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Interstitial Fluid Sphingosine-1-Phosphate in Murine Mammary Gland and Cancer and Human Breast Tissue and Cancer Determined by Novel Methods

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Abstract The tumor microenvironment is a determining factor for cancer biology and progression. Sphingosine-1-phosphate (S1P), produced by sphingosine kinases (SphKs), is a bioactive lipid mediator that regulates processes important for cancer progression. Despite its critical roles, the levels of S1P in interstitial fluid (IF), an important component of the tumor microenvironment, have never previously been measured due to a lack of efficient methods for collecting and quantifying IF. The purpose of this study is to clarify the levels of S1P in the IF from murine mammary glands and its tumors utilizing our novel methods. We developed an improved centrifugation method to collect IF. Sphingolipids in IF, blood, and tissue samples were measured by mass spectrometry. In mice with a deletion of SphK1, but not SphK2, levels of S1P in IF from the mammary glands were greatly attenuated. Levels of S1P in IF from mammary tumors were reduced when tumor growth was suppressed by oral administration of FTY720/fingolimod. Importantly, sphingosine, dihydrosphingosine, and S1P levels, but not dihydro-S1P, were significantly higher in human breast tumor tissue IF than in the normal breast tissue IF. To our knowledge, this is the first reported S1P IF measurement in murine normal mammary glands and mammary tumors, as well as in human patients with breast cancer. S1P tumor IF measurement illuminates new aspects of the role of S1P in the tumor microenvironment.

Keywords Cancer · Endothelial cells · Interstitial fluid · Mass spectrometry · Sphingolipids · Sphingosine-1-phosphate

Introduction

The tumor microenvironment is a determining factor in cancer biology and progression [1]. Although it has been long known that the lymphatic system is the initial pathway for metastasis in many cancers including mammary cancer, recent findings

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suggest new mechanisms for how cancer cells gain access to the lymphatic system and how they manipulate their microenvironment to establish metastasis. An increasing number of proteins in the tumor microenvironment are now known to play important roles in tumor progression [2–4]. Interstitial fluid that bathes the tumor and stromal cells is considered as an important part of the tumor microenvironment not only as the initial route of metastasis, but also as a supplier of factors that promote tumor metastasis.

Sphingosine-1-phosphate (S1P) is a potent bioactive signaling molecule that regulates many physiological and pathological processes involved in immune cell trafficking, inflammation, vascular homeostasis, and cancer progression [5–8]. S1P is generated by sphingosine kinases (SphK1 and SphK2), and is then secreted, exerting its functions by binding to five specific G protein–coupled receptors (S1PR1–5) in autocrine, paracrine, and/or endocrine manners, a process known as "inside-out" signaling [9–11]. "Inside-out" signaling refers to the process by which S1P produced inside cells is secreted by transporters and signals through its receptors on the outside of cells. The "inside-out" signaling of S1P plays important roles in cancer cell pathophysiology [12]. Though we have shown that SphK1 is the significant contributor to extracellular S1P while SphK2 contributed to intracellular S1P of mammary cancer cells [12], to date the relative contribution of each SphK to secreted S1P has never been definitively demonstrated in an in vivo setting.

Recently studies from our laboratory have demonstrated that S1P produced by SphK1 in cancer cells promotes mammary cancer progression by stimulating angiogenesis, lymphangiogenesis, and subsequently lymph node metastasis [13], We have also shown that S1P produced by up-regulation of SphK1 and subsequent activation of the S1PR1 receptor play an essential role in maintaining persistent activation of the important transcription factors NF-kB and Stat3 in a feedforward amplification loop that links chronic inflammation and colitis associated carcinogenesis [14]. Despite this emerging understanding of importance of S1P in cancer cell signaling, the role of S1P in the tumor microenvironment, particularly in the interstitial fluid (IF), remains unclear. This is in part because of difficulties presented by collecting and analyzing IF, a barrier that once surmounted, is expected to provide important insights into the tumor microenvironment and how tumors develop and respond to therapy.

