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Original Article

PAF receptor antagonist Ginkgolide B inhibits tumourigenesis and angiogenesis in colitis-associated cancer

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Abstract: Platelet activating factor (PAF), a potent pro-inflammatory phospholipid, has been found to trigger tumor growth and angiogenesis through its G-protein coupled receptor (PAFR). This study was aimed to investigate the potential role of PAF in azoxymethane (AOM)/dextran sulfate sodium (DSS) induced colitis-associated cancer (CAC), using PAFR antagonist Ginkgolide B (GKB). We found GKB up-regulated serum level of PAF-AH activity. As assessed by disease activity index (DAI), histological injury scores, leukocytes infiltration, and expression of pro-inflammatory cytokines, GKB ameliorated colonic inflammation and decreased tumor number and load in mice. GKB also decreased expression of vascular endothelial growth factor (VEGF) and microvessel density (MVD) in tumor. These results suggest that PAFR antagonist might be a potential therapeutic strategy for CAC.

Keywords: PAF, Ginkgolide B, colitis-associated cancer, angiogenesis, VEGF

Introduction

Inflammatory bowel disease (IBD) is a chronic intestinal inflammatory process with two main phenotypes: ulcerative colitis (UC) and Crohn's disease (CD). IBD is considered as one of the top three risk conditions for colorectal cancer, and this risk increases with the duration of inflammation [1]. To date, the mechanisms by which IBD promotes colorectal cancer have not been fully elucidated. Increasing evidences suggested that inflammation induced angiogenesis contribute to CAC [2, 3].

Angiogenesis is one of the essential processes in tumor growth and metastasis. Angiogenesis based targeted therapy is considered as milestone for cancer treatment. Angiogenesis also takes part in inflammatory diseases by increasing influx of inflammatory cells and nutrient supply, activating cytokines, chemokines, and matrix metalloproteinases, et al [4]. Microvessel density and expression of VEGF level and its receptors are increased in IBD mucosa [5]. Human intestinal microvascular endothelial cells (HIMECs) isolated from UC and CD tissue showed enhanced capacity of adhering leukocytes [6]. Serum and mucosa level of VEGF was significantly increased in patients with IBD and correlated with disease activity [7-9]. However, the role of angiogenesis in CAC remains poorly understood.

Platelet activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a potent pro-inflammatory mediator, can recruit and activate leukocytes, prompt release of cytokines and chemokines and induce angiogenesis [10]. PAF exerts its biological activities in various tissues through a G-protein coupled receptor (PAFR) [11]. In UC and CD, PAF is up-regulated in the inflammatory colonic mucosa, and is considered to be involved in the pathogenesis of IBD [12, 13]. PAF level is elevated in colonic tissue in colitis mice model, and PAFR antagonist can significantly alleviate colonic inflammation [14]. On the other hand, PAF could stimulate migration of HIMECs, and promote angiogenesis and vascular permeability by activating VEGF expression [15-18]. In breast, prostate cancer, and

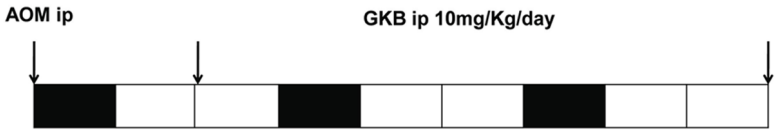

Figure 1. Experimental protocol of CAC model. All mice received i.p. injected AOM on day 1 followed by 3 cycles of 2% DSS in the drinking water for 7 days and then distilled water for 14 days. Mice in control group received no drug treatment; Vehicle treated group received vehicle (4% ethanol + 5% Tween 80 + 5% polyethylene glycol 400) i.p. injection after day 14; GKB treated group received 10mg/kg/day of GKB (*Sigma-Aldrich, St Louis, MO, USA*) i.p. injection after day 14. (n=8 per group). GKB, Ginkgolide B; PEG, polyethylene glycol; CAC, *colitis-associated cancer;* AOM, axozymethane; DSS, dextran sulfate sodium.

Kaposi's sarcoma, PAFR antagonist could inhibit tumor growth by inhibition of tumor angiogenesis [16, 19, 20]. Thus, these research suggest that PAF may contribute to CAC by stimulating inflammation related angiogenesis.

To date, the pro-angiogenesis effect of PAF in inflammation and cancer is confirmed, whereas no evidence of PAF taking part in CAC development is found. This study is aimed to investigate the role of PAF in tumorigenesis and angiogenesis of CAC by administration of GKB in mice.

