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First report of detection of lysophosphatidic acids (LPAs) and analysis of LPA quantity in a human embryo-conditioned medium

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Abstract: **Background.** Lysophosphatidic acid (LPA) receptor 3 expressed in the uterus plays a critical role in uterine receptivity for the embryo. We investigated whether human embryos produce LPA by analysis of a human embryo-conditioned medium (HECM). **Methods.** LPAs in HECM were extracted by the Bligh-Dyer method. Extracted LPAs were converted to trimethylsilyl (TMS) derivatives. Gas chromatography (GC)/ selected ion monitoring (SIM) and GC / mass spectrometry (MS) were used to analyze LPAs derivatized with TMS. Monitoring and quantitative analysis of LPAs were performed using GC/SIM with $[M-15]^+$ ion derived from LPAs. Identification of LPAs was confirmed by GC/MS. **Results.** LPA-C16:0, 16:1, 18:0, 18:1 and 18:2 were detected in HECM. The concentration of LPAs was calculated by comparing each detected peak area with a corresponding standard LPA. **Conclusion.** This study is the first study in which five molecular species of LPAs produced by human embryos were detected in HECM and their quantities analyzed.

Key words: LPA, Embryo-conditioned medium, Blastocyst transfer, Cross-talk

Introduction

Communication between the developing embryo and maternal tissues (cross- talk) while the embryo travels though the fallopian tubes and becomes implanted in the endometrium plays an important role in successful embryo implantation [1]. The embryo produces several factors during its development to signal its presence to the maternal organism, and several embryonic factors that modulate endometrial receptivity have been reportedly detected in an embryo-conditioned culture medium [2–8].

In blastocyst transfer (BT), cross-talk is absent until the blastocysts are transferred and the pregnancy rate using BT is limited to around 40% [9]. We recently reported [10, 11] that injection of a human embryo-conditioned medium (HECM) into the uterine cavity 3 days prior to frozen-thawed BT improved the implantation and pregnancy rates of assisted reproductive technology patients in comparison with BT without injection of HECM. Our clinical studies indicate that some embryonic substances existing in HECM enhance endometrial receptivity, leading to improved implantation and pregnancy rates.

Recent studies have shown that lysophosphatidic acid (LPA) signaling influences embryo spacing and uterine receptivity in mice [12, 13]. Deletion of LPA3 in mice led to uneven embryo spacing, possibly caused by a defect in uterine contraction, and delayed implantation caused by a defect in uterine receptivity. The lipid signaling system is also an integral part of the establishment and maintenance of pregnancy in pigs [14]. Liszewska *et al.* [15] investigated the LPA pathway during early pregnancy in sheep. Quantitative RT-PCR showed that the LPA-generating enzyme of autotaxin was expressed in the endometrium and conceptus. More recently, Achache *et al.* [16] reported that LPA3 levels were comparatively lower in patients with recurrent implantation failure. Although these studies show that LPA3 is crucial for the implan-

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tation of embryos, no study has yet demonstrated that the human embryo produces LPA as a ligand of LPA3. Thus, in this study, we investigated whether LPA exists in HECM as a potential cross-talk signal from the embryo to endometrium.

Materials and Methods

Preparation of HECM

The HECM used in this study was prepared as follows. In the oocyte retrieval cycle, patients were stimulated using a standard GnRH agonist / FSH protocol or an antagonist / FSH protocol. Ovulation was triggered when the second leading follicle was >18 mm in diameter. Oocytes were retrieved transvaginally under ultrasonographic guidance 36 h after human chorionic gonadotropin (hCG) administration. The oocytes were fertilized via a conventional method.

The embryos were initially cultured in BlastAssist System 1 (MediCult, Jyllinge, Denmark). From day 2, depending on the number of cleaved embryos and the morphological grade of the embryos for any given patient, cleaved embryos were cultured until day 5 of the blastocyst stage in BlastAssist System 2 (MediCult, Jyllinge, Denmark). Up to four embryos were cultured in groups under mineral oil in a 50 *μ*l droplet of culture medium. Then the embryo-conditioned medium of BlastAssist System Medium 2 was preserved at –20°C until further analyses were performed. In total, 2,590 *μ*l of HECM originating from 188 embryos of 45 patients was examined. The percentages of patients with indicated causes of infertility were: 44.4%, tubal factor; 22.2%, male factor; 11.1%, endometriosis; 6.7%, PCO; and 15.6%, unexplained factors. The mean age of the patients was 36.3 ± 4.3 years.

Chemicals

Oleoyl-L-α-lysophosphatidic acid sodium salt (LPA-C18:1) as the reference standard of LPA was obtained from Sigma-Aldrich (St. Louis, MO). BSTFA [N,O-Bis (trimethylsilyl) trifluoroacetamide] +1% TMCA (chlorotrimethylsilane) was also purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical reagent grade. Purified water was obtained from a Milli-Q water system (Millipore, Milford, MA).

Extraction of LPAs from HECM

LPAs were extracted using the Bligh-Dyer method [17]. In brief, HECM was mixed with 0.5 ml of water, 2.5 ml of methanol and 1.25 ml of chloroform in a glass tube. After shaking the mixture gently, chloroform (2.5 ml) and 0.5 M HCl (1.0 ml) were added and shaken, and then the mixture was centrifuged to allow phase separation. The lower chloroform phase was carefully transferred to another glass tube. Chloroform (2.5 ml) was added to the remaining phase, which was shaken and centrifuged again. The separated chloroform phase was collected and mixed with the earlier separated chloroform in the same glass tube.

