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**PRODUCTION OF LYOPHOSPHATIDIC ACID IN BLISTER FLUID :
INVOLVEMENT OF A LYOPHOSPHOLIPASE D ACTIVITY**

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Short title : LYSOPHOSPHATIDIC ACID IN BLISTER FLUID

Abbreviations :

- Lysophosphatidic acid : LPA
- Phospholipase A₂ : PLA₂
- Phosphatidic acid : PA
- Lysophospholipase D : LPLD
- Lysophosphatidylcholine : LPC
- Autotaxin : ATX
- LPA1 receptor : LPA1-R
- LPA2 receptor : LPA2-R
- LPA3 receptor : LPA3-R

Keywords :

lysophosphatidic acid, blister fluid, wound healing, lysophospholipase D, skin

ABSTRACT

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 during wound healing, or in other situations. Since re-epithelialization occurs after blister rupture, the presence of LPA in blister fluids was investigated.

Using a radioenzymatic assay, LPA was detected in 33 blister fluids originating from 24 bullous dermatoses, and at higher concentrations than in plasma. LPA concentration was independent of the type of dermatoses. In parallel, blister fluids contained a lysophospholipase D (LPLD) activity but no detectable phospholipase A₂ activity. The expression of the LPLD autotaxin (ATX) and of LPA1-receptor were greatly increased in blister skin when compared to 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-receptor, play an important role in re-epithelialization occurring after blister rupture.

INTRODUCTION

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 (Baker *et al*, 2002; Xu *et al*, 1998 ; Merchant *et al*, 1991), 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 A₂ (PLA₂)-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). Umezo-Goto *et al* (Umezu-Goto *et al*, 2002), Tokumura *et al* (Tokumura *et al*, 2002) and Ferry (Ferry *et al*, 2003) purified LPLD activity from bovine serum, human plasma and adipocytes respectively, and demonstrated that it corresponded to 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 W, 2002), and different situations, such as cancers, are associated with LPA production (Baker *et al*, 2002; Xu *et al*, 1998; Merchant *et al*, 1991). However, its precise mechanism of action 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* (Piazza *et al*, 1995) demonstrated that LPA induced proliferation and differentiation. The fact that LPA is released from activated platelets (Eichholtz *et al*, 1993), as well as the presence of active phospholipases in the skin (Mazereeuw-Hautier *et al*, 2000; Maury *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 decided to investigate LPA in blister fluid.

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 aetiologies 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 suction blister. This fluid has been qualified as a “filtrate” of serum since the concentration of each compound was smaller than in 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). In the present study, we report the presence of LPA in human skin blister fluid and show evidence for the involvement of a LPLD activity, corresponding to ATX, in the synthesis of blister fluid LPA. We also show that the expression of LPA receptors was regulated during bullous formation. In parallel, we demonstrate an effect of LPA on keratinocyte migration, that might contribute to the effect of LPA in skin wound healing.

MATERIALS AND METHODS

Collection of blister fluid and blood

33 samples from 24 patients (13 females and 11 males, mean age was 53.96 ± 30.44 years, range 2-90 years) suffering from different bullous dermatosis were investigated between December 2001 and November 2002. There were 6 different aetiologies 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), dystrophic epidermolysis bullosa (n:1). The subjects of our study gave informed 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). Final diagnosis was based on anamnesis, clinical manifestations, routine histology, detection of autoantibodies using direct and indirect immunofluorescence, and viral culture and serologies. Collection of fluid was realized before the beginning of any treatment, except for 7 of the 10 patients with bullous pemphigoid who were already under steroid therapy (topical application of Diprolene^R (clobetasol) 30 g/j, n:2 or oral route of Solupred^R (prednisolone) 0.50 to 1.50 mg/kg/j, n:5). The blisters originating from patients under steroid therapy were only used for LPA quantification experiments and not for the determination of the metabolic origin of LPA. 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 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 EDTA conditioned tubes.

Collection of skin biopsies

Eight biopsies were taken between January and June 2003 from 4 other patients (3 females and 1 male, mean age 81 years, range 77-91 years) suffering from bullous dermatosis (bullous pemphigoid (n:3), second-degree burn (n:1)). For each 4 patient, two biopsies were performed: one containing the entire blister and one containing the uninvolved 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 previously described (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 [¹⁴C] oleoyl-CoA, recombinant rat LPA acyl-transferase selectively catalyses the transformation of LPA into [¹⁴C] PA. Products of the reaction were separated by one-dimensional thin-layer chromatography and autoradiographed. Each spot of [¹⁴C] PA was scraped off and counted with 3 ml scintillation cocktail. Radioactivity was converted to nanomoles and the concentration of LPA was calculated.

