of the liver

Cellular component	Expression	Function	References
Hepatocytes	P2Y1, P2Y2, P2Y4, P2Y6, P2Y13, NTPDase8 (apical membrane)	Glycogen metabolism Insulin resistance	108–111
Cholangiocytes	P2Y1, P2Y2, P2Y4, P2Y6, P2X2, P2X3, P2X4, P2X6	Bile (anion) secretion Nucleotide salvage Canalicular contraction Interaction with hepatocytes Adenosine resorption from bile	115,117
Endothelial cells	P2Y1, P2Y2, P2Y6, P2X4, P2X7 NTPDase1	NO release Secretion of prostaglandins E2	96–102
Vascular smooth muscle cells	P2Y1, P2Y2, P2Y6, P2X4, P2X7	Portal vein contraction	48,50,164,174
Hepatic stellate cells	P2Y2, P2Y4, P2Y6, NTPDase2	Secretion of Prostaglandin F2 and D2 Cell contraction	60,83–85
Portal fibroblasts	NTPDase2	Hepatic fibrosis	48,92,93,95
Kupffer cell/macrophages	P2Y1, P2Y2, P2Y4, P2Y6, P2X1, P2X4, P2X7, NTPDase1	Killing of intracellular pathogens Secretion of prostaglandin E2 Interleukin 6	120–134
Liver associated lymphocytes: NK, NKT, T cells, B cells	P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y14 P2X1, P2X2, P2X4, P2X7, NTPDase1	Modulate Concanavalin A mediated hepatitis	135–145
Neutrophils	P2Y2	Chemotaxis	174