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.