Here we introduce simple and reproducible methods to measure the levels of sphingolipids including S1P in small volume of interstitial fluid from healthy mammary glands and tumor using a modified centrifugation method combined with liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). Using our new method, we are able for the first time to demonstrate the contributions of SphK1 and SphK2 to secreted S1P in vivo, and have been able to provide definitive evidence that S1P is increased in breast tumor interstitial fluid and that this increase is ameliorated by treatment with the prodrug FTY720, concomitantly with suppression of tumor growth.

Materials and Methods

Reagents

Internal standards were purchased from Avanti Polar Lipids (Alabaster, AL) and added to samples in 20 μl ethanol:methanol:water (7:2:1) as a cocktail of 500 pmol each. The HPLC grade solvents were obtained from VWR (West Chester, PA).). FTY720 was from Cayman Chemical Company (Ann Arbor, MI).

Animals

All animal studies were conducted in the Animal Research Core Facility at VCU School of Medicine in accordance with institutional guidelines. Experiments without breast tumor implantation utilized SphK1$^{-/-}$ and SphK2$^{-/-}$ mice since they are well characterized, and because we have previously found that SphK2$^{-/-}$ mice demonstrate compensatory higher expression of SphK1 in the tissues [14]. These knockout mice were kindly provided by Dr. Richard L. Proia of National Institute of Diabetes and Digestive and Kidney Diseases [15, 16]. We obtained each knockout mouse with littermate WT from heterozygous parents. Experiments with 4T1 breast tumor implantation, used syngeneic Balb/c female mice at 8–10 weeks of age (Harlan, Indianapolis, IN).

Tumor Growth

4T1-luc2 cells, a mouse mammary gland derived adenocarcinoma cell line that has been engineered to express luciferase (Caliper Life Sciences, Perkin Elmer, Waltham, MA), were cultured in RPMI Medium 1640 with 10 % fetal bovine serum. 4T1-luc2 cells (1×10^5 cells in 10 μl RPMI) were implanted in the 2nd chest mammary gland under direct vision as previously described [13, 17]. The tumor burden of 4T1-luc2 cell tumors was determined by measurement of bioluminescence with the IVIS Imaging System (Xenogen, Perkin Elmer). Where indicated, tumor-bearing mice were randomized 2 days after implantation into two treatment groups treated with saline or FTY720 (p.o. 1 mg/kg/day).

Human Tissue Samples from Patients with Breast Cancer

Breast cancer tissue samples were collected from 7 patients who had invasive tumors larger than 1.5 cm and underwent surgical resection in Niigata University Medical and Dental

Hospital. This study protocol was approved by the Institutional Review Board of Niigata University Medical and Dental Hospital, and informed consent was obtained from all the patients. Cancerous tissue, peri-tumor normal breast tissue and normal breast tissue distant from the cancer were collected from surgical specimens immediately after operation, excised and frozen in liquid nitrogen. Peri-tumor normal breast tissue was defined as tissue within 1 cm from the gross edge of tumor, and distant from tumor was defined as tissue more than 2 cm from the gross edge of tumor. All tissue samples were stored at −80 °C.

Construction of the IF Collection Tube

Based on the previous "nylon basket" approach to the collection of IF developed by Wiig et al., [3, 18, 19] an IF collection tube was constructed by gluing Spectrum/Mesh nylon filters (20 μM mesh, 55 μM thick, Spectrum Labs. Inc., Rancho Domingguez, CA) to the bottom of Wizard Minicolumn Inserts (Promega, Madison, WI) after removing the original bottom filter. These were then placed on top of the spin columns (Fig. 1).

Collection of IF from Tissues

Animals were sacrificed by exsanguination, blood was collected, and tissues were harvested for IF collection by an established method [3, 18, 19] with some modifications. Briefly, tissue was excised, blotted gently, and placed in pre-weighed tubes on ice. Tubes were re-weighed to determine tissue weight and the tissue was sectioned several times with scissors. The samples were then transferred into the inserts with nylon mesh, and placed into the pre-weighed centrifuge tubes. The tubes were centrifuged at 106 × g for 10 min at 4 °C and the IF accumulated below the filter. The volume of IF was quantified by weight. PBS containing phosphatase inhibitors (100 μl) was added to the IF and the tubes were centrifuged at 1000 × g for 10 min at 4 °C to remove any contaminating cells (Fig. 1).

