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Hepatic oval (stem) cell expression of EDG receptors for lysophosphatidic acid in mouse chronic liver injury

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Running title : LPA receptors in hepatic oval cells

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Abbreviations: EDG, endothelial differentiation gene; LPA, lysophosphatidic acid; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine.

Abstract.

Growth factor lysophosphatidic acid (LPA) regulates cell proliferation and differentiation, increases motility, and survival in several cell types, mostly via G-protein coupled receptors encoded by Endothelial Differentiation Genes (EDG). We show herein that hepatic oval (stem) cell proliferation, induced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) in a mouse model of chronic liver injury, was associated with the expression of LPA1, LPA2 and LPA3 receptor subtypes; only LPA1 receptor protein was detectable in normal liver by western blot. In the injured liver, enhanced LPA1 receptor was identified predominantly in oval cells along the portal tract, proliferating ductular epithelial cells and in small cells, which were located in the nearby parenchyma and formed clusters. Interestingly, the LPA1 receptor was co-expressed in DDC-treated livers with the stem cell antigen SCA-1, suggesting that this receptor may be associated with bone marrow-derived progenitors. All three receptors for LPA were detected mostly in small cells in the vicinity of the portal tract, and co-localized with the A6 antigen, a marker of ductular oval cells. In addition, hepatic levels of endogenous LPA were significantly higher in DDC-fed mice compared to normal animals. We propose that the expression of diverse LPA receptors may be a necessary part of the mechanism responsible for activation of oval cells during liver injury. As a result, LPA and its analogs may represent critical endogenous mediators, which regulate survival, increase motility and modulate proliferation and differentiation of hepatocyte progenitors in regenerating liver.

Introduction

Proliferation of putative hepatic stem cells has been observed in both experimental animal models of liver injury and human pathologic ductular reactions (1-4). Small hepatocyte-like oval cells originated from ductules in response to various toxic or carcinogenic agents appear to represent a progeny of the facultative hepatic stem (oval) cell compartment (5-7). A mouse model of chronic liver disease induced by feeding of mice with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) has attracted particular attention (8, 9). The most intriguing characteristics of the liver from these mice have been the appearance and progressive accumulation of small oval cells with a positive staining for A6 antigen, a marker of proliferating ductular epithelial cells(10).

The roles for peptide growth factors, such as transforming growth factor-α (TGF-α), in hepatic stem cell proliferation and differentiation have been studied (11, 12). In contrast, the biological significance of unique phospholipid growth factors such as lysophosphatidic acid (LPA) in stem cell biology, is largely unknown. The cellular effects of LPA are mediated predominantly via G-protein coupled receptor subfamily encoded by endothelial differentiation genes (EDG) (13-16). There are three major receptor subtypes, which preferentially bind LPA : LPA1 (former EDG2), LPA2 (EDG4) and LPA3 (EDG7) (16). LPA induces proliferation of hepatic stellate cells (HSC) (17, 18), and increases motility and migration of human hepatoma cell line (19). In contrast, growth inhibition by LPA was reported in primary rat hepatocytes (20). Our recent studies revealed the presence of LPA1 and LPA2 receptors in the proliferating AML12 murine hepatocyte cell line, which overexpress TGF-α (21, 22). In these hepatocytes, LPA protected the cells from apoptosis induced by TNFα/D-galactosamine or *Clostridium difficile* exotoxin exposure. Herein we report the expression of LPA receptors and their co-localization with A6 antigen in the injured murine liver. Moreover, we found co-expression of the hepatic LPA1

receptor and the SCA-1 antigen, a hematopoetic stem cell marker. These data demonstrate for the first time that the induction of the receptors for LPA may be a necessary part of mechanism responsible for activation of hepatic stem cells in regenerating liver, thereby implicating LPA as an important growth factor involved in the recovery from hepatic injury.

Materials and Methods.

