Lysophosphatidic acid, a growth factor-like lipid, in the saliva

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Abstract **Lysophosphatidic acid is a multifunctional phospholipid mediator and elicits a variety of biological responses in vitro and in vivo. Evidence is accumulating that lysophosphatidic acid plays important physiological roles in diverse mammalian tissues and cells. In the present study, we first examined whether lysophosphatidic acid is present in human saliva. We found that a significant amount of lysophosphatidic acid is present in the saliva (0.785 nmol/ml). The predominant fatty acyl moiety of lysophosphatidic acid was 18:1n-9 + n-7 followed by 18:0 and 16:0. A small amount of lysoplasmanic acid, an alkyl ether-linked analog of lysophosphatidic acid, was also detected in the saliva (0.104 nmol/ml). We found that physiologically relevant concentrations of lysophosphatidic acid induced accelerated growth of cells of mouth, pharynx, and esophagus origin in vitro. Lysophosphatidic acid also induced rapid increases in the intracellular free Ca^{2+} concentrations in these cells. We obtained evidence that lysophosphatidic acid receptor mRNAs are actually present in these cells. These results strongly suggest that lysophosphatidic acid is involved in wound healing in the upper digestive organs such as the mouth, pharynx, and esophagus.**—Sugiura, T., S. Nakane, S. Kishimoto, K. Waku, Y. Yoshioka, and A. Tokumura. **Lysophosphatidic acid, a growth factor-like lipid, in the saliva.** ***J. Lipid Res.*** **2002.** 43: **2049–2055.**

Supplementary key words lysophosphatidic acid • growth factor • molecular species • mouth • esophagus • pharynx • tissue injury • carcinoma • saliva

Lysophosphatidic acid (acyl LPA) is a common intermediate in the biosynthesis of glycerolipids in diverse mammalian tissues and cells. In addition to the role as a metabolic intermediate, acyl LPA is known to act as a potent lipid mediator. Acyl LPA has been shown to induce a variety of biological responses in vitro and in vivo, such as hypertension (1), smooth muscle contraction (2), platelet activation (3–5), cell proliferation (6, 7), wound healing (8), neurite retraction (9, 10), cell survival (11–13), accelerated development of embryos (14), stimulation of ovum transport in oviducts (15), regulation of endothelial permeability (16), haptotactic migration of monocytes (17), and tumor cell invasion (18). Lysoplasmanic acid (alkyl LPA) is also known to exhibit biological activities similar to those of acyl LPA (19–22). Evidence is accumulating that LPAs play important physiological and pathophysiological roles in various biological systems in mammals (23–29).

The detailed mechanism of action of LPAs remained uncertain until recently, although it had long been assumed that there exist specific binding sites for LPAs. Recently, several investigators have provided direct evidence that LPAs interact with specific receptors expressed in various mammalian tissues and cells, thereby eliciting biological responses (29). Thus far, three separate types of LPA receptors have been identified: LPA_1 (encoded by the Edg2 gene), LPA_2 (encoded by the Edg4 gene), and LPA_3 (encoded by the Edg7 gene) (30–33). These LPA receptors are seven transmembrane, G protein-coupled receptors, and there are homologies between these receptors and the sphingosine-1-phosphate receptors. A number of investigators have already studied the intracellular signaling pathways for LPAs; LPAs have been shown to induce actin rearrangement, inhibition of adenylyl cyclase, activation of phospholipase C, activation of mitogen-activated protein kinase, stimulation of serum response elements, and DNA synthesis (23–29).

Despite increasing information concerning the biological activities and the mechanism of action of LPAs, available information concerning the actual level of LPAs in

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Abbreviations: FAB, fast atom bombardment; GC, gas chromatography; LPA, monoradyl-*sn*-glycero-3-phosphate (lysophosphatidic acid, lysoplasmanic acid); MS, mass spectrometry; RT, reverse transcription; TMS, trimethylsilyl. Fatty acids are designated in terms of the number of carbon atoms: number of double bonds, e.g., 16:0 for palmitic acid.

