Production of Lysophosphatidic Acid in Blister Fluid: Involvement of a Lysophospholipase D Activity

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Lysophosphatidic acid (LPA) is present in abundance in serum resulting from platelet activation and is also found in other biological fluids. LPA controls numerous cellular responses and plays a role in specific functions such as wound healing, especially in the skin. Nevertheless, its presence in the skin has never been investigated. Since re-epithelialization occurs after blister rupture, we tested the presence of endogenous LPA in blister fluid and investigated a possible mechanism for its biosynthesis and biological functions. Using a radioenzymatic assay, LPA was detected in 33 blister fluids originating from 24 bullous dermatoses, and at higher concentrations than in plasma. In parallel, blister fluids contained a lysophospholipase D (LPLD) activity but no detectable phospholipase A_2 activity. The expressions of the LPLD autotaxin (ATX) and of LPA1–receptor (LPA1-R) were greatly increased in blister skin when compared with normal skin. Finally, LPA was found to have a positive effect on the migration of cultured keratinocytes. These results show that LPA is present in blister fluid synthesized by the LPLD ATX. Due to its ability to enhance keratinocyte migration, LPA in blister fluid could, via the LPA1-R, play an important role in re-epithelialization occurring after blister rupture.

Key words: blister fluid/lysophosphatidic acid/lysophospholipase D/skin/wound healing
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Lysophosphatidic acid (LPA) is the simplest phospholipid found in nature. It is present in abundance in serum resulting from platelet activation. LPA is also found in other biological fluids such as plasma (Baker *et al*, 2002) and ascite fluids (Xu *et al*, 1998) of patients suffering from ovarian cancer, aqueous humor, and lacrimal gland fluid (Liliom *et al*, 1998), follicular fluid (Tokumura *et al*, 1999), saliva (Sugiura *et al*, 2002), extracellular fluid of adipose tissue (Valet *et al*, 1998), and arthritis inflammatory fluids (Fourcade *et al*, 1995). It has been demonstrated that LPA may be generated by various cells including cancer cells (Merchant *et al*, 1991; Xu *et al*, 1998; Baker *et al*, 2002), fibroblasts (Fukami and Takenawa, 1992) and adipocytes (Valet *et al*, 1998). Nevertheless, the precise cellular origin of LPA in biological fluids still remains unclear (Gaits *et al*, 1997; Pages *et al*, 2001). Furthermore, the pathways involved in LPA production are still a matter of debate. There are two main pathways: the phospholipase A_2 (PLA_2)-dependent deacylation of phosphatidic acid (PA) and the lysophospholipase D (LPLD)-dependent hydrolysis of lysophosphatidylcholine (LPC) (Gaits *et al*, 1997; Pages *et al*, 2001; Aoki *et al*, 2002). It has been previously proved that LPLD activity was involved in LPA production in fluids such as rat plasma (Tokumura *et al*, 1998), human follicular fluid (Tokumura *et al*, 1999), and in the extracellular medium of adipocytes (Gesta *et al*, 2002). Umezu-Goto *et al* (2002), Tokumura *et al* (2002), and Ferry *et al* (2003) purified LPLD activity from bovine serum, human plasma, and adipocytes, respectively, and demonstrated that it was caused by a soluble form of autotaxin (ATX). ATX is a tumor cell mobility factor, originally isolated from melanoma cell supernatants, and belonging to the ecto-nucleotide pyrophosphatase/phosphodiesterase family (Moolenaar, 2002). LPA acts via interaction with specific G-protein-coupled receptors belonging to the endothelium differentiation gene family: LPA1-receptor (LPA1-R: Edg-2), LPA2-receptor (LPA2-R: Edg-4), and LPA3-receptor (LPA3-R: Edg-7) (Chun *et al*, 2002). Pharmacological specificity and tissue distribution may differ from one subtype to another (Contos *et al*, 2000). LPA controls numerous cellular responses such as proliferation, differentiation, migration, and apoptosis, and plays a role in specific functions such as wound healing (Liliom *et al*, 1998; Sturm *et al*, 1998; Hines *et al*, 2000). LPA is involved in the pathophysiology of arteriosclerosis (Siess, 2002), and different conditions, such as cancers, are associated with LPA production (Merchant *et al*, 1991; Xu *et al*, 1998; Baker *et al*, 2002). Its precise mechanism of action, however, is currently unknown. In the skin, it has been demonstrated that LPA plays a role in tissue repair and regeneration processes. Using cultured human keratinocytes, Piazza *et al* (1995) demonstrated that LPA induced proliferation and differentiation. The fact that LPA is released from activated

