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Lysophosphatidic Acid Stimulates Lymphangiogenesis in Human Lymphatic Endothelial Cells

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Abstract

Lymphangiogenesis is the process by which new lymphatic vessels sprout and grow from existing vessels whether under developmental, immunological, or cancerous conditions. Proper lymphatic vessel formation is important in working alongside normal angiogenesis in order to help regulate the body's tissue fluid as well as aid in immunosurveillance. Various factors regulate lymphangiogenesis such as members of the vascular endothelial growth factor family (VEGF). Another factor that has recently been identified to play a role in lymphangiogenesis is the bio-active phospholipid lysophosphatidic acid (LPA) however the molecular mechanism by which LPA regulates lymphangiogenesis has not been well characterized. In this study, human lymphatic endothelial cells (HLECs) were treated with LPA in the presence or absence of VEGF and the late stage effects of lymphangiogenesis were examined. Preliminary evidence suggests that VEGF and LPA induces proliferation in HLECs, however there is no increase in this stimulation when both factors are added together. A Matrigel tube formation assay revealed that LPA induces an increase in cellular extensions as well as in tubule length as compared to the control.

Introduction

Lymphatic vessel formation, lymphangiogenesis, is known to occur during normal development and during tissue stress such as inflammation and wound healing. In an inflammatory response, there is an excess collection of fluid near the wound. Lymphatic vessels grow into the damaged area to relieve the fluid and to allow more leukocytes or white blood cells to enter the afflicted area [1]. Thus, lymph vessel formation is vital for regulating the body's tissue fluid and recruiting leukocytes as part of the body's immune response.

One important mediator of the inflammatory response is the bio-active phospholipid lysophosphatidic acid (LPA). LPA belongs to a family of lipid of growth factors that is present in low concentrations in serum and biological fluids but is found in higher concentrations at sites of inflammation and tumor growth [2]. LPA has been shown to modulate inflammatory responses through the stimulation of chemokines, cytokines, and cytokine receptors expression as well as regulate cytoskeletal rearrangement and migration of target cells. LPA evokes its biological effects through binding to G-protein coupled receptors. There are five known receptors for LPA, $LPA_{1\text{-}5}$[3]. It has previously been found that binding to LPA_2 induces the production of pro-angiogenic factors such as VEGF, Interleukin-6 (IL-6) and Interleukin-8 (IL-8) [4].

In this study, I will look at the effect of LPA on lymphangiogenesis using human lymphatic endothelial cells (HLECs). Using an in vitro assay, I measured the ability of HLECs to proliferate in the presence of LPA in the presence or absence of vascular endothelial growth factor (VEGF). Additionally, a BD Matrigel tube formation assay was used to measure the effect of LPA on the ability of HLECs to extend and form rudimentary tubes.

LPA Structure

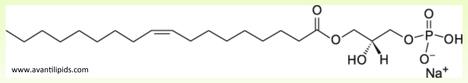

Figure 1. Diagram of the structure of lysophosphatidic acid

Literature Cited

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3. Lin, C.-H., Lu, J., and Lee, H. "**Interleukin-1β Expression Is Required for Lysophosphatidic Acid-Induced Lymphangiogenesis in Human Umbilical Vein Endothelial Cells**" *International Journal of Inflammation* 2011, (2010):
4. Zhao, C., Fernandes, M. J., Prestwich, G. D., Turgeon, M., Battista, J. D., Clair, T., Poubelle, P. E., and Bourgoin, S. G. "**Regulation of Lysophosphatidic Acid Receptor Expression and Function in Human Synoviocytes: Implications for Rheumatoid Arthritis?**" *Molecular Pharmacology* 73, no. 2 (2008): 587–600

Proliferation Assay

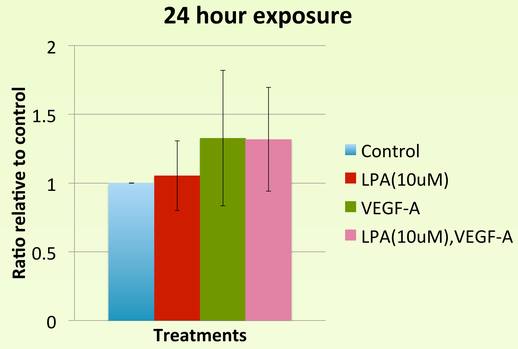


Figure 2. Proliferation assay results following 24 hour exposure to factors. Following serum starvation, 3,000 HLECs were plated in each fibronectin-coated well of a 96-well plate in EGM2-MV media under the following conditions: control (EGM2-MV media alone), LPA (10uM), LPA (1uM), VEGF-A, or both LPA (10uM) and VEGF-A. The cells were then incubated at 37°C for 24 hours and a cell proliferation assay was performed using a Cell Counting Kit-8 (Dojindo). Briefly, a water soluble tetrazolium salt, WST-8, was added to each well and allowed to incubate for 4 hours. WST-8 is reduced by dehydrogenases in the cells producing an orange colored formazan dye which can be detected by measuring the absorbance at 450 nm using a microplate reader. The amount of dye generated is directly proportional to the number of living cells in that well. Multiple assays were performed and the ratio of absorbance relative to the control was determined. The average of this ratio for the multiple assays is shown here. There is no significant difference between the number of viable cells between the different treatment groups.