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mammalian tissues and fluids is still limited. Previously, we reported that substantial amounts of acyl LPA as well as small amounts of alkyl LPA are present in rat brain (22) and hen egg yolk and white (34). Das and Hajra (35) also demonstrated the occurrence of acyl LPA in various rat organs. Furthermore, several investigators have provided evidence that acyl LPA is present in significant amounts in blood plasma (36–39), serum (38–40) and follicular fluids (41). It is apparent, however, that sufficient amounts of analytical data have not hitherto been accumulated regarding the levels of LPAs. In order to better understand the physiological and pathophysiological roles of LPAs in living animals, it is essential to determine the exact levels of LPAs in various mammalian tissues and fluids under various conditions. It is also important to determine the subclass composition as well as molecular species composition of naturally occurring LPAs, because certain species of LPA preferentially interact with certain receptor subtypes (42).

In this study, we focused on the saliva. The reasons are as follows: *1*) the epithelial cells of the mouth, pharynx and esophagus are in direct contact with saliva, and *2*) epithelia of these organs are often injured microscopically or macroscopically by ingested materials. We found that human saliva contains a relatively high amount of acyl LPA and a small amount of alkyl LPA. We also found that acyl LPA stimulates the growth of cells of mouth, pharynx and esophagus origin in vitro at physiologically relevant concentrations. The possible physiological and pathophysiological implications of the occurrence of LPAs in the saliva are discussed.

MATERIALS AND METHODS

Chemicals

Arachidonic acid, heptadecanoic acid, and the *sn*-glycero-3-phosphocholine cadmium chloride complex were obtained from Sigma (St. Louis, MO). Phospholipase D (*Streptomyces chromofuscus*) was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Dicyclohexylcarbodiimide was obtained from Wako Pure Chem. Ind. (Osaka, Japan). Dimethylaminopyridine was from the Aldrich Chemical Co. (Milwaukee, WI). 1(2)-Heptadecanoyl-*sn*-glycero-3-phosphate [acyl LPA (17:0)] and 1(2)-octadecyl-*sn*-glycero-3-phosphate [acyl LPA (18:1n-9)] were prepared according to previously described methods (22). All other chemicals were of reagent grade.

Cells

EC-GI-10 cells (esophagus sqamous cell carcinoma) were obtained from Riken Gene Bank (Saitama, Japan). Detroit 562 cells (pharynx sqamous cells carcinoma) and SCC-9 cells (tongue sqamous cell carcinoma) were obtained from the American Type Culture Collection (Rockville, MD).

Purification of LPAs from human saliva

Saliva was obtained from six young healthy volunteers. Saliva (80 ml), obtained from individual donors, was then mixed with 100 ml of chloroform and 200 ml of methanol and shaken vigorously. Butylated hydroxytoluene (final 0.05%) was added to avoid lipid peroxidation and acyl LPA (17:0) (10 nmol) was added as an internal standard. To make two phases, chloroform (100 ml) and water (100 ml) were added as previously described (the final ratio of chloroform-methanol-water, 2:2:1.8, v/v/v). After vigorous shaking, the mixture was left to stand at 4°C for 12 h. The mixture was then centrifuged to allow phase separation. We confirmed that LPAs were exclusively recovered from the upper phase but not from the lower phase (22); the upper phase was carefully transferred to glass tubes and washed with chloroform. A total of 200 ml of chloroform and a total of 1.6 ml of 12 M HCl were then added to the upper phases. After vigorous shaking and centrifugation, the lower phase was carefully aspirated. The upper phase was washed twice with chloroform, and the lower phases were taken and combined with the initial lower phase. The combined lower phase was evaporated to dryness under vacuum and the residue was dissolved in chloroform-methanol (1:2, v/v). The LPAs were purified by TLC using chloroform-acetone-methanol-acetic acid-water (4.5:2:1:1.3:0.5, v/v/v/v) as the solvent system in a developing tank sealed with nitrogen gas. The areas corresponding to the LPAs were scraped off the TLC plates into a glass tube. The LPAs were extracted from the silica gel by a modified method of Bligh and Dyer where HCl was added (the final concentration of HCl in the upper phase, 0.07 M) prior to the phase separation. LPAs were further purified by TLC using chloroform-methanol-25% ammonia (65:35:5, v/v) as the solvent system and then by TLC using chloroform-acetone-methanol-acetic acid-water (4.5:2:1:1.3:0.5, v/v/v/v) as the solvent system (22).

