Examination of lipid profiles in abdominal fascial healing using MALDI-TOF to identify potential therapeutic targets

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ABSTRACT

Background: Failure of fascial healing in the abdominal wall can result in incisional hernia, which is one of the most common complications after laparotomy. Understanding the molecular healing process of abdominal fascia may provide lipid markers of incisional hernia or therapeutic targets that allow prevention or treatment of incisional hernias.

Purpose: This study aims to investigate temporal and in situ changes of lipids during the normal healing process of abdominal fascia in the first postoperative week.

Methods: Open hemicolectomy was performed in a total of 35 Wistar rats. The midline fascia was closed identically for all rats using a single continuous suturing technique. These animals were sacrificed with equal numbers (n=5) at each of 7-time points (6, 12, 24, 48, 72, 120, and 168 h). The local and temporal changes of lipids were examined with mass spectrometry imaging and correlated to histologically scored changes during healing using hematoxylin and eosin staining.

Results: Two phosphatidylcholine lipid species (PC O-38:5 and PC 38:4) and one phosphatidylethanolamine lipid (PE O-16:1, 20:4) were found to significantly correlate with temporal changes of inflammation. A phosphatidylcholine (PC 32:0) and a monosialodihexosylganglioside (GM3 34:1;2) were found to correlate with fibroblast cell growth.

Conclusion: Glycerophospholipids and gangliosides are strongly involved in the normal healing process of abdominal fascia and their locally fluctuating concentrations are considered as potential lipid markers and therapeutic targets of fascial healing.

Introduction

Incisional hernia (IH) is one of the most common complications after laparotomy, caused by failed healing of abdominal fascia [1–3]. The incidence of IH following laparotomy is around 13% within two post-operative years [4] and related costs are estimated at billions of Euros for Europe alone [5,6]. Despite the significant importance of normal fascial healing in preventing IH, few studies have illustrated the temporal healing process, especially on the molecular level, in the early post-surgery period.

Generally, cellular and molecular events proceed sequentially with four stages: haemostasis, inflammation, proliferation, and remodelling. Despite wound healing of virtually all soft tissues passing through these stages, cells and molecules in fascial healing differ from other specific tissues [7]. Previous studies have illustrated that the healing rate of abdominal fascial incisions varies from skin wounds [8–10].

Abbreviations: LPA, Lyso phosphatidic acid; AA, Arachidonic acid; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; GM3, Monosialodihexosylganglioside; LPC, Lyso phosphatidylcholine; PA, Phosphatidic acid; CerPE, Ceramide phosphophylethanoleamine; SM, Sphingomyelin; MMPE, Monomethyl-phosphatidylethanolamine; PI, Phosphatidylinoisitol; CI, Cardiolipin.

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Controversy exists with regard to the healing rate between these two injured tissues. Lemonnier et al. have reported that fascial fibroblasts in culture have longer cell-doubling times, larger cell volume, and higher glucose requirements, compared to dermis fibroblasts [10]. European Hernia Society guidelines recommend the use of slowly absorbable sutures for the closure of abdominal fascial incision versus rapidly absorbable sutures because of the high risk of incisional hernia occurrence when using rapidly absorbable sutures [11,12]. In contrast, sutures supporting skin wounds can be removed within one to two weeks postsurgery. However, Michael and Dubay have concluded that abdominal fascial incisions regain tensile strength faster than the cutaneous wound in a rat model [8,9].

Lipids, acting as signalling molecules, as well as structural components of cell membranes, play a critical role in cell structure and function [13,14]. Lipid mediators can affect the cellular biology related to wound healing. For example, diacylglycerol is essential for cellular processed during wound healing [15]. LPA enhances the reepithelialisation in the early healing stages of the ear skin in a mouse model [16]. AA promotes skin wound healing in in vitro experiments [17], and eicosanoids derived from arachidonic acid may modulate inflammatory reactions during the healing process [18].

Analysis of lipid profiles during wound healing has benefited from recent developments in mass spectrometry [19]. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry imaging (MSI) can analyze and localize lipids directly from their native tissues and enables label-free visualization of lipid spatial distribution [20,21]. Compared to commonly used methods of lipid analysis, like thin-layer chromatography or high-performance liquid chromatography, MALDI-TOF MSI for lipid analysis is less time-consuming while conserving the spatial distribution of molecules [22]. Furthermore, compared to electrospray ionization, MALDI is more sensitive and less affected by impurities [22]. A previous study has employed MALDI-TOF MSI to identify lipids in the skin wound healing process [23].