This combined lower phase was dried under a nitrogen gas flow. The residue was treated with 25 ml of BSTFA and 25 ml of pyridine at 70°C for 60 min and converted to trimethylsilyl (TMS) derivatives following the method of Tokumura *et al*. [18].

To obtain the reference standard TMS derivatives of LPA (TMS-LPA), 100 ng of LPA-C18:1 dissolved in culture medium was prepared using the same procedure as that described above.

Gas chromatography(GC)selected ion monitoring(SIM) and GC/mass spectrometry(MS)analysis of the TMS derivatives of LPSs(TMS-LPAs)

The TMS derivatives were analyzed using a gas chromatograph (HP-6890, Agilent Technologies Inc., SANTA CLARA, CA) equipped with a fused silica column (SPB-1, Supleco, Bellefonte, PA). Electron ionization (70 eV) mass spectra of the LPAs derivatized with TMS were obtained using a JMS-700V mass spectrometer (JEOL. Ltd., Tokyo, Japan, accelerating voltage, 10 kV; ionizing current, 100 mA). The column temperature program was 10°C/min from 150°C to 300°C, and the temperature of the injection port was 250°C. A splitless mode injection of 1ml of the TMS derivatives was subjected to individual analysis.

Electron ionization of TMS-LPAs generates characteristic ions common to all LPAs at *m/z* 299.1, 129.1 and 73.1, the TMS derivatives of *sn*-glycero-3-phosphate ($C_9H_{24}O_5PSi_2$), glycerol ($C_6H_{13}OSi$) and TMS (C_3H_9Si), respectively [18]. The individual LPAs provide specific values of $[M-15]^+$ at *m/z* corresponding to their fatty acyl chain lengths and molecular weights. The derivatized LPA-C16:0, C16:1, 18:0, 18:1 and 18:2 showed peaks of $[M-15]^+$ ions at *m/z* 611.3, 609.3, 639.4, 637.3 and 635.3, respectively [18].

GC/SIM analysis is a sensitivity enhancement technique that is used when a very small amount of substance is expected, and it was employed to determine whether HECM contained LPAs by using two monitoring ions of derivatized LPAs. One was an ion at *m/z* 299.1 derived from the TMS derivative of *sn*-glycero-3-phosphate. The other was an $[M-15]^+$ ion at a specific *m/z* as described above corresponding to an individual LPA.