Materials and methods

Animals

Eight-week-old female C57/BL6 mice weighing about 18-20 g were obtained from the Experimental Animal Center of Sun Yat-Sen University, Guangzhou, China and mice were housed in a room with controlled humidity and temperature in collective cages. The animals were supplied a pelleted basal diet and drinking water. This study was approved by the Ethical Committee for Animal Care and Use of Zhongshan School of Medicine, Sun Yat-sun University according to an approved protocol.

Inducement of CAC and drug administration

We used AOM (Sigma Aldrich, St. Louis, MO, USA) and DSS (MP Biomedicals, Santa Ana, CA, USA) establishing CAC model as previously described [21]. The procedure is shown in **Figure 1**. Animals were sacrificed under anesthesia (1:1, v/v of xylazine 2%-ketamine 10%) 9 weeks after the injection of AOM.

Evaluation of CAC

Disease activity index (DAI) was determined in accordance with the method described by Murthy et al. [22]. After the mice were killed, colon tissues were removed immediately and the length of colons were measured, and then colons were opened longitudinally, flushed with cold PBS to remove faecal material. The numbers of colon tumors were counted and load per mice (assessed by diameters) were measured with a calliper, and then fixed in 10% buffered formalin, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E). Images were captured by a digital camera (Nikon, Tokyo, Japan) at × 100 magnification. Histological injury scores of H&E-stained specimens were calculated by two investigators who were blinded to the treatment according to previously established criteria [23].

Measurement of PAF-AH activity

Blood samples were collected by orbital bleeding under anesthesia. Serum PAF-AH levels were measured by mouse PAF-AH ELISA kit (Cusabio Biotech, Wuhan, China) according to the manufacturer's instructions. After color development, the absorbance was measured at 450 nm using a microplate reader (Thermo Scientific, Waltham, MA, USA). The results of PAF-AH activity were expressed by picogram per milliliter.

Myeloperoxidase activity assay

MPO activity was measured by ELISA. Colon samples were weighed and homogenized by a tissue homogeniser in nine volumes of PBS, and then centrifuged at 2,000 ×g for 20 min at 4°C. MPO activity in the resulting supernatant was assayed by mouse MPO ELISA kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. MPO activity was determined by measuring the absorbance at 450 nm using a microplate reader (Thermo Scientific, Waltham, MA, USA). The results were expressed by units of MPO per milligram tissue.

ELISA for cytokines

The colons were harvested and homogenates were made as mentioned above. The concen-