This study aims to investigate temporal lipid changes correlated with cellular changes during normal fascial healing in the first postoperative week in rats. Rat models have been chosen in this study to mimic human healing as they are genetically similar to humans [24] and it is ethically impossible to isolate functionally healed fascia from patients.

Methods

Animal experiment

A total of 35 adult male Wistar rats (n = 35), weighing 250–300 g, were obtained from Harlan UK Ltd (Bicester, UK). The animal experiment was approved by the Dutch Central Committee (2014–2012), complying with the Dutch Animal Experimental Act. Two animals were housed in one Macro cage (EU type IV, 1800 cm²) filled with hygiene animal bedding with enrichments of cardboard and wooden blocks, fitted with a 12-hour light-night circle and maintained at 25 °C. Animals were permitted to access food (10 mm Sniff rat/mouse sterilized food compressed into pellets) and drink (acidified water) ad libitum. Surgeries were performed in a standard operation room in a randomized manner starting the morning of each operation day. All animals were healthy and did not receive any medication or treatments prior to the experiment. Following at least one week of acclimatization, animals were anesthetized with 2% isoflurane (Isoflo®, Zoetis) and disinfected with 2% iodine solution. Buprenorphine 0.05 mg/kg was administered to all animals prior to the surgical procedures. A midline incision around 5 cm was made on the abdominal skin and then, separately, a 5 cm midline incision was made on the fascia until the peritoneal cavity. After ascending colon resection and anastomosis, the fascia was closed continuously with Vicryl™ 4-0 suture (Ethicon Inc; Johnson&Johnson, Somerville, NJ). Buprenorphine 0.05 mg/kg was subcutaneously applied to all animals for pain relief every 8 to 12 h until no signs of pain were observed. The welfare of all animal was evaluated using a 12 item welfare sheet, including activity, behavior, gait, posture, physical condition, fur/skin, hydration, breathing, faeces/urine, surgical wound, edema, and necrosis. At each of seven time points (6, 12, 24, 48, 72, 120, and 168 h), five animals were sacrificed by cardiac puncture with complete blood draw under anaesthesia. Buprenorphine 0.05 mg/kg was subcutaneously administered to all animals half an hour prior to sacrifice. All animals received the identical surgical procedure. Samples from rats, including fascial incision, sutures and surrounding muscle, were immediately snap frozen with liquid nitrogen and stored at −80 °C until measurement.

Sample preparation

A cryo-microtome (Leica CM 1860 UV, Leica Biosystems, Wetzlar, GE) was pre-equilibrated to −20 °C more than half an hour before cutting. Samples were sectioned at 10 µm thickness and thaw mounted on pre-cooled Indium Tin Oxide coated (ITO) slides. Slides were dried in desiccator for 15 min prior to matrix application.

Twelve layers of 7 mg/ml norharmane matrix solution (2: 1 chloroform/methanol (v: v)) were applied to the tissue sections using a HTX TM-sprayer (HTX Technologies, Chapel Hill, NC) at a 30 °C nozzle temperature and 0.120 mL/min flow rate. Norharmane and chloroform were obtained from Sigma Aldrich (Zwijndrecht, the Netherlands). Methanol was obtained from Biosolve BV (Valkenswaard, the Netherlands).

MSI data acquisition

All sections were measured with Bruker Rapiflex MALDI-TOF/TOF Tissueutyper (Bruker Daltonik GmbH, Bremen, Germany) in reflector mode, in both ionization modes scanning a mass range of m/z 400–2000 at pixel size of 50 µm × 50 µm. First, tissue sections were analysed using a 20 µm × 20 µm scan pixel area with a 50 µm × 50 µm spacing in the negative ionization mode without offset, and subsequently, measured in the positive ionization mode with an offset of 20 µm × 20 µm, using the same spacing. Red phosphorus spots were pipetted on each individual slide to calibrate the instrument. Calibration was conducted prior to each analysis.

Two consecutive sections from a 120-hour sample with sufficiently different cell types were chosen to perform high spatial resolution (10 µm) mass spectrometry imaging experiments in both ionization modes, separately, using the same matrix application and MSI instrument as mentioned above. The scan area measured 8 µm × 8 µm per pixel.

MALDI-MS/MS lipid identification was performed on selected consecutive sections from the 120-hour group using a Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to a MALDI source (Spectro-glyph, Kennewick, USA). MS1 spectra in both ionization modes were scanned over a mass range of m/z 400–2000 with 550 ms of injection time. MS2 data of seven masses was acquired at 120,000 mass resolution while moving the stage continuously, with 30 scans averaged for each precursor. The normalized collision energy ranged from 20 to 30 eV with an isolation window of ± 0.7 Da. The matching of MS1 values and MS2 fragments (when available) for lipid assignments was conducted with the online ALEX123 database (http://alex123.info/ALEX123/MS.php), using a mass tolerance of 3 ppm and 5 ppm, respectively.

