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ScienceDirect

Journal of Nutritional Biochemistry 93 (2021) 108635

Journal of Nutritional Biochemistry

Gamma-glutamyl carboxylated Gas6 facilitates the prophylactic effect of vitamin K in inhibiting hyperlipidemia-associated inflammatory pathophysiology via arresting MCP-1/ICAM-1 mediated monocyte-hepatocyte adhesion

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Received 17 August 2020; received in revised form 14 January 2021; accepted 10 March 2021

Abstract

Role of growth arrest-specific 6 (Gas6), member of vitamin K (VK)-dependent protein family in hyperlipidemia-associated inflammation remains unresolved. To address this, blood samples were collected from hyperlipidemic subjects and age-matched healthy controls and observed that gamma-glutamyl carboxylated Gas6 (Gla-Gas6) but not total Gas6 were significantly lower while pro-inflammatory markers, MCP-1 and ICAM-1 were remarkably higher in hyperlipidemic subjects compared to control. Correlation analyses demonstrated that Gla-Gas6 levels were inversely correlated with MCP-1 and ICAM-1 but positively with plasma VK in hyperlipidemic subjects but not in control. This suggests that boosting VK level might ameliorate the hyperlipidemia-associated inflammatory pathophysiology via augmenting Gla-Gas6. Further studies with high fat diet (HFD)-fed mice demonstrated that VK supplementation (1, 3, and 5 μg/kg BW, 8 weeks) dose-dependently reduced both hepatic and plasma levels of MCP-1 and ICAM-1 while elevating that of Gla-Gas6 but not total Gas6 in HFD-fed mice. Cell culture studies with gamma-glutamyl carboxylase (enzyme causes VK-dependent carboxylation of Gas6) knockdown hepatocytes and monocytes dissected the direct role of Gla-Gas6 in inhibiting high palmitic acid (0.75 mM)-induced inflammation via arresting MCP-1/ICAM-1 mediated hepatocyte-monocyte adhesion. The present study demonstrated an important role of Gla-Gas6 in facilitating the prophylactic effect of VK against hyperlipidemia associated inflammation.
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Keywords: Hyperlipidemia; Inflammation; Hepatocyte-Monocyte adhesion; Vitamin K; Gamma-glutamyl carboxylated growth arrest-specific protein 6 (Gla-Gas6).

1. Introduction

Growth arrest-specific protein 6 (Gas6), a member of vitamin K dependent protein (VKDP) family has been found to play crucial roles in regulating various cellular activities, including cell migration, adhesion, cell growth, and survival [1–3]. Beside this, several studies have also shown that Gas6 can act as an anti-inflammatory agent by inhibiting the expression of various pro-inflammatory markers, such as monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and E-selectin and preventing the adhesive interaction of leucocyte to endothelial cells under different pathophysiological conditions [4–8]. It has been widely established that being a VKDP, Gas6 requires vitamin K (VK) mediated post-translational carboxylation to become biologically active [9–12]. Using VK as a cofactor, the enzyme γ-glutamyl carboxylase (GGCX) catalyzes the conversion of specific glutamic acid (Glu) residues present in the N-terminus region of Gas6 to γ-carboxyglutamic acid (Gla) and thus generates carboxylated Gas6 (Gla-Gas6) [13].

Hyperlipidemia, a medical condition characterized by elevated blood levels of triglycerides, total cholesterol, cholesterol esters, and phospholipids, is considered as a well-established risk factor for the development of atherosclerosis, cardiovascular disease, and fatty liver disease [14]. Furthermore, hyperlipidemia is linked with the incidence of chronic inflammatory events stimulating the release of various pro-inflammatory cytokines, like IL-6, TNF-α, and interleukin 8 (IL-8) and chemokines, like MCP-1, which regulate the expression of different cell adhesion molecules (CAM) including vascular cell adhesion molecule-1 (VCAM-1) and ICAM-1

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https://doi.org/10.1016/j.jnutbio.2021.108635
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thus leading to severity of the pathological conditions [15]. Our recent study has demonstrated that Gla-Gas6 but not Gas6 plays a major role in preventing hyperlipidemia associated impaired hepatic lipid metabolism via regulating AMPK/SREBP1/PPARα signaling cascade [9]. Although, lots of research works have been done concerning the physiological roles of Gas6, yet no literature is available demonstrating the association of Gla-Gas6 with the hyperlipidemia-linked inflammation. Therefore, the present study has been undertaken to examine the protective role played by VK among the hyperlipidemic population via augmenting the Gla-Gas6 plasma level.