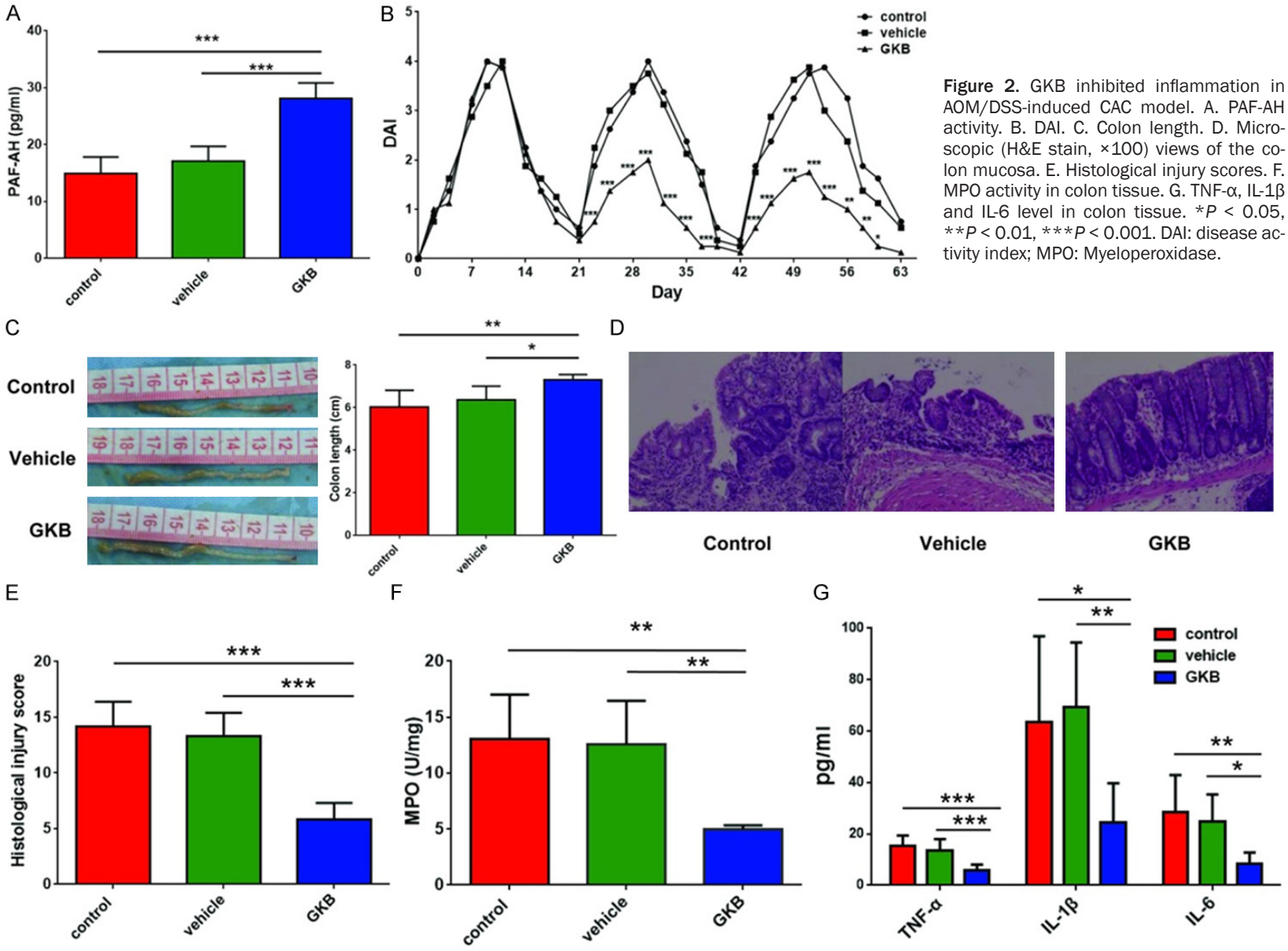

Figure 2. GKB inhibited inflammation in AOM/DSS-induced CAC model. A. PAF-AH activity. B. DAI. C. Colon length. D. Microscopic (H&E stain, ×100) views of the colon mucosa. E. Histological injury scores. F. MPO activity in colon tissue. G. TNF-α, IL-1β and IL-6 level in colon tissue. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. DAI: disease activity index; MPO: Myeloperoxidase.

Table 1. Correlations between PAF-AH and TNF-α, IL-1β, IL-6, tumor number, tumor load, MVD

	P-value
PAF-AH versus TNF-α	0.001**
PAF-AH versus IL-1β	0.048*
PAF-AH versus IL-6	0.011*
PAF-AH versus tumor number	< 0.001***
PAF-AH versus tumor load	0.002**
PAF-AH versus MVD	< 0.001***

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

trations of TNF-α, IL-1β and IL-6 in supernatants of homogenized colon tissue were measured by mouse ELISA kits for TNF-α, IL-1β and IL-6 (BioLegend, SanDiego, CA, USA) according to the manufacturer's instructions. After color development, the absorbance was measured at 450 nm using a microplate reader (Thermo Scientific, Waltham, MA, USA). The results of TNF-α, IL-1β and IL-6 activity of supernatant were expressed by picogram per milliliter.

Immunohistochemistry for CD31

Immunohistochemistry for CD31 was performed on formalin-fixed, paraffin-embedded tissue sections using the mouse polyclonal antibody (dilution, 1/100; Abcam, Cambridge, UK) and following the protocols. Stained tumor sections were examined under a light microscope, and images were captured by a digital camera (Nikon, Tokyo, Japan) at ×200 magnification. For each tumor section, 5 fields with the greatest density of positively stained vessels were evaluated. MVD was defined as the number of positively stained vessels per high-power field.

Quantitative real time PCR

Total RNA was isolated from tissue samples with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was reverse transcribed by M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol. Forward (CATCTTCAAGCCGTCCTGTGT) and reverse (CAGGGCTTCATCGTTACAGCA) primers were designed as sequence-specific primers for the detection of VEGF mRNA expression. Real-time PCR was performed with GoTaq qPCR Master Mix (Promega, Madison, WI, USA) on a Mini-Opticon Real-Time PCR detection instrument (Bio-Rad, Hercules, CA, USA) using the SyBr Green detection protocols. Samples were amplified with the following program: initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation for 15 s at 95°C and annealing/elongation for 60 s at 60°C. All PCRs were run in triplicate, and control reactions without template were included.