Mild alkaline hydrolysis of LPAs

The LPAs (250–280 nmol), purified from human saliva, were treated with 0.2 M NaOH in 90% methanol or 90% methanol alone (control) for 20 min, as previously described (22, 34). The LPAs remaining were then extracted by a modified method of Bligh and Dyer (43), where HCl was added to the extraction mixture prior to the phase separation to acidify the upper phase. The lower phase was transferred to another tube, and the upper phase was washed twice with chloroform. The combined lower phase was evaporated to dryness. The amounts of the LPAs (alkali-stable or total) were determined by measuring the lipid phosphorus (22, 34).

Analysis of the fatty acyl moiety of acyl LPA by gas chromatography

The fatty acyl moiety of the acyl LPA was converted to fatty acid methyl esters by treating the purified acyl LPA with 0.5 M methanolic sodium methoxide. The resultant fatty acid methyl esters were extracted and analyzed using a gas chromatograph (GC8A, Shimadzu, Kyoto, Japan) equipped with a fused silica column (SP2330, Supelco, Bellefonte, PA).

Fast atom bombardment mass spectrometry analysis of LPAs

The structures of the LPAs were confirmed by fast atom bombardment mass spectrometry (FAB MS) using a JEOL JMS-SX102A mass spectrometer. A 2:1 mixture of thioglycerol and dithiothreitol/dithioerythreitol (3:1) was used as the matrix, as previously described (22, 34).

GC/MS analysis of the trimethylsilyl derivatives of LPAs

The LPAs were treated with 50 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane and 50 μl of pyridine at 60°C for 30 min and converted to the tri-trimethylsilyl (TMS) derivative essentially according to the method of Tokumura et al. (44). The electron impact ionization (70 eV) mass spectra of the triTMS derivatives of the LPAs were obtained using a JEOL JMS-SX102A MS (accelerating voltage, 10 kV; ionizing current, 300 μA) coupled with a GC equipped with a fused silica

column (DB-1, 30 m × 0.25 mm I.D., 0.25 μm thickness, J&W Scientific, Folsom, CA). The column temperature was increased from 200°C to 320°C at the rate of 10°C/min, and the temperature of the injection port was 300°C (34).

Effects of acyl LPA on the growth of EC-GI-10, Detroit 562, and SCC-9 cells in vitro

EC-GI-10 esophagus carcinoma cells were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS). Detroit 562 pharynx carcinoma cells were incubated in Eagle's minimun essential medium supplemented with 10% FBS. SCC-9 tongue carcinoma cells were cultured in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium containing 0.4 μg/ml of hydrocortisone supplemented with 10% FBS. These cells were plated in 24-well plates at the density of 3×10^4 cells/well and incubated for 24 h. The cells were then washed twice with phosphate-buffered saline and further incubated in the medium without FBS for 48 h in the presence of various concentrations of acyl LPA (18:1n-9). After the removal of the medium, the cells were washed and treated with 0.25% trypsin for 2 min. Then, the cells were suspended in phosphate-buffered saline (final 0.5 ml). The suspended cells were counted using a hemocytometer. The viability was checked by the Trypane blue dye exclusion test. The viability was above 99% in each experiment.