Abbreviations: ATX, autotaxin; BSA, bovine serum albumin; LPA, lysophosphatidic acid; LPA1-R, LPA1-receptor; LPA2-R, LPA2-receptor; LPA3-R, LPA3 receptor; LPC, lysophosphatidylcholine; LPLD, lysophospholipase D; PA, phosphatidic acid; PLA_2, phospholipase A_2

platelets (Eichholtz *et al*, 1993), as well as the presence of active phospholipases in the skin (Maury *et al*, 2000; Mazereeuw-Hautier *et al*, 2000), strongly supports this hypothesis. This is also supported by the fact that topical application of LPA to a wound model of mouse or rat skin promotes wound healing (Demoyer *et al*, 2000; Balazs *et al*, 2001). Nevertheless, the presence of LPA has never been investigated in the skin, during wound healing, or in other situations. Since re-epithelialization of the underlying wound bed occurs after blister rupture, we tested the presence of endogenous LPA in blister fluid and investigated a possible mechanism for its biosynthesis and biological functions in various dermatoses.

Skin blisters (Diaz and Giudice, 2000) are formed as a result of a breakdown of tissue integrity, with detachment of cellular junctions and fluid accumulation. There are several etiologies of blister formation (hypersensitivity, physical injury, autoimmunity, hereditary, virus infection), and the blisters can occur at different levels within the epidermis. The blisters can be intraepidermal (eczema, burn, varicella-zoster virus infection), or subepidermal (bullous pemphigoid, toxic epidermal necrolysis, dystrophic epidermolysis bullosa). Some biochemical features of blister fluid have already been documented in succion blister. This fluid has been qualified as a "filtrate" of serum since the concentration of each compound was smaller than in the serum and was dependent on its molecular weight (Volden *et al*, 1980). Blister fluid also contains local products of cell injury and inflammation (Grando *et al*, 1989a; Ono *et al*, 1995; D'Auria *et al*, 1999). The presence of LPA in blister fluid has never been demonstrated.

Results

LPA is present in blister fluids In order to determine the presence of LPA in blister fluid, LPA was quantified using a radioenzymatic assay. Micromolar (mean $\pm$ SEM, 0.60 ± 0.0087 μM, range 0–1.90) concentrations of LPA were detected in blister fluids originating from all patients except for one, who was suffering from toxic epidermal necrolysis. The results for LPA concentrations are shown in Table I. No statistical correlation could be established between LPA concentration and the following characteristics, respectively: age (Spearman, $p = 0.33$), sex (Kruskal–Wallis, $p = 0.95$), aetiology of bullous diseases (Kruskal–Wallis, $p = 0.054$), blister duration (Spearman, $p = 0.41$), indirect immunofluorescence (Kruskal–Wallis, $p = 0.7$), eosinophilia (Kruskal–Wallis, $p = 0.69$) or steroid treatment for bullous pemphigoid (Kruskal–Wallis, $p = 0.07$).

We wanted to determine whether LPA present in blister fluid originated from plasma or was produced locally within the blister. We therefore compared LPA concentration in blister fluid to LPA concentration in plasma originating from the same individuals ($n = 2$). We found that plasma concentrations were 9-fold lower than blister fluid concentrations (plasma: 0.07 μM *vs* blister 0.4 μM and 0.064 μM *vs* 1.2 μM for the two patients, respectively) (data not shown). This result clearly shows that LPA is produced in blister fluid and does not originate from plasma. In blister fluid, LPA was present in the supernatants and absent in the pellet after ultra-centrifugation and cell removal (data not shown), indicating LPA solubility.

Since LPA is present in blister fluid, we decided to investigate the presence of two possible precursors: PA and LPC. The amount of PA and LPC present in blister fluids ($n = 5$ and 10, respectively) was found to be 1.1 ± 0.2 and 2.8 ± 0.2 μM, respectively (mean $\pm$ SEM). These results indicate that blister fluid not only contained LPA but also its precursors: PA and LPC.