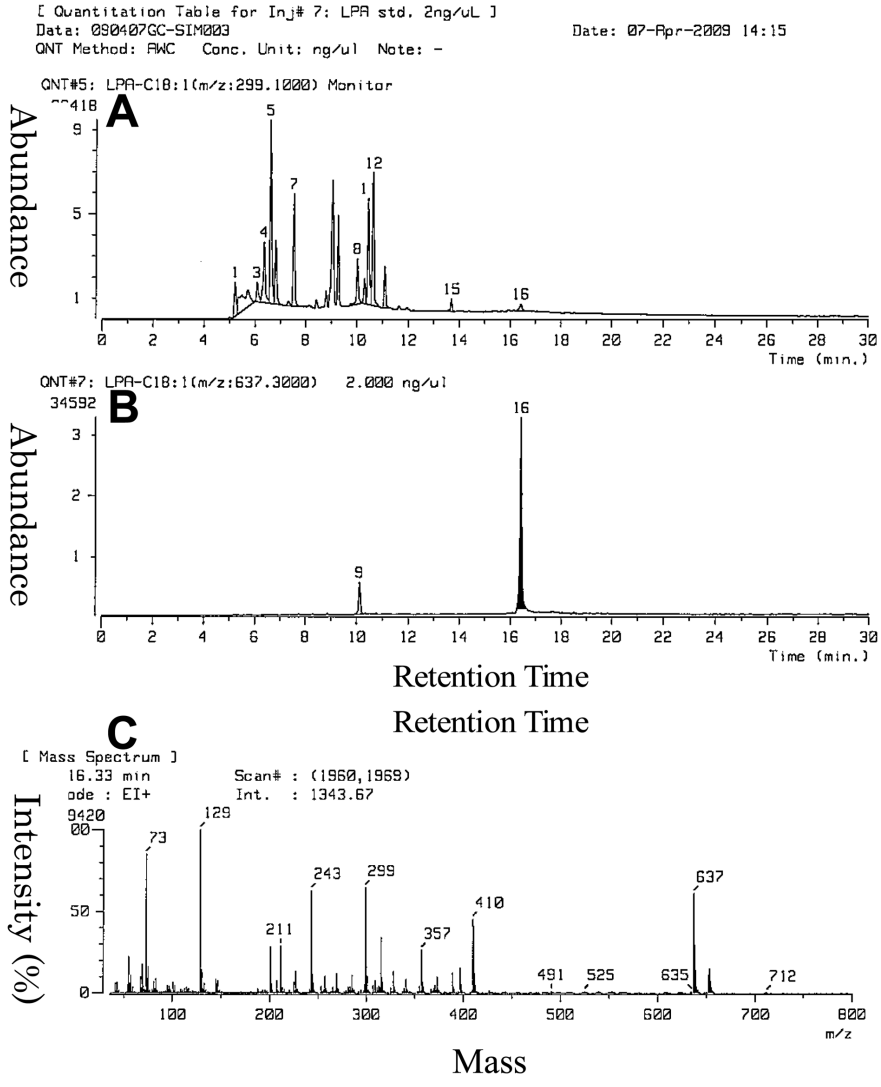

Fig. 1. GC/SIM and GC/MS analysis of synthetic LPA-C18:1 as the reference standard.
(A) GC/SIM analysis of HECM at *m/z* 299.1. (B) GC/SIM analysis of HECM using the $[M-15]^+$ ion at *m/z* 637.3. (C) GC/MS scanning at 16.33 min of retention time. The four peaks at *m/z* 73 (73.1), 129 (129.1), 299 (299.1) and 637 (637.3) were generated by the reference standard. Numbers in parentheses indicate theoretical mass values equivalent to TMS derivatives of LPA-C18:1. GC, gas chromatography; SIM, selected ion monitoring; MS, mass spectrometry; LPA, lysophosphatidic acid; HECM, human embryo conditioned medium; TMS, trimethylsilyl.

LPAs were quantitatively analyzed by GC/SIM of $[M-15]^+$ derived from LPAs. To quantify the LPAs in HECM, experimental values of the peak area were compared to the value obtained from the standard of LPA. To confirm the identification of individual molecular species of LPAs, the observed mass spectrum was examined to determine whether three characteristic mass values (at *m/z* 299.1, 129.1 and 73.1) and one specific mass value of $[M-15]^+$ ion showed peaks in GC/MS analysis.

Informed consent was obtained from the patients, and the entire procedure was examined and approved by the Institutional Review Board of Hanabusa Women's Clinic.

Results

GC/SIM and GC/MS analysis of the TMS derivative of reference standard LPA

Figure 1A shows the retention time (Rt) peak of 16.33 min of TMS-LPA-C18:1, which is a characteristic ion derived from glycero-3-phosphate, appearing at *m/z*

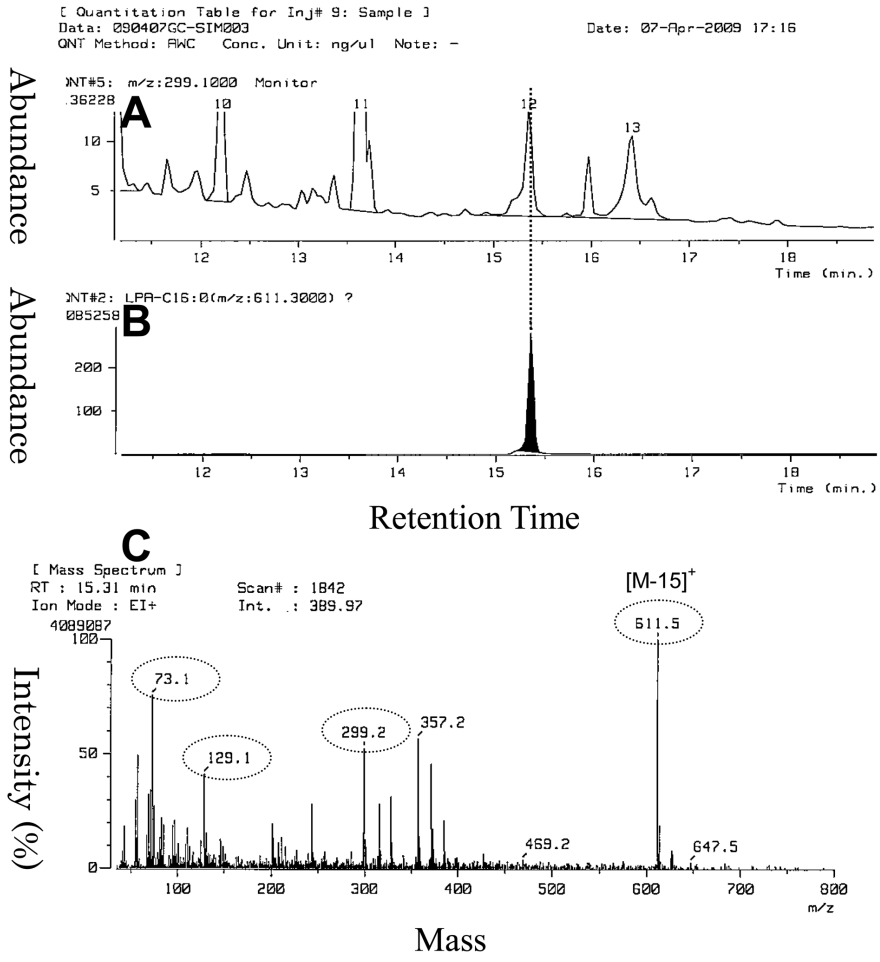

Fig. 2. Detection of LPA-C16:0 in HECM by GC/SIM and GC/MS analysis.
(A) GC/SIM analysis of HECM at *m/z* 299.1. (B) GC/SIM analysis of HECM using the $[M-15]^+$ ion at *m/z* 611.3. (C) GC/MS scanning at 15.31 min of retention time. The four ions at *m/z* 73.1, 129.1, 299.2 (299.1) and 611.5 (611.3) were generated from LPA-C16:0. Numbers in parentheses indicate theoretical mass values equivalent to TMS derivatives of LPA-C16:0. LPA, lysophosphatidic acid; HECM, human embryo-conditioned medium; GC, gas chromatography; SIM, selected ion monitoring; MS, mass spectrometry; TMS, trimethylsilyl.

