Poster No. C037

ENZYCHEM LIFESCIENCES

KRIBB Korea Research Institute of Bioscience & Biotechnology

Anti- metastatic effect of PLAG via interference of neutrophil elastase/PAR2/EGFR signaling on A549 lung cancer orthotopic implantation model

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ABSTRACT

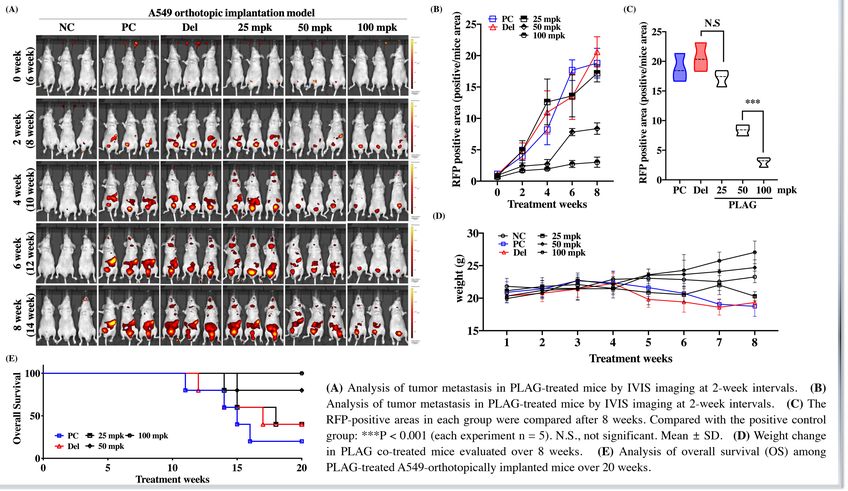
Background: A recent studies report that tumor-infiltrating neutrophil (TIN) has potential on malignant tumor progress like as metastasis. 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) is a synthetic lipid-based small molecular compound. PLAG has prevents chemotherapy-induced neutropenia by attenuating neutrophil extravasation via down-regulation of adhesion molecules, inflammatory cytokines & chemokines. In this study, we investigated whether PLAG has an anti-metastatic effect on A549 lung cancer orthotopic implantation model via modulating TIN.

Results: In the orthotopic implantation model, metastatic tumor to GI-track and brain was easily detected by IVIS in the positive control and delivery groups. It was confirmed that metastasis to GI-track was 73% decreased in 100 mpk PLAG treated group compared with positive control. Also, the metastasis to the brain was decreased by about 92% in PLAG treated group compared with positive control. Primary lung tumor growth was retarded by PLAG with dose dependent manner. The size of primary tumor is much smaller than positive control and alveolar tissue of lung was closed to that of normal mice in 100 mpk PLAG treated group. In contrast, massive neutrophil infiltration in positive and delivery groups was detected by IHC, but significantly reduced that observed with PLAG administration. Moreover, p-EGFR was concomitantly detected with similar pattern of neutrophil infiltration. During *in vitro* assay, the spheroid A549 lung cancer cells scattered by neutrophil, but it was effectively hindered by PLAG treatment. Neutrophil-stimulated cancer in the non-direct contact method showed an enhanced metastatic activity, this phenomenon was significantly reduced in the PLAG group. The neutrophil stimulated that expression of EMT markers such as Snail, whereas it was effectively reduced by PLAG. Trans-activation between tumor cells and neutrophil was mediated by neutrophil elastase (NE). NE activates PAR2 of cancer cells and subsequently induces p-EGFR for metastatic progress. The suppression of metastasis by PLAG was mediated through regulation of the NE-PAR2 pathway. PAR2-activated by NE stimulates cleavage of HB-EGF through the ARR2/clathrin complex trafficking. However, intracellular trafficking and degradation of PAR2 are accelerated by PLAG treatment.

Conclusion: In this study, the neutrophil recruitment into tumor was observed in the primary lung tumor and metastasis into GI and brain but PLAG dramatically reduced it. These results suggest that neutrophil educate tumor cells for enhancing metastasis by EGFR transactivation, however, PLAG effectively interfered these reactions. There are no available anti-metastatic drugs yet, PLAG is very suitable candidates for anti-metastatic drugs via modulating of TIN activation.