Metabolic origin of LPA in blister fluid We then decided to elucidate the metabolic origin of LPA produced in blister fluid. It has been demonstrated in other tissues that LPA could be produced by a soluble extracellular LPA synthesis activity (Tokumura *et al*, 1999; Gesta *et al*, 2002). In order to test the presence of such an activity in blister fluids, incubation of the fluids was performed at 37°C for 6 h. After incubation, the concentration of LPA had doubled (mean SEM: 1.70 ± 0.72 *vs* 3.40 ± 1.05 μM) ($n = 5$) (Wilcoxon, $p < 0.05$) (Fig 1), showing the presence of a soluble extracellular LPA synthesis activity. We then studied the two major pathways possibly involved in soluble LPA synthesis: the PLA_2-dependent deacylation of PA, and the LPLD-

Table I. Lysophosphatidic acid (LPA) concentrations in blister fluids

Bullous dermatoses	No. of patients	No. of samples	LPA (μM) Mean ($\pm$ SEM)	Range
Eczema	3	4	0.8 ± 0.3	0.2–1.6
Burn	5	5	0.9 ± 0.27	0.4–1.9
Bullous pemphigoid	10	10	0.4 ± 0.09	0.2–1.2
No treatment	3	3	0.68 ± 0.26	0.41–1.2
Under steroid treatment	7	7	0.32 ± 0.04	0.16–0.5
Varicella-zoster virus infection	2	2	1.1 ± 0.42	0.7–1.5
Toxic epidermal necrolysis	3	11	0.5 ± 0.15	0–1.6
Dystrophic epidermolysis bullosa	1	1	0.2	

Quantification of LPA was performed using a radioenzymatic assay as described in Materials and Methods.

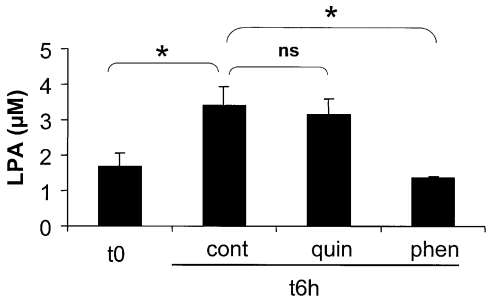

Figure 1
Soluble extracellular lysophosphatidic acid (LPA) synthesis activity in blister fluid and influence of quinacrine and phenanthrolin. LPA was quantified in blister fluids from five patients, before (t0) and after 6 h (t6 h) incubation at 37°C without (cont) or with quinacrine (quin) at 100 μM or phenanthrolin (phen) at 100 μM. Values are means ± SEM from four independent repetitive assays realized for each patient. Statistical significance was considered when $p<0.05$ (*), ns, not statistically significant.

dependent hydrolysis of LPC (Gaits *et al*, 1997; Pages *et al* 2001; Aoki *et al*, 2002). From the same five patients, we showed that the intrinsic LPA synthesis activity was not modified after 6 h of incubation at 37°C with quinacrine (Fig 1). In conjunction, no PLA_2 activity could be detected in blister fluids ($n=32$), except for one patient suffering from toxic epidermal necrolysis (data not shown). In contrast, after 6 h of incubation at 37°C with phenanthrolin, LPA synthesis activity was completely abolished ($n=5$) (Fig 1). In parallel, LPLD activity was 0.018 ± 0.005 pmol per min per mg protein, range 0–0.9, ($n=10$) (data not shown).

Since LPLD activity can be catalyzed by ATX (Tokumura *et al*, 2002; Umezu-Goto *et al*, 2002; Ferry *et al*, 2003), the expression of ATX transcripts was investigated in blister skin using real-time RT-PCR, and was compared with normal skin originating from the same individuals ($n=4$). As shown in Fig 2, ATX mRNA was found in blister skin and was approximately four times more abundant than in normal skin (Wilcoxon, $p<0.05$). This result strongly suggested that ATX might be responsible for LPLD activity in blister fluid, and that skin expression of ATX was induced during the formation of the blister.

Skin expression of LPA-receptors The biological responses of LPA being mediated by specific receptors (Contos *et al*, 2000; Chun *et al*, 2002), the expression of these receptors was also investigated in blister skin and compared to normal skin originating from the same individuals ($n=4$). As shown in Fig 2, mRNA of the three LPA receptor subtypes (LPA1-R, LPA2-R, LPA3-R) was detected in blister skin. When compared with normal skin, LPA1-R mRNA from blister skin was 6.42 times more abundant (Wilcoxon, $p<0.05$), whereas LPA2-R mRNA and LPA3-R mRNA were 4.30 and 2.50 times (not statistically significant (Wilcoxon, $p=0.14$ and 0.15)) less abundant, respectively. In parallel, no significant alteration in the level of hypoxanthine phosphoryl transferase mRNA (used as a housekeeping gene) was observed. These results showed that blister formation is accompanied by the regulation of LPA receptors expression, and particularly by a consistent upregulation of the LPA1-R subtype.