299.1. The peak of the $[M-15]^+$ ion at *m/z* 637.3, specific to TMS-LPA-C18:1, was detected at the same Rt as shown in Fig. 1B. The peak area value at *m/z* 637.3 of the Rt of 16.33 min was 16.4979 when 1 *μ*l of the TMS derivatives of standard LPA (equivalent to 2.0 ng of LPA-C18:1) was used in the GC/SIM analysis (Fig. 1B). GC/MS scanning indicated four peaks at *m/z* 73, 129, 299 and 637, which were confirmed as LPA-C18:1 derivatized with TMS (Fig. 1C). These results show that if LPAs exist in HECM, they can be detected by observing both the ion at *m/z* 299.1 and the $[M-15]^+$ ion in GC/SIM analysis. LPAs can also be quantified by comparing the actual value of the peak area with the value obtained from the TMS derivative of standard LPA in GC/SIM analysis.

The results confirm that both GC/SIM and GC/MS are suitable analytical methods for the detection of LPAs derivatized with TMS prepared from HECM.

Identification and quantity of LPAs in the embryo-conditioned medium

TMS-LPA-C16:0 prepared from HECM was analyzed by GC/SIM and GC/MS as shown in Fig. 2 The peak of *m/z* 299.1 was detected at Rt 15.31 min corresponding to TMS-LPA-C16:0 (Fig. 2A). The peak of the specific $[M-15]^+$ ion at *m/z* 611.3 derived from LPA-C16:0 was observed at the same Rt of 15.31 min (Fig. 2B), whereas

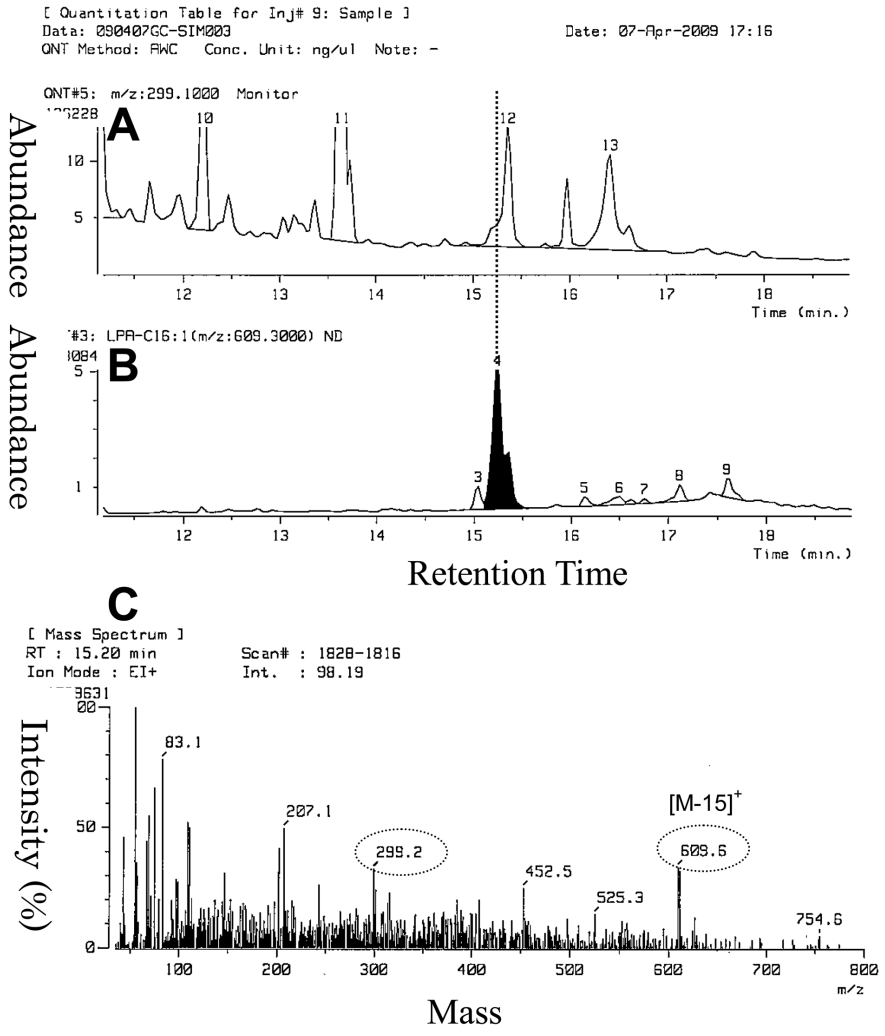

Fig. 3. Detection of LPA-C16:1 in HECM by GC/SIM and GC/MS analysis.
(A) GC/SIM analysis of HECM at *m/z* 299.1. (B) GC/SIM analysis of HECM using the $[M-15]^+$ ion at *m/z* 609.3. (C) GC/MS scanning at 15.20 min of retention time. The characteristic ions at *m/z* 129.1 and 73.1 generated by LPA-C16:1 were barely confirmed by mass spectrometry. LPA, lysophosphatidic acid; HECM, human embryo conditioned medium; GC, gas chromatography; SIM, selected ion monitoring; MS, mass spectrometry; TMS, trimethylsilyl.