Effects of acyl LPA on the intracellular free Ca^{2+} concentrations of EC-GI-10, Detroit 562 and SCC-9 cells

Cells were cultured on a cover glass (diameter 12 mm) in 24-well plates. The cells were further incubated in the medium without FBS for 24 h. The cells were then incubated in 25 mM Hepes-Tyrode's solution ($-Ca^{2+}$) (pH 7.4) containing 5 μM Fura-2/AM and 0.02% Cremophor EL (Sigma, St. Louis, MO) at room temperature for 90 min. After rapid washing with 25 mM Hepes-Tyrode's solution, the cover glass was placed in a cuvette filled with 3 ml of 25 mM Hepes-Tyrode's solution. $[Ca^{2+}]_i$ was estimated using a CAF-100 Ca^{2+} analyzer (JASCO, Tokyo, Japan) (22). $CaCl_2$ was added 4–5 min before the measurement (the final concentration of Ca^{2+} in the cuvette, 1 mM). Acyl LPA (18:1n-9) was dissolved in 6 μl of dimethyl sulfoxide and added to the cuvette.

Reverse transcription-PCR

Total RNA was isolated from various cell lines using ISOGEN (Nippon Gene Co., Tokyo, Japan). One microgram of the total RNA was treated with DNase I (Invitrogen, 1 U) for 15 min at room temperature in a 10 μl reaction mixture containing 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$ and 50 mM KCl. After the mixture was heated at 70°C for 10 min, the cDNA was synthesized by incubating at 42°C for 60 min in a 20 μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 0.75 μM dNTPs, 10 μg/ml oligo$(dT)_{18}$ primer, and 200 U Superscript II reverse-transcriptase (Invitrogen Corp, Carlsbad, CA). After inactivation of the enzyme by heating at 98°C for 5 min, 0.4 μl of the reaction mixture was used for PCR. The sequences of the PCR primer sets were: 5′-CAATCGAGAGGCACATTACGGT-3′ (sense) and 5′-GATGTGAGCATAGAGAACCACC-3′ (antisense) for Edg2 (257 bp); 5′-AGACTGTTGTCATCATCCTGGG-3′ (sense) and 5′-AAGGGTGGAGTCATCAGTGGGT-3′ (antisense) for Edg4 (331 bp); 5′-CTGCTCATTTTGCTTGTCTGGG-3′ (sense) and 5′-CCACAACCATGATGAGGAAGGC-5′ (antisense) for Edg7 (175 bp); and 5′-CAGAGCAAGAGAGGCATCCT-3′ (sense) and 5′-AGGATCTTCATGAGGTAGTC-3′ (antisense) for β-actin (404 bp). Amplification of each gene was conducted with 30 cycles, except for that of the β-actin gene with 25 cycles, consisting of 1 min denaturation at 94°C, 1 min annealing (54°C, 58°C, 57°C, and 55°C for Edg2, Edg4, Edg7, and β-actin, respectively), and 1 min elongation at 72°C in a 20 μl reaction mixture containing 66.7 mM Tris-HCl (pH 8.8), 16.7 mM $(NH_4)_2SO_4$, 6.67 mM $MgCl_2$, 10 μM 2-mercaptoethanol, 6.67 μM EDTA, 167 μg/ml BSA, 1 μM sense and antisense primers, 15 mM dNTPs, 1 U Taq polymerase (Takara Shuzo, Kyoto, Japan), and 0.4 μl of reverse transcription (RT) reaction mixture. After amplification, 10 μl aliquots of the reaction mixture were electrophoresed on a 2% agarose gel. The gel was then stained with ethidium bromide and photographed under an UV lamp.

TABLE 1. Subclass composition of LPAs obtained from human saliva

Subclass	%	nmol/ml
Acyl LPA	88.3[a]	0.785[b]
Alkyl LPA	11.7[a]	0.104[c]
Total	100.0	0.889

[a] Subclass composition of LPAs was determined by mild alkaline hydrolysis as described in Materials and Methods. The data are the means of two separate experiments which gave similar results.

[b] Fatty acyl moieties of acyl LPA were converted to fatty acid methyl esters and analyzed by GC. The data are the means of six determiners.

[c] The amount of alkyl LPA was calculated from the amount of acyl LPA and subclass compositions of LPAs.