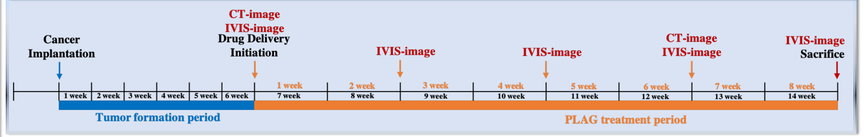
RESULTS

1. Inhibition of cancer metastasis by PLAG in the A549-orthotopically implanted mouse model

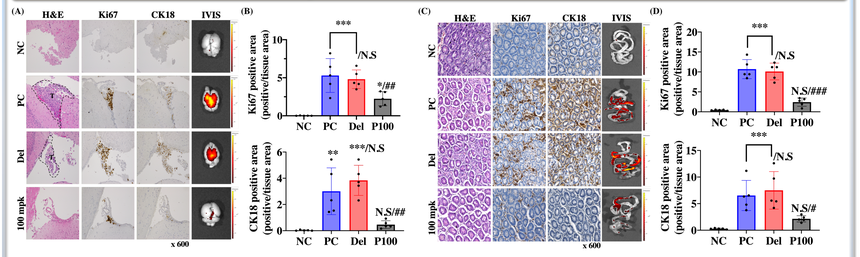


(A) Analysis of tumor metastasis in PLAG-treated mice by IVIS imaging at 2-week intervals. **(B)** Analysis of tumor metastasis in PLAG-treated mice by IVIS imaging at 2-week intervals. **(C)** The RFP-positive areas in each group were compared after 8 weeks. Compared with the positive control group: ***P < 0.001 (each experiment n = 5). N.S., not significant. Mean ± SD. **(D)** Weight change in PLAG co-treated mice evaluated over 8 weeks. **(E)** Analysis of overall survival (OS) among PLAG-treated A549-orthotopically implanted mice over 20 weeks.

EXPERIMENTAL DESIGN

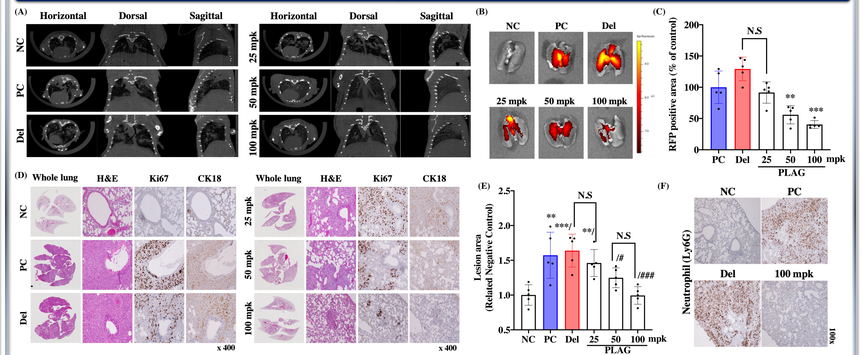


2. Treatment with 100 mpk PLAG reduced metastasis to the brain and intestines



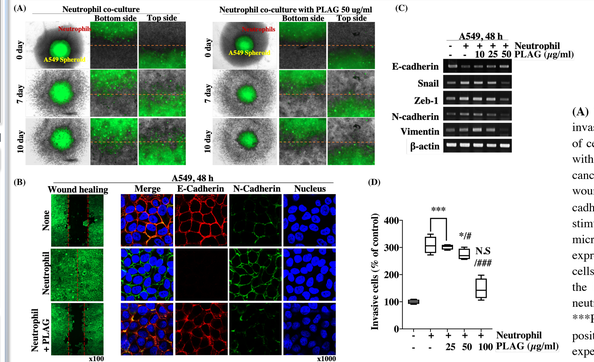
(A) Inhibition of tumor metastasis into the brain in PLAG-treated mice was verified by immunohistochemical staining with anti-Ki67 and anti-CK18 antibodies. **(B)** The area of cancer metastasis to the brain region was evaluated using human-specific antibodies against Ki67 and CK18. Compared with the negative control group: *P < 0.05, **P < 0.01, ***P < 0.001. N.S., not significant. Compared with the positive control group: ##P < 0.01 (each experiment n = 5). N.S., not significant. Mean ± SD. **(C)** Inhibition of tumor metastasis into the intestines in PLAG-treated mice was verified by immunohistochemical staining with anti-Ki67 and CK18 antibodies. **(D)** The area of cancer metastasis to the gastrointestinal tract was evaluated using human-specific antibodies against Ki67 and CK18. Compared with the negative control group: *P < 0.05, **P < 0.01, ***P < 0.001. N.S., not significant. Compared with the positive control group: ##P < 0.01 (each experiment n = 5). N.S., not significant. Mean ± SD.