Western blot analysis

Western blot analysis was performed to assay VEGF protein expression in tumor. In short, equal amounts of isolated protein were added to electrophoresis sample buffer and boiled according to the manufacturer's guidelines. Proteins were separated by SDS-PAGE on precast gels (10% acrylamide; NuSep, AUS) and transferred to a nitrocellulose membrane, which was incubated with specific antibodies against VEGF (dilution, 1/150; Abcam, Cambridge, UK) and GAPDH (dilution, 1/5000; Abcam, Cambridge, UK). Detection of specific bands was performed with the ECL Western blotting analysis system (Abcam, Cambridge, UK).

Statistical analysis

Data were expressed as the mean ± standard error and analyzed using SPSS 17.0 statistical software (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Administration of GKB inhibited inflammation in AOM/DSS-induced CAC model

Increased amount of PAF could stimulate synthesis of PAF acetylhydrolase (PAF-AH), which would in turn deactivate PAF to keep a balanced serum level of PAF. Therefore, serum PAF-AH level could act as an indicator for PAF signaling [24]. Meanwhile, PAF-AH plays protective role in inflammatory diseases, such as atherogenesis [25], asthma [26], and CD [27].

To confirm that GKB could inhibit PAF signaling in vivo, serum PAF-AH activity was tested by ELISA. After 7 weeks of GKB treatment, serum PAF-AH activity was significantly higher in GKB treated group than that in control group and vehicle treated group ($P < 0.001$ vs. control group, $P < 0.001$ vs. vehicle treated group) (**Figure 2A**).

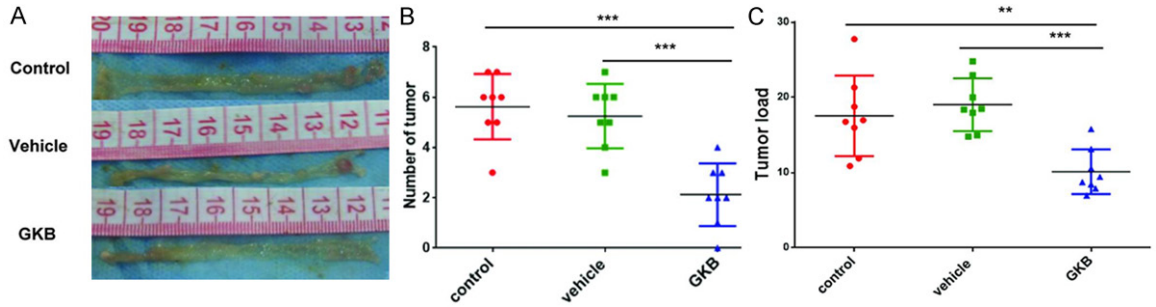

Figure 3. GKB inhibited tumorigenesis in AOM/DSS-induced CAC model. A. Macroscopic views of the colonic tumor. B. Number of colonic tumors per mouse (multiplicity). C. tumor load (sum of tumor diameters) per mouse **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

