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1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) exhibits the therapeutic effect in chemoradiation-induced oral mucositis mouse model

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Abstract

Oral mucositis is a common complication of chemoradiation therapy and is often accompanied by erythema, ulceration, pain, weight loss. It could delay remission and limit the effectiveness of cancer therapy and increase the risk of infections. However, no specific therapy for protection against mucositis is currently available. In previous study, PLAG (1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol, acetylated diglyceride) was shown to exert a therapeutic effect with pegfilgrastim to treat chemotherapy-induced neutropenia by modulating neutrophil transmigration and chemotherapy-induced megakaryocyte/erythrocyte progenitor decrease was significantly alleviated following PLAG administration. In this study, we investigated the therapeutic effect of PLAG in 5-fluorouracil (5-FU) and radiation-induced oral mucositis mouse model. Following 5-FU (50 mg/kg) injection on Day 0 and 2, mice were exposed to whole-body irradiation with 1 Gy of gamma radiation on Day 2. In order to facilitate the risk of infection, tongues were scratched 0.2 cm wound at using the tip of an 18-gauge needle at an equal force and depth on Day 4. PLAG was orally administered at 250 mg/kg/day. 5-FU/γ-radiation/scratching-induced oral mucositis mice exhibited ulceration, fibrosis, and festering wounds and had still not fully recovered. PLAG increased mice survival rate and decreased body-weight loss. PLAG treatment group decreased the ulcer formation and diminished the degree of wound festering form and inhibited mucositis-induced inflammatory responses in the serum on Day 7. Therefore, PLAG administration significantly reduced CRIOM and recovered scar better quicker.

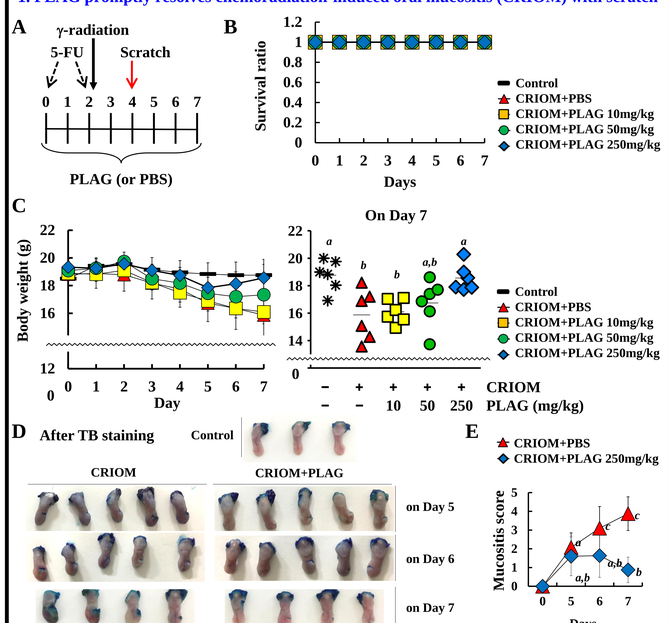
For head only radiation, custom-made lead shield was used for mice to limit the radiation to the head. After 5-FU (100 mg/kg) injection, mouse's head were received 20 Gy of x-ray, and PLAG was administrated with 250 mg/kg daily. In toluidine blue (TB) staining, PLAG suppressed chemoradiation-induced oral mucositis. In histological analysis, CRIOM-induced epithelium damage was protected by PLAG administration. Inflammation-related cytokines were significantly limited by PLAG administration. In HaCaT cells, 5-FU/x-ray significantly rapidly increased intracellular ROS generation. However, PLAG increased ROS in short time and might be eliminate DAMP (Damage-associated molecular pattern). Following 24 hr x-radiation, the apoptosis ratio was significantly decreased by PLAG treatment. From these data, PLAG could be therapeutically useful in reducing the complications associated with chemotherapy and radiation, and thus may be an excellent supplementary agent for anti-cancer therapy.

