MEK / ERK inhibitor effectively impact generalized lymphatic anomaly (GLA) cells growth through EGFR / MEK /ERK signaling pathway

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Abstract
Background Generalized lymphatic anomaly is characterized by diffuse or multicentric proliferation of dilated lymphatic vessels resembling common lymphatic malformation. Studies on GLA are frequently hampered by a lack of appropriate models to test the effects of potential treatments or decipher the mechanism of pathology. Moreover, diverse phenotypes observed with GLA require a large number of samples to be analyzed to obtain statistically informative results. Due to the very limited experimental material, most of the research is restricted to single case report.

Methods We first time used two-step endothelial cell isolation technique (step 1: single cells were first sorted with a-human CD31 magnetic beads; step 2: collected CD31 Pos cells from step1 were sorted with a-human PDPN magnetic beads) to generate two GLA-LEC cell lines, and purified normal-LEC from normal liver tissue in the same case. To characterize the aberrant phenotype of generalized lymphatic anomaly lymphatic endothelial cells (GLA-LEC#1, and GLA-LEC#2). We investigated GLA-LECs growth curve, cell cycle, apoptosis, and sprouting angiogenesis in vitro. Matrigel plug assay was applied in immunodeficient mice to monitor the GLA-LECs formed vasculature in vivo. Rapamycin and dual MEK / ERK inhibitor were tested to investigate the efficacy on inhibiting GLA-LEC proliferation and downstream signaling pathway.

Results We have successfully purified GLA-LECs from GLA tissues with > 99% purity. These cells also expressed the lymphatic markers lymphatic vessel endothelial hyaluronan receptor (LYVE-1) and podoplanin (PDPN). GLA-LECs showed significantly higher proliferation rate compared to normal-LECs in both cases. Cell cycle analysis of cell distribution suggested that compared with normal-LECs, GLA-LECs showed increased proportion of cells in S phase and less G0/G1 phase. When GLA-LECs and normal-LECs apoptosis induced by serum deprivation, more Annexin V positive population of endothelial cells were observed in normal-LECs but not GLA-LECs. Hyper-activated epidermal growth-factor receptor (EGFR) signaling was observed in both cases of GLA-LECs, endogenously highly expression of EGF receptor and EGF induced phosphorylation of EGFR (phosphor Y1068) were found in both GLA cell lines. GLA-LECs are sensitive to both rapamycin and MEK / ERK dual inhibitor treatment. In vivo, by using Matrigel plug assay, we found both GLA-LECs and immortalized GLA-LEC (SV40) grew
robust vessel-like structure.

Conclusions In vitro, both GLA-LECs cell lines are highly proliferative as compared with normal-LECs. Rapamycin and dual MEK/ERK inhibitor dose-dependently inhibited GLA-LECs proliferation. In vivo, GLA-LECs showed angiogenic phenotype, and grew robust vessel-like structure in immunodeficient mice.

Full-text Due to technical limitations, full-text HTML conversion of this manuscript could not be completed. However, the manuscript can be downloaded and accessed as a PDF.

Figures
Figure 1

Establishment primary generalized lymphatic anomaly lymphatic endothelial cell lines (GLA-LEC). (A) Confluent GLA-LEC#1 and GLA-LEC#2 viewed under phase-contrast microscopy exhibit the typical cobblestone morphology. (B) Fluorescence microscopy showing positive immunoreactivity for CD31, Prox-1 (C), and LYVE-1 (D). Scale bar, 100μm.
GLA-LECs showed highly proliferative with less apoptotic phenotype in vitro (A) Cell proliferation of Normal-LEC#1 and GLA-LEC#1 was examined by CyQUANT. 1,000 cells (in 96-well plate) are seeded per well, every other day fluorescent dye binding to cellular DNA content was added to each well. Signal was collected as fluorescent unit. **p < 0.01, ****p < 0.0001, N=3. (B) Normal-LEC#1 and GLA-LEC#1 cell populations residing in G0/G1, S and G2/M. ****p < 0.0001, N=8. (C) Normal-LEC#1 and GLA-LEC#1 cells were serum-deprived for 48 hours followed by incubation in Annexin V-binding buffer containing PE-conjugated Annexin V and 7-aminoactinomycin D (7-AAD) at room temperature for 15 minutes in the dark. Labeled cells were subsequently analyzed by flow cytometry. ****p < 0.0001, N=6. (D) Western blot for phosphorylated and total EGFR and ERK in GLA-LEC#1 serum-starved overnight and exposed to 100 ng/ml EGF for 10 minutes. -actin is used as loading control. (E) Cell proliferation of Normal-LEC#2 and GLA-LEC#2 was examined by CyQUANT. (F) Normal-LEC#2 and GLA-LEC#2 cell populations residing in G0/G1, S and G2/M. ****p <
0.0001, N=3. (G) Normal-LEC#2 and GLA-LEC#2 cells were serum-deprived for 48 hours followed by Annexin V / 7-AAD incubation, subjected by flow cytometry. *p < 0.05, N=4. (H) Western blot for phosphorylated and total ERK in GLA-LEC#2 serum-starved overnight and exposed to 100 ng/ml EGF for 10 minutes.