Quantification of LPA was also performed after 6 hours of incubation of blister fluid at 37°C with 100 µM of quinacrine (Biomol, Plymouth Meeting, PA)), or phenanthroline (Sigma-Aldrich, St Louis, MO).

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 PLA₂ or bacterial PLD respectively. Incubation was realized at 37°C for 90 min with pancreatic PLA₂ at 1,92 UI / ml (Sigma-Aldrich, St Louis, MO), or bacterial PLD from *Streptomyces chromofuscus* at 1UI/ ml (Sigma-Aldrich, St Louis, MO).

Assay for PLA₂ activity in blister fluids

PLA₂ activity was determined by measuring the rate of release of pyrenyldecanoic acid from the pyrene-labelled 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 hour at room temperature with 1 ml reaction buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 10 % (w/v) fatty acid free bovine serum albumin, and the substrate (final concentration 2 µM). The reaction was initiated by the addition of 10 mM CaCl₂. Fluorescence measurements were performed with a spectrofluorometer (SFM 25 Kontron) 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 [¹⁴C] palmitoyl-lysophosphatidylcholine (NEN, 55.8

mCi/mmmole) at 0.0025 $\mu\text{Ci}/\mu\text{l}$ 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, PA), and separated using $\text{CHCl}_3/\text{MeOH}/\text{NH}_4\text{OH}$ (60/35/8) as a migration solvent. The plate was autoradiographed overnight at -80°C using a Biomax-MS film (Kodak, 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.

Expression of LPA-receptors in skin biopsies by Real Time RT-PCR

Total RNA were isolated from skin biopsies using the RNA STAT-60 kit (AMS Biotechnology, UK) and Rneasy mini protocol for RNA Cleanup (Quiagen, Valencia, CA). Total RNA (1 μg) were reverse transcribed for 60 min at 37°C using the Superscript II reverse transcriptase (Invitrogen, Carlsbad, California) 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, CA). Fluorescence was monitored and analyzed in a GeneAmp 7000 detection system instrument (Applied Biosystems, Forster city, CA). Analysis of the 18S ribosomal RNA was performed in conjunction

using the ribosomal RNA control taqman assay kit (Applied Biosystems, Forster city, CA) in order to normalize gene expression. Results were expressed according to the δ CT method as $2^{(Ct^{18S}-Ct^{gene})}$, where Ct corresponds to the number of cycles needed to generate a fluorescent signal above a predefined threshold.

Primer design was optimized by using the Primer Express software (Perkin Elmer Life Sciences, Boston, MA). The oligonucleotides used were:

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'-TGACACTGGCAAACAATGCA -3', antisense : 5'-GCTTGCGACCTTGACCATCT-3'.

Cell Migration assay of keratinocytes

HaCaT cells, a spontaneously transformed, non-tumorigenic human keratinocyte cell line, were cultured in RPMI 164 (Gibco, Langley, OK) with 10% fetal bovine serum at 37°C, with 5% CO₂. 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 to 12- μ m pore size uncoated polycarbonate membranes (Poretics Osmonic, California). The lower chambers were filled with

various concentrations of LPA (0 to 5 μ M) in RPMI containing 1% bovine serum albumin, or with human epidermal growth factor (Sigma-Aldrich, St Louis, MO) (35 ng/ml in RPMI 1% bovine serum albumin) as a positive control, or with the medium alone (RPMI 1% bovine serum albumin) as a negative control. The upper chambers were filled with cells in suspension in RPMI (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, PA). The migration was evaluated by counting the stained migrating cells at the bottom surface of the membrane by light microscopy (Axioskop 20, Zeiss (Thornwood, NY)). At least, three different fields were evaluated and the average was calculated. Stimulation by LPA was expressed as a migration-index and represented the percentage of migrated cells with LPA divided by the negative control. All experiments were carried out in triplicate and repeated at least three times.

Protein determination

Protein concentration was determined by the DC Protein assay (Biorad, Hercules, CA) with bovine serum albumin as standard.