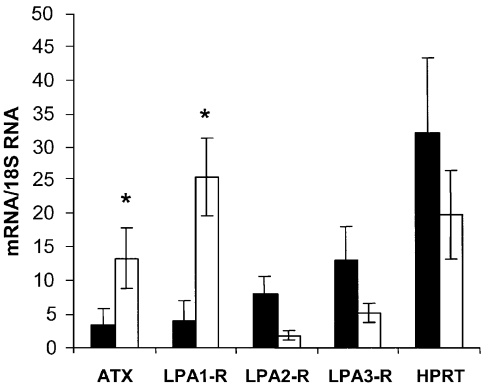

Figure 2
Expression of lysophosphatidic acid (LPA) receptor subtypes and autotaxin (ATX) in skin biopsies using real-time PCR. Normal skin (filled bars) and blister skin (unfilled bars) were both obtained from the same individuals suffering from bullous dermatoses (four patients). Total RNA was isolated and mRNA encoding LPA1-receptor (LPA1-R), LPA2-receptor (LPA2-R) and LPA3-receptor (LPA3-R), autotaxin (ATX), and hypoxanthine phosphoribosyl transferase (HPRT) was quantified using real-time PCR as described in Materials and Methods. Values are means ± SEM from four independent repetitive assays realized for each patient. mRNA in normal skin was compared to RNA in blister skin (*$p<0.05$).

Effects of LPA on HaCaT cells' migration In the literature, LPA has been involved in wound healing by inducing keratinocyte proliferation and differentiation. Since migration takes part in wound healing and LPA induces epithelial cells migration (Liliom *et al*, 1998; Sturm *et al*, 1998; Hines *et al*, 2000), we decided to investigate the effect of LPA on keratinocyte migration.

LPA was found to have a positive concentration-dependent effect on keratinocyte migration. The effect increased according to LPA concentrations and was statistically different from control at concentrations of 1 μM or above, showing a plateau at 2 μM (Fig 3) (Spearman,

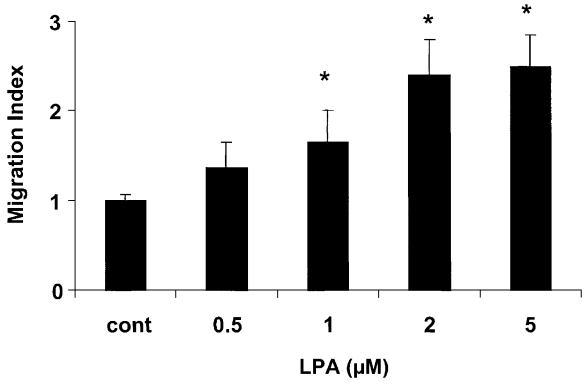

Figure 3
Effect of lysophosphatidic acid (LPA) on keratinocyte migration. LPA was solubilized in phosphate-buffered saline using 1% bovine serum albumin prior to addition of RPMI-1% bovine serum albumin. HaCaT cells were treated or not (control (cont): RPMI containing 1% bovine serum albumin) to increased concentrations of LPA. Cell migration was assessed using modified Boyden chambers as described in Materials and Methods. The migration was expressed as a migration index, and represented the percentage of migrated cells with LPA compared to the percentage of migrated cells with the control. Values are means ± SEM from three independent repetitive experiments realized with three independent pools of cells. The migration index obtained after treatment by various concentrations of LPA was compared with migration index of the control (*$p<0.05$).

$p < 0.05$). This result suggests that LPA in blister fluid enhances keratinocyte migration.

Discussion

This work shows the presence of LPA in blister fluid. LPA is a bioactive phospholipid mediating its activity via stimulation of specific G-protein-coupled receptors.