Using the plasma samples from both hyperlipidemic subjects and age-matched healthy control individuals and high fat diet (HFD)-fed hyperlipidemic animals, this study for the first time examined the hypothesis that circulating Gla-Gas6 deficiency may be associated with hyperlipidemia-linked inflammatory pathophysiology and VK supplementation might lessen the inflammation via upregulating the levels of Gla-Gas6. Furthermore, by using both hepatocyte and monocyte cell culture models and GGCX knockdown studies, the present work also investigated the direct role of Gla-Gas6 in mediating the beneficial effect of VK in preventing inflammation against high palmitic acid (PA) exposure. The outcome of the study will be helpful in understanding the biochemical mechanism underlying beneficial effect of Gla-Gas6 in mediating the positive effect of VK in preventing the incidence of inflammatory pathophysiology associated with hyperlipidemia.

2. Materials and methods

2.1. Materials

Anti-CD11a (#ab186873), anti-CCR2 (#ab203128), anti-GGCX (#ab197982), and anti-beta-actin (#ab49900) were obtained from Abcam, Inc. (Cambridge, MA, USA). Anti-ICAM-1 (#sc-107) was procured from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). Anti-CCR2 (#PA5-23037) and anti-CCR2-DyLight650 (#PA5-23039) were purchased from Invitrogen (Carlsbad, California, US). Anti-CD11a-FITC (#SAB4700380) and anti-CD54-PE (#SAB4700328) were purchased from Sigma-Aldrich (St. Louis, Missouri, US). Anti-Gla (REF 3570) was obtained from BioMedica Diagnostics (Stamford, Connecticut, US). γ-carboxylated Gas6 (Gla-Gas6, APA204Hu01) was purchased from Cloud-Clone Corp. (Katy, TX, USA). siRNA oligos against human GGCX (oligo name SASI_HS01_00088048 & SASI_HS01_00088048_AS; oligo ID 8024752694-000020 & 8024752694-000030 respectively) and rat GGCX (oligo name SASI_RN01_00070053 & SASI_RN01_00070053_AS, oligo ID 8024752694-000080 & 8024752694-000090 respectively) were procured from Sigma-Aldrich (St. Louis, Missouri, US).

2.2. Blood collection from hyperlipidemic and age-matched healthy control subjects

Human subjects were enrolled for the study following the protocol approved by the Institutional Ethics Committee for Human Experimentation. From the human subjects informed written consents were obtained before collecting the blood samples. Studies with human subjects were performed by following the protocol described earlier [9]. Subjects with hyperlipidemia (n=22) and age-matched healthy controls (n=19) enrolled under this study were the volunteers attending the clinical center, CSIR-NEIST, Jorhat. Hyperlipidemic population was selected based on the aberrantly high plasma profile of triglyceride (>200 mg/dL or 2.25 mM) and total cholesterol (>200 mg/dL or 5.1 mM). Volunteers were excluded from this study if they had any history of sickle cell disease, smoking habits, uncontrolled hypertension, hypothyroidism, hyperthyroidism, treatment with lipid lowering medication, anticoagulant, or insulin. Volunteers were also omitted if they showed signs of coagulation complications, hepatic dysfunction, or renal dysfunction. Pregnant women or women with nursing infants were also not considered under this study. Subjects taking any supplemental vitamins were excluded. Blood samples were collected from the volunteers after overnight fasting (8 h). Blood containing sodium citrate (3.2%) vials and serum tubes were immediately delivered to the CSIR-NEIST clinical center for testing of prothrombin time and chemistry profiles respectively. Blood containing EDTA vials were taken to the research laboratory followed by separation of plasma via centrifugation at 3000 rpm for 15 min. The plasma samples were then transferred to cryo vials and stored at -80°C for further use.