Statistical analysis

Data were expressed as mean \pm SEM. Comparison was performed by means of Student's test. Statistical significance was considered when $p < 0.05$.

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, $0.60 \pm 0.0087 \mu\text{M}$, range 0 – 1.90) concentrations of LPA were detected in blister fluids originating from all patients except for one, suffering from toxic epidermal necrolysis. The results for LPA concentrations are shown in Table 1. No statistical correlation could be established between LPA concentration and the following characteristics: age, sex, aetiology of bullous diseases, blister duration, indirect immunofluorescence, eosinophilia or steroid treatment for bullous pemphigoid.

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 (n:2). We found that plasma concentrations were 9-fold lower than blister fluid concentrations (plasma : $0.07 \mu\text{M}$ vs. blister $0.4 \mu\text{M}$ and $0.064 \mu\text{M}$ vs. $1.2 \mu\text{M}$ 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 n:10 respectively) was evaluated to 1.1 ± 0.25 and $2.8 \pm 0.18 \mu\text{M}$ respectively. 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 hours. After incubation, the concentration of LPA had doubled (mean \pm SEM: 1.70 ± 0.72 vs. 3.40 ± 1.05 μ M) (n: 5) ($p < 0.005$) (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 PLA₂-dependent deacylation of PA, and the LPLD-dependent hydrolysis of LPC (Gaits *et al*, 1997; Pages *et al* 2001; Aoki *et al*, 2002). From the same 5 patients, we showed that the intrinsic LPA synthesis activity was not modified after 6 hours of incubation at 37°C with quinacrine, an inhibitor of PLA₂ (McCrea *et al*, 1985) (Fig 1). In conjunction, no PLA₂ activity could be detected in blister fluids (n:32), except for 1 patient suffering from toxic epidermal necrolysis (data not shown). In contrast, after 6 hours of incubation at 37°C with phenanthrolin, previously demonstrated to inhibit LPLD activity (Tokumura *et al*, 1998; Gesta *et al*, 2002), LPA synthesis activity was completely abolished (n:2) (Fig 1). In parallel, LPLD activity was 0.018 ± 0.005 pmol/min/mg protein, range 0-0.9, (n:10) (data not shown).

Since LPLD activity can be catalysed by ATX (Umezu-Goto *et al*, 2002; Tokumura *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 to normal skin. As shown in Figure 2, ATX mRNAs were found in blister skin and were approximately 4-times more abundant than in normal skin ($p < 0.05$). 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 (Chun *et al*, 2002; Contos *et al*, 2000) the expression of these receptors was also investigated in blister skin and was compared to normal skin. As shown in Figure 2, mRNA of the 3 LPA receptor subtypes (LPA1-R, LPA2-R, LPA3-R) were detected in blister skin. When compared to normal skin, LPA1-R mRNA from blister skin were 6.42 times more abundant ($p < 0.05$), while LPA2-R mRNA and LPA3-R mRNA were 4.30 and 2.50 times (not statistically significant) less abundant, respectively. In parallel, no significant alteration in the level of hypoxanthine phosphoryl transferase mRNA (used as an housekeeping gene) was observed. These results showed that blister formation is accompanied by regulation of LPA receptors expression, and particularly by a consistent up-regulation of the LPA1-R subtype.

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 dose-dependent effect on keratinocyte migration. The effect increased according to LPA concentrations and was statistically

different from control at concentrations of 1 μ M or above, showing a plateau at 2 μ M (Fig 3). This result suggests that LPA in blister fluid enhances keratinocyte migration.

DISCUSSION

The present 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 proliferation and differentiation of keratinocytes. Sauer (Sauer *et al*, 2004) showed that LPA mediates keratinocyte growth arrest and chemotaxis. The LPA effects 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. LPA concentrations found in blister fluid were in accordance with the reported concentrations responsible for interactions between LPA and its receptors. 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 skin expresses LPA receptor subtypes (LPA1-R, LPA2-R, LPA3-R). Since Amano (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, it 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, up-regulation 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 directly involved in 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 humour (Liliom *et al*, 1998) (0.785, 1 and 0.2 μM , respectively), and lower than in plasma (0.08 to 0.2 μM) (Tokumura *et al*, 1998). This last observation suggests that, conversely 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 PLA₂-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 PLA₂ in LPA synthesis in blister fluid. This hypothesis was also supported by the absence of detectable PLA₂ activity in blister fluid. In contrast, our data strongly support the fact that LPA synthesis activity corresponds to a LPLD activity, since it is blocked by phenanthroline, 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 skin from blister.