Histological staining after MSI

All measured tissue sections were stained with haematoxylin and eosin (H&E) and mounted with coverslips. After air-drying overnight, digital H&E image was recorded using a Mirax digital slide scanner (Zeiss, Jena, Germany). The inflammatory score and fibroblast score were determined by an experienced pathologist of Maastricht University (M.G.) without knowledge conditions to which the tissues were subjected. The microscopically assessed inflammation and fibroblast scores

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were described using a 0–5 scale (0, not present; 1, slightly present; 2, more than slightly present; 3, moderately present; 4, more than moderately present; 5, abundantly present). The inflammation scores refer to the total amount of granulocytes and macrophages. The histological scores were tested with linear regression or generalized linear model, when applicable. Statistical analysis of histological scores was conducted using IBM SPSS Statistics for Windows (version 25.0, Armonk, NY, USA, IBM Corp.).

**MSI data processing**

Recalibrations for both the negative and positive ionization mode datasets were performed using FlexAnalysis v3.4 (Bruker Daltonik GmbH, Bremen, Germany) in linear correction mode using m/z 885.6 and m/z 782.6 as calibrants, respectively. H&E images were co-registered with the MSI data using FlexImaging 4.1 software (Bruker Daltonik GmbH, Bremen, Germany), and connective tissues in the fascial incision and adjacent partial muscles were annotated with histological structure. After recalibration, co-registration, and annotation, all data were imported into the SCiLS Lab 2019b (SCiLS GmbH, Bremen, Germany) and root mean square normalized.

The average spectra from each dataset was exported to mMass 5.5.0 for peak-picking with the following parameters: (1) signal-to-noise (S/N) ratio of 7.0, peak-picking height of 75 and 90 for positive and

![Fig. 1. Representative MSI and H&E images. Visualization of m/z 722.5 PE O-16:1 20:4 at different time points in negative ionization mode (A). Magnifications of two representative tissue sections from 6-hour (B) and 168-hour group (C). Co-registered, hematoxylin and eosin stained (H&E) images with yellow lines representing MALDI-TOF scanned regions and green dashed lines representing regions of fascial incision (D and E). Average spectra in negative ion mode and magnification at mass range m/z 700–810 (F).](image-url)
negative ionization mode, respectively; (2) baseline correction precision of 35 for both positive and negative ionization mode with relative offset at 0; (3) Deisotoping: maximum charge of 1, isotope mass tolerance of 0.1 m/z, isotope intensity tolerance of 70%, and isotope mass shift of 0.0. Linear regression analysis ($P$ value threshold = 0.05) was performed in R (v3.5) to model the relationship between the intensity of any m/z species and the healing time. P-values were corrected for multiple testing using the Benjamini-Hochberg method.

Pearson correlation analysis (threshold ≥ 0.4) among different annotated cell regions (typical clusters of smooth muscle cells, granulocytes and macrophages, and fibroblasts) in 10 μm high spatial resolution MSI datasets were conducted using the significant masses we found in the linear regression analysis to obtain m/z species co-localised to inflammation and fibroblast regions.

Results

Animal experiment

No complications, including dehiscence of the fascial incision and skin wound, anastomotic leakage, and wound infection, were identified after surgery in 34 out of 35 rats. One rat from the 120-hour group developed ileus, which potentially can affect normal fascial healing negatively because of increased abdominal pressure, and was subsequently excluded from further MSI and histological analysis.

Linear regression analysis for 50 μm spatial resolution MSI data

Representative MSI and H&E images are displayed in Fig. 1. A total of 33 MSI negative mode MSI datasets and 31 MSI positive mode datasets were included for MSI data analysis. Three samples were excluded from further MSI data analysis, including one sample from the 168-hour group in both negative and positive ionization MSI data, one from the 48-hour group and one from the 72-hour group in positive ionization mode due to sample contamination. On average, 174 and 176 of the species were identified using linear regression analysis to identify consistent trends over time ($P$ value threshold = 0.05) in negative and positive ionization modes, respectively. A total of 18 and 35 of the m/z species were identified using linear regression analysis to identify consistent trends over time ($P$ value threshold = 0.05) in negative and positive ionization modes, respectively (Supplementary Figure 1). Representative m/z values identified with a temporal increase and decrease are presented in Fig. 2. The identification of these m/z species are shown in Supplementary Table 1.