RESULTS

First, we examined whether human saliva contains LPAs. We found that significant amounts of LPAs are included in human saliva (total 0.889 nmol/ml) (**Table 1**). A large part of the LPAs was accounted for by acyl LPA (88.3%) and a small part was accounted for by alkyl LPA (11.7%). The fatty acid composition of acyl LPA is shown in **Table 2**. The major fatty acyl constituent of acyl LPA was 18:1n-9 + n-7 (40.1%) followed by 18:0 (28.5%) and 16:0 (17.9%). We also found that a small amount of 18:2n-6 (8.0%) was present as a fatty acyl constituent. On the other hand, the levels of the C20 and C22 polyunsaturated fatty acid-containing species such as 20:4n-6-containing species were very low.

Next, we examined the structures of the LPAs obtained from human saliva by FAB MS. **Figure 1** shows the negative ion mode mass spectrum of the LPAs: *m/z* 395 for alkyl LPA (16:0)/acyl LPA (15:0) ($[M-H]^-$), *m/z* 407 for acyl LPA (16:1n-7) ($[M-H]^-$), *m/z* 409 for acyl LPA (16:0) ($[M-H]^-$), *m/z* 433 for acyl LPA (18:2n-6) ($[M-H]^-$), *m/z* 435 for

TABLE 2. The amount and the fatty acid composition of acyl LPA obtained from human saliva

Acyl Moiety	nmol/ml	%
16:0	0.140 ± 0.078	17.9
16:1n-7	0.034 ± 0.031	4.3
18:0	0.223 ± 0.160	28.5
18:1n-9 + n-7	0.315 ± 0.144	40.1
18:2n-6	0.063 ± 0.036	8.0
20:4n-6	0.009 ± 0.004	1.1
Others	0.001 ± 0.004	0.1
Total	0.785 ± 0.403	100.0

Fatty acyl moieties of acyl LPA were converted to fatty acid methyl esters and analyzed by GC as described in Materials and Methods. The data are the means ± SD of six determinations.

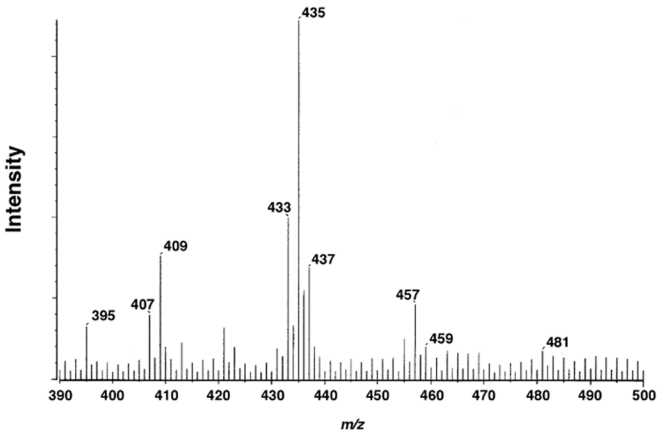

Fig. 1. Negative ion mode mass spectrum of monoradyl-*sn*-glycero-3-phosphate (lysophosphatidic acid, lysoplasmanic acid) (LPA) obtained from human saliva. LPAs obtained from human saliva were analyzed by fast atom bombardment (FAB) MS as described in Materials and Methods.

acyl LPA (18:1n-9 + n-7) ($[M\text{-}H]^-$), *m/z* 437 for acyl LPA (18:0) ($[M\text{-}H]^-$), *m/z* 457 for acyl LPA (20:4n-6) ($[M\text{-}H]^-$), *m/z* 459 for acyl LPA (20:3n-6 + n-3) ($[M\text{-}H]^-$) and *m/z* 481 for acyl LPA (22:6n-3) ($[M\text{-}H]^-$).

The structures of the LPAs were further examined by GC/MS. **Figure 2** shows the mass chromatograms of the TMS derivatives of the major molecular species of acyl LPA obtained from human saliva: *m/z* 611 for acyl LPA (16:0) ($[M-CH_3]^+$), *m/z* 635 for acyl LPA (18:2n-6) ($[M-CH_3]^+$), *m/z* 637 for acyl LPA (18:1n-9 + n-7) ($[M-CH_3]^+$), and *m/z* 639 for acyl LPA (18:1n-9 + n-7) ($[M-CH_3+2H]^+$) and acyl LPA (18:0) ($[M-CH_3]^+$).

