Research Highlight

Twofer anti-vascular therapy targeting sphingosine-1-phosphate for breast cancer

Kazuaki Takabe, Akimitsu Yamada, Omar M. Rashid, Barbara J. Adams, Wei-Ching Huang, Tomoyoshi Aoyagi, Masayuki Nagahashi

Division of Surgical Oncology, Department of Surgery, Virginia Commonwealth University School of Medicine and Massey Cancer Center, Richmond, VA 23298-0011, United States

Corresponding to: Kazuaki Takabe, MD, PhD, FACS. Division of Surgical Oncology, Department of Surgery, Virginia Commonwealth University School of Medicine and Massey Cancer Center, PO Box 980011, West Hospital, Room 7-402, 1200 East Broad Street, Richmond, VA 23298-0011, United States. Email: ktakabe@vcu.edu.

Submitted Apr 28, 2012. Accepted for publication Jul 12, 2012.
DOI: 10.3978/j.issn.2227-684X.2012.07.01
Scan to your mobile device or view this article at: http://www.glandsurgery.org/article/view/1007/1191

Twofer is an American expression to achieve two objectives with one action, such as to kill two birds with one stone. It is said to originate from the shortening of "two for the price of one". This is what we discovered when the sphingosine-1-phosphate (S1P) signaling pathway is targeted in breast cancer (1); inhibition of S1P production results in suppression of both arms of the angiogenic process, generation of blood vessels that supply blood to tumors, and lymphatic vessels that drain interstitial fluid from them.

S1P is a bioactive lipid mediator, which is now well established as a key regulatory molecule in cancer through its ability to promote cell proliferation, migration, invasion, and angiogenesis (2). S1P is generated inside the cell by two sphingosine kinases, SphK1 and SphK2 (2). SphK1 is located in the cytoplasm close to the cell membrane, thus S1P generated by SphK1 is readily available to be exported out of the cells where it regulates many functions by binding to and signaling through its specific G protein-coupled receptors ($S1P_{1\text{-}5}$) (3). This process known as "inside-out" signaling explains the autocrine and paracrine actions of S1P (2). We previously published that SphK1, but not SphK2, is involved in S1P export from breast cancer cells mediated by the ATP-binding cassette transporters, following estradiol stimulation (4). Our findings are in agreement with the reports from others that also demonstrated that SphK1 is the key kinase that is associated with cancer biology (5). For instance, expression of SphK1, which is up-regulated in breast cancer, is associated with resistance to chemotherapy, and correlates with poor prognosis (5). We have also demonstrated that SphK1 expression can be up-regulated by lysophosphatidic acid (6) via ERK1 signaling pathway (7) and stimulate cell mobility.

Angiogenesis, generation of new blood vessels from pre-existing ones, is a crucial component of the tumor microenvironment that determines the growth and progression of tumors by providing them oxygen, nutrition, and conduit for invaded cells to metastasize. Recent growing evidence implicates the S1P signaling pathway as one of the most important mechanisms of angiogenesis. Expression of $S1P_1$ is up-regulated in the tumor vasculature during angiogenesis, and down-regulation of its expression was effective in inhibiting angiogenesis and tumor growth *in vivo*, suggesting that the $S1P_1$ is also a critical component of the tumor angiogenic response (8). The fact that neutralization of extracellular S1P with anti-S1P antibody shows a significant inhibition of angiogenesis, tumor growth, and metastasis, further confirms that S1P plays a dominant role in this process (9). Finally, the defective vascular maturation observed in $S1P_1$-deficient mice, which die *in utero* from massive hemorrhage due to immature vessel development, highlights a fundamental role for S1P signaling in vasculogenesis during development as well (10).

In contrast to angiogenesis, only a few studies so far have examined the involvement of the S1P signaling pathway in lymphangiogenesis, the generation of new lymphatic vessels from pre-existing ones. Anelli *et al*. showed that S1P can induce *in vitro* endothelial tube formation and cell migration in endothelial cell lines derived from vascular

and lymphatic systems at nanomolar concentrations (11). Yoon *et al.* reported that S1P induced lymphangiogenesis *in vitro* via the $S1P_1$ receptor in lymphatic endothelial cells (LECs) (12). Interestingly, LEC-specific deletion of SphK1 in the SphK2 knockout mouse, which means total knockout of S1P generation specifically in LECs, inhibited lymphatic vessel maturation, which suggests that SphKs and S1P in LECs are required for the proper development of lymphatic vessels (13). This led us to hypothesize that S1P play an important role in tumor-induced lymphangiogenesis *in vivo* as well.