Statistical analysis of histological score data

The inflammation scores and the fibroblast score increased significantly over time using linear regression (slope, 0.017, $P < 0.001$) and generalized linear model ($P < 0.001$), respectively (Fig. 3).

Correlation coefficient analysis based on 10 μm MSI data

A total of eight out of 53 of the m/z species were found to correlate with inflammation scores or fibroblast cells with a correlation coefficient of at least 0.4. All of those eight m/z signals, except m/z 838.7, were identified using high-mass resolution MS1 and MS2 experiments, leaving five unambiguously identified lipids, comprising three PC lipid species (PC 32:0, PC O-38:5 and PC 38:4), one PE lipid (PE O-16:1 20:4) and one sphingolipid (GM3 34:1:2). Tables 1 and 2 show the lipid identifications in detail. All MS/MS spectra are shown in Supplementary Figs. 3-9. Two PC lipid species (PC O-38:5 and PC 38:4) and a PE lipid (PE O-16:1 20:4) were correlated with inflammation scores. PC 32:0 and GM3 34:1:2 were found to correlate with fibroblast cells. Representative MSI images of PE O-16:1 20:4 and PC O-38:5 in negative and positive ionization mode are shown in Fig. 4. MS1 images of the other three lipids are shown in Supplementary Figure 2.

Discussion

Molecular changes in abdominal fascia caused by surgical injury are essential for fascial regeneration [25,26]. Due to very limited comparability within previously published animal models mimicking incisional hernia in patients [27], revealing the molecular changes in normal fascial healing over time could provide a better understanding of IH. This study illustrates the temporal changes of lipids correlated to inflammatory response and fibroblast growth in acutely injured fascia. These lipids are potential lipid markers of IH in further animal IH models, or therapeutic targets to promote fascial healing and prevent IH.

Virtually all wound healing passes through similar healing stages [28]. In skin wound healing, the inflammatory response begins within a few hours after an acute injury, and the proliferative stage occurs from 4 to 21 days [29]. In our study, focused on fascial healing, the inflammation score increased over time and fibroblast proliferation started to increase at the middle of the first postoperative week. This indicates that the healing stages of inflammation and proliferation in the early fascial healing are comparable to skin wound healing stages. At the molecular level, several studies have revealed growth factors [30,31], chemokines [32], cytokines [31,33], and various phospholipid membrane-derived lipid mediators that are involved in the skin wound healing process, including LPA [34], sphingolipids [35], and eicosanoids [36]. Still, much remains unknown about the molecular mechanism of fascial healing. It is reported that an abdominal fascial incision heals separately from the adjacent skin wound [8]. Dubay et al. have demonstrated that abdominal fascial fibroblasts have a significant increase in cell proliferation compared to dermis fibroblasts in a seven-day cell culture [9]. Swarming-like collective cell migration of fibroblasts is exclusive and requires N-cadherin upregulation in fascia, which is lacking in upper skin wound healing [37]. Therefore, the underlying molecules activated during inflammation and proliferation stages during fascial healing should be different from skin wound healing.

The important role of lipids in initiating an acute inflammatory response is widely acknowledged [38]. PCs are one of the most abundant biological components in cell membrane, belonging to a class of phospholipids with choline as a head group [39,40]. PE can be converted to PE through PE methyltransferases and is involved in the biosynthesis of PC [41,42]. PC and PE, as two major phospholipids, are key players in

Fig. 2. Representative m/z values identified with a temporal increase and decrease. The x-axis represents time in hours, and the y-axis represents the relative intensity of the m/z species.
cell survival and development. Abnormal variation of PC, PE, or the PC/PE ratio can influence the energy metabolism of organelles, and is linked to various pathological progressions [43]. In our study, the two PC lipid species (PC O-38:5 and PC 38:4) and PE O-16:1_20:4 increased significantly during the first postoperative week, in parallel with an increase in the inflammation score during the normal fascial healing. The correlation coefficients of these lipid species that correlate with inflammation scores exceeded 0.4, suggesting that these lipids are indicators of the inflammatory response in fascial healing. Furthermore, previous evidence has shown that exogenous PC treatments can promote mucosal healing in ulcerative colitis in patients [44,45] and may promote surgical wound healing [46]. PCs can be isolated from natural sources or obtained using synthetic or semi-synthetic methods and have excellent biocompatibility [47]. PCs correlated to the inflammatory response in our findings could be applied directly to promote fascial healing in the early postoperative period, although further studies are needed.

