Hepatocellular carcinoma (HCC) occurs in fibrotic liver as a consequence of underlying cirrhosis. The goal of this study was to investigate how the interaction between HCC cells and stromal fibroblasts affects tumor progression. We isolated and characterized carcinoma-associated fibroblasts (CAFs) and paired peritumoral tissue fibroblasts (PTFs) from 10 different patients with HCC and performed coculture experiments. We demonstrated a paracrine mechanism whereby HCC cells secrete lysophosphatidic acid (LPA), which promotes transdifferentiation of PTFs to a CAF-like myofibroblastic phenotype. This effect is mediated by up-regulation of specific genes related to a myo/contractile phenotype. After transdifferentiation, PTFs expressed α-smooth muscle actin (α-SMA) and enhanced proliferation, migration, and invasion of HCC cells occur. A pan-LPA inhibitor (α-bromomethylene phosphonate [BrP]-LPA), or autotaxin gene silencing, inhibited this PTF transdifferentiation and the consequent enhanced proliferation, migration, and invasion of HCC cells. In vivo, PTFs coinjected with HCC cells underwent transdifferentiation and promoted tumor progression. Treatment with BrP-LPA blocked transdifferentiation of PTFs, down-regulated myofibroblast-related genes, and slowed HCC growth and progression. Patients with larger and metastatic HCC and shorter survival displayed higher serum levels of LPA. Analysis of microdissected tissues indicated that stroma is the main target of the LPA paracrine loop in HCC. As a consequence, α-SMA–positive cells were more widespread in tumoral compared with paired peritumoral stroma. Conclusion: Our data indicate that LPA accelerates HCC progression by recruiting PTFs and promoting their transdifferentiation into myofibroblasts. Inhibition of LPA could prove effective in blocking transdifferentiation of myofibroblasts and tumor progression. (HEPATOL 2011;54:920-930)

In Western countries, hepatocellular carcinoma (HCC) develops in patients with liver cirrhosis and is the final stage of a disease that can last for many years. It is generally accepted that HCC originates from hepatocytes but grows and advances while fully embedded in a surrounding microenvironment.
with a rich content of myofibroblasts, fibroblasts, and other cell types due to the underlying cirrhosis. Liver myofibroblasts, derived from quiescent fibroblasts and hepatic stellate cells activated by the chronic injury, can be recognized by their expression of α-smooth muscle actin (α-SMA). Myofibroblasts have been detected at the advancing edge of several different malignancies as the predominant phenotype in the cancer-associated fibroblast (CAF) population.

Although the origin of CAFs is still controversial, their immunophenotypical characterization, which primarily includes α-SMA and excludes epithelial and endothelial common markers, is widely accepted. CAFs differ from peritumoral tissue fibroblasts (PTFs) not in terms of somatic mutations but rather molecular and functional differences in modulating neighboring cancer cells. However, the paracrine cross-talk between HCC and stromal fibroblasts such as CAFs or PTFs is still poorly understood.

HCC is recognized as a highly heterogeneous tumor because of its complex multistep pathogenesis, which is further complicated by the preexisting liver cirrhosis. For this reason, the identification of a proper biological target is essential in order to design suitable molecular-oriented therapy.

Lysophosphatidic acid (LPA) is a potent bioactive lipid, produced by hydrolysis of lysophosphatidylcholine by autotaxin (ATX). ATX, which is also known as ectonucleotide pyrophosphatase/phosphodiesterase family member 2, is an enzyme that was first identified as an autocrine motility factor because it is capable of promoting migration of melanoma cells. ATX is an important mediator of tumor progression and plays a key role in cancer progression either as a motile factor or by producing LPA. LPA is a bioactive lipid implicated in several functions, including proliferation, apoptosis, migration, and cancer cell invasion. It was shown recently that the ATX/LPA pathway that activates LPA receptor 1 (LPA1) promoted cell invasion in an in vitro experimental model of HCC.

In this study, we demonstrate that secretion of LPA by HCC cells promotes transdifferentiation of stromal peritumoral fibroblasts to myofibroblasts, and that this accelerates tumor progression. Consistently, LPA is shown to be increased in patients with more advanced disease and, finally, myofibroblasts are more expressed in HCC compared with paired peritumoral tissue.

