PLAG prevents the loss of circulating neutrophils in the chemotherapy induced neutropenia model

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Abstract

Chemotherapy-induced neutropenia (CIN) is a common dose-limiting toxicity in cancer patients undergoing cytotoxic chemotherapy. It is generally understood that myelosuppression is the major mechanism resulting in CIN. However, an excessive extravasation (diapedesis) of neutrophils in response to tissue damage-caused by anti-cancer agents is also a possible mechanism. In this study, we have investigated the effects of 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) for the maintenance of circulating neutrophil in the gemcitabine induced neutropenia. In CIN mouse model using anticancer drugs, PLAG effectively protected the loss of circulating neutrophils as similar to CXCR2 antagonist, reparixin. And succeeded in vitro neutrophil migration assay also revealed that chemo-attractive activity of neutrophil induced by treatment of anticancer drugs was completely interrupted by PLAG addition. Chemokine (C-X-C motif) ligand 2 (CXCL2) and CXCL8 were effectively induced by treatment of gemcitabine with dose dependent manner in monocyte cell lines and mainly mediated via the signal transducer and activator of transcription 3 (STAT3) signaling pathway. PLAG effectively attenuated gemcitabine induced CXCL2 and CXCL8 expression via interrupting STAT3 activity and also regulated gemcitabine induced macrophage 1 antigen (MAC-1) and L-selectin expression, which means that PLAG interrupted the acceleration of neutrophil extravasation under CIN. Also we have verified that PLAG analogues and metabolites were not affected on neutrophil migration unlike PLAG, suggesting that acetylated diacylglycerol has a key role to regulate on neutrophil migration. Collectively, modulating effect of PLAG on excessive neutrophil extravasation by chemotherapy via attenuating STAT3/CXCL2(8) pathway could be used as very powerful regimen for preventing of neutropenia during diverse chemotherapy.

Introduction

- Neutropenia, the decreasing of circulated neutrophil in blood, is a critical side effect in chemotherapy. Neutropenic patients are vulnerable to infection of pathogen. **Aapro MS. *et al* (2006). Eur J Cancer 42: 2433–2453.**
- Gemcitabine is a DNA analog and causes generation of reactive oxygen species (ROS). **Chen SH. *et al* (2014). Biochimie 103:71-9.**
- Accumulation of ROS induces diverse inflammatory responses, such as, expression of chemokines. **Mittal M. et al (2014). Antioxid Redox Signal 20(7):1126-67.**
- Chemokines, CXCL2 and CXCL8, are produced from the damaged tissues by chemotherapeutic agent, gemcitabine, which accelerates neutrophil transmigration. **Song Y. *et al* (2015). Biochem Biophys Res Commun 2015;458(2):341-6.**
- Signal Transducer and Activator of Transcription 3 (STAT3) is a important factor on neutrophil migration, involved on expression of chemokines and adhesion molecule. **Zhang S. *et al* (2015). Am J physiol Lung Cell Mol Physiol. 2015;308(11):L1159-67**
- MAC-1 and L-selectin are key molecule in neutrophil transmigration. And chemokine induces that expression of adhesion molecules. **Takami M. *et al* (2002). J Immunol 2002;168(9):4559-66.**

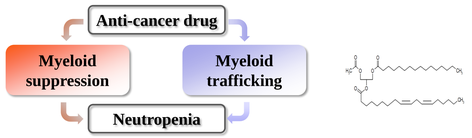


Diagram of CIN (chemotherapy-induced neutropenia)

PLAG

Method

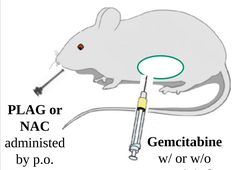
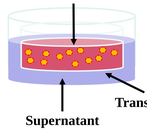


Diagram of mice model

HL-60 (5 x 10^6cells/100μl) was suspended in RPMI1640 w/ or w/o SB225002

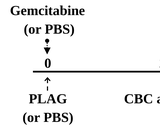


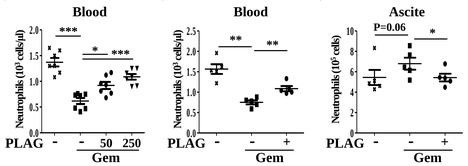
(cultured medium from THP-1 treated with **Gemcitabine** w/ or w/o **PLAG**, **NAC or S3I** for 24hr)

Diagram of transwell assay

Result

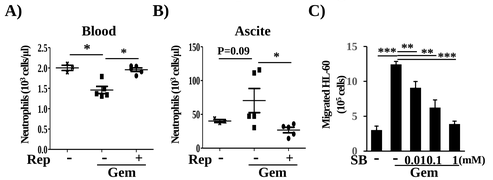
1. PLAG attenuates gemcitabine induced neutropenia via repression of neutrophil transmigration.