Introduction

- PLAG (1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol) is an acetylated form of diacylglycerol and a mono-acetyl-diglyceride that was first isolated from the antlers of sika deer. PLAG can be chemically synthesized using glycerol, palmitic acid, and linoleic acid, and the synthetic form has been confirmed to be identical with the naturally isolated form. **Yang HO *et al.*, (2004) Biol Pharm Bull 27(7):1121-1125.**
- PLAG reduces the incidence of gemcitabine-induced neutropenia in pancreatic cancer patients. **Oh D *et al.*, (2015) World J Oncology 6(4):410-415.**
- PLAG was shown to exert a therapeutic effect with pegfilgrastim to treat chemotherapy-induced neutropenia by modulating neutrophil transmigration. **Yoo N., *et al* (2016) Cancer Letters 377(1), 25-31**
- PLAG plays a role in differentiating HSCs toward MEP and alleviating chemotherapy-induced bone marrow cell reduction. Thus PLAG shows its potential to augment the therapeutic effect of anti-cancer drugs-induced thrombocytopenia. **Lee HR *et al.*, (2018) Thrombosis Research 161:84-90.**
- PLAG plays an anti-inflammatory role in chemotherapy-induced oral mucositis and subsequently, promotes the healing of ulceration and reduces inflammation. PLAG enhances recovery from 5-FU-induced oral mucositis. **Lee HR *et al.*, Frontiers in Oncology (2016) 6: 209.**

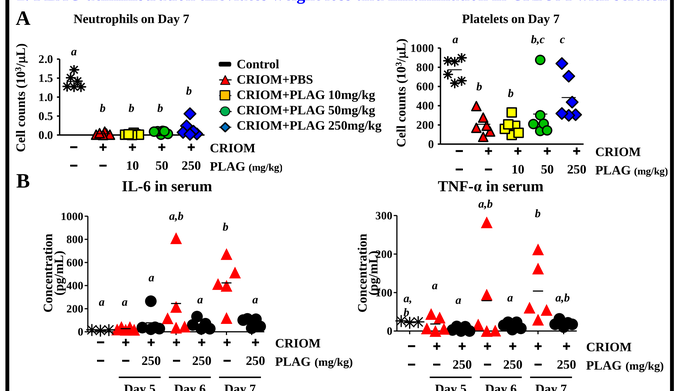
Results

1. PLAG promptly resolves chemoradiation-induced oral mucositis (CRIOM) with scratch



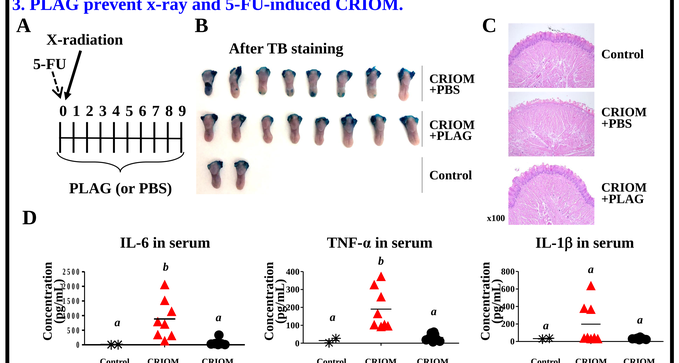
The 8-10 weeks female Balb/c mice (Koatech Co.) were administered intraperitoneally 5-FU (50 mg/kg, Sigma Aldrich) on Day 0 and 2. PLAG (Enzychem Lifesciences Co.) was administered orally with 10, 50, 250 mg/kg once daily. Whole body was received 1Gy of gamma radiation on Day 2. For the scratching group, mice were anesthetized with 2,2,2-tribromoethanol (150 mg/kg, Sigma Aldrich) by intraperitoneal injection, and then a 0.5-cm^2 area of the tongue was scratched using the tip of an 18-gauge needle (Koreavaccine) at an equal force and depth on Day 4. Each group contained seven mice. Experimental design of the study was represented in **Figure 1A**. There is no difference in survival ratio (**Figure 1B**). When body weigh was recorded daily, the chemoradiation and scratch induced the severe damage on mice (**Figure 1C**). PLAG administration reversed weight loss and recovered to control on Day 7. Following 1% toluidine blue (TB, Sigma Aldrich) staining, which is visualized mucosal lesions, CRIOM groups was shown the ulcer formation, fester wound and infection (**Figure 1D**). However, PLAG administrated group represented the decreased ulcer and diminished wound festering, and finally showed the rapid tongue restoration on Day 7. Mucositis score was significantly decreased by PLAG administration (**Figure 1E**). These data indicate that PLAG has a significant therapeutic effect against chemoradiation-induced oral mucositis and cachexia. Data represent one experiment performed in triplicate. Results are presented as mean±s.e.m. Statistical analysis was performed by one-way ANOVA, followed by Duncan's post hoc test. Different letters (*a*, *b*, *c*) show values having significant difference at $p<0.05$.