Materials and Methods

Samples of HCC and paired adjacent liver tissue were obtained from 10 patients (Supporting Table 2) undergoing liver resection. Approval for the study was obtained from the local ethics committee, and patients gave prior written informed consent for the use of their tissues. Peritumoral and HCC tissues were minced with scalpels in a tissue culture dish and then enzymatically dissociated in Dulbecco’s modified Eagle’s medium/F12 medium supplemented with 0.1 % bovine serum albumin, 100,000 U/L penicillin G, 100 mg/L streptomycin, 1.0 g/mL fungizone, 500 units/mL collagenase D (Invitrogen), and 100 U/mL hyaluronidase (Calbiochem) at 37°C for 16 hours. The suspension was then centrifuged at 500 rpm (80g) for 5 minutes to separate the epithelial and fibroblast cells. Fibroblasts in the supernatant were pelleted by way of centrifugation at 800 rpm (100g) for 10 minutes, followed by two washes with Dulbecco’s modified Eagle’s medium/F12 medium. Fibroblast antigen-positive cells were isolated from the cell pellet through positive selection using anti-fibroblast MicroBeads and the MS Column (Miltenyi Biotec) according to the manufacturer’s instructions. Isolated cells were resuspended in Iscove’s modified Dulbecco’s medium supplemented with 20% fetal bovine serum (Invitrogen) and 5 µg/mL insulin and plated in 25 cm² tissue culture flasks. The cultures were then incubated at 37°C at 5% CO₂. Primary cultures of PTFs and CAFs isolated from human HCC tumors were immunophenotypically characterized by way of positive immunostaining for fibroblast markers and distinguished from tumor cells by negative staining for pan-cytokeratin, Hep-1, E-cadherin, and α-fetoprotein (Supporting Fig. 1). Purity of the isolated fibroblast population, assessed by way of immunostaining, was >99%. Cells from passages 3-10 were used for all experiments.

Full descriptions of additional Materials and Methods are given in the Supporting Information.

Results

First, we stained HCC and matching peritumoral tissues with an anti-α-SMA antibody to detect stromal myofibroblasts. We found that α-SMA-positive cells were mostly present in the fibrotic septa of the peritumoral cirrhotic tissue, whereas in tumor tissues α-SMA-positive cells were mainly expressed within the tumor stroma (Fig. 1A). We then isolated and further characterized CAFs and PTFs from 10 different patients using a panel of epithelial and mesenchymal antigens (Fig. 1B,C and Supporting Fig. 1A).

Consistent with the microscopic observation, the number of vimentin-positive cells was similar in PTFs and CAFs preparations, whereas the number of α-SMA-positive cells was much higher (P < 0.0001) in CAFs...
compared with PTFs (Fig. 1D and Supporting Fig. 1B). No staining was observed in either cell population for pan-cytokeratin, nor for other epithelial or vascular markers (Fig. 1C), thus indicating the absence of contamination by other cell types. These results were reproducible in all the different preparations (six out of 10 are shown).
The different expression of α-SMA between CAFs and PTFs also reveals different functions. CAFs display a greater ability to contract collagen gel \((P < 0.0001)\) and to proliferate more efficiently over time up to 14 days \((P < 0.001)\) compared with PTFs (Fig. 1 E,F and Supporting Fig. 1C,D). These results were consistently reproduced in all the different cell preparations.

In coculture experiments, both CAFs and PTFs stimulated Huh7 proliferation with the same efficiency in a three-dimensional collagen gel after 4 \((P < 0.05)\) and 7 \((P < 0.05)\) days (Supporting Fig. 2). However, in the same experiments, the addition of α-bromomethylene phosphonate [BrP]-LPA, a pan-LPA inhibitor, blocked the proliferation rate of Huh7 cells \((P < 0.05)\) stimulated by the presence of PTFs or CAFs (Fig. 2A). Notably, under the same experimental conditions, there was only a trend toward an increased proliferation of PLC/PRF/5 cells upon CAFs treatment, whereas BrP-LPA abolished the CAFs-dependent PLC/PRF/5 proliferation (Fig. 2B). In cell motility...
experiments, Huh7 cells migrated more efficiently in the presence of PTFs compared with control \((P < 0.05)\), and even more efficiently in the presence of CAFs. However, the presence of BrP-LPA significantly inhibited tumor migration \((P < 0.05)\) (Fig. 2C). PLC/PRF/5 cells showed a similar trend, although the efficiency of migration was much lower than that of Huh7 cells (Fig. 2D). In addition, invasion of Huh7 cells through Matrigel was increased in the presence of PTFs and was blocked when BrP-LPA was added. The invasive capability of Huh7 cells was enhanced in the presence of both PTFs and CAFs and was inhibited in the presence of BrP-LPA (Fig. 2E). Conversely, PLC/PRF/5 showed a poor invasive capacity through Matrigel in the presence of either PTFs or CAFs. Therefore, BrP-LPA did not display any effect on these cells (Fig. 2F). No toxicity effect was observed at used concentration on Huh7 cells, PLC/PRF/5 cells, PTFs, or CAFs as evaluated by MTT assay (Supporting Fig. 3). In conclusion, PTFs and CAFs increased the aggressive phenotype in Huh7 cells but not in PLC/PRF/5 cells.