Prior to our investigation, we have discovered that there were several issues that needed to be addressed in order to determine the role of S1P in tumor-induced angiogenesis and lymphangiogenesis *in vivo*. Since S1P has such a profound role in the immune response (14), the most commonly used traditional *in vivo* metastatic breast cancer models implant xenograft human breast cancer cells into immune compromised mice which thus ignore the host immune response to cancer were deemed an inappropriate model to investigate the role of S1P in cancer biology (15). Thus, we decided to utilize a syngeneic model that implants mouse breast cancer cells into immune intact mice. At this point, we found that there is no consensus in the method of implantation of the cells. We have identified that more than 200 genes, 75% of these are known to be related to cancer biology, were differentially expressed between tumors after most commonly used subcutaneous implantations and tumors after orthotopic implantations in the mammary fat pad of the same cell-line, 4T1. By bioluminescence technology, we also identified that most commonly practiced orthotopic implantation of breast cancer cells into abdominal mammary glands results in direct invasion to the abdominal wall that results in abdominal carcinomatosis, which clearly does not mimic human breast cancer progression. In conclusion, we established an orthotopic method via implantation into the chest mammary gland under direct visualization using 4T1-luc cells to monitor its progression with bioluminescence technology (15). We have found that this syngeneic orthotopic chest mammary gland implantation model readily develops lymph node metastases followed by lung metastases, which more accurately recapitulates human breast cancer progression than subcutaneous models (1). Utilizing this animal model, we found that circulating S1P levels correlated with tumor burden. Interestingly, serum S1P levels were found to be significantly elevated in Stage IIIA breast cancer patients, who have developed lymph node metastasis compared with that of age/ethnicity-matched healthy volunteers, which implicate that our findings using our animal model may be applicable in human patients (1).

Tumor-induced angiogenesis and lymphangiogenesis are usually evaluated by histological determinations of microvessel density, which rely on selective morphometric analyses (e.g., vessel counts, vascular morphology etc.) (16). The strengths of the morphometric analyses are that it can evaluate the location of the vessels as well as the morphology of the vessels during tumor progression. The limitations include variable sites of tissue sectioning, variable immunostaining techniques, different vessel density quantification methods, and the lack of standardization in the estimation of angiogenesis and lymphangiogenesis (16). To compliment this approach and overcome some of these limitations, we have developed a novel approach using flow cytometry to quantify both blood endothelial cells (BECs) and LECs from the same sample to simultaneously evaluate both angiogenesis and lymphangiogenesis (1). Flow cytometry provides quantification data on how many BECs and LECs exist in the given sample, which strongly reinforces the morphometric data.

Another issue was the development of an assay to evaluate the role of S1P on angiogenesis and lymphangiogenesis *in vivo*. We utilized SK1-I as a SphK1-specific pharmacological inhibitor that we have previously reported (17,18). We have demonstrated *in vitro* utilizing the tube formation assay that Ang2 induced angiogenesis and lymphangiogenesis are both suppressed by SK1-I, implicating that there is a cross talk between Ang2 and S1P signaling pathway. The question was whether this is the case *in vivo* as well. Matrigel plug assay, of which the matrigel mixed with the compound of interest are implanted subcutaneously, is most commonly used to examine the vasculogenesis *in vivo*. However, this assay is criticized because the vasculogenesis induced is multi-directional, and thus the quantification may be inaccurate. Directed *in vivo* angiogenesis assays (DIVAA), which implant a one-side-open capsule that contains only 20 microliter of Matrigel, was reported to allow only uni-directional angiogenesis and provide a simple and quantitative method *in vivo* (19). We have adapted the DIVAA, by combining it with fluorescence activated cell sorting, and developed a novel assay to simultaneously quantify both angiogenesis and lymphangiogenesis, which we named DIVAA/FACS method (1). Utilizing this DIVAA/FACS method, we have demonstrated that exogenous S1P enhanced angiogenesis and lymphangiogenesis, while inhibition of SphK1 by SK1-I completely blocked Ang2

induced angiogenesis and lymphangiogenesis *in vivo*, which was consistent with our *in vitro* results.

Utilizing our newly established syngeneic orthotopic implantation model, we have discovered that pharmacological inhibition of SphK1 with SK1-I significantly decreased S1P levels both in the serum and tumor, with tumor volume evaluated by tumor size, weight, bioluminescence, and mitotic activity shown by Ki67 staining. Furthermore, both lymph node and lung metastases were significantly suppressed by SK1-I treatment. Finally, combining both morphometric analysis and flow cytometry, both angiogenesis and lymphangiogenesis were suppressed by SK1-I, not only around the primary tumor, but also in lymph nodes that are distant from the tumor. This result indicates that S1P plays a key role not only in "tumoral lymphangiogenesis", but also in "lymph node lymphangiogenesis", which we speculate to actively promote metastasis via the lymphatics.

In conclusion, we have discovered that the S1P signaling pathway plays a critical role both in angiogenesis and in lymphangiogenesis induced by tumor, using SphK1-specific inhibitor, SK1-I. We believe that targeting the SphK1 and S1P signaling pathway can be a novel modality for the treatment of breast cancer by suppression of both arms of angiogenic processes, angiogenesis and lymphangiogenesis.

Acknowledgements

Kazuaki Takabe is supported by United States National Institute of Health (R01CA160688, K12HD055881, R01CA154314, and R01DK057543) and Susan G. Komen for the Cure (Investigator Initiated Research Grant (222224). Masayuki Nagahashi is a Japan Society for the Promotion of Science Postdoctoral Fellow.
Disclosure: The authors declare no conflict of interest.

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Cite this article as: Takabe K, Yamada A, Rashid OM, Adams BJ, Huang WC, Aoyagi T, Nagahashi M. Twofer anti-vascular therapy targeting sphingosine-1-phosphate for breast cancer. Gland Surg 2012;1(2):80-83. DOI: 10.3978/j.issn.2227-684X.2012.07.01