$[M-15]^+$ ions obtained from other molecular species of LPAs were not detected. In addition to the $[M-15]^+$ ions at *m/z* 611.5, GC/MS scanning from 35 to 800 also showed the characteristic ions derived from TMS-LPA-C16:0 at *m/z* 73.1, 129.1 and 299.1 (Fig. 2C). These data confirm that LPA-C16:0 existed in the HECM.

By using the same analysis procedure, LPA-C16:1, 18:0, 18:1 and 18:2 in HECM were identified (Figs. 3, 4, 5 and 6). The peaks of ions at *m/z* 299.1 were recognized at Rt of 15.23, 16.58, 16.39 and 16.35 min corresponding to TMS-LPA-C16:1, 18:0, 18:1 and 18:2, respectively (Figs. 3A, 4A, 5A and 6A). The peaks of $[M-15]^+$ ions at *m/z* 609.3, 639.4, 637.3 and 635.3 derived from LPA-C16:0, C16:1, 18:0, 18:1 and 18:2, respectively, were observed at the same Rt as those noted above (Figs. 3B, 4B, 5B and 6B). GC/MS analysis showed three characteristic ions other than the specific $[M-15]^+$ ion of each LPA (Figs. 4C, 5C and 6). In Fig. 3C, the characteristic ions at *m/z* 73.1 and 129.1 were barely observed above the noise, though their peaks appeared with weak intensity in GC/MS scanning.

Actual values of the peak areas of $[M-15]^+$ ions were obtained from GC/SIM analysis of each TMS derivative of LPA-C16:0, 16:1, 18:0, 18:1 and 18:2 in HECM. The

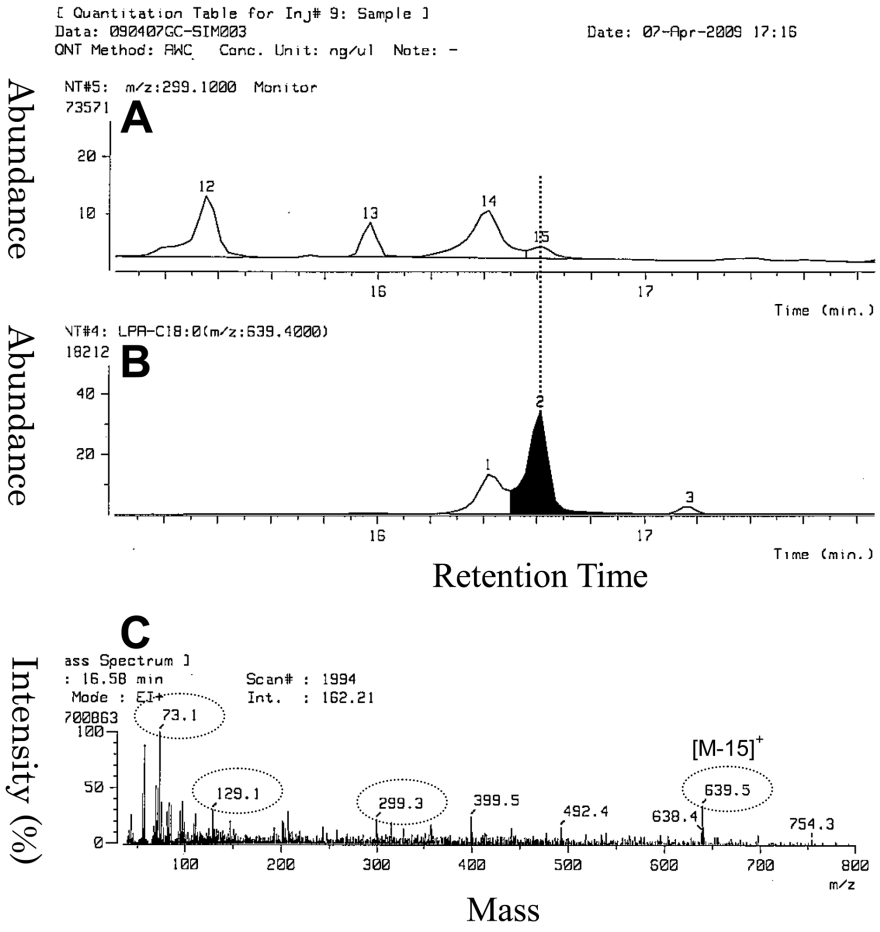

Fig. 4. Detection of LPA-C18:0 in HECM by GC/SIM and GC/MS analysis.
(A) GC/SIM analysis of HECM at *m/z* 299.1. (B) GC/SIM analysis of HECM using the $[M-15]^+$ ion at *m/z* 639.5. (C) GC/MS scanning at 16.58 min of retention time. The four ions at *m/z* 73.1, 129.1, 299.3 (299.1) and 639.5 (639.4) were generated from LPA-C18:0. Numbers in parentheses indicate theoretical mass values equivalent to TMS derivatives of LPA-C18:0. LPA, lysophosphatidic acid; HECM, human embryo-conditioned medium; GC, gas chromatography; SIM, selected ion monitoring; MS, mass spectrometry; TMS, trimethylsilyl.

concentration of LPAs in ng/μl was calculated by comparing each actual value with a value obtained from standard LPA-C18:1. The concentration in nmol/ml was calculated using the molecular weight of each LPA based on the concentration in ng/μl. The concentration of five detected LPAs varied from 0.2 to 7.0 nmol/ml (Table 1).