To gain a better insight into the molecular mechanisms underlying the paracrine cross-talk between stromal and HCC cells, we studied the paracrine action of LPA. We first measured the concentrations of secreted LPA in conditioned medium from PTFs and CAFs and in two different HCC cell lines (Huh7 and PLC/PRF/5). High levels of LPA were detected in Huh7 cells compared with PLC/PRF/5 cells, CAFs, and PTFs \((P < 0.0001)\) (Fig. 3A). We then analyzed the messenger RNA (mRNA) expression levels of LPA receptors 1-5 in the same cells. We found that among the LPA receptors investigated, LPA receptor 1 was the most strongly expressed, being mainly expressed by CAFs and PTFs compared with Huh7 cells (Fig. 3B). In agreement with the LPA levels, ATX expression levels were more abundant in Huh7 compared with PLC/PRF/5 cells, CAFs, and PTFs \((P < 0.0001)\) (Supporting Fig. 4A). In conclusion, Huh7 cells produced LPA and ATX, whereas PLC/PRF/5 cells, PTFs, and CAFs did not, and CAFs and PTFs only expressed LPA receptors.

To investigate the functional role of PTFs or CAFs in the cross-talk between stromal and HCC cells, we challenged PTFs to migrate in the presence of Huh7- and PLC/PRF/5-conditioned medium (CM). In the presence of Huh7-CM, PTFs migrated efficiently to the same extent as in the presence of LPA. This effect was already evident after 12 hours but was stronger after 72 hours. BrP-LPA blocked this migration (Fig. 3C). On the contrary, no PTF migration was observed in the presence of PLC/PRF/5-CM. Therefore, BrP-LPA did not display any effect on this coculture, whereas the addition of LPA still promoted strong migration (Fig. 3D).

Moreover, the number of \(\alpha\)-SMA–positive cells was increased in PTFs migrating in the presence of Huh7-CM and LPA, but was strongly reduced by BrP-LPA \((P < 0.05)\). This effect was particularly evident after 72 hours (Fig. 3E). On the contrary, the number of \(\alpha\)-SMA–positive cells was not increased in PTFs migrating in the presence of PLC/PRF/5-CM, but was strongly increased when exogenous LPA was added (Fig. 3F). Similar results were obtained with Hep3B and HLE, LPA-producing and nonproducing, respectively, as shown in Supporting Fig. 5. In conclusion, Huh7 cells recruited and activated PTFs through the secretion of LPA, but PLC/PRF/5-CM did not.

To confirm the role of LPA as a paracrine mediator of stromal–tumor interaction, we knocked down the ATX gene, a major LPA-producing enzyme, in Huh7 cells and performed coculture experiments. ATX-silenced cells secreted low levels of LPA compared with control \((P < 0.0001)\), as evaluated by enzyme-linked immunosorbent assay (ELISA) measurement of LPA in Huh7-CM (Supporting Fig. 4B). More importantly, low levels of LPA in ATX-silenced Huh7 determined a significant reduction of tumor proliferation and migration in cocultures with CAFs and PTFs compared with control \((P < 0.05)\). The addition of exogenous LPA to ATX-silenced cells partially restored their capability to proliferate and migrate (Supporting Fig. 4C,D).

To further study the effect of LPA in this context, we stimulated PTFs and CAFs with LPA. As shown above, the number of \(\alpha\)-SMA–positive cells was higher in the CAF population than in the PTF population. However, treatment with LPA strongly increased the number of \(\alpha\)-SMA–positive cells in the PTF population but not in the CAF population \((P < 0.0005)\). Moreover, treatment with BrP-LPA blocked this effect (Fig. 4A,B). Consistently, LPA increased the ability of PTFs to contract collagen gel compared with control \((P < 0.001)\). This effect was mild on CAFs \((P < 0.05)\), a phenotype with an intrinsic ability to contract collagen gel (Fig. 4C). Furthermore, LPA significantly stimulated proliferation of PTFs over time, but not of CAFs \((P < 0.0001)\) (Fig. 4D). To explain the phenotypic changes in PTFs induced by LPA, we investigated the behavior of several genes under LPA stimulation. Among the investigated genes, we identified a gene signature responsible for the transdifferentiation of PTFs to a CAF-like myofibroblastic phenotype (Fig. 4E,F).