Cell Counting and Immunoblot

Cell numbers were determined in IF without centrifugation, after centrifugation at 106 × g, 1000 × g, 10,000 × g by counting the numbers under microscopy. Protein from lymph node (LN) tissue extracts or from IF were quantified by western blotting with anti-actin antibody and stained with Ponceau S to visualize proteins. Densitometry of the blot was assessed using Image J software, and the relative level of actin was normalized with equal protein amount of LN and IF.

Quantitation of Sphingolipids by LC-ESI-MS/MS

Lipids were extracted from IF, blood, or tissue samples and sphingolipids were quantified by liquid chromatography, electrospray ionization-tandem mass spectrometry (LC-ESIMS/MS, 4000 QTRAP, ABI) as described previously [12, 20, 21].

Statistical Analysis

Results were analyzed for statistical significance with a two-tailed Student's t-test, with $P < 0.05$ considered significant. Experiments were repeated at least three times with consistent results.

Results

An Improved Method to Collect IF from Tissues

Although it has been suggested that S1P levels are relatively low in IF compared to cells, this has not been verified experimentally [22]. We modified an established method by Wiig et al. [3, 18, 19] and developed a new IF collection tube to enable efficient collection of IF from small tissue samples for sphingolipid measurements (Fig. 1). The recovery of IF was low from tissues weighing less than 200 mg, while the volume of IF collected was proportional to the weight of tissues weighing more than 200 mg (Fig. 2a). To protect S1P from degradation, buffer containing phosphatase inhibitors was added to the IF, and a subsequent centrifugation at 1000 × g was used to remove contaminating cells (Fig. 2b). To examine whether collected IF contained cells or components of broken cells, 10 μg of protein in IF from lymph node tissue and the same amount of protein extracted from lymph node tissue were separated by SDS-PAGE and immunoblotted with an antibody to actin, the major intracellular protein. Actin was barely detectable in the IF (Fig. 2c). Densitometric analysis of the actin band revealed that the IF contained less than 0.3 % of the actin protein as compared to the same amount of protein extracted from lymph node tissue (Fig. 2c). Repeated analyses of IF samples demonstrated minimal variation (i.e. tight error bars), also indicating low contamination.

Effect of Deletion of SphK1 or SphK2 on Sphingolipid Levels in Blood

As we have an interest in investigating the level and function of S1P in the various fluid compartments of the body, we initially investigated the different contributions of SphK1 and SphK2 to S1P levels in whole blood and serum using knockout mice. In agreement with previous reports [15, 23–25], levels of S1P and dihydro-S1P (DHS1P) in blood as well as in serum of SphK1$^{-/-}$ mice were lower than those

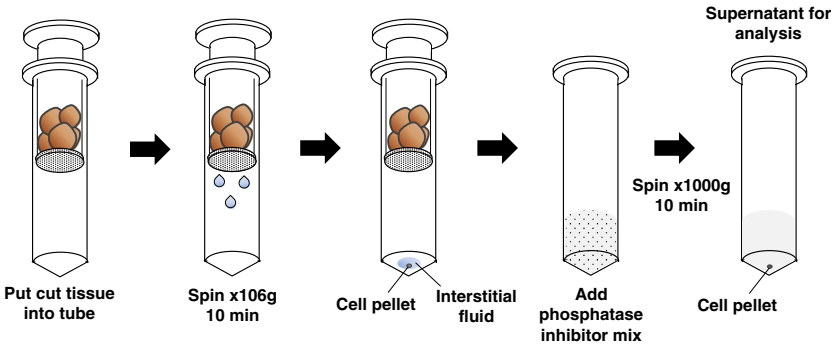

Fig. 1 Scheme for the isolation of IF from tissues. Excised tissue was placed inside pre-weighed inner tubes with nylon mesh. The tubes were centrifuged at 106 × g for 10 min at 4 °C allowing IF accumulation in the bottom of the tube together with a very small cell pellet. After weighing to determine the volume of IF, phosphatase inhibitor mix was added and the tubes were centrifuged at 1000 × g for 10 min at 4 °C to remove any contaminating cells