Materials. DDC (3,5-diethoxycarbonyl-1,4-dihydrocollidine) was a product of Sigma-Aldrich (St. Louis, MO). A6 rat anti-mouse monoclonal antibody was a generous gift from Dr. Valentina Factor (Laboratory of Chemical Carcinogenesis, NIH). Rabbit polyclonal antibody against the C-terminal part of LPA1 and the N-terminal part of LPA2 were purchased from Exalpha Biologicals, Inc. (Boston, MA). Mouse monoclonal antibody against N-terminal fragments of LPA1 and LPA3 were gifts from Dr. John S. Kenney, (Antibody Solutions, Palo Alto, CA). SCA-1 monoclonal antibody were purchased from Pharmingen (San Diego, CA). Radiolabeled [^{14}C]oleoyl-CoA (specific radioactivity 55 mCi/mmol) was purchased from New England Nuclear (Boston, MA).

Induction of oval cell proliferation in vivo. Induction of mouse chronic liver injury accompanied by oval cell proliferation was performed using the procedure described by Preisegger et al. (10). Mice (5-week-old, C57BL/6J) were given the standard grain-based diet (NIH-31) containing 0.1 % DDC (BioServ, Inc. Frenchtown, NJ) and allowed food and water *ad libitum*. Five weeks after initiation of the diet, mice were sacrificed, and livers were processed as described below.

Western blot analysis of EDG receptors. For Western blot analyses of EDG receptors, liver specimens were excised, thoroughly rinsed with cold PBS and homogenized on ice in Western blot buffer as described previously in detail (21). Samples (50 μg protein) were subjected to SDS-PAGE (12% gel), and electroblotted onto polyvinylidene difluoride membranes. Membranes were blocked in 10 mM tris (pH 7.5), 100 mM NaCl, 0.1% Tween-20 containing 5% nonfat dry

milk for 60 min at room temperature. After overnight incubation with primary antibodies (1 μg/ml, 4° C), proteins were visualized using a goat anti-rabbit antibody conjugated to HRP and a chemiluminescense detection system.

Immunohistochemistry of A6, SCA-1 antigen, and EDG receptors. Liver samples were frozen in OCT (-80^0 C), sectioned at 5 μm and fixed in -20° acetone for 10 min followed by 3 washes with PBS. The slides were sequentially blocked with serum, avidin and biotin, washed with PBS and incubated with anti-mouse A6 (1:10) or SCA-1 (1:100) antibody for 1 hour at room temperature. Slides were washed 2 times with PBS, and proteins were visualized using a rat Vectastatin-ABC-alkaline phosphatase detection kit. For LPA1 and LPA2 receptor immunohistochemistry, frozen 5 μm liver sections were fixed in ice-cold 2 % formaldehyde in PBS for 8 min on ice. Samples were washed 3 times with 0.1 % saponin in Earle Buffered Salt Solution (EBBS/S, saponin-washing buffer) for 3 minutes each and incubated for 20 minutes in serum blocking solution with 0.1% saponin added (Vectastatin rabbit ABC-alkaline phosphatase detection kit, Vector Laboratories, CA). Endogenous biotin was blocked with a biotin blocking kit, and following a wash with EBSS/S, sections were incubated with the receptor antibody (10 μg/ml) overnight at 4^oC. Samples were washed with EBSS/S and incubated with biotinylated secondary antibody (ABC Kit) with saponin for 30 minutes. Following incubations, slides were washed 2 times with EBSS/S and proteins were visualized using Vectastatin-ABC-alkaline phosphatase detection kit with Vector blue substrate. Nuclei were counter-stained with Nuclear Fast Red. For LPA3, monoclonal antibody was used at a 1:10 dilution. The samples were processed as above; in addition to biotin blocking, endogenous peroxide was blocked using peroxide blocking Kit. The detection system was the M.O.M-HRP Kit from Vector Laboratories. Nuclei were counter-stained with Meyer's hematoxyline.