2. PLAG administration alleviates weight loss and inflammation in CRIOM with scratch



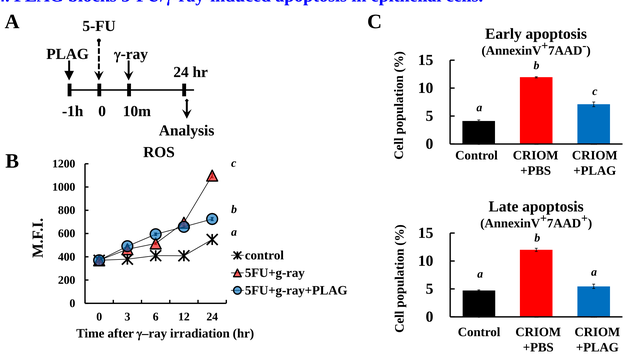
Mice (n=5~6 per group) were treated as described in Figure 1A, the circulating neutrophils and platelet in blood were counted by complete blood count analysis (BC 5300 Mindray analyzer, Shenzhen Mindray Bio-medical Electronics). Although neutrophils has no deference among LPS-treated gropus, the number of platelet was increased in PLAG-treated mice than LPS alone (**Figure 2A**). Following tongue scratch, the serum samples were acquired and analyzed ELISA assay. Inflammatory cytokine, IL-6 and TNF-α were significantly increased by LPS and were suppressed by PLAG administration as control level (**Figure 2b**). Our data showed that PLAG blocks the inflammatory response. Data represent one experiment performed in triplicate. Results are presented as mean±s.e.m. Statistical analysis was performed by one-way ANOVA, followed by Duncan's post hoc test. Different letters (*a*, *b*, *c*) show values having significant difference at $p<0.05$.

3. PLAG prevent x-ray and 5-FU-induced CRIOM.



Mice (7-9 weeks, Balb/c mice, KAIST) were administered intraperitoneally 5-FU (100 mg/kg, Sigma Aldrich). After 1 hr, mice head received 20 Gy using x-ray irradiator (X-RAD 320, 1.8 Gy/min). Custom-made lead shields were used for mice to limit the radiation to the heads. PLAG (Enzychem Lifesciences Co.) was administered orally with 250 mg/kg once daily. Experimental design of the study was represented in **Figure 3A**. Mice were scarified 9 days after head-only radiation and the isolated tongues were stained 1% toluidine blue (TB, Sigma Aldrich). PLAG administrated mice were shown no mucositis and ulcer in tongues (**Figure 3B**). In H&E staining, CRIOM-induced epithelium damage was markedly protected by PLAG administration on Day 9 (**Figure 3C**). On Day 9, IL-6, TNF-α, and IL-1β in serum were significantly increased in LPS treatment group (**Figure 3D**). PLAG/LPS treated mice were shown the decreased inflammatory cytokines as control level. Data represent one experiment performed in triplicate. Results are presented as mean±s.e.m. Statistical analysis was performed by one-way ANOVA, followed by Duncan's post hoc test. Different letters (*a*, *b*) show values having significant difference at $p<0.05$.

4. PLAG blocks 5-FU/γ-ray-induced apoptosis in epithelial cells.



HaCaT (human keratinocyte cell line) was preincubated with 100 μg/ml of PLAG for 1 hr, and then stimulated with 100 ng/mL of 5-FU. Cell plates were received 7 Gy of gamma radiation and incubated in CO_2 incubator. Experimental design of the study was represented in **Figure 4A**. On 3, 6, 12, and 24 hr, cells were fixed and stained with CM-H2DCFDA (Invitrogen) for intracellular ROS. For flow cytometric analysis, cells were washed and were analyzed with a FACSVerse flow cytometer (BD Biosciences), and data were processed with FlowJo software (Tree Star, OR, USA). PLAG increased intracellular ROS level at 3 and 6 hr, and decreased at 24 hr (**Figure 4B**). However, 5-FU/γ-ray rapidly increased up to 24 hr. These reflected cell apoptosis ratio using annexinV and 7AAD staining assay. On 24 hr, 5-FU/γ-ray-induced apoptosis was significantly decreased by PLAG treatment (**Figure 4C**). These means that PLAG stimulates the faster generation of ROS and promotes cell homeostasis. Data represent one experiment performed in triplicate. Results are presented as mean±s.e.m. Statistical analysis was performed by one-way ANOVA, followed by Duncan's post hoc test. Different letters (*a*, *b*) show values having significant difference at $p<0.05$.

Summary

- **PLAG increased mice survival rate and decreased body-weight loss in CRIOM mice model.**
- **PLAG decreased the ulcer formation, diminished the degree of wound festering form, and significantly recovered scar better quicker.**
- **PLAG protected 5-FU/γ-ray-induced apoptosis in epithelial cells.**