found in wild type (WT) littermates (Fig. 3a and 3c). In contrast, and in agreement with others [14, 26], S1P and DHS1P levels in blood as well as serum of SphK2$^{-/-}$ mice were higher than those of their WT littermates, most likely due to compensatory up-regulation of SphK1 that produces S1P and DHS1P in the SphK2$^{-/-}$ animals [14] (Fig. 3b and 3d). Hence, SphK1, rather than SphK2, appears to contribute to the S1P levels in whole blood and serum. Levels of sphingosine (Sph) and dihydro-Sph (DHSph) in blood and serum are much lower than the phosphorylated sphingoid bases in both knockouts and WT mice, as were previously reported [21, 26–29].

S1P Levels Are Higher in Mammary Gland IF than in Mammary Gland Itself

Next, it was of interest to examine the contribution of SphK1 and SphK2 to levels of bioactive sphingolipids in IF compared to the tissue it was collected from. We examined their levels in IF from mammary gland compared to the tissue itself. S1P and DHS1P levels in mammary glands were much lower than those of Sph and DHSph and there were no major differences in S1P levels between the SphK1 knockouts and their littermate controls (Fig. 4a), yet there were significant decreases in levels of DHS1P and Sph in SphK1$^{-/-}$ mice. S1P levels were slightly increased in SphK2 knockout mice compared to their littermate controls, while levels of Sph were not changed (Fig. 4b). Importantly, substantial concentrations of S1P and DHS1P were found in IF from mammary glands, which were approximately 10-fold higher than those in the tissue. Moreover, deletion of SphK1 greatly reduced levels of both phosphorylated sphingoid bases as well as Sph and DHSph (Fig. 4c). In contrast, deletion of SphK2 did not affect their levels in IF significantly (Fig. 4d).

Levels of Bioactive Sphingolipids in Breast Tumor IF Correlate with Tumor Growth

We previously showed in a syngeneic mouse breast cancer model in which 4T1-luc2 murine mammary cancer cells were orthotopically implanted into the chest mammary glands of

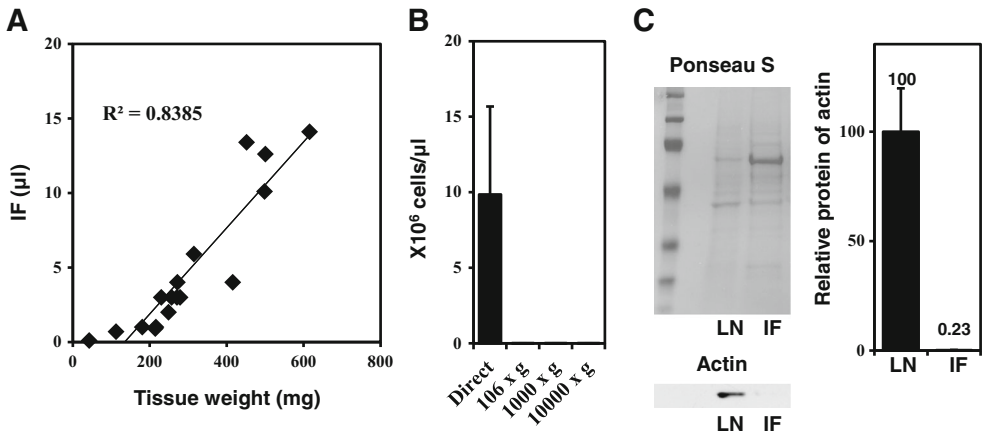

Fig. 2 Collection of interstitial fluid (IF) from tissues. **a** IF was collected from different amounts of 4T1 breast tumor tissue. Correlation between the tumor tissue weight and the volume of IF collected is shown. **b** Removal of contaminating cells from IF. Cell numbers in IF were determined after centrifugation as indicated. **c** 10 µg of protein from lymph node tissue extracts (LN) or from IF were analyzed by western blotting with anti-actin antibody (lower panel) and stained with Ponceau S to visualize proteins (upper panel). Densitometry of the blot was assessed using Image J software, and the relative level of actin was normalized with equal protein amount of LN and IF