Several authors have concluded that LPA plays a role in skin wound healing (Piazza *et al*, 1995; Demoyer *et al*, 2000; Balazs *et al*, 2001) by enhancing the proliferation and differentiation of keratinocytes. Sauer *et al* (2004) showed that LPA mediates keratinocyte growth arrest and chemotaxis. The effects of LPA on wound healing have also been studied in intestinal epithelial cells (Liliom *et al*, 1998; Sturm *et al*, 1998; Hines *et al*, 2000), where LPA induced migration and proliferation. After blister formation, re-epithelialization of the underlying wound bed occurs after blister rupture. Re-epithelialization involves a sequential program of events in order to close a wound as quickly as possible (Yamaguchi and Yoshikawa, 2001). An increase in the rate of migration of keratinocytes over the wounded area, as well as an increase in the rate of proliferation of basal cells, occurs. Simultaneously, cells migrate into the upper epidermal layers, ultimately undergoing terminal differentiation to form the stratum corneum and repairing the barrier function of the skin. The concentrations of LPA found in our study (mean $\pm$ SEM, 0.60 $\pm$ 0.0087 μM, range 0–1.90) were high enough to induce potential biological effects since the usual EC_{50} are between 5 and 150 nM (Pages *et al*, 2001). Therefore, LPA may take part in wound healing via effects on cell proliferation, differentiation, and migration. This hypothesis is supported by our data showing that LPA increases keratinocytes migration in culture, and that the skin expresses LPA receptor subtypes (LPA1-R, LPA2-R, LPA3-R). Since Amano *et al* (2004) demonstrated that LPA stimulates laminin 5 expression, the effect of LPA on migration could be mediated by this protein, known to be involved in the adhesion and migration of keratinocytes. Whereas the specific contribution of each LPA-receptor subtype in the biological activity of LPA in keratinocytes remains to be determined, its is interesting to notice that the expression of the LPA1-R subtype was substantially increased in skin from blisters when compared to normal skin, whereas the expression of LPA2-R and LPA3-R was not altered or was even decreased. Even if the underlying mechanisms remain unclear, upregulation of the LPA1-R suggests that this receptor plays a predominant role in the biological activity of LPA in skin during blister formation. Therefore, LPA1-R might represent a pharmacological target for skin wound-healing therapy.

In our study, LPA was present in blister fluids originating from all bullous dermatoses but no correlation could be established between the level of LPA and the characteristics of the blister. This result may be explained by the small number of cases and their heterogeneity, or could mean that LPA is a consequence of the blister rather than being directly involved in the pathogenesis of the bullous disease.

LPA concentration in blister fluid (0.6 μM) was close to that found in other biological fluids such as saliva (Sugiura *et al*, 2002), lacrimal gland, and aqueous humor (Liliom *et al*, 1998) (0.785, 1, and 0.2 μM, respectively), and higher than in plasma (0.08 to 0.2 μM) (Tokumura *et al*, 1998). This last observation suggests that, in contrast to what was described for other blister fluid constituents, such as fatty acids, organic acids, and aminoacids, LPA does not result from a filtration of plasma but rather from a local synthesis. This hypothesis is supported by the demonstration of the presence of a LPA synthesis activity, as well as of LPA precursors (PA and LPC) in blister fluid.

Two major pathways might be involved in LPA synthesis: the PLA_2-dependent deacylation of PA and the LPLD-dependent hydrolysis of LPC. Since LPA synthesis activity in blister fluid is not blocked by quinacrine, we excluded the possibility of the involvement of PLA_2 in LPA synthesis in blister fluid. This hypothesis was also supported by the absence of detectable PLA_2 activity in blister fluid. In contrast, our data strongly suggest that LPLD activities are responsible for endogenous LPA formation since it is blocked by phenanthrolin, an ion chelator previously demonstrated to inhibit LPLD activity in plasma and adipose tissue (Tokumura *et al*, 1998; Gesta *et al*, 2002). Furthermore, an easily measurable LPLD activity was found in blister fluid. Based on the literature, the only enzyme demonstrated to be able to catalyze a soluble LPLD activity is ATX, suggesting that this enzyme could be responsible for LPA synthesis in blister fluid.

This is in accordance with the demonstration of a relatively high level of expression of ATX in the skin from blister, compared with human adipose tissue where LPA is particularly abundant (mRNA/18SRNA: 13.35 *vs* 40) (Boucher *et al*, 2005).

Interestingly, ATX expression was more abundant in blister skin than in normal skin. Blister formation can be considered as tissue injury where cell composition and functions are altered, leading to the release of several components including mediators of inflammation (Grando *et al*, 1989a; Ono *et al*, 1995; D'Auria *et al*, 1999), enzymes such as tryptases, myeloperoxydases, or proteases, and their inhibitors (Grando *et al*, 1989b; D'Auria *et al*, 2000). This type of skin injury could be responsible for the upregulation of ATX expression and therefore could lead to the synthesis of LPA. Such a relation between tissue injury and LPA production has been previously reported. LPA was indeed found to be released by epithelial cells in aqueous humor after corneal injury (Liliom *et al*, 1998) or during intracranial hematoma in a pig model (Tigyi *et al*, 1995).

In conclusion, LPA is produced in blister fluid by an LPLD activity. Our data suggest that ATX is the source of LPLD activity. LPA in blister fluid might act via LPA-receptors, especially LPA1-R, and have a role in skin wound healing, in particular, via an effect on keratinocyte migration.

Since ATX is involved in LPA production, this enzyme could represent an interesting target to enhance skin wound healing.