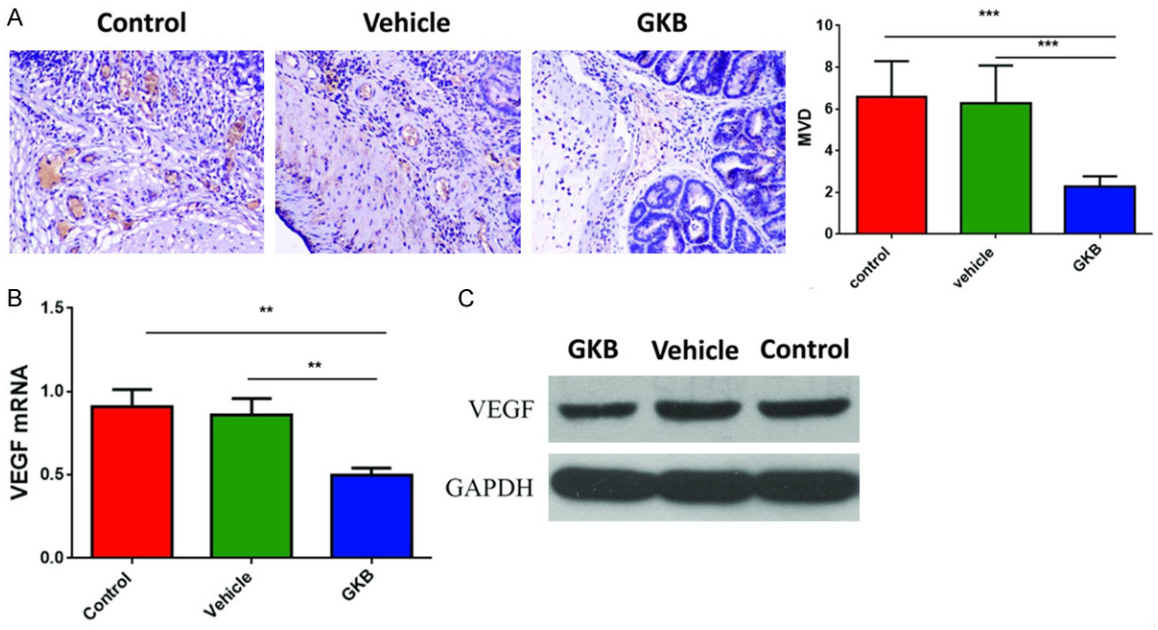

Figure 4. GKB inhibited tumor angiogenesis in AOM/DSS-induced CAC model. A. Microscopic (×200) views of immunohistochemistry for CD31 stained colon tumor and numbers of microvessel density of colon tumor. B. Q-PCR evaluation of VEGF mRNA. C. Western blot analysis evaluation of VEGF protein **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

To analyze the role of PAF in AOM/DSS-induced CAC model, we first examined objective alterations after administration of PAFR antagonist. DAI, assessed by weight loss, stool consistency, hemoccult or gross bleeding, was significant decreased in GKB treated group after day 21 (**Figure 2B**). Inflammation caused shortening of the colon was markedly ameliorated in GKB treated group than in control group (*P* = 0.008 vs. control group, *P* = 0.048 vs. vehicle treated group) (**Figure 2C**). For microscopic examination, glandular distortion and inflammatory cells infiltration were found in submucosa (**Figure 2D**), and degrees of mucosal destruction were assessed by histological injury score. We found GKB treated group showed a significant decreased histological injury score compared with the control group and vehicle treated group (*P* < 0.001 vs. control group, *P* < 0.001 vs. vehicle treated group) (**Figure 2E**).

Leukocytes infiltration is one of key events in chronic intestinal inflammation and can serve as an indicator for local inflammation. Assessed by MPO activity, we observed that leukocytes infiltration was significantly decreased in GKB treated group compared with control and vehicle treated group (*P* = 0.002 vs. control group, *P* = 0.003 vs. vehicle treated group) (**Figure 2F**).

We also examined pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in colonic mucosa. As

assessed by ELISA, expression of TNF-α, IL-1β and IL-6 were significantly decreased in colon tissue in GKB treated group compared with control ($P < 0.001$, $P = 0.017$ and $P = 0.003$ respectively) and vehicle treated group ($P = 0.001$, $P = 0.006$ and $P = 0.021$ respectively) (**Figure 2G**). To further prove the influence of PAF signaling on colonic inflammation, we performed correlation analysis between expression of inflammatory cytokines and PAF-AH, the indicator for PAF signaling. We found TNF-α, IL-1β and IL-6 were negatively correlated with activity of PAF-AH by correlation analysis. ($P = 0.001$, $P = 0.048$ and $P = 0.011$ respectively) (**Table 1**). Taken together, these results suggest that PAFR antagonist could suppress inflammation in AOM/DSS-induced CAC model.

GKB inhibited tumorigenesis in AOM/DSS-induced CAC model

Intraperitoneal injection of mutagenic agent AOM with repeated oral administration of pro-inflammatory agent DSS could produce mice tumor model. Most tumors distributed in distal one-third of colon (**Figure 3A**). After 7 weeks of GKB treatment, tumor number and load (sum of all tumor diameter per mouse) were both significantly reduced in GKB treated group (tumor number: $P < 0.001$ vs. control group, $P < 0.001$ vs. vehicle treated group; tumor load: $P = 0.004$ vs. control group, $P = 0.001$ vs. vehicle treated group) (**Figure 3B**, **3C**). Correlation analysis showed that tumor number and load were negatively correlated with activity of PAF-AH by correlation analysis. ($P < 0.001$ and $P = 0.002$, respectively) (**Table 1**).