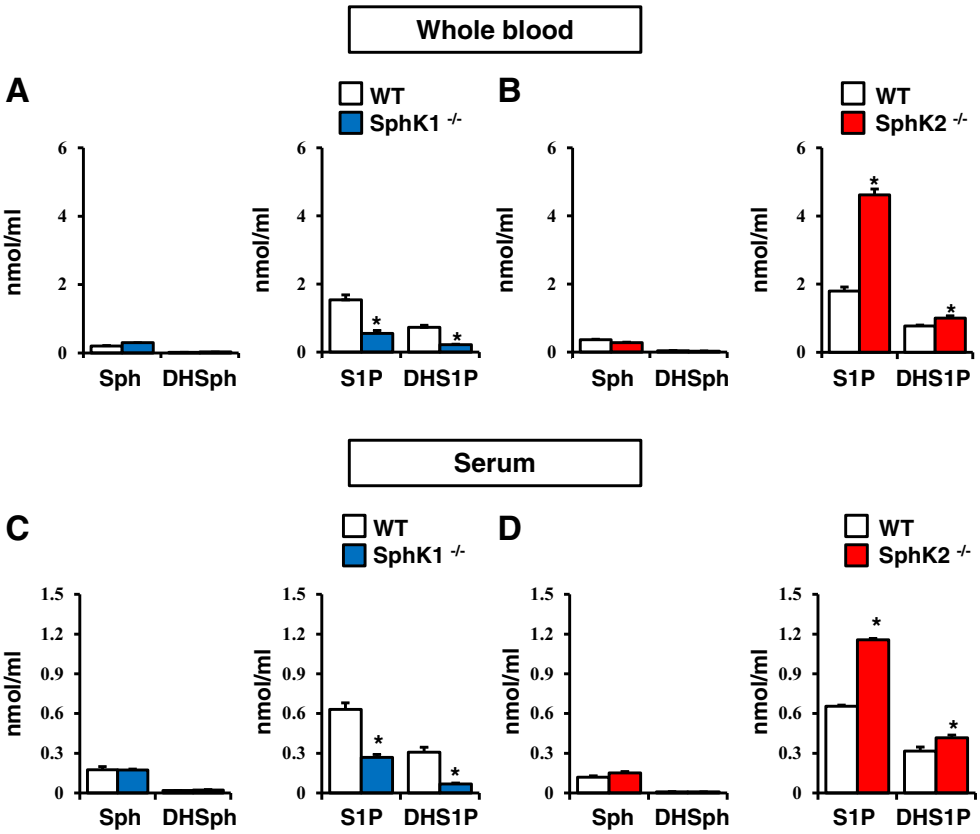

Fig. 3 Levels of sphingolipids in blood and serum from SphK1$^{-/-}$ and SphK2$^{-/-}$ mice and littermate wild type mice. Levels of sphingosine (Sph), dihydro-Sph (DHSph), S1P, and dihydro-S1P (DHS1P) in blood (**a, b**), and serum (**c, d**) from 2 month old SphK1$^{-/-}$ mice (*blue bars*), SphK2$^{-/-}$ (*red bars*), and their respective WT littermates (*white bars*) were determined by LC-ESI-MS/MS. Mean ± SEM ($n = 4$). *, $P < 0.05$