3. PLAG treatment inhibited the growth of A549 human lung cancer in mice



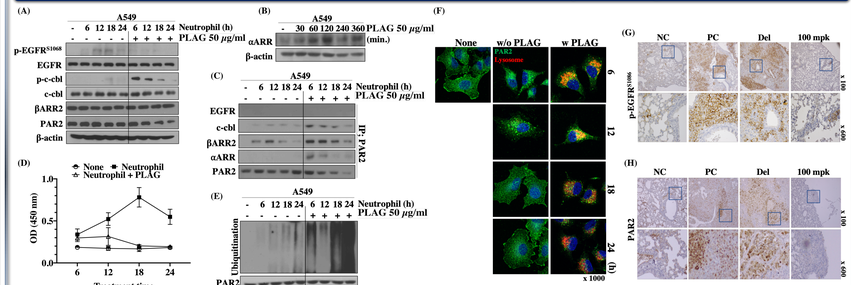
(A) Orthotopic tumor cell proliferation in mouse lungs determined by CT imaging. **(B, C)** Orthotopic tumor cell proliferation in mouse lungs determined by IVIS imaging. Compared with the negative control group: **P < 0.01, ***P < 0.001 (each experiment n = 5). N.S., not significant. Mean ± SD. **(D)** Tumor growth in PLAG-treated lung tissues evaluated by H&E staining and immunohistochemical staining with anti-Ki67 and anti-CK18 antibodies 14 weeks after implantation. **(E)** Evaluation of the main tumor region in PLAG-treated lung tissues after implantation with A549 cells. The volume of alveolar space occupied by tumor cells was measured and compared with that in negative control mice using Image J. Compared with the negative control group: **P < 0.01, ***P < 0.001. N.S., not significant. Compared with the positive control group: #P < 0.05, ##P < 0.01 (each experiment n = 5). N.S., not significant. Mean ± SD. **(F)** Labeling with anti-neutrophil antibody showed that PLAG treatment reduced the numbers of tumor-infiltrating neutrophils in the orthotopic lung tumors.

4. Inhibitory effect of PLAG on metastasis in the tumor-infiltrating neutrophil microenvironment



(A) The inhibitory effect of PLAG in the spheroid invasion assay of A549 cells was calculated on the basis of cells scattering, which is mediated by direct contact with neutrophils. **(B)** Inhibitory effect of PLAG on cancer cell mobility was evaluated and analyzed by wound healing assay. Modulation of E-cadherin and N-cadherin by PLAG in the presence of neutrophil stimulation in A549 cells observed by confocal microscopy. **(C)** Modulation of EMT-related gene expression in PLAG and neutrophil co-treated A549 cells was evaluated by RT-PCR. **(D)** PLAG inhibited the invasive activity of A549 cells in the presence neutrophil. Compared with negative controls: *P < 0.05, ***P < 0.001. N.S., not significant. Compared with positive controls: #P < 0.05, ###P < 0.001 (each experiment n = 5). Mean ± SD.

5. PLAG inhibition of EGFR transactivation mediated by PAR2 degradation



(A) Inhibition of the EGFR signaling pathway and PAR2 degradation by PLAG treatment in neutrophil-stimulated A549 cells evaluated by Western blot. **(B)** Induction of αARR expression by PLAG treatment was confirmed by Western blotting. **(C)** Changes in complex formation between endocytosis-related proteins and PAR2 degradation following PLAG treatment. **(D)** Changes in HB-EGF secretion following PLAG treatment and neutrophil stimulation in A549 cells. **(E)** Validation of the PAR2 ubiquitination-inducing effect of PLAG in neutrophil-activated A549 cells. **(F)** Lysosomal PAR2 degradation in PLAG-treated cells was verified by confocal microscopy. **(G)** Phosphorylation of EGFR **(H)** and expression of PAR2 in the lung tissues of A549-orthotopically implanted mice were prominently reduced by PLAG treatment.

CONCLUSION