Materials and Methods

Collection of blister fluid and blood Thirty-three samples from 24 patients (13 females and 11 males; mean age was 53.96 $\pm$ 30.44 y, range 2–90 y) suffering from different bullous dermatosis were investigated between December 2001 and November 2002.

There were six different etiologies of bullous dermatoses: bullous pemphigoid (n = 10), second-degree burn (n = 5), eczema (n = 3), toxic epidermal necrolysis (n = 3), varicella–zoster virus infection (n = 2), and dystrophic epidermolysis bullosa (n = 1). The medical ethical committee of the University of Toulouse approved all the described studies. The subjects of our study gave their informed written consent, and investigations were carried out in accordance with the Declaration of Helsinki as revised in 2000 (http://www.wma.net/e/policy/b3.htm). Some fluids were collected at several clinical stages from some patients suffering from toxic epidermal necrolysis (n = 2) or eczema (n = 1). The final diagnosis was based on anamnesis, clinical manifestations, routine histology, detection of autoantibodies using direct and indirect imunofluorescence, and viral culture and serologies. Collection of fluid was realized before the beginning of any treatment, except for seven of ten patients with bullous pemphigoid who were already under steroid therapy (topical application of Diprolene (clobetasol) 30 g per D, n = 2 or oral route of Solupred (prednisolone) (0.50–1.50 mg per kg per D, n = 5). The blisters originating from patients under steroid therapy were only used for LPA quantification and $sPLA_2$ activity experiments, and not for the determination of the metabolic origin of LPA. The identification of the different types of blisters used for the various experiments is shown in Table II. The blisters were punctured with a needle, and the fluid was collected in a syringe. In order to determine whether LPA was soluble or associated with cell particles, fluids were subjected to ultra-centrifugation (100,000 $\times$ *g* for 5 min at 37°C). Fluids were immediately stored at −20°C until further analysis.

In cases of requirement of blood sample for medical reasons, human plasma was collected in ethylenediaminetetraacetic acid (EDTA)-conditioned tubes.

Collection of skin biopsies Eight biopsies were taken between January and June 2003 from four additional patients (three females and one male, mean age 81 y, range 77–91 y) suffering from bullous dermatosis (Table II). Two biopsies were taken from each patient: one containing the entire blister, "blister skin", and one containing the uninvolved skin, "normal skin". Biopsies were immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

LPA quantification in blister fluid Quantification of LPA was performed using a radioenzymatic assay as described previously (Saulnier-Blache *et al*, 2000). In short, lipids contained in blister fluids were extracted with 1 volume of 1-butanol followed by evaporation of the solvent under nitrogen. In the presence of [^{14}C] oleoyl-CoA, recombinant rat LPA acyl-transferase selectively catalyzes the transformation of LPA into [^{14}C] PA. Products of the reaction were separated by one-dimensional thin-layer chromatography and autoradiographed. Each spot of [^{14}C] PA was scraped off and counted with 3 mL scintillation cocktail. Radioactivity was converted to nanomoles, and the concentration of LPA was calculated. We used 1-oleoyl-LPA (Sigma-Aldrich, St Louis, Missouri) as a standard (Saulnier-Blache *et al*, 2000). This method has been previously validated as a quantitative assay (Saulnier-Blache *et al*, 2000). We verified that the assay was also quantitative using blister fluids.

In order to determine the presence of intrinsic LPA synthesis activity and its metabolic pathway (PLA_2 or LPLD), quantification of LPA was also performed after 6 h of incubation of blister fluid at 37°C with or without 100 μM of quinacrine (Biomol, Plymouth Meeting, Pennsylvania) an inhibitor of PLA_2 (McCrea *et al*, 1985), or 100 μM of phenanthrolin (Sigma-Aldrich), previously demonstrated to inhibit LPLD activity (Tokumura *et al*, 1998; Gesta *et al*, 2002).

PA and LPC quantification in blister fluid As previously described (Gesta *et al*, 2002), the amount of PA and LPC present in blister fluid was determined by quantifying the amount of LPA generated after treatment by pancreatic PLA_2 or bacterial PLD, respectively. Incubation was realized at 37°C for 90 min with pancreatic PLA_2 at 1.92 UI per mL (Sigma-Aldrich), or bacterial PLD from Streptomyces chromofuscus at 1 UI per mL (Sigma-Aldrich). This method has been previously validated as a quantitative assay (Saulnier-Blache *et al*, 2000). We verified that the assay was also quantitative using blister fluids.