Fibroblasts contribute significantly to wound healing, creating extracellular matrix components and contracting the wound [48]. Lipid signalling can affect fibroblast proliferation [49,50]. The plasma membrane of fibroblasts contains micrometer-scale patches enriched with sphingolipids that can metabolise to signalling molecules regulating cell survival and proliferation [51,52]. Prostaglandin F2α can stimulate fibroblast proliferation via the prostaglandin receptor, separately from growth factor-beta [53]. In our study, PC 32:0 and GM3 34:1;2 were linked to temporal changes of the fibroblasts, indicating that these two lipids could be fibroblast biomarkers in fascial healing. Additionally, a study has demonstrated that proliferating fibroblasts prefer exogenous lipids as the source of membrane lipids rather than de novo synthesis [54]. GM3 analogues can be synthesized conveniently from commercially available substances [55]. Application of PC 32:0 or GM3 34:1;2 could be a novel strategy to promote fibroblast growth in fascial incision, although further research is needed. It should be noted that GM3, belonging to gangliosides, is involved in mediating growth factor signalling in human skin. GM3 is observed to be a driver of impaired wound

Table 1
Four m/z species were correlated to temporal changes in inflammation.

| Observed m/z by TOF | Pearson correlation coefficient | Observed m/z by Orbitrap | Lipid assignment | MS experiment level for ID | Ion mode | Mass error (in ppm) |
|-------------------|-----------------------------|--------------------------|------------------|---------------------------|----------|-------------------|
| 722.5             | 0.57                        | 722.5146                 | PE O-16:1_20:4-H<sup>+</sup> | MS2 | NEG | +2.2 |
| 794.7             | 0.66                        | 794.6037                 | PC O-38:5 + H<sup>+</sup> | MS2 | POS | -2.7 |
| 832.6             | 0.46                        | 832.5811                 | PC 38:4 + Na<sup>+</sup> | MS2 | POS | -1.9 |
| 838.7             | 0.46                        | /                        | /                | /             | POS | /    |

Table 2
Four m/z species were correlated to temporal changes in fibroblast score.

| Observed m/z by TOF | Pearson correlation coefficient | Observed m/z by Orbitrap | Lipid assignment | MS experiment level for ID | Ion mode | Mass error (in ppm) |
|-------------------|-----------------------------|--------------------------|------------------|---------------------------|----------|-------------------|
| 734.6             | 0.80                        | 734.5685                 | PC 32:0 + H<sup>+</sup> | MS2 | POS | -1.3 |
| 756.6             | 0.81                        | 756.5505                 | PC 32:0 + Na<sup>+</sup> | MS2 | POS | -1.2 |
| 772.6             | 0.75                        | 772.5236                 | PC 32:0 + K<sup>+</sup> | MS2 | POS | -2.2 |
| 1151.7            | 0.45                        | 1151.7059                | GM3 34:1:2-H<sup>+</sup> | MS2 | NEG | +0.0 |

Fig. 3. A, representative H&E images at each time point. B, inflammation and fibroblast scores at each time point. Bar plot, mean ± standard deviation.
healing, and depletion of GM3 can result in improved wound healing in diabetic mice [56,57]. However, in our studies, GM3 34:1;2 increased over time, positively linked to an increase in fibroblasts. This could be explained by tissue-specific involved molecules that differ between fascial healing and skin wound healing.

Animal models have intrinsic limitations. However, a previous study has demonstrated the molecular comparability between rats and humans regarding healing and scarring using a gene expression comparison [24]. Furthermore, regarding an IH model, histological results between rats and humans are comparable [58]. Therefore, it is possible that the present results could be translated to humans, however further in vivo human confirmation and validation would be necessary.

Conclusion

In summary, five lipids, including PE O-16:1_20:4, PC O-38:5, PC 38:4, PC 32:0, and GM3 34:1;2, were found to be significantly involved in fascial healing and have the potential to be lipid markers and/or therapeutic targets for the prevention of IH in patients, although further studies evaluating the functional effect of these lipids in fascial healing are needed.

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Ethical approval

The Dutch Animal Experimental Committee of Maastricht University approved the animal experiment in this study under project proposal 2014–120.

Informed consent

Not applicable

Author contributions

Hong Liu and Jianhua Cao have contributed equally to this work and are shared first authors.

All authors meet the criteria based on the International Committee of Medical Journal Editors (ICMJE).

Declaration of Competing Interest

None of the authors has any conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2021.06.002.

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Fig. 4. Representative MSI images of [PE O-16:1_20:4-H]~(A) and [PC O-38:5 + H]~(B) with weak denoising at 10 µm × 10 µm spatial resolution in negative and positive ionization mode, respectively. Magnification of these tissue sections, and their co-registered hematoxylin and eosin stained (H&E) images, are shown in C and D. Representative inflammation regions are indicated in yellow.