Assay of hepatic LPA levels. LPA is measured by radioenzymatic assay using recombinant LPA acyltransferase (rLPAAT) as described previously (23). Briefly, dry lipid extract is resuspended in 200 µl of reaction medium (1 µl of [^{14}C]oleoyl-CoA, 20 µl of 200 mM Tris (pH 7.5), 10 µl of rLPAAT, 8 µl of 500 µM sodium orthovanadate, and 161 µl of H_2O containing Tween 20 at 1 mg/ml) and incubated for 120 min at 20°C. The reaction was stopped by addition of 400 µl of $CHCl_3$–methanol–12 M HCl, 40:40:0.26 followed by vigorous shaking and centrifugation at 3,000xg for 10 min. The lower $CHCl_3$ phase was evaporated under nitrogen, resuspended in 20 µl of $CHCl_3$–methanol, 1:1, supplemented with internal standard LPA and phosphatific acid (PA) and separated by TLC using $CHCl_3$–methanol–NH_4OH–H_2O, 65:25:0.9:3 as a solvent system. [^{14}C]PA spots were scanned, scraped and counted with 3 ml of scintillation cocktail.

Results

Induction of hepatic expression of EDG receptors for LPA in chronic liver injury. Expression of LPA1, LPA2 and LPA3 receptor proteins in the liver was first characterized by western blot analyses. All these proteins have a molecular weight (MW) of 41-43 kDa calculated from the deduced amino acid sequence. The LPA1, but not LPA2 or LPA3 antibody revealed immunopositive proteins with the relevant MW in normal mouse liver (Fig. 1). Hence, western blot analysis showed that the levels of the LPA receptor protein in intact control liver are either very low (LPA1) or not detectable (LPA2 and LPA3). Feeding of mice with DDC for five weeks resulted in the dramatic increase in the expression of the LPA1, and induction of expression of LPA2 and LPA3 proteins (Fig 1).

Immunohistochemistry of hepatic EDG receptors for LPA.

A routine histological analysis of liver tissue revealed the proliferation of interlobular bile ducts, which formed wide lumens. The expected features of marked cholestasis with bile deposits in bile ducts and ductules, portal tract fibrosis, and inflammatory infiltration, were observed in the livers of DDC-treated mice.

In normal mouse liver, the LPA1 receptor protein was barely detectable, and probably restricted to non-parenchymal cells with the occasional immunopositive staining in ductular epithelium (Fig. 2 a). No LPA1-positive immunoreactivity was found in hepatocytes. In DDC-induced liver damage, the LPA1 receptor was expressed in small cells, which were located in the vicinity of the portal tracts, and which spread into parenchyma and formed clusters (Fig. 2 b, see inset). In contrast to LPA1, the LPA2 and LPA3 receptor proteins were essentially not detectable in normal liver by immunohistochemistry (Fig. 2 c, e); only a very rare LPA2-positive ductular staining was observed (Fig. 2 c). DDC treatment resulted in the appearance of periportal LPA2 positive small cell collectives similar to, but to a lesser extent than LPA1 reaction (Fig. 2 d). No LPA3 receptor immunoreactivity was found in normal liver (Fig. 2 e), while in DDC-treated mice, LPA3 receptor protein was expressed and localized predominantly in ductular epithelium (Fig. 2 f).

Hepatic LPA1 and LPA2 receptors are co-localized with the A6 antigen, and co-expressed with the SCA1 antigen in DDC-treated mice.

No positive A6 staining was found in the liver tissue of control mice, and in the liver samples from DDC-treated mice incubated without primary A6 antibody (data not shown). Mice maintained on DDC-containing diet exhibited very high levels of A6 antigen-positive staining in the liver, including bile duct and ductular epithelum cells (Fig. 3 a, arrow). Remarkably, DDC diet induced the proliferation of A6 positive small oval cells in the periphery of portal tracts, and extensive accumulation of these cells in the hepatic parenchyma (Fig. 3 a, arrowhead). The cells harboring A6 antigen formed clusters in the liver parenchyma in the surrounding area of portal tracts.