Table II. Identification of the different types of blister fluid used for the various experiments

Type of experiments	Type of bullous dermatoses studied
LPA quantification in blister fluids	All type of bullous dermatosis
LPA quantification in plasma	Lyell (n = 2)
PA quantification	Lyell (n = 2)
	Bullous pemphigoid (n = 2)
	Burn (n = 1)
LPC quantification	Lyell (n = 3)
	Bullous pemphigoid (n = 2)
	Burn (n = 3)
	Eczema (n = 1)
	Varicella-zoster virus infection (n = 1)
LPA synthesis activity	Lyell (n = 2)
	Bullous pemphigoid (n = 2)
	Burn (n = 1)
Inhibition of LPA synthesis activity by quinacrine	Lyell (n = 2)
	Bullous pemphigoid (n = 2)
	Burn (n = 1)
PLA_2 activity	All type of bullous dermatosis
Inhibition of LPA synthesis activity by phenanthrolin	Lyell (n = 2)
	Bullous pemphigoid (n = 2)
	Burn (n = 1)
LPLD activity	Lyell (n = 3)
	Bullous pemphigoid (n = 2)
	Burn (n = 3)
	Eczema (n = 1)
	Varicella-zoster virus infection (n = 1)
Real-time PCR	Bullous pemphigoid (n = 3)
	Burn (n = 1)

The blisters originating from patients under steroid therapy were only used for LPA quantification and PLA_2 activity experiments, and not for the rest of the experiments.

n, number of patients; LPA, lysophosphatidic acid; PA, phosphatidic acid; LPC, lysophosphatidylcholine; PLA_2, phospholipase A_2; LPLD, lysophospholipase D.

Assay for PLA_2 activity in blister fluids PLA_2 activity was determined by measuring the rate of release of pyrenyldecanoic acid from the pyrene-labeled phospholipid 1-hexadecanoyl-2-(1-pyrene-decanoyl-)-*sn*-glycero-3-phosphoglycerol, ammonium salt (Invitrogen, Carlsbad, California), as previously described (Radvanyi *et al*, 1989), with minor modifications. Blister fluid samples of 10 μL were incubated for 1 h at room temperature with 1 mL reaction buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM

Table III. Oligonucleotides used to study the expression of ATX and LPA-receptors by RT-PCR

Gene	Oligonucleotide sequences
Human ATX	
Sense	5′-GGACCAACATCTCCGGATCTT-3′
Antisense	5′-GGAGGTCCAGCCTCTTGAAGT-3′
Human LPA1-R	
Sense	5′-TGGGCCATTTTCAACTTGGT-3′
Antisense	5′-TCTGGCGAACATAGCCAAAGA-3′
Human LPA2-R	
Sense	5′-TCATCATGGGCCAGTGCTACT-3′
Antisense	5′-GTGGGAGCTGAGCTCTTTGC-3′
Human LPA3-R	
Sense	5′-TGGGCCATCGCCATTTT-3′
Antisense	5′-GAGCAGGCAGAGATGTTGCA-3′
Human hypoxanthine phosphoribosyl transferase	
Sense	5′-TGACACTGGCAAAACAATGCA-3′
Antisense	5′-GCTTGCGACCTTGACCATCT-3′

ATX, autotaxin; LPA1-R, LPA1-receptor; LPA2-R, LPA2-receptor; LPA3-R, LPA3-receptor.

EDTA, 10 % (wt/vol) fatty acid-free bovine serum albumin (BSA), and the substrate (final concentration 2 μM). The reaction was initiated by the addition of 10 mM $CaCl_2$. This method has been previously validated as a quantitative assay (Radvanyi *et al*, 1989). We verified that the assay was also quantitative using blister fluids. Fluorescence measurements were performed with a spectrofluorometer (SFM 25 Kontron; Kontron, Downers Grove, Illinois) at excitation and emission wavelengths of 345 and 398 nm, respectively.

Measurement of LPLD activity in blister fluid LPLD activity was measured by conversion of radiolabeled LPC into radiolabeled LPA as previously described (Gesta *et al*, 2002) with minor modifications. A solution of [^{14}C] palmitoyl-lysophosphatidylcholine (NEN, 55.8 mCi per mmole) at 0.0025 μCi per μL in DMEM supplemented with 1% fatty acid-free BSA was first prepared, and 20 μL of this solution was incubated with 500 μL of blister fluid plus 1 μL of sodium orthovanadate 0.5 mM for 90 min at 37°C. At the end of the incubation period, phospholipids were extracted with 500 μL of 1-butanol, evaporated, spotted on a silica gel 60 TLC glass plate (VWR, West Chester, Pennsylvania), and separated using $CHCl_3$/MeOH/NH_4OH (60/35/8) as a migration solvent. The plate was autoradiographed overnight at −80°C using a Biomax-MS film (Kodak, Rochester, New York) in order to localize [^{14}C]LPA spots, which were scraped off and counted with 3 mL of scintillation cocktail. Activity was expressed as pmol [^{14}C]LPA released per min per mg of protein. Tokumura (Tokumura *et al*, 1998) validated the quantitative nature of the assay. In our study, we verified that the assay was also quantitative using blister fluids.