The $[M-15]^+$ ion showed a value (16.4979) of peak area when 1 μl of the TMS derivatives of standard LPA (equivalent to 2 ng of LPA-C18:1) was injected for GC/SIM analysis. Actual values of peak area of $[M-15]^+$ ions were obtained from GC/SIM analysis of each TMS derivatives of LPA-C16:0, 16:1, 18:0, 18:1 and 18:2 in HECM. Concentrations of LPAs in ng/μl were calculated by comparing the actual value of the $[M-15]^+$ ions with the value of standard LPA. Concentrations in nmol/ml were calculated using each molecular weight of LPA. (The molecular weights of the LPAs are as follows: LPA-C16:0 = 407.2, LPA-16:1 = 405.2, LPA-18:0 = 435.2, LPA-18:1 = 433.2 and LPA-18:2 = 431.2)

Abbreviations: LPAs, lysophosphatidic acids; HECM, human embryo conditioned medium; GC, gas chromatography; SIM, selected ion monitoring.

The medium without embryo culture was analyzed to confirm that the LPAs were produced by the developing embryo in the culture medium. No molecular species of LPA were detected by GC/SIM (Fig. 7). This confirmed that LPA did not exist in the medium before embryo culture (control medium).

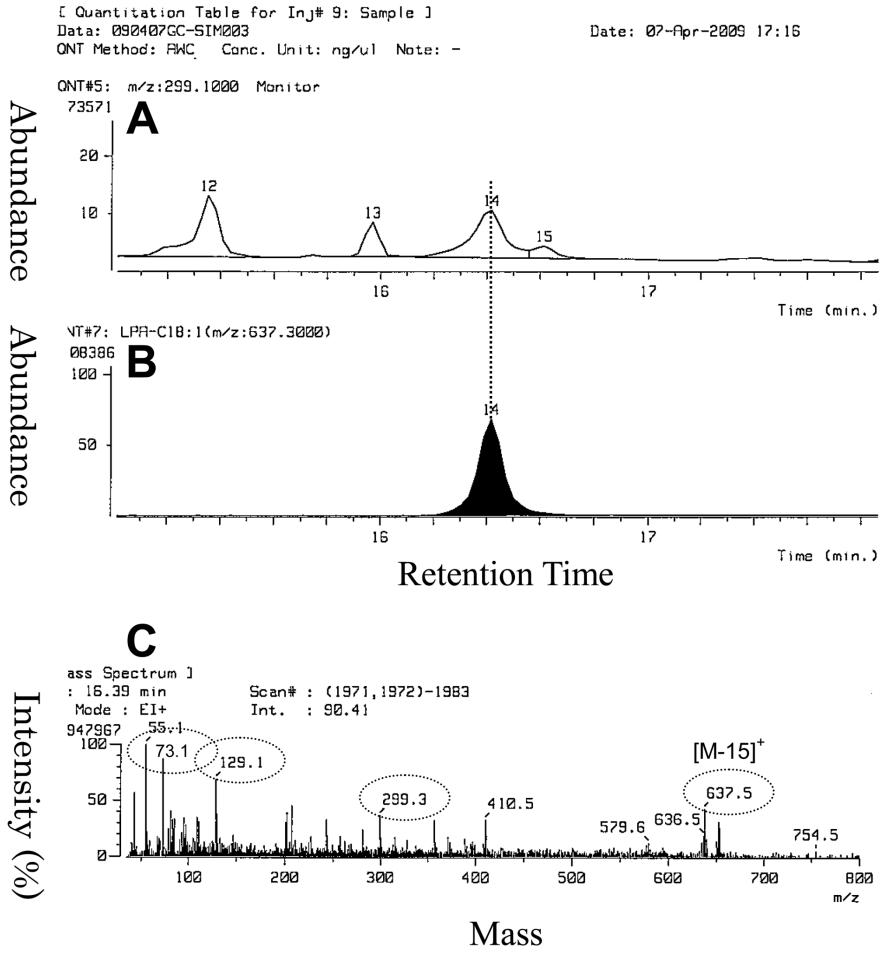

Fig. 5. Detection of LPA-C18:1 in HECM by GC/SIM and GC/MS analysis.
(A) GC/SIM analysis of HECM at *m/z* 299.1. (B) GC/SIM analysis of HECM using the $[M-15]^+$ ion at *m/z* 637.5. (C) GC/MS scanning at 16.39 min of retention time. The four ions at *m/z* 73.1, 129.1, 299.3 (299.1) and 637.5 (637.3) were generated from LPA-C18:1. Numbers in parentheses indicate theoretical mass values equivalent to TMS derivatives of LPA-C18:1. LPA, lysophosphatidic acid; HECM, human embryo-conditioned medium; GC, gas chromatography; SIM, selected ion monitoring; MS, mass spectrometry; TMS, trimethylsilyl.

These results indicate that five molecular species of LPA-C16:0, 16:1, 18:0, 18:1 and 18:2 were produced in the human embryo-conditioned medium.