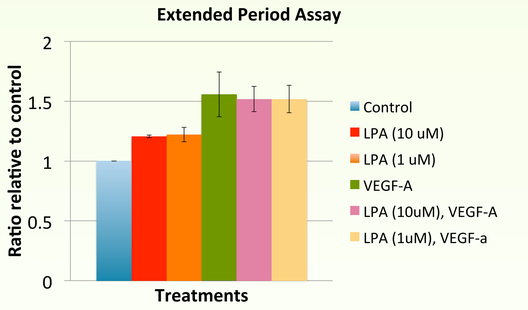
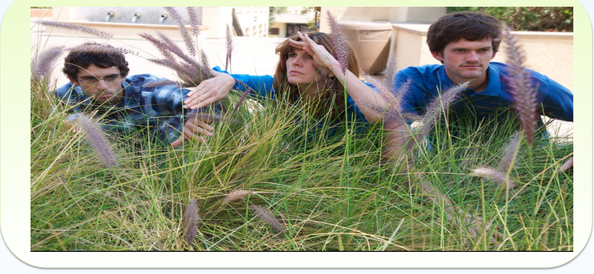


Figure 3. Increase in proliferation detected following a longer incubation time. The proliferation assay was performed as in figure 2 with the following differences: 24 hours after the initial set up, an additional "boost" of LPA and/or VEGF was added to the culture media. Cells were allowed to incubate for an additional 48 hours before the absorbance was determined and analyzed as before. Preliminary results from two assays suggest that LPA exposure as well as VEGF exposure induces an increase in proliferation relative to the control.



Acknowledgments

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Matrigel™ Lymphangiogenic Tube Formation Assay

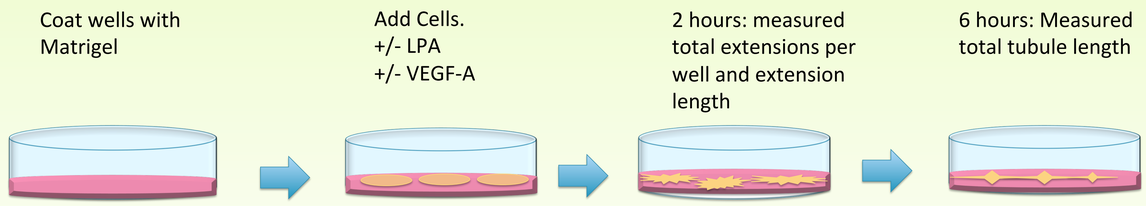

Figure 4. Schematic illustration of BD Matrigel™ Matrix Lymphangiogenic tube formation assay. 40 µl of BD Matrigel™ Matrix (reconstituted basement membrane, BD Biosciences) was added to a series of wells in a 96-well tissue culture plate and allowed to solidify by incubating at 37 C for 30 minutes. HLECs were plated at 10,000 cells/well in growth media (EGM2-MV) under one of the various conditions: growth media control, 10uM LPA, 1 uM LPA, 50 mg/ml VEGF, or both 1 uM LPA and VEGF-A. Cells were then incubated at 37 C and using a Nikon Ti-260 microscope were observed and photographed at various timepoints. At 2 hours, the number and length of cell extensions per field of view was measured. At 6 hours total tubule length was determined.