Expression of LPA-receptors and ATX in skin biopsies by real-time RT-PCR We studied the expression of LPA-receptors and ATX using real-time PCR, as previously validated by Moniotte *et al* (2001). Total RNA was isolated from skin biopsies using the RNA STAT-60 kit (AMS Biotechnology, Abington, Oxon, UK) and Rneasy mini protocol for RNA Cleanup (Quiagen, Valencia, California). Total RNA (1 μg) was reverse transcribed for 60 min at 37°C using the Superscript II reverse transcriptase (Invitrogen) in the presence of random hexamers. A minus RT reaction was performed in conjunction, as a control, to ensure the absence of genomic DNA contamination. Real-time RT-PCR was performed starting with 26 ng cDNA with 300 nM (human LPA2-R) or 900 nM (human ATX, human LPA1-R, human LPA3-R, and human hypoxanthine phosphoribosyl transferase as a control) concentrations of both sense and antisense primers in a final volume of 25 μL, using the SYBR green TaqMan Universel PCR Master Mix (Applied Biosystems, Forster City, California). Fluorescence was monitored and analyzed in a GeneAmp 7000 detection system instrument (Applied Biosystems). Analysis of the 18S ribosomal RNA was performed in conjunction with the use of the ribosomal RNA control taqman assay kit (Applied Biosystems) in order to normalize gene expression. Results were expressed according to the δCT method as $2^{(Ct18S-Ctgene)}$, where Ct corresponds to the number of cycles needed to generate a fluorescent signal above a predefined threshold (Moniotte *et al*, 2001).

Primer design was optimized by using the Primer Express software (Perkin Elmer Life Sciences, Boston, Massachusetts). The oligonucleotides used for these experiments are shown in Table III.

Cell migration assay of keratinocytes HaCaT cells (a gift from Fusenig, Heidelberg, Germany), a spontaneously transformed, non-tumorigenic human keratinocyte cell line, were cultured in RPMI 164 (Gibco, Langley, UK) with 10% fetal bovine serum at 37°C, with 5% CO_2. After confluence, the medium was changed to a medium without serum for 24 h. Cell migration assays were performed in modified Boyden chambers containing 8–12 μm pore size uncoated polycarbonate membranes (Poretics Osmonic, Livermore, California). LPA was solubilized in phosphate buffered saline containing 1% BSA. The lower chambers were filled with various concentrations of LPA (0–5 μM) in RPMI containing 1% BSA, or with human epidermal growth factor (Sigma-Aldrich) (35 ng per mL in RPMI containing 1% BSA) as a positive control (Barrandon and Green, 1987), or with the medium alone (RPMI containing 1% BSA) as a negative control. The upper chambers were filled with cells in suspension in RPMI containing 1% BSA (1×10^6 per mL). After 45 min of incubation at 37°C, the filter was removed and the top surface of the membrane was wiped to remove non-migrating cells. The membrane was fixed in ethanol and stained with RAL 555 fast-staining kit (VWR, West Chester, Pennsylvania). The migration was evaluated by counting the stained migrating cells at the bottom surface of the membrane by light microscopy (Axioskop 20, Zeiss (Thornwood, New York)). At least three different fields were evaluated, and the average was calculated. The migration was expressed as a migration index and represented the percentage of migrated cells with LPA compared with the percentage of migrated cells with medium alone (RPMI 1% BSA).

Protein determination Protein concentration was determined by the DC Protein assay (Biorad, Hercules, California) with BSA as the standard.

Statistical analysis Data were expressed as mean ± SEM from four independent repetitive experiments realized for each patient's sample of blister fluid or skin biopsy. For experiments of migration, three independent repetitive experiments were performed using three independent pools of cells. Statistical analysis was performed using SAS 6.0 and non-parametric tests (Wilcoxon matched-pairs signed-rank test, Spearman's rank-order correlation coefficient, and Kruskal–Wallis one-way of variance by ranks). Statistical significance was considered when $p < 0.05$.

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