The LPA1 and LPA2 receptors were co-localized with the A6 antigen on the serial sections of liver tissue. There was focal coincidence of A6 and LPA1 immunoreactivity in the periportal small cell clusters, bile duct epithelium and bile ductular epithelium (Fig. 3 b). LPA2 receptor immunoreactivity was identified primarily in bile duct epithelial cells (Fig.3 c). In DDC-treated levers, stem cell antigen SCA-1 was also expressed intensely and was found to reside in duct and ductular epithelium, and to a lesser extent, in small cells in the parenchyma nearby portal tracts (Fig. 4 a). LPA1 receptors were co-expressed with the SCA-1 antigen in similar locations as indicated on the serial liver sections (Fig. 4 b), suggesting the possible association of LPA1 with bone marrow-derived hepatic stem cells during liver regeneration.

Hepatic levels of LPA. Lipids were extracted from liver specimens of mice fed with DDC-containing diet for five weeks and normal animals (control diet). LPA levels were significantly higher in DDC-treated mice: 679.0 $\pm$ 41.2 pmol/g of liver weight, compared to control mice: 428.9 $\pm$ 29.7 pmol/g of liver weight (Fig. 5). The LPA levels in murine plasma were shown to be 170 $\pm$ 50 pmol/ml (23). Thus, it appears that even in normal mice, hepatic levels of endogenous LPA may be slightly higher than in plasma.

Discussion

The novel and most important findings of these studies are: (i) induction of LPA1, LPA2 and LPA3 receptors (Fig. 1 and 2) in mouse model of liver injury, which is accompanied by ductular proliferation, and (ii) co-localization of LPA1 receptors and A6 antigen; both are expressed in proliferating ductules and small cells, likely originated from ductular epithelium (Fig. 3). LPA1 receptors were also co-expressed with the stem cell antigen SCA-1 (Fig. 4).

These observations raise the important questions as to exactly what type of hepatic cells express LPA receptors, whether this expression is related to particular pathological conditions, and what factors are responsible for the induction of LPA receptors? The present data suggest

that LPA receptors might be expressed by activated hepatic stem cells (oval cells and, possibly small hepatocytes). On the other hand, the vast majority of mature hepatocytes in the intact mouse liver apparently do not express LPA receptor proteins.

Thus, we propose that the increase in the hepatic LPA1 expression and induction of expression of LPA2 and LPA3 proteins in DDC-treated mice may be related predominantly to activated hepatocyte progenitors (oval cells), and possibly primed hepatocytes entering cell cycle. It was shown that mitogenic activation of T lymphocytes changed the pattern of expression of EDG receptors and cell responsiveness to LPA. Non-stimulated T cells expressed predominantly LPA2, whereas in mitogen-activated T cells, co-dominance of the LPA1 receptor type was observed (24). An increase in the ratio of LPA1 to LPA2 shifted the effect of LPA from suppression to generation of IL2 by T lymphocytes. Therefore, regulated changes in the ratio of the EDG receptor levels have been suggested to be a mechanism of control of cell responsiveness to LPA. Moreover, co-localization of LPA1 receptors and A6 antigen in ductular cells in DDC-fed mice (Fig. 3) suggests that expression of LPA1 receptors may be associated with the activation of oval cells in response to liver injury. Furthermore, LPA1 receptors have also been shown to co-express with SCA-1 antigens (Fig. 4), marker of hematopoietic stem cells (25). In addition, expression of SCA-1 antigen was obsereved in fetal liver (26). Taken together, these data support our notion that EDG receptor expression may be associated with activation and proliferation of hepatic oval cells.

Little is known about the roles for LPA and its receptors in the liver. However, several findings in other systems propose such a role by comparison. For example, in developing brain, the LPA1 receptor is abundantly expressed at sites of neurogenesis, namely in the ventricular zone of cerebral cortex. The LPA1 expression was restricted to the timeframe of neuroblasts differentiation into mature neurons (27). Similarly, Schwann cells expressed the LPA1 only

within a limited period of differentiation from the precursor to myelinated cells (28). Likewise, LPA receptors may be expressed in hepatic cells in transitory state, particularly in oval cells and/or small hepatocytes upon their activation and differentiation into mature hepatocytes in regenerating liver.