To test the reliability in vivo of the mechanism described in vitro, we assayed the tumorigenicity of
Huh7 cells in a xenograft model of HCC. Huh7, injected alone, formed tumors within 3 weeks after injection, with a further increase of the tumor mass in the next 3 weeks. However, when co-injected with CAFs, Huh7 cells formed larger tumors faster ($P < 0.01$), after only 2 weeks. Furthermore, Huh7 cells co-injected with PTFs provoked a greater development of tumors ($P < 0.05$), whereas treatment with BrP-LPA dramatically reduced tumor growth after the first three drug administrations ($P < 0.01$) (Fig. 5A). In tumors originated by co-injection of Huh7 cells with PTFs, we detected a large number of $\alpha$-SMA–positive cells (control group). Conversely, in tumors originated from the same cells but treated with BrP-LPA, the number of $\alpha$-SMA–positive cells was significantly decreased (treated group) (Fig. 5B). Next, we evaluated...
whether the gene signature identified in cultured PTFs stimulated with LPA was affected in mice following treatment with BrP-LPA. We found that genes that were up-regulated \textit{in vitro} were inhibited in BrP-LPA-treated tumors (Fig. 5C). To ensure the specificity of our model, we investigated the expression of human...
and mouse α-SMA in xenografted HCC originated by coinjection of Huh7 with PTFs and by the same coinjection then treated with BrP-LPA. h-α-SMA was more strongly expressed than mouse α-SMA, as measured by real-time polymerase chain reaction (PCR), and in drug-treated animals the human isoform of α-SMA but not the murine isoform was down-regulated, suggesting that injected PTFs were still present and functionally active at the end of the experiment, and also that the presence of host/resident myofibroblasts did not significantly affect results (Fig. 6D). In conclusion, we demonstrated that LPA secreted by
HCC cells recruits and activates PTFs, orchestrating their differentiation to a CAF-like myofibroblastic phenotype which, in turn, accelerates HCC progression. Finally, we aimed to validate these findings in HCC patients. We therefore analyzed LPA serum levels in 60 patients with HCC (30 patients with and 30 without metastases), and in 50 patients with liver cirrhosis. We found that LPA serum levels were higher in HCC compared with liver cirrhosis patients ($P < 0.05$). Among HCC patients, LPA serum levels were significantly ($P < 0.05$) higher in those with metastasis compared with those without ($P = 0.0492$) or early disease ($P = 0.0208$). Expression levels of ATX, a major LPA-producing enzyme, are increased during HCC progression. Data from the Wurmbach cohort (GSE6764) showing that ATX expression levels are increased in HCC tissues of patients with macrovascular invasion ($n = 10$) compared with microvascular invasion ($n = 7$) and to HCC patients with very early ($n = 7$; $P = 0.0492$) or early disease ($n = 10$; $P = 0.0208$) and was more strongly expressed in tumoral compared with paired nontumoral tissues ($n = 126$; $***P < 0.0001$). Error bars show the SEM.

Fig. 6. Clinical evidence of the role of LPA in patients with HCC. (A) LPA levels in human serum samples from healthy donors (HD), patients with liver cirrhosis (LC), and patients with HCC with (HCC M+) and without metastasis (HCC M-) measured by way of LPA ELISA colorimetric assay. Data are shown as box and whisker plots, and $P$ values were calculated by way of two-tailed Student $t$ test.*$P < 0.05$. **$P < 0.001$. (B) Box and whisker plot showing the correlation between LPA serum levels and tumor size. Serum LPA levels are increased in HCC patients with larger tumors compared with those with smaller tumors. $P$ values were calculated by way of two-tailed Student $t$ test. **$P < 0.01$. (C) LPA expression levels are correlated with survival in HCC patients. In log-rank analyses, low serum LPA levels ($\leq 2 \mu M$) predict a longer survival of HCC patients than high serum levels ($>2 \mu M$). **$P = 0.0065$ (log-rank test). (D) Expression levels of ATX, a major LPA-producing enzyme, are increased during HCC progression. Data from the Wurmbach cohort (GSE6764) showing that ATX expression levels are increased in HCC tissues of patients with macrovascular invasion ($n = 10$) compared with microvascular invasion ($n = 7$; **$P = 0.0096$) and to HCC patients with very early ($n = 7$; $P = 0.0492$) or early disease ($n = 10$; $P = 0.0208$). (E,F) Data from the Wang cohort (GSE14520) showing increased expression levels of ATX in HCC tissues ($n = 129$) compared with peritumoral tissues ($n = 126$; **$P < 0.0001$) and the Chen cohort (GSE3500; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE3500) showing increased expression levels of ATX in HCC tissues ($n = 79$) compared with peritumoral tissues ($n = 69$; **$P < 0.0001$). Error bars show the SEM.
gene was significantly expressed in tumoral compared with paired nontumoral tissues (Fig. 7D). This is consistent with publicly accessible microarray data published by Wang (Fig. 7E). Taken together, these data show that the stromal component represents the main target of LPA in patients with HCC, and that α-SMA-positive cells are predominant within the tumor stroma, as shown by the increased expression of the ACTA2 gene.