We then examined the effects of acyl LPA on the growth of cells of the upper digestive system origin in vitro. Here, we investigated the effects of acyl LPA (18:1n-9) on the growth of EC-GI-10 esophagus carcinoma cells, Detroit 562 pharynx carcinoma cells, and SCC-9 tongue carcinoma cells. In this experiment, FBS was omitted from the culture medium because FBS is known to contain a large amount of LPA. As shown in **Fig. 3A**, the number of EC-GI-10 cells increased to some extent (from 4.4×10^4 cells/well to 6.1×10^4 cells/well) in the absence of FBS or LPA after a 48 h culture period. Noticeably, the addition of acyl LPA (18:1n-9) markedly enhanced the cell growth. The effect was observed from as low as 0.3 μM acyl LPA (18:1n-9) and the maximal effect was observed with 3 μM acyl LPA (18:1n-9). The number of cells that increased during 48 h culture in the presence of 3 μM acyl LPA (18:1n-9) was more than twice the number of cells that increased in the absence of acyl LPA (18:1n-9). Similar facilitative effects of acyl LPA on cell growth were observed with Detroit 562 cells (Fig. 3B) and SCC-9 cells (Fig. 3C). The number of Detroit 562 cells and SCC-9 cells that proliferated during the 48 h culture in the presence of 3 μM acyl LPA (18:1n-9) were 1.8-fold and 3.3-fold higher than the control levels (cultured in the absence of acyl LPA), respectively.

To determine whether the effects of acyl LPA (18:1n-9) are mediated through the LPA receptors, we next examined the effects of acyl LPA (18:1n-9) on $[Ca^{2+}]_i$ in these cells; LPA-induced Ca^{2+} transients observed in many cell types are assumed to be mediated through the LPA receptors. As shown in **Fig. 4A**, the addition of 1 μM acyl LPA (18:1n-9) induced a rapid increase in $[Ca^{2+}]_i$ in the EC-GI-10 cells. The response was observed from 10 nM acyl LPA and reached a plateau at around 1 μM (data not shown). Similar Ca^{2+} transients were observed with Detroit 562 cells (Fig. 4B) and SCC-9 cells (Fig. 4C). These results strongly suggest that these cells express functional LPA receptors on their cell surface.

Finally, we examined whether these cells actually contain the LPA receptor mRNA. As shown in **Fig. 5**, LPA_1 (Edg2) mRNA was detected in all the cell types examined here (EC-GI-10 cells, Detroit 562 cells and SCC-9 cells). LPA_2 (Edg4) mRNA was also detected in these cells. On the other hand, the levels of LPA_3 (Edg7) mRNA were very low in these cells, especially in the EC-GI-10 cells and Detroit 562 cells. It is thus conceivable that LPA mainly interacts with LPA_1 (Edg2) and LPA_2 (Edg4), thereby eliciting biological responses in these cells.

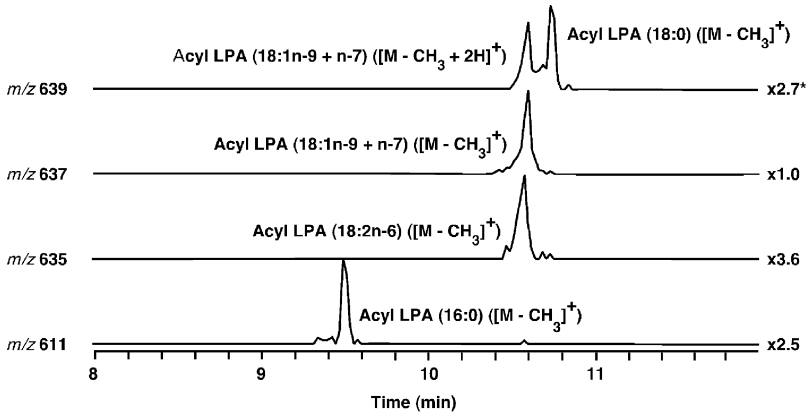

Fig. 2. Mass chromatograms of the trimethylsilyl (TMS) derivatives of several major species of acyl LPA obtained from human saliva. The TMS derivatives of acyl LPA were analyzed by gas chromatography (GC)/MS as described in Materials and Methods. *Magnification for the intensity of ion.