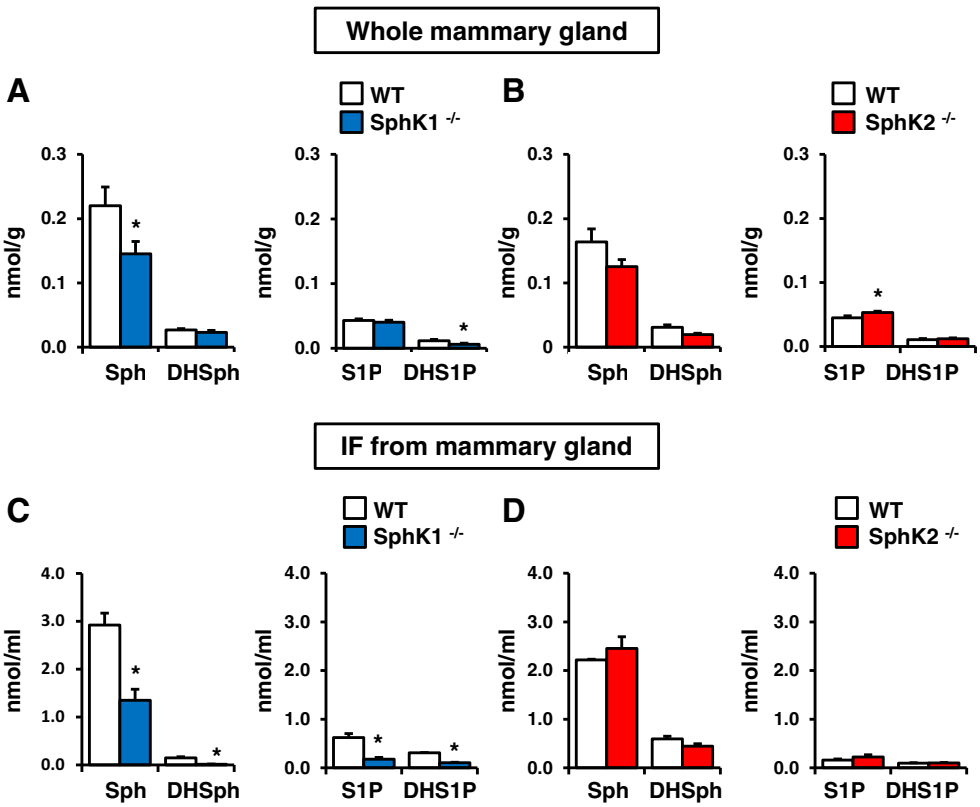

Fig. 4 Levels of bioactive sphingolipid metabolites in normal mammary glands and mammary gland IF from SphK1$^{-/-}$ and SphK2$^{-/-}$ mice and corresponding littermate wild type (WT) mice. Sphingosine (Sph), dihydro-Sph (DHSph), S1P, and dihydro-S1P (DHS1P) in mammary glands (**a, b**), and IF from the mammary glands (**c, d**) from SphK1$^{-/-}$ mice and WT littermates (**c**), SphK2$^{-/-}$ mice and their WT littermates (**d**) were determined by LC-ESI-MS/MS. Data are mean ± SEM ($n = 4$). *, $P < 0.05$

immunocompetent mice that S1P levels are increased in tumors and correlated with tumor growth [13]. Because we [14] and others [30, 31] have shown that oral administration of FTY720 reduces tumorigenesis, and because of the known effects of FTY720 on S1P signaling, we treated 4T1 tumor bearing mice with FTY720 and examined correlations between tumor burden and levels of bioactive sphingolipids in tumor IF. FTY720 greatly reduced tumor growth, as demonstrated by in vivo bioluminescence and tumor volume measurements (Fig. 5a–c), levels of S1P and DHS1P in tumor IF were significantly decreased compared to saline treated animals (Fig. 5d).

S1P Levels Are Higher in Breast Cancer IF than in Normal Breast Tissue IF from Human Patients

Next, we examined the levels of sphingolipids in IF from human patients with breast cancer to examine whether the observation seen in animal models is also applicable to the human patients. For this purpose, we obtained IF from breast tumor tissue and normal breast tissue from two different areas (peri-tumoral area and distant area from the tumor) in each patient with breast cancer and determined levels of sphingolipids in the fluid. Importantly, Sph, DHSph, and S1P levels, but not DHS1P, were significantly higher in the breast tumor tissue IF than in the normal breast tissue IF (Fig. 6). There is no significant difference in levels of Sph, DHSph, S1P or DHS1P between IF from normal breast tissue that is distant from tumor and that from peri-tumor normal breast tissue (Fig. 6).