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, myeloperoxidases, or proteases and their inhibitors (D'Auria *et al*, 2000; Grando *et al*, 1989b). This type of skin injury could be responsible for the up-regulation of ATX expression and therefore 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 intra cranial hematoma in a pig model (Tigyi *et al*, 1995).

In conclusion, LPA is produced in blister fluid by a LPLD activity that probably corresponds to ATX. 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.

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Bullous dermatoses	No. patients	No. samples	LPA (μM) Mean (\pm SEM)	Median (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	

Table 1 : Lysophosphatidic acid (LPA) concentrations in blister fluids.

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

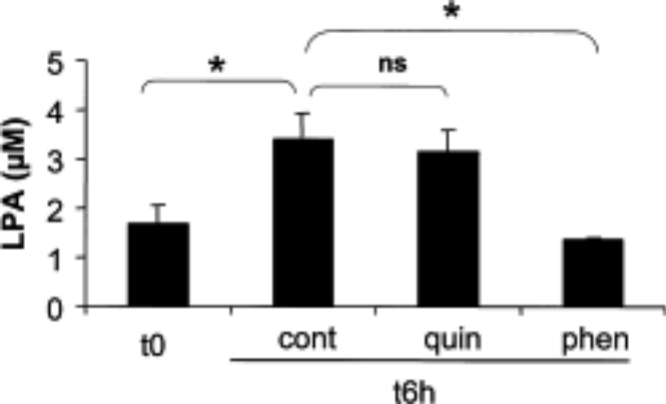
FIGURES LEGENDS

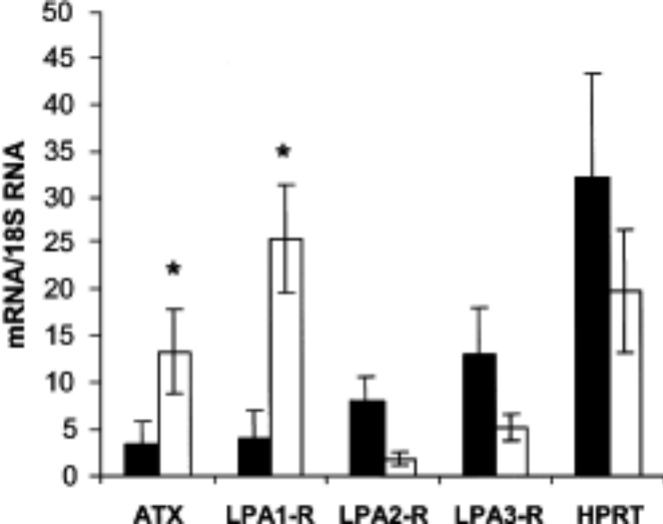
Fig 1: Soluble extracellular lysophosphatidic acid (LPA) synthesis activity in blister fluid and influence of quinacrine and phenanthrolin. LPA was quantified in blister fluids before (t0) and after 6 hours (t6h) incubation at 37°C without (cont) or with quinacrine (quin) at 100 µM (n:5) or phenanthrolin (phen) at 100 µM (n:2). Values are means ± SEM from n separate experiments. *: p < 0.05.

Fig 2: Expression of lysophosphatidic acid (LPA) receptor subtypes and autotaxin (ATX) in skin biopsies.

Blister skin and normal skin were both obtained from patients suffering from bullous dermatoses. Total RNA were isolated and mRNA encoding LPA1- (LPA1-R), LPA2- (LPA2-R) and LPA3- (LPA3-R) receptors, and autotaxin (ATX), and hypoxanthine phosphoribosyl transferase (HPRT) were quantified using real time PCR as described in Materials and Methods. Values are means ± SEM from 4 separate experiments. *: p < 0.05.

Fig 3 : Effect of lysophosphatidic acid (LPA) on keratinocyte migration. HaCaT cells were treated or not (cont) to increased concentrations of LPA and cell migration was assessed using modified Boyden chambers as described in Materials and Methods. Values are means ± SEM from 3 separate experiments. *: p < 0.05.





Migration Index