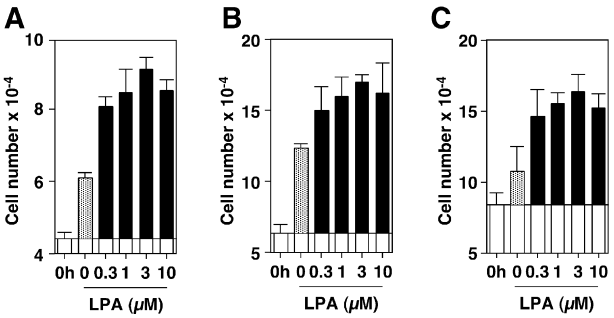

Fig. 3. Effects of acyl LPA (18:1n-9) on the growth of EC-GI-10 esophagus carcinoma cells (A), Detroit 562 pharynx carcinoma cells (B), and SCC-9 tongue carcinoma cells (C). Cells were incubated in medium without FBS in 24-well plates for 48 h in the presence of various concentrations of acyl LPA (18:1n-9). After the removal of the medium, the cells were treated with 0.25% trypsin. The suspended cells were counted using a hemocytometer as described in Materials and Methods. 0 h, before incubation. The data are the means ± SD of four determinations.

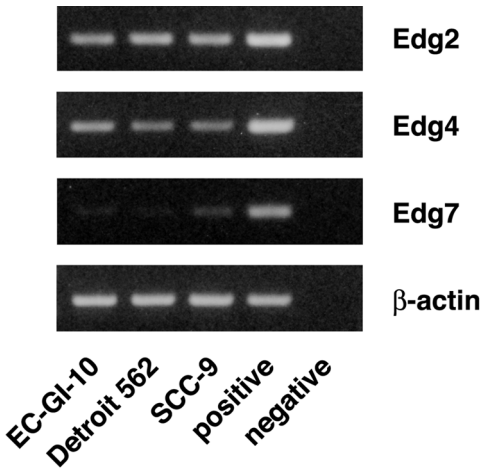

Fig. 5. Reverse transcription (RT)-PCR analysis of the LPA receptor mRNA. The total RNAs were isolated from EC-GI-10 cells, Detroit 562 cells and SCC-9 cells. RT-PCR analysis of the mRNAs for LPA receptors (Edg2, Edg4 and Edg7) and β-actin was performed as described in Materials and Methods. PCR for the positive control was carried out using cDNA corresponding to each gene.

DISCUSSION

Acyl LPA is a unique lysophospholipid with diverse biological functions (1–18) and has received increasing attention in recent years (23–29). Alkyl LPA is also known to exhibit biological activities similar to those of acyl LPA (19–22). Both acyl LPA and alkyl LPA have been shown to exist in several mammalian organs such as the brain (22, 35). Noticeably, a number of investigators have demonstrated that biological fluids such as blood plasma and serum contain significant amounts of acyl LPA (36–40). Acyl LPA is also found to occur in ascites fluids (45) and follicular fluids (41). However, no data have hitherto been available concerning the saliva. This is the first report showing the occurrence of LPAs in the saliva. The level of acyl LPA in the saliva (0.785 nmol/ml) was almost comparable to the level of acyl LPA in the blood plasma (0.61–0.74 nmol/ml) (39).