Table 1
Diet composition for experimental mice.

Nutrients	Control diet		High Fat Diet (HFD)	
	gm (%)	kcal (%)	gm (%)	kcal (%)
Fat	4	10	24	45
Protein	19	20	24	20
Carbohydrate	67	70	41	35
Ingredients	gm	kcal	gm	kcal
Casein	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	550	2200	0	0
Maltodextrin	150	600	0	0
Fructose	0	0	345.6	1382
Cellulose	50	0	50	0
Soybean Oil	25	225	25	225
Beef Tallow	20	180	177.5	1598
Mineral Mix	10	0	10	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate	16.5	0	16.5	0
Di Calcium Phosphate	13	0	13	0
Vitamin Mix	10	40	10	40
Choline Bitartrate	2	0	2	0
Total	1055	4057	858.1	4057
kcal/gm	**3.8**		**4.7**	

As the values are very important to imply the differences in calori content in both the diets, they were mentioned in bold.

2.3. Estimation of plasma profile of lipid, Gla-Gas6, total Gas6, VK, ICAM-1, and MCP-1 levels among human subjects

Plasma profile of triglyceride and total cholesterol were estimated using specific kits from Robonik Pvt. Ltd. (Mumbai, India).

Circulatory level of Gla-Gas6 was determined following the method described earlier by performing affinity purification of the total Gla proteins from the plasma samples [9]. Briefly, monoclonal antibody against Gla residues was coupled to a sepharose CL-4B column (Sigma) and the purified total Gla proteins was used to estimate the levels of Gla-Gas6 by using Gas6 ELISA kit from R&D Systems (Minneapolis, MN, USA). Plasma level of total Gas6 was determined by using the same Gas6 ELISA kit.

Plasma VK (Phylloquinone) levels were assessed using HPLC (Waters) equipped with a C30 column (Accucore, 3 × 100 mm, 2.6 μm) followed by a post-column online zinc metal reactor and fluorometric detector (λex 248 and λex 430) [16].

Plasma MCP-1 and ICAM-1 levels were measured by using ELISA kits from R&D Systems (Minnesota, US).

2.4. Animal studies

2.4.1. Animals

The animal study was performed following the same procedures mentioned earlier [9]. Male Swiss albino mice (5 weeks old, 25–30 g) were procured from animal research facility, Bose Institute, West Bengal, India. Mice were kept in the animal care facility maintaining standard environmental conditions (12:12 h light-dark cycle, 22–24°C). Animal experiments were performed following the guidelines of the Institutional Animal Ethical Committee (IAEC), Bose Institute. Approval was also obtained from both CPCSEA (Committee for the Purpose of Control & Supervision on Experiments on Animals), Ministry of Environment & Forests, New Delhi, India (1796/PO/Ere/S/14/CPCSEA) and IAEC for conducting this study.

2.4.2. Animal experimental design

The whole animal experiment was conducted for a duration of 16 weeks. Experimental mice were divided into seven groups by computer-generated randomization with each group consisting of five mice. The mice were fasted overnight and weight was then checked the next day just before starting the experiment. VK (Phylloquinone) solution was prepared in 0.1% olive oil (OO) and 100 μL of the stock solution was administered per 100 g BW of the experimental mice daily. The animal diet composition has been presented in Table 1. Treatments were given to the mice as follows:

Control: Mice were fed a low fat diet (10% kcal from fat) for 16 weeks and OO was administered at a dose of 100 μL/100 g BW daily by oral gavage for last 8 weeks.

VK-5: Mice were fed a low fat diet for 16 weeks and 5 μg/kg BW dose of VK was administered daily by oral gavage for the last 8 weeks.

HFD: Mice were fed a high fat diet (45% kcal from fat) for 16 weeks.

HFD+OO: Mice were fed HFD for 16 weeks and 100 μL/100 g BW dose of OO was administered daily by oral gavage for the last 8 weeks.

HFD+VK-1, HFD+VK-3, HFD+VK-5: Mice were fed HFD for 16 weeks and VK was administered at a dose of 1, 3, and 5 μg/kg BW respectively daily by oral gavage for the last 8 weeks.