Discussion

To the best of our knowledge, this study is the first study in which LPA-C16:0, C16:1, 18:0, 18:1 and 18:2 were detected in HECM and their quantities determined. Our findings show that human embryos produce LPA.

In our preliminary study, cytokines such as interleukin-1, interleukin-6 and vascular endothelial growth factor were not detected in HECM using an enzyme-linked immunosorbent assay (data not shown). Thus, we thought that embryonic substances (other than cytokines) that modulate endometrial receptivity may exist in the conditioned medium.

Recent studies have shown that LPA is crucial for the implantation of embryos. Their results also suggest that embryo implantation and spacing are regulated by LPA3-mediated LPA signaling, which influences uterine receptivity in mice [12, 13]. More recently, Achache *et al.* [16] demonstrated that human endometrial expression of LPA3 is significantly lower in patients with repeated implantation failure. However, no study had detected LPA produced by embryos as a ligand of LPA3. LPAs comprise a family of small phospholipid molecules containing a phosphoglycerol backbone and a single fatty acyl

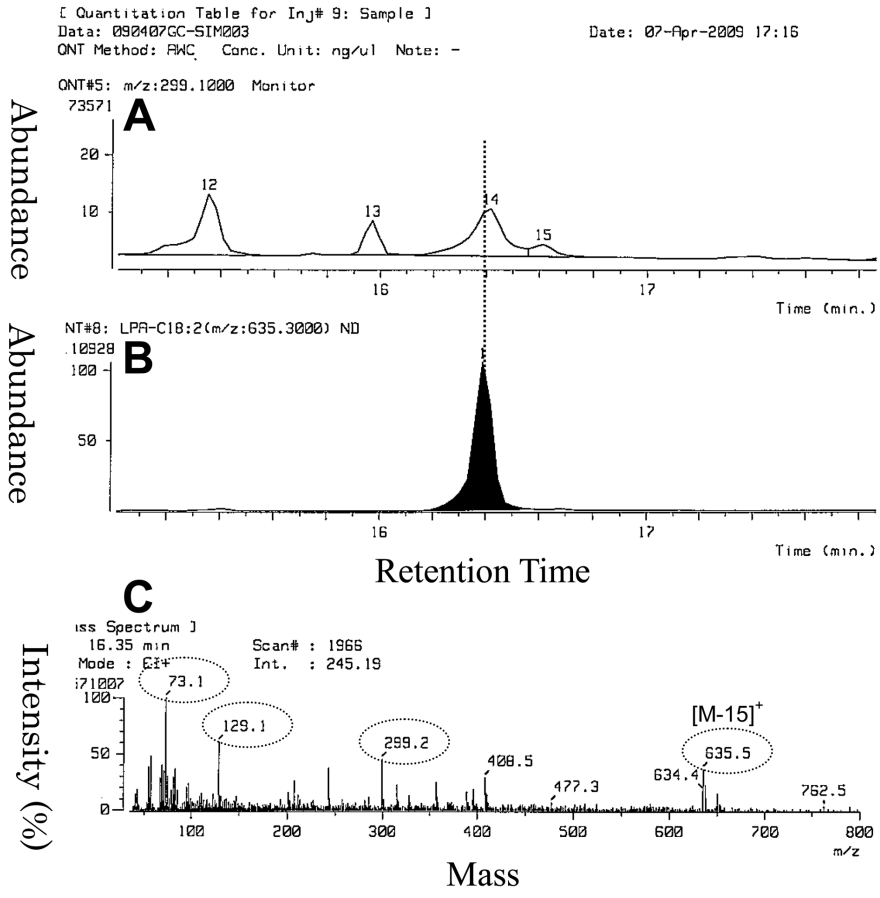

Fig. 6. Detection of LPA-C18:2 in HECM by GC/SIM and GC/MS analysis.
(A) GC/SIM analysis of HECM at *m/z* 299.1. (B) GC/SIM analysis of HECM using the $[M-15]^+$ ion at *m/z* 635.5. (C) GC/MS scanning at 16.35 min of retention time. The four ions at *m/z* 73.1, 129.1, 299.2 (299.1) and 635.5 (635.3) were generated from LPA-C18:2. Numbers in parentheses indicate theoretical mass values equivalent to TMS derivatives of LPA-C18:2. LPA, lysophosphatidic acid; HECM, human-embryo conditioned medium; GC, gas chromatography; SIM, selected ion monitoring; MS, mass spectrometry; TMS, trimethylsilyl.

chain, which varies in its *sn*-1 or *sn*-2 position and in its length and degree of saturation. Recently, Bandoh *et al.* [19] reported that LPA3 could only be activated by unsaturated acyl LPA, and showed that there is an order of magnitude preference for *sn*-2- over *sn*-1-substituted LPA at acyl chain lengths between 16 and 20 carbons. Chan reported that immature mouse dendritic cells migrated preferentially in response to unsaturated LPA, and that LPA3 was important in this response [20]. In the present study, we detected five LPA species that included three unsaturated LPAs: LPA-C16:1, 18:1 and 18:2.