The subclass composition of LPAs obtained from human saliva resembles the subclass composition of LPAs obtained from rat brain. Alkyl LPA accounted for 11.7% of the total LPA in human saliva (Table 1) and 10.6% in rat brain (22). The fatty acid composition of acyl LPA obtained from human saliva (Table 2) also resembles the fatty acid composition of acyl LPA obtained from rat brain (22). The predominant fatty acyl constituent was 18:1n-9 + n-7 followed by 18:0 and 16:0 in both cases. The difference noted between the fatty acid composition of acyl LPA in human saliva and that in rat brain is that human saliva acyl LPA contained a higher amount of 18:2n-6 and a lower amount of 20:4n-6 compared with rat brain acyl LPA. The fatty acid composition of human saliva acyl LPA also somewhat resembles that of human blood plasma acyl LPA (39), although saliva acyl LPA contained a higher amount of the 18:1-containing species and a lower amount of 18:2-containing species compared with the blood plasma acyl LPA.

The occurrence of LPAs in the saliva is quite noticeable in view of the fact that LPAs, especially acyl LPA (46), act as potent cell proliferators. As shown in Fig. 3, physiologically relevant concentrations of acyl LPA markedly enhanced the growth of cells of esophagus, pharynx, and tongue origin in vitro. This strongly suggests the possibility that LPAs in the saliva are involved in wound healing of the upper digestive organs in vivo. The epithelia of these organs are often injured by ingested materials such as food, hot drinks, and alcohol-containing drinks. The saliva secreted from salivary glands (~1.0–1.5 l/day) always keep the surface of the mouth, pharynx, and esophagus moist; saliva-derived LPAs could be effective and helpful in healing the injured epithelium of these tissues by inducing the restoration of the tissues. Alternatively, saliva LPAs may protect the epithelium through inducing prolonged survival of the cells in the epithelium, because LPAs have been shown to enhance the survival of several types of cells in vitro (11–13).

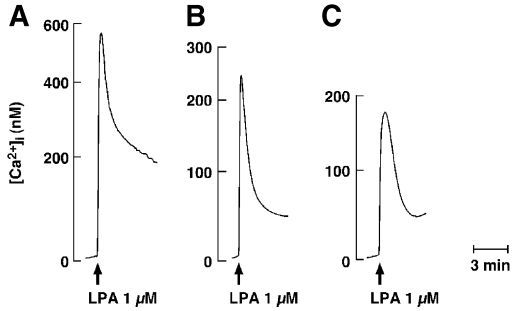

Fig. 4. Effects of acyl LPA (18:1n-9) on $[Ca^{2+}]_i$ in EC-GI-10 cells (A), Detroit 562 cells (B) and SCC-9 cells (C). Cells, cultured on a cover glass, were then incubated in 25 mM Hepes-Tyrode's solution ($-Ca^{2+}$) (pH 7.4) containing 5 μM Fura-2/AM and 0.02% Cremophor EL at room temperature for 90 min. After the addition of $CaCl_2$ (final 1 mM), $[Ca^{2+}]_i$ was estimated using a CAF-100 Ca^{2+} analyzer. Acyl LPA (18:1n-9) (final 1 μM) was dissolved in dimethyl sulfoxide and added to the cuvette as described in Materials and Methods.

Various types of protein growth factors, such as the epidermal growth factor and nerve growth factor, are also present in the saliva (47). The relative importance of these growth factors and LPAs in wound healing is not known. Whatever the relative importance, however, it seems very likely that LPA plays important protective and restorative roles in cooperation with these growth factors in the mouth, pharynx and esophagus; the levels of LPAs in the saliva are sufficient to induce various biological responses such as the stimulation of cell growth (Table 2 and Fig. 3).

In addition to the roles in the digestive organs, saliva LPAs may play essential roles in wound healing in other tissues such as the skin, especially in animals. It is interesting that animals heal the wounds on their bodies and legs by licking. Hutson et al. (48) have provided clear evidence that the licking of wounds accelerates wound healing in mice. Li et al. (49) demonstrated that topically-applied nerve growth factor accelerates wound contraction in experimental animals. It is plausible that not only protein growth factors but also LPAs in the saliva play important roles in healing external wounds in animals.

In conclusion, we found that human saliva contains various molecular species of LPAs. The levels of LPAs present in the saliva are sufficient to induce various biological responses such as the accelerated growth of cells of mouth, pharynx, and esophagus origin. These results suggest that LPAs in the saliva play important physiological and pathophysiological roles in wound healing in these tissues in vivo.

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