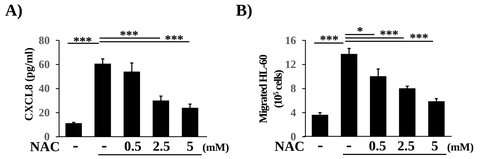
Gemcitabine was administered intraperitoneally in a dose of 50 mg/kg. For the group receiving PLAG, was administered 50 or 250 mg/kg of PLAG through oral and measured number of neutrophil in blood and ascite. The bars represent the mean ± SD. Student's t-test was performed to determine the p values, and p values less than 0.05 were considered statistically significant. *p<0.05, **p<0.01, ***p<0.005.

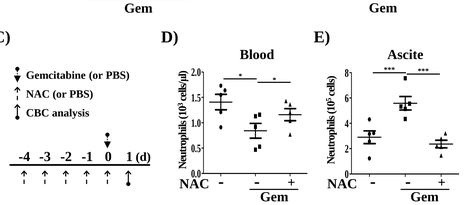
2. CXCR2 mediated chemotaxis is a key role in gemcitabine induced neutrophil transmigration.



(A, B) Gemcitabine was administered intraperitoneally in a dose of 50 mg/kg. For the group receiving 30 mg/kg of reparixin, CXCR2 antagonist was administered by intraperitoneal injection and after 24h, number of neutrophil in blood **(A)** and ascite **(B)** were measured by CBC analysis. **(C)** The supernatant of gemcitabine treated THP-1 cells was placed in the bottom chamber. And HL-60 cells were seeded in the upper chamber and treated with various concentrations of CXCL8 antagonist, SB 225002 (0.01, 0.1, 1 mM). Migrated HL-60 cell was counted after 24hr incubation in assay chamber. The bars represent the mean ± SD. Student's t-test was performed to determine the p values, and p values less than 0.05 were considered statistically significant. *p<0.05, **p<0.01, ***p<0.005.

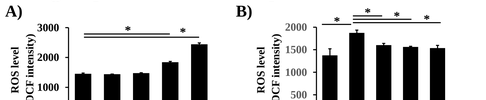
3. NAC, ROS scavenger, attenuates gemcitabine induced chemokine expression and neutrophil transmigration via repression of ROS generation.

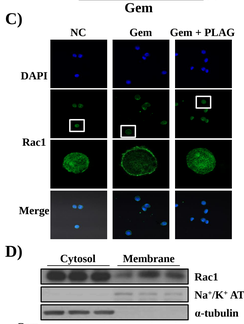




(A, B) THP-1 cells were stimulated with gemcitabine (10μg/ml) with various concentrations of NAC, ROS scavenger (0.5, 2.5, 5 mM) **(A)** The cultured supernatant was harvested at 24h and the protein level of CXCL8 was measured by ELISA. **(B)** The supernatant of gemcitabine treated THP-1 cells was placed in the bottom chamber. And HL-60 cells were seeded in the upper chamber. Migrated HL-60 cell was counted after 24hr incubation in assay chamber. **(C, D, E)** Gemcitabine was administered intraperitonially in a dose of 50 mg/kg. For the group receiving NAC, was administered 250 mg/kg of NAC through oral and measured number of neutrophil in blood and ascite. The bars represent the mean ± SD. Student's t-test was performed to determine the p values, and p values less than 0.05 were considered statistically significant. *p<0.05, **p<0.01, ***p<0.005.

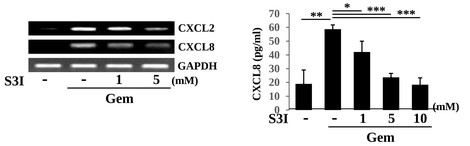
4. PLAG represses gemcitabine induced ROS generation through repression of NOX complex formation.

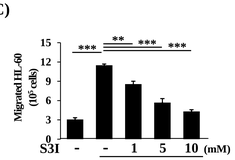




(A, B) THP-1 cells were stimulated with gemcitabine (10μg/ml) w/ or w/o various concentrations of PLAG. ROS generation was measured by flow cytometry analysis in THP-1 was performed using CM-H2DCFDA, general oxidative stress indicator **(C, D)** Rac-1 localization. **(C)** Localization of Rac-1 was visualized by using ZEN imaging software. **(D)** Western blot analyses of cytosolic and membrane fractions from THP-1 cells. And detection of Rac-1 in the cytosolic or membrane fraction. To determine even protein loading and purity of cytosolic and membrane fraction, probed with α-tubulin and Na^+/K^+ ATPase antibody. The bars represent the mean ± SD. Student's t-test was performed to determine the p values, and p values less than 0.05 were considered statistically significant. *p<0.05, **p<0.01, ***p<0.005.