Figure 3

GLA-LECs showed increased sprouting angiogenesis in vitro (A) Photomicrographs of normal-LEC#1 and GLA-LEC#1 on Cytodex microcarriers in three-dimensional fibrin gels. (B) Number of sprouts and total length of sprouts were quantified by ImageJ. ****p < 0.0001, N=25. (C) Photomicrographs of normal-LEC#2 and GLALEC# 2 on Cytodex microcarriers in three-dimensional fibrin gels. (D) Quantitative analysis of number of sprouts and total length of sprouts. ****p < 0.0001, N=30. Scale bar = 150 μm.
In vivo angiogenesis assay of GLA-LEC. (A) GLA-LEC#1 (2×106) was injected subcutaneously into immunodeficient mice (NOD. Cg-Prkdcscp1/s12rgtm1Wjl/Sz). After 10 days, Matrigel plug were removed from both control (Matrigel alone) and GLA-LEC#1 group. (B) Hematoxylin and Eosin staining revealed that grafts with GLA-LECs contain robust vessel-like structure, but not in control sample. (C) To identify the patient derived GLA-LEC#1, immunofluorescence staining by anti-human CD31 (in green) and anti-mouse CD31 (in red) antibodies were conducted. In most of the vessel-like structure, endothelial cells are patient derived GLA-LEC#1, but not host derived mouse endothelial cells.
GLA-LECs are sensitive to Rapamycin and MEK / ERK dual inhibitor treatment. (A, E) GLALEC# 1 and GLA-LEC#2 were treated by different dose of rapamycin (10ng/ml, 25ng/ml, 50ng/ml, 100 ng/ml). CyQUANT assay was used to determine the cell proliferation. ***p < 0.001, N=3. (C, G) Western blotting was applied to study downstream signaling pathway involved in rapamycin treatment. (B, F) GLA-LEC#1 and #2 were treated by different dose of dual MEK / ERK inhibitor RO5126766 (10nM, 50nM, 250nM, 1000nM). CyQUANT assay was used to determine the cell proliferation. ***p < 0.001, N=3. Western blotting was applied to investigate the downstream signaling pathway involved in MEK / ERK inhibitor treatment.
GLA-LEC#1 immortalized with SV40 large T antigen. (A, B) Retrovirus was used to overexpress SV40 T antigen in GLA-LEC#1. Both immunocytochemistry and western blotting were used to determine SV40 large T expression in the generated cell line. (C) Cumulated population doubling levels of the parental GLA-LEC#1 compared with GLA-LEC#1 (SV40).

(D) Later passage (passage 22), SV40 LT antigen is still can be detected and stably expressed in the immortalized GLA-LEC (SV40) cell line. (E) In later passage (passage 22), GLA-LEC#1 showed increased number of SA-β-gal-stained cells, whereas the GLA-LEC (SV40) did not show any positive staining. (F, G) In vivo GLA-LEC (SV40) Matrigel plug assay. With different cell density (0.5×10⁶ and 2×10⁶), different time point (Day5 and Day10), GLA-LEC (SV40) showed robust vessel-like structures detected by antihuman CD31 antibody immunofluorescence staining. Scale bar, 100μm.
Supplementary Files

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