The VK doses were selected based upon our previous studies [17]. Food intake was noted on daily basis and body weight was measured weekly. After completion of 16 weeks mice were fasted overnight, weighed, and euthanized by exposing to isoflurane. Blood was collected in EDTA tubes via heart puncture with a 191/2-gauge needle. Plasma was separated from the blood samples via centrifuging at 3000 rpm for 15 min. Liver tissues removed from the mice were perfused with cold saline to remove leftover blood and instantly stored at −80°C till further use.

2.4.3. Estimation of plasma levels of lipid, sICAM-1, MCP-1, Gla-Gas6, total Gas6, and assessment of kidney and liver functions in the experimental animals

Plasma levels of triglyceride and total cholesterol as well as liver (alanine aminotransferase, ALT) and kidney (creatinine) functions were studied using assay kits from Robonik Pvt. Ltd. (Mumbai, India). MCP-1 and soluble ICAM-1 (sICAM-1) levels in the plasma samples were examined using ELISA kits from Sigma and R&D Systems, Inc. (Minneapolis, MN) respectively. All appropriate controls and standards were used as specified in kit protocol.

Plasma profile of Gla-Gas6 and total Gas6 were estimated following the similar methods as described in Section 2.3.

2.5. Cell culture studies

2.5.1. Cell culture

The rodent hepatocyte cell line, CC1 was obtained from Sigma. These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 5.5 mM glucose, 10% (v/v) heat-inactivated FBS, 1% non-essential amino acid (NEAA), 100 U/mL penicillin, 100 μg/mL streptomycin, 12 mM sodium bicarbonate, 20 mM HEPES, and 2 mM glutamine in a humidified atmosphere containing 5% (v/v) CO_2 and 37°C as mentioned earlier [9].

Human THP-1 monocytes were procured from NCCS, Pune, India. THP-1 was maintained in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine as described earlier [18]. The cell line was maintained at 37°C in a humidified atmosphere containing 5% (v/v) CO_2.

2.5.2. Preparation of BSA conjugated palmitate solution

To expose the hepatocytes and monocytes to hyperlipidemic condition, palmitic acid (PA) solution was prepared as a conjugate with fatty acid free-bovine serum albumin (BSA) following the protocol mentioned earlier [19]. In brief, sodium palmitate was dissolved in 50% (v/v) ethanol followed by diluting in DMEM containing 2% (w/v) BSA and the solution was incubated at 37°C for 2 h with continuous stirring to prepare BSA-conjugated PA solution (6:1 molar ratio of palmitate:BSA, which is close to the ratio observed in human serum) [20].

2.5.3. Treatment of hepatocytes and monocytes with PA, VK, and Gla-Gas6

Hepatocytes and monocytes were first treated with PA with or without VK (Phylloquinone). Cells were supplied with different concentrations of VK (1, 5, and 10 nM) for 2 h followed by PA (0.75 mM) exposure for next 20 h. The working solutions of VK was prepared in sterile DMSO. Control cells were supplied with DMEM/RPMI containing 2% BSA with or without VK (10 nM) as mentioned above. For studying the effect of Gla-Gas6, PA-treated cells were supplied with or without Gla-Gas6 (100 ng/mL). The concentrations of PA [9], VK [17], and Gla-Gas6 [9,21] were chosen based upon previous literatures.

2.5.4. Surface analysis of cell membrane proteins by flow cytometry

Surface expressions of CD11a, CCR2, and ICAM-1 were performed using flow cytometry according to the protocol described earlier [18]. After treatment, cells were washed in FACS buffer (PBS with the addition of 10% fetal bovine serum and 0.1% sodium azide), centrifuged, suspended in FACS buffer supplied with FITC conjugated anti-CD11a (Sigma) (1:5 dilution) or anti-ICAM-1 (Sigma) (1:5 dilution) or anti-CCR2 (Invitrogen) (1:50 dilution) primary antibodies and incubated for 1 h at 37°C. After 1 h, cells were washed in FACS buffer followed by suspending in 500 μL PBS for flow cytometry analysis. A minimum of 10,000 cells were analyzed in each sample by using a flow cytometer (CytoFLEX, Beckman Coulter) equipped with multicolor analysis capability. Gates were set to ignore dead cells, cell debris, and cells of abnormal size and shape. Isotype controls were examined to confirm no non-specific binding. Flow cytometry results were expressed as mean fluorescence intensity (MFI) per 10,000 cells.