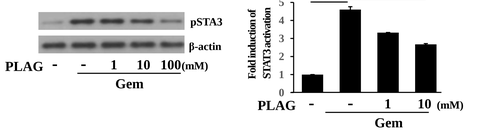
5. Gemcitabine induces chemokine expression and neutrophil transmigration via up-regulation STAT3 activation.

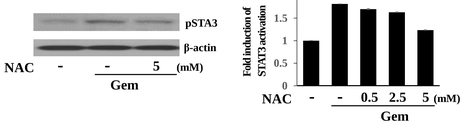




THP-1 cells were stimulated with gemcitabine (10μg/ml) with various concentrations of S3I-201, STAT3 inhibitor (1, 5, or 10 mM) **(A)** CXCL2 or CXCL8 was measured by RT-PCR **(A)** and ELISA **(B)**. β-actin was used as an internal control. **(C)** The supernatant of gemcitabine treated THP-1 cells was placed in the bottom chamber. And HL-60 cells were seeded in the upper chamber. Migrated HL-60 cell was counted after 24hr incubation in assay chamber. The bars represent the mean ± SD. Student's t-test was performed to determine the p values, and p values less than 0.05 were considered statistically significant. *p<0.05, **p<0.01, ***p<0.005.

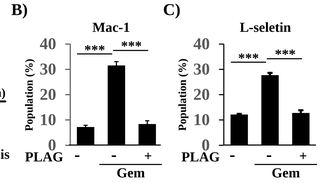
6. Gemcitabine induced STAT3 activation attenuated by PLAG and NAC.





(A, B) THP-1 cells were stimulated with gemcitabine (10μg/ml) with PLAG at various concentrations (1, 10, or 100μg/ml). **(A)** The cells were harvested, extracted total proteins, and phosphorylated STAT3 detected by western blot. β-actin was used as an internal control. **(B)** Reporter construct containing luciferase gene regulated by STAT3 activity was transfected to A549 cells and the effect of PLAG on expression of the luciferase gene was analyzed by reporter assay. **(C, D)** THP-1 cells were stimulated with gemcitabine (10μg/ml) with NAC at various concentrations (0.5, 2.5, or 5μg/ml). **(C)** The cells were harvested, extracted total proteins, and phosphorylated STAT3 detected by western blot. β-actin was used as an internal control. **(D)** Reporter construct containing luciferase gene regulated by STAT3 activity was transfected to A549 cells and the effect of PLAG on expression of the luciferase gene was analyzed by reporter assay. The bars represent the mean ± SD. Student's t-test was performed to determine the p values, and p values less than 0.05 were considered statistically significant. *p<0.05, **p<0.01, ***p<0.005.

7. PLAG down-regulates adhesion molecules in mouse PBMC undergoing chemotherapy.



Gemcitabine was administered intraperitoneally in a dose of 50 mg/kg. For the group receiving PLAG, was administered 250 mg/kg of PLAG through oral and flow cytometry analysis in blood was performed using Alexa Fluor 488-conjugated anti-Gr-1, PE-Cy7-conjugated anti-CD11b, and APC-conjugated CD11b (MAC-1 alpha) or APC-conjugated L-selectin. GR-1$^+$CD11b$^+$ cells were analyzed for APC-conjugated CD11b (MAC-1 alpha) **(B)**, and APC-conjugated L-selectin **(C)**. The bars represent the mean ± SD. Student's t-test was performed to determine the p values, and p values less than 0.05 were considered statistically significant. *p<0.05, **p<0.01, ***p<0.005.

Conclusion

- Circulating neutrophils were transmigrated by chemotherapeutic agent, gemcitabine, and which was attenuated by PLAG.
- ROS generation and chemokine-CXCR2 interaction are key roles in chemotherapeutic agent, gemcitabine, induced neutrophil transmigration.
- PLAG represses gemcitabine induced formation of NOX complex via inhibition of Rac1 localization to membrane.
- PLAG and NAC down-regulate neutrophil transmigration via repression of STAT3 mediated chemokine expression. And PLAG also down-regulates expression of adhesion molecules, Mac-1 and L-selection, in neutrophils.
- In this study, we suggest that chemotherapeutic agent induced chemokine mediated transmigration is a possible cause in chemotherapy induced neutropenia. And PLAG has an effect on CIN via down-regulation of ROS-STAT3 mediated chemokines expression and chemokine induced adhesion molecule expression.