**Discussion**

Although the onset and growth of HCC occurs in tissues with a rich content of local myofibroblasts, the cross-talk between tumor and stroma has been little investigated, despite that fact that in mammary gland development, myofibroblasts play a crucial role in allowing the penetration of end buds into the fat pad during branching morphogenesis or local tumor spread. In this study, we demonstrate that the interaction between HCC and stroma plays a key role in tumor progression, and that in patients this interaction occurs in more advanced disease. We based our conclusions on the following data: (1) CAFs stimulated proliferation, migration and invasion of HCC cells; (2) HCC cells secreted LPA, which promoted transdifferentiation of PTFs to a CAF-like myofibroblastic phenotype through the up-regulation of genes related to a contractile phenotype; (3) this recruitment and transdifferentiation was blocked by inhibiting LPA secretion; (4) PTFs co-injected with HCC cells accelerated tumor growth and progression, but an LPA inhibitor blocked PTF transdifferentiation and slowed HCC growth and progression; and (5) patients with higher LPA concentrations had larger, metastatic HCC and worse survival.

Myofibroblasts have recently been implicated in HCC progression, but the molecular mechanisms...
regulating the interaction between HCC and cells and myofibroblasts are still unknown. We demonstrate for the first time that LPA is involved in the reciprocal cross-talk between HCC cells and resident fibroblasts, leading to tumor progression. In particular, HCC cells activate resident fibroblasts (PTFs), which acquire a contractile capability and express α-SMA, sustained by the up-regulation of specific contractile-related genes giving rise to a myofibroblast-like phenotype. This occurs through a paracrine mechanism, because HCC cells secrete LPA and PTFs express LPA receptors that are absent in HCC cells. LPA is implicated in different malignancies, and has recently been shown to induce HCC cell invasion by increasing the production of matrix metalloproteinase-9.12 Once PTFs have assumed a myofibroblast-like phenotype, in coculture experiments they increase the proliferation, migration, and invasion of HCC cells in vitro and promote tumoral progression in vivo. We did not investigate mediators of the back cross-talk from myofibroblasts toward HCC cells, but the central role of LPA is further demonstrated by the fact that by blocking LPA with a pharmacological inhibitor or with ATX-silencing, the increased proliferation, migration, and invasion of HCC cells is abrogated. In vivo, this is even more striking, because after treating animals with an LPA inhibitor, we found a down-regulation of the genes supporting the myofibroblast phenotype and a lower number of activated PTFs, whereas HCC progression decreased. LPA is seen to have a central role in orchestrating the tumor–stroma interaction. This finding is consistent with a previous work showing an alteration of the phospholipid in HCC, where ATX displays a crucial role in the inflammatory peritumoral reaction by interacting with the tumor necrosis factor α/nuclear factor κB pathway.16 Furthermore, the inhibition of this pathway reduces the pro-tumorigenic activity of CAFs in skin cancer.17

In HCC patients, we found increased serum LPA in those with a worse clinical outcome, as also suggested by an analysis of publicly accessible microarray data13 and by Wang (Personal Communication; http://www.ncbi.nlm.nih.gov/gds?term=gse14520). Our findings are further confirmed by previous work reporting increased levels of LPA in tissues, bile, and serum and in more advanced stages of disease.13,18 Finally, we also demonstrate that LPA receptors are mainly expressed in stroma rather than epithelium of HCC; consistent with our experimental data, the ACTA2 gene was also more strongly expressed in tumoral tissues than in paired peritumoral tissues. This is supported by analysis of the publicly accessible microarray data from Wang (Personal Communication, http://www.ncbi.nlm.nih.gov/gds?term=gse14520). These data recapitulate those proposed in an in vivo model of breast cancer.19

In conclusion, our results indicate that LPA plays a central role in orchestrating the cross-talk between HCC cells and resident stromal fibroblasts, and that this promotes HCC progression.

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References