The present study also determined the quantity of LPAs in the embryo-conditioned medium, and found that they were higher than those reported in human plasma by Xiao Y *et al.* [21]. Furthermore, the quantity of LPAs in the embryo-conditioned medium was compatible with the LPA concentration that is needed induce chemotaxis of immature murine dendritic cells [20]. The influence

Table 1. Quantitative analysis of LPAs in HECM by GC/SIM

Molecular species	Value of peak area	Concentration of LPAs	
		(ng/μl)	(nmol/ml)
LPA-C16:0	1210.9626	2.8	7.0
LPA-C16:1	41.9263	0.1	0.2
LPA-C18:0	188.5961	0.4	1.0
LPA-C18:1	487.2762	1.1	2.6
LPA-C18:2	537.2205	1.3	2.9

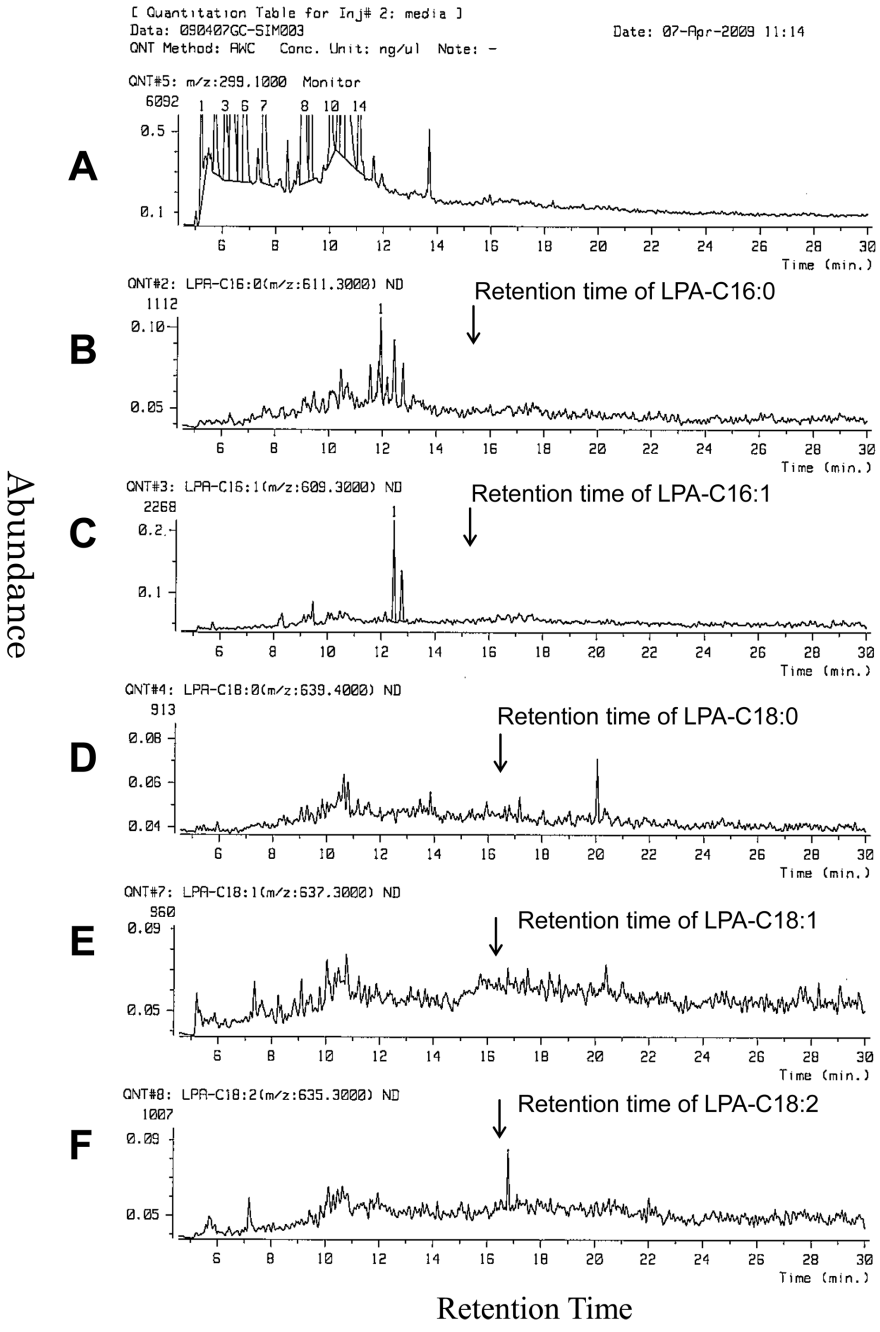

Fig. 7. Detective analysis of LPAs in blank medium (without embryo culture) by GC/SIM.
The medium without embryo culture was treated using the same procedure as that used to prepare TMS derivatives of LPAs. The peak of LPAs generated characteristic ion at *m/z* 299.1 was not detected between 15 to 17 min of retention time (A). No specific $[M-15]^+$ ion derived from LPA-C16:0 (B), 16:1 (C), 18:0 (D), 18:1 (E) and 18:2 (F) was detected by GC/SIM analysis. LPA, lysophosphatidic acid; TMS, trimethylsilyl; GC, gas chromatography; SIM, selected ion monitoring.

of the quantity of LPAs on implantation requires further study.

The results of the present study indicate that the five LPA species including three unsaturated LPAs produced by embryos are candidate embryonic signals to the endometrium for implantation.

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