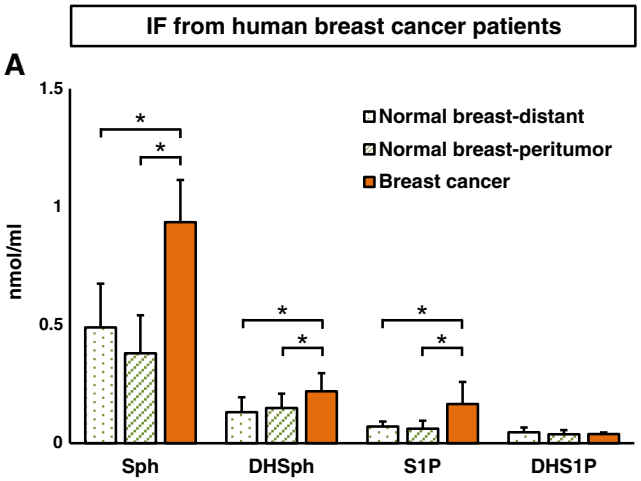

Fig. 6 Levels of bioactive sphingolipids in IF from breast tumor and normal breast tissue of patients with breast cancer. **a** IF was obtained from breast cancer tissue and normal breast tissue obtained from distant area from tumor and peritumor area. Levels of sphingosine (Sph), dihydro-Sph (DHSph), S1P, and dihydro-S1P (DHS1P) in the IF from tumor and normal breast tissue were determined by mass spectrometry. Data are mean ± SEM. *, $P < 0.05$

Discussion

High levels of S1P in blood are critical for maintenance of the tone and integrity of the vascular endothelium. The S1P gradient between high levels in the circulation and the low levels in tissues due to the presence of S1P degrading activity from phosphatases and S1P-lyase is important for immune cell trafficking [22]. It has been generally assumed that S1P levels in IF of lymphoid tissues are very low so that S1PR1 on lymphocytes can sense the S1P gradient as they exit into the blood. Previous studies have suggested that S1P secreted by tumor cells plays an important role in tumor progression and metastasis [13]. However, there are no reports on S1P in IF, due in part to the difficulties in collection. To our knowledge, this is the first report of the measurement of S1P and DHS1P in tumor IF in murine normal mammary glands and mammary tumors, as well as in human patients with breast cancer.

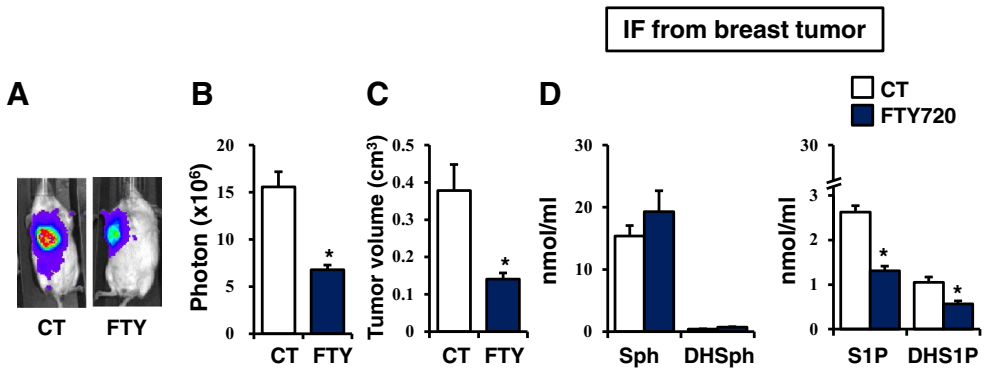

Fig. 5 Levels of bioactive sphingolipids in breast tumor IF correlate with tumor growth. 4T1-luc2 cells were surgically implanted in mammary glands. Tumor-bearing mice were randomized into 2 groups and treated daily by gavage with saline (*open bars*) or FTY720 (1 mg/kg, *black bars*). **a** Representative IVIS images of 4T1 breast tumors on day 14 after implantation. **b** Tumor burden determined by in vivo bioluminescence. Data are means ± SEM ($n = 5$). *, $P < 0.05$. **c** Tumor size was determined and volume calculated with the cylinder formula. Data are means ± SEM. *, $P < 0.05$. **d** Levels of sphingosine (Sph), dihydro-Sph (DHSph), S1P, and dihydro-S1P (DHS1P) in 4T1 breast tumor IF from saline or FTY720 treated mice were determined by mass spectrometry. Data are mean ± SEM. *, $P < 0.05$