Oval cell activation and proliferation is a result of complex and well-orchestrated set of events when, in response to severe liver injury, hepatic cells release growth factors, cytokines and chemokines creating a specific microenvironment, which primes quiescent pool of hepatic stem cells (29). The release of bioactive lipids, such as LPA and S1P, from activated cells of any type in the liver *milieu exterier* is also highly probable, but not yet well characterized . Recruitment of activated macrophages and platelets also might contribute to local accumulation of LPA in injured sites (30). Although, we have shown the increased levels of LPA in damaged liver, the source(s) of this mediator in the damaged liver is not clear. Coordinated expression of LPA receptors might render oval cells to become responsive to LPA in appropriate pathophysiological situation, when activity of this mediator is required for control of cell survival, proliferation, differentiation and motility (22). However, direct pathophysiological signals, which induce expression of LPA receptors in oval cells remain to be specified. It remains to be established how LPA affects oval cell survival, proliferation, differentiation or motility, and what are the receptor-mediated LPA signaling cascades in hepatic stem cells during their activation and maturation.

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Legends to Figures.

Figure 1. Western blot analysis of LPA receptor proteins expression. Liver samples (50 μg protein each) were subjected to SDS-PAGE and probed with LPA1, LPA2 and LPA3 antibody as described in **Materials and Methods.** Representative blots of two normal and DDC-treated mouse liver samples out from 4 animals in each group, are presented.

Figure 2. Immunohistochemistry of LPA receptor proteins. Liver sections from normal **(a, c, e)** and DDC-fed mice **(b, d, f)** were processed and stained for LPA1 **(a, b),** LPA2 **(c, d)** and LPA3 **(e, f)**. Blue staining **(a-d)** and brown staining **(e, f)** represent LPA receptor-immunopositive material. Arrows indicate the accumulation of LPA receptor immunoreactivity in periportal area of liver parenchyma. Note the appearance of LPA1-positive cell clusters in the vicinity of the portal tract. **f-**arrow shows LPA3 immunoreactivity in portal tract bile duct epithelium. **b**, inset: LPA1-positive cell clusters in proliferating bile ductules**. a-f**, magnification x400; **b, inset,** magnification x600.

Figure 3. Co-localization of A6 antigen, LPA1 and LPA2 in DDC-fed mice. Serial liver sections were processed and stained for A6 antigen **(a),** LPA1 **(b)** and LPA2 **(c)** as indicated in **Materials and Methods.** Blue staining characterizes A6 antigen and LPA receptor immunoreactivity. Arrows show the presence of A6 antigen, LPA1 and LPA2 receptors in bile duct and bile ductular epithelium. Arrowheads indicate the immunopositive cells in the liver parenchyma. Magnification x400.

Figure 4. Co-expression of SCA-1 antigen and LPA1 receptor in DDC-treated mice.

Serial liver sections were processed as described in **Materials and Methods** and stained for SCA-1 antigen **(a)** and LPA1 receptor **(b)**. Blue staining represents SCA-1 and LPA1 receptor immunoreactivity. Arrows indicate a localization of SCA-1 and LPA1 receptor preferably in bile duct and bile ductular epithelium and in small cells forming clusters in periductular area. Note: a significant sinusoidal localization of SCA-1 antigen **(Fig. 4 a)**. Magnification x400.

Figure 5. Hepatic levels of LPA in normal and DDC-treated mice.

LPA was extracted from liver tissues using n-butanol. Typically, ~0.5 g of the liver was homogenized in 3.0 ml of n-butanol. LPA was assayed in lipid extract and calculated as described in detail under **Materials and Methods**. Data are expressed as pmole of LPA per gram of crude liver. Nonpaired t-test was employed for the statistical analysis.