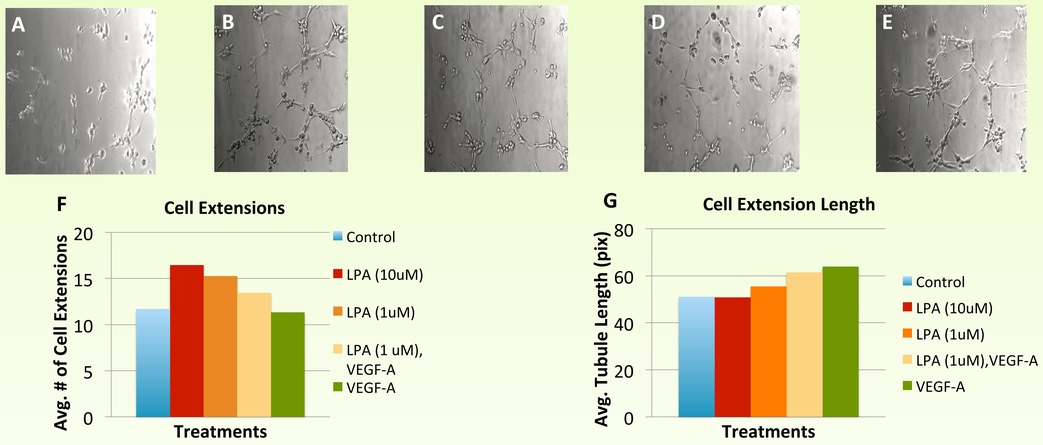

Figure 5. LPA induces formation of cellular extensions of HLEC after two hours in Matrigel™ lymphangiogenesis assay. To measure the induction of lymphangiogenesis in HLECs in the presence of LPA, HLECs were plated in triplicate on BD Matrigel™ Matrix as described in figure 4 under the following conditions: (A) growth media, (B) 10 uM LPA, (C) 1 uM LPA, (D) VEGFA, and (E) 1uM LPA and VEGFA. Treatments were done in duplicate and three different fields of view per well were photographed after 2 hours and the average of both the (F) number of cellular extensions and (G) the length of extensions per field of view was determined. At 1uM, LPA induces a slight increase in the number of cellular extensions per field of view well as the overall length of cellular extensions as compared to growth media alone. Exposure to 10uM LPA induced an even greater number of cellular extensions but did not result in an overall increase in extension length as compared to the control. Conversely, VEGFA treatment did not result in a greater number of cellular extensions as with growth media alone but did induce longer extension length. Treatment with both LPA and VEGFA displayed results in between LPA and VEGFA treatment alone.

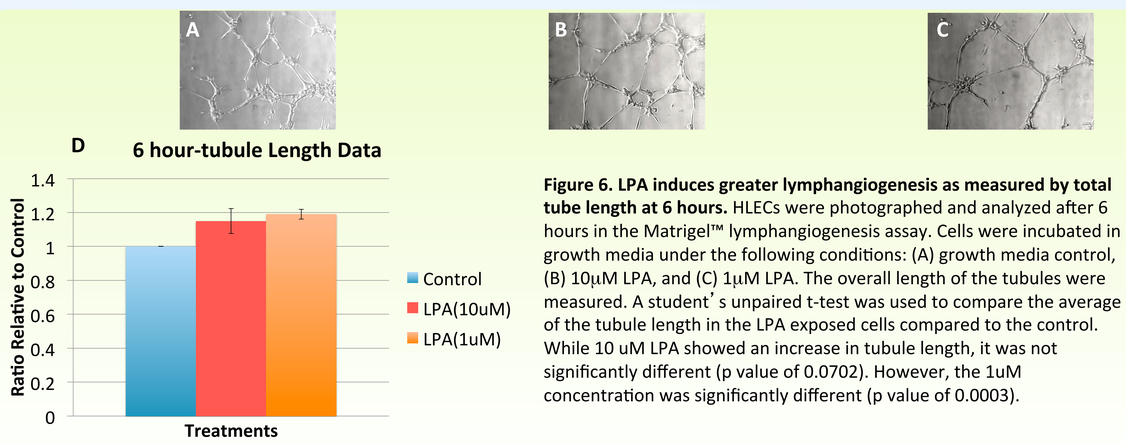

Figure 6. LPA induces greater lymphangiogenesis as measured by total tube length at 6 hours. HLECs were photographed and analyzed after 6 hours in the Matrigel™ lymphangiogenesis assay. Cells were incubated in growth media under the following conditions: (A) growth media control, (B) 10µM LPA, and (C) 1µM LPA. The overall length of the tubules were measured. A student's unpaired t-test was used to compare the average of the tubule length in the LPA exposed cells compared to the control. While 10 uM LPA showed an increase in tubule length, it was not significantly different (p value of 0.0702). However, the 1uM concentration was significantly different (p value of 0.0003).

Conclusions

- Preliminary evidence suggests LPA stimulates proliferation in HLECs
- No significant co-stimulatory response detected with both LPA and VEGF-A.
- Results from Matrigel lymphangiogenic tube formation assay suggest
  - At two hours, compared to control, 1 µM LPA exposure resulted in an increase in both cell extension number and length while 10 µM LPA induced more extensions but no difference between extension length was found.
  - At 6 hours, there was a significant difference in tube length between 1 µM LPA and the control with a p value of 0.0003.