Wiig et al. previously described a method to collect IF without causing cellular damage using low speed centrifugation of tissues on nylon mesh which required a large amount of tissue [3, 18, 19]. In order to collect IF from a smaller amount of tissue, we improved this method by designing a more efficient IF collection tube with a smaller nylon mesh surface area, thereby reducing loss of IF from absorption of the fluid by the mesh. Based on our experience, we recommend the use of at least 400 mg tissue for reproducible collection of IF. Nevertheless, LC-ESI-MS/MS is sufficiently sensitive to accurately measure sphingolipids in a volume of IF of less than 10 μl. As was reported by Wiig et al. [3, 18], we also found negligible contamination of IF with cellular components from contaminating or broken cells as shown by the extremely low amounts of actin.

Using the simple method for collection of IF that we have described, we have been able to validate previous assumptions regarding extracellular S1P as well as discover several new insights into the role of S1P in the tumor microenvironment. Levels of S1P in normal mammary glands are known to be relatively low, much lower than Sph; however, we have found high concentrations of bioactive sphingolipids (reaching 0.6 μM S1P and 0.2 μM DHS1P) to be present in IF from normal mammary glands. Sphingolipid metabolites in mammary gland IF from SphK1$^{-/-}$ mice were significantly decreased, suggesting that it is SphK1 that plays a pivotal role in regulating levels of these metabolites in IF from normal mammary glands. Though in vitro studies have suggested that it is SphK1 and not SphK2 that is the major contributor of secreted S1P, this is the first study to validate this in an in vivo setting.

FTY720 is a pro-drug approved for treatment of multiple sclerosis. It is phosphorylated in vivo to FTY720-phosphate, a S1P mimetic that modulates S1PR functions [32]. However, we [14] and others [30, 31] have shown that FTY720 also potent anti-cancer activities. In agreement, we found that oral administration of FTY720 greatly reduced breast tumor growth in a syngeneic model. Importantly, S1P and DHS1P levels in tumor IF were significantly decreased by FTY720 administration and correlated with the reduction of tumor growth. While this observation further supports the notion that S1P may have an important role within the tumor microenvironment, it also provides an important insight into the possible mechanisms of action of FTY720 on cancer progression. Though FTY720 in its phosphorylated form is known to have its immunosuppressive effects as a functional antagonist of S1PR1, inducing internalization and degradation of S1PR1 and prolonged receptor downregulation, it has also been shown that FTY720 inhibits SphK1 and induces its proteasomal degradation [33, 34]; therefore, the lower levels of S1P in the tumor IF from tumor bearing mice treated with FTY720 compared to saline treated animals could also be due to inhibition or reduction of SphK1 in the breast cancer cells. SphK1 is known to be upregulated in many cancers including breast [35–39] and we have shown that tumor bearing mice have increased systemic S1P [13] and may communicate with the host via the systemic SphK1/S1P axis to regulate lung metastasis/colonization [40]. Our findings suggest the possibility that S1P secreted from tumor cells to IF may be important for metastasis by stimulating S1P signaling important for cancer progression and highlights its important role in the tumor microenvironment. Further studies to investigate the roles of tumor IF in cancer progression is necessary to address this issue.

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Author Contribution MN and KT conceived the study. MN, AY, TA, WCH, KPT, BA, carried out experiments. HM developed the IF collection tube. JCA performed mass spectrometry analysis. MN and TK wrote the manuscript with assistance from OMR, TW, SS and SM.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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