2.5.5. sICAM-1 and MCP-1 assays

Soluble ICAM-1 (sICAM-1) and MCP-1 levels in the treatment supernatant of hepatocytes were measured by using ELISA kits from Sigma and R&D Systems, Inc. (Minneapolis, MN) respectively. All appropriate controls and standards were used as specified in the kit's protocol.

2.5.6. Monocyte-hepatocyte adhesion

Hepatocytes were seeded and allowed to grow to confluent monolayers. Cells were treated with different conditions as mentioned above. Simultaneously, monocytes were supplied with 8 μM CellTracker Green CMFDA (Invitrogen, Eugene, OR, USA) and then treated with different conditions at similar concentrations provided to CC1. After completion of 22 h treatment, 1 million monocytes were loaded to the hepatocytic monolayers followed by incubating for 30 min at 37°C. The non-adherent cells were then removed by washing with DMEM and the adherent cells were lysed in 0.2% Triton-X for evaluation [18]. The fluorescent intensity of the monocytes added to the hepatocytes was measured at excitation 485 nm and emission 528 nm. The flow cytometry result was expressed as mean fluorescence intensity (MFI).

2.5.7. siRNA mediated gene silencing studies

GGCX and control siRNA were procured from Sigma (St. Louis, MO) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) respectively. Hepatocytes and monocytes were transiently transfected with 100 nM siRNA complex using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) following the method described previously [9]. After transfection of 24 h, cells were treated with VK (10 nM) against high PA exposure as mentioned above. To establish the effect of Gas6, GGCX siRNA transfected cells were also supplemented with or without Gla-Gas6 (100 ng/mL) against high PA exposure.

2.5.8. Immunoblotting analysis

Immunoblotting was performed following the same method described in our earlier literature [9]. In brief, after completion of treatment period, tissues or cells were lysed in radioimmunoprecipitation assay (RIPA) buffer comprising of protease and phosphatase inhibitors (1 mM PMSF, 5 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mM EDTA, 10 mM NaF, and 1 mM $NaVO_4$). Cell lysates were removed by centrifugation at 15000 rpm for 30 min and protein estimation was performed by BCA assay (Pierce/ThermoScientific, Rockford, IL). Approximately the same amount of proteins (~20-40 μg) from all samples were used for immunoblotting with anti-CD11a (1:1000 dilution), anti-CCR2 (1:500), anti-ICAM-1 (1:500), anti-GGCX (1:1000), and beta-actin (1:30000) primary antibodies and appropriate HRP conjugated secondary antibody (1:5000 dilution). The immunoblots were developed by using enhanced chemiluminescence (ECL) substrate (BioRad) and the band intensities were evaluated by using the histogram option of Adobe Photoshop CS6.

2.6. Statistical analysis

Student's t-test and one-way ANOVA were performed to statistically analyze the data from human, animal, and cell culture studies using Sigma Stat statistical software (San Jose, CA, USA) [9]. The treatment groups were compared using the Newman–Keuls method. p value less than 0.05 was considered to be significant for a statistical test.

3. Results

3.1. Plasma Gla-Gas6, total Gas6, VK, inflammatory markers (ICAM-1 and MCP-1), and lipid levels in age-matched healthy controls and subjects with hyperlipidemia

Similar distributions of M/F ratio (14/5 vs. 15/7), age (52.57±1.79 vs. 53±1.51 years), body weight (58.84±2.88 vs. 56.13±2.81 kg), and BMI (21.27±0.86 vs. 21.70±1.03 kg/m2) were recorded between healthy controls and hyperlipidemic subjects. The above values are presented as control population vs. hyperlipidemic population.

It was observed that the levels of triglyceride (1.80±0.25 vs. 3.15±0.30 mM), total cholesterol (2.20±0.08 vs. 5.67±0.37 mM), ICAM-1 (9.82±1.69 vs. 16.43±0.93 ng/mL), and MCP-1 (54.80±4.25 vs. 60.66±5.09 ng/mL) were significantly higher in hyperlipidemic subjects compared to controls. The above values are presented as control population vs. hyperlipidemic population.