GKB inhibited tumor angiogenesis in AOM/DSS-induced CAC model

Given the pro-angiogenic effect of PAF in some types of human cancer and chronic inflammation [19, 28], we wondered whether PAF also exert angiogenic role in CAC models. As assessed by CD31 immunohistochemical staining, MVD were significant decreased in tumors from GKB treated group than in control and vehicle treated group ($P < 0.001$ vs. control group, $P < 0.001$ vs. vehicle treated group) (**Figure 4A**). VEGF is one of the key factors greatly affect angiogenesis. As examined by qPCR and western blot, mRNA and protein levels of VEGF were significantly suppressed by GKB, compared with control group and vehicle treated group ($P = 0.003$ vs. control group, $P = 0.007$ vs. vehicle treated group) (**Figure 4B**, **4C**). Correlation analysis showed that MVD ($P < 0.001$) were negatively correlated with the activity of PAF-AH (**Table 1**).

Discussion

The mechanism of how colitis prompting CAC is still not clear. Previous studies showed that PAF is one of the most potent lipid inflammatory mediators in inflammatory diseases and cancers [12, 13, 16, 19, 20, 29]. However, no research has explored the function of PAF in the development of CAC. In this study, we found that inhibition of PAF by GKB markedly suppressed colitis and reduced tumor number and load in AOM/DSS-induced CAC. In addition, GKB reduced MVD and decreased mRNA and protein level of VEGF in tumor, suggesting that GKB might inhibit CAC by suppressing angiogenesis. Thus, PAFR antagonist may be a novel strategy for the treatment of CAC. To our knowledge, this is the first study characterizing the relationship between PAF and CAC and explaining the pro-angiogenesis role of PAF in CAC model.

Theoretically, PAFR antagonist GKB can increase serum level of PAF by blockade of PAFR and result in increased PAF-AH activity, which could in turn degrade excessive amount of PAF [24]. In this study, GKB could effectively block PAFR in mice, as indicated by higher activity of PAF-AH after administration of GKB. Previous study showed the pro-inflammatory role in several disease [25-27]. Consistent with these findings, activity of PAF-AH was positively correlated with MPO activity and pro-inflammatory cytokines, suggesting the pro-inflammatory role of PAF in AOM/DSS-induced CAC model. Infiltrating leukocytes, over-expressed inflammatory cytokines and enhanced microvascular permeability form a feedback loop and boost the inflammatory response [30]. In the present study, we observed that MPO activity, the index of leukocytes infiltration, was significantly decreased in GKB treated mice, suggesting that inhibition of PAF signaling could suppress the recruitment of leukocytes. Pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, are over-expressed in patients with UC and CD and play vital role in development of CAC [31-34]. In our study, administration of GKB could significantly decrease expression of TNF-α,

IL-1β and IL-6. Therefore, inhibition of PAF biological activity by PAFR antagonist can effectively ameliorate inflammation in AOM/DSS-induced CAC model.

Inhibit PAF signaling by PAFR antagonist could inhibit breast, prostate cancer, Kaposi's sarcoma [16, 19, 20]. We found GKB significantly reduced the tumor number and load in CAC model, which were negatively correlated with the activity of PAF-AH. These results suggest that inhibition of PAF bioactivity might play a preventive role in CAC.

Angiogenesis contributes greatly to inflammation, and is one of the essential processes in the growth and metastasis of solid tumors [4]. PAF was found to regulate angiogenesis in breast, prostate cancer, Kaposi's sarcoma [16, 19] and rheumatoid arthritis [28]. In our study, mice treated with GKB showed significant decreased tumor MVD compared with control group and vehicle treated group, and activity of PAF-AH was significant correlated with MVD. PAF exerts its pro-angiogenic effects mainly through VEGF. PAF could stimulate expression of VEGF and promote angiogenesis in human endometrial epithelial cells, which could be abrogated by PAFR antagonist [35-38]. VEGFR-signaling was found promote growth of tumor cells in CAC, providing a molecular linkage between angiogenesis and CAC [39]. In this study, we found that blockade of PAF signaling was able to inhibit mRNA and protein level of VEGF, which could explain the reduction of MVD and tumor load after administration of GKB. These results suggest PAF may prompt CAC by stimulating angiogenesis.

In this study, PAFR antagonist GKB successfully inhibited inflammation and angiogenesis, and resulted in suppression of CAC in AOM/DSS mice model. Although the detailed mechanism needs further investigation, the current study implicates treatment with PAFR antagonist might serve as a novel therapeutic strategy for CAC.

Acknowledgements

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Disclosure of conflict of interest

None.

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