On the contrary, Gla-Gas6 (22.17±0.89 vs. 17.62±0.73 pg/mL) and VK levels (5.82±0.86 vs. 3.28±0.32 nM) were found to be significantly lower in subjects with hyperlipidemia compared to that of age-matched healthy controls. The plasma level of total Gas6 (1.87±0.01 vs. 1.88±0.01 ng/mL) was found to be similar among both the groups. All the studied population showed no signs of coagulation associated complications (data not given).

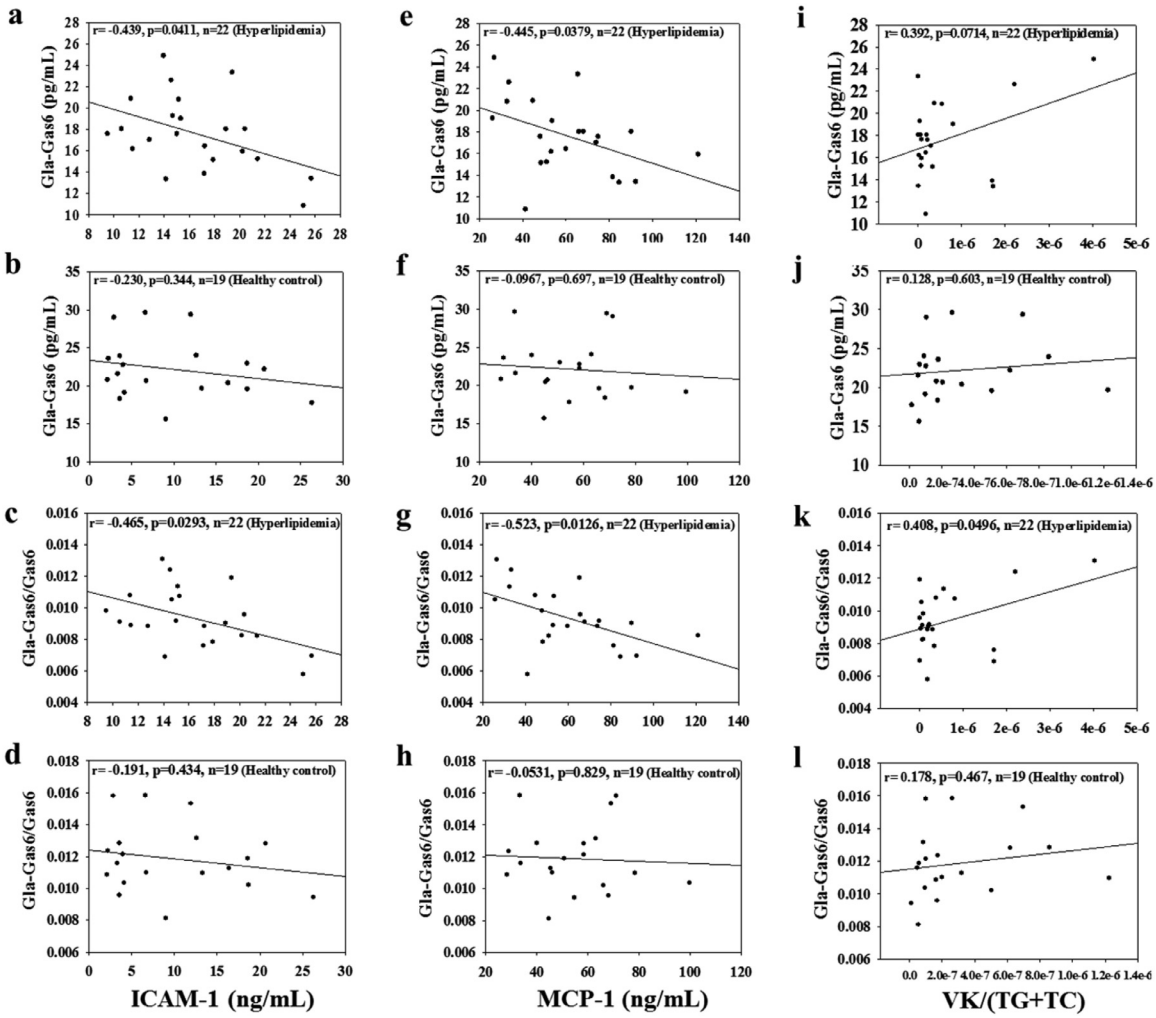

Fig. 1. The correlation study demonstrates the relationship between plasma levels of (a) Gla-Gas6 (pg/mL) and ICAM-1 (ng/mL), (c) Gla-Gas6/total Gas6 and ICAM-1 (ng/mL), (e) Gla-Gas6 (pg/mL) and MCP-1 (ng/mL), (g) Gla-Gas6/total Gas6 and MCP-1 (ng/mL), (i) Gla-Gas6 (pg/mL) and VK/(TG+TC), and (k) Gla-Gas6/total Gas6 and VK/(TG+TC) in hyperlipidemic subjects; (b) Gla-Gas6 (pg/mL) and ICAM-1 (ng/mL), (d) Gla-Gas6/total Gas6 and ICAM-1 (ng/mL), (f) Gla-Gas6 (pg/mL) and MCP-1 (ng/mL), (h) Gla-Gas6/total Gas6 and MCP-1 (ng/mL), (j) Gla-Gas6 (pg/mL) and VK/(TG+TC), and (l) Gla-Gas6/total Gas6 and VK/(TG+TC) among healthy control subjects.

3.2. Association of plasma Gla-Gas6 with ICAM-1, MCP-1, and VK among healthy control and hyperlipidemic population

Figure 1 (a-l) shows the relationships among Gla-Gas6, ICAM-1, MCP-1, and VK in healthy control and subjects with hyperlipidemia. Results demonstrated that plasma levels of Gla-Gas6 exhibits a statistically significant negative correlation with the circulating levels of both ICAM-1 (Fig. 1a) (r=-0.439, p=0.0411) and MCP-1 (Fig. 1e) (r=-0.445, p=0.0379) among the hyperlipidemic subjects but not in healthy control. Similarly, a significant inverse correlation was also observed between the levels of Gla-Gas6/total Gas6 and ICAM-1 (Fig. 1c) (r=-0.465, p=0.0293) or MCP-1 (Fig. 1g) (r=-0.523, p=0.0126) among the hyperlipidemic population. Interestingly, a significant positive correlation was observed between Gla-Gas6/total Gas6 and VK (Fig. 1k) (r=0.408, p=0.0496) among the hyperlipidemic subjects. While performing the correlation analyses, we expressed the concentration of circulating VK after normalizing with total plasma lipid i.e. the sum of total cholesterol and triglyceride (TC+TG) levels since VK is a lipid soluble vitamin. These results altogether suggest that boosting circulatory VK status may have a positive effect on elevating Gla-Gas6 level which might reduce the plasma levels of ICAM-1 and MCP-1 among subjects with hyperlipidemia. The regression analysis presented here was determined while controlling for body weights using multiple linear regression analysis.

3.3. Effect of VK supplementation on Gla-Gas6 and the signaling pathways of inflammation in HFD-fed mice

Figure 2 demonstrates the effect of VK supplementation on the plasma levels of Gla-Gas6, total Gas6, and inflammatory cytokines and the protein expressions of inflammatory molecules in liver tissues of both normal and HFD-fed mice. Results demonstrated that plasma Gla-Gas6 levels were significantly reduced; however, the levels of MCP-1 and sICAM-1 were significantly increased in HFD-fed mice compared to those seen in control diet-fed mice. Interestingly, VK administration in HFD-fed mice dose-dependently (1, 3, and 5 μg/kg BW) elevated the levels of Gla-Gas6 and decreased the levels of MCP-1 and sICAM-1. Different treatments did not cause any change in the plasma levels of total Gas6 in all the groups of experimental animals.

Investigating the signaling pathways of inflammation, it has been observed that the protein expression of ICAM-1 was significantly elevated in the hepatic tissues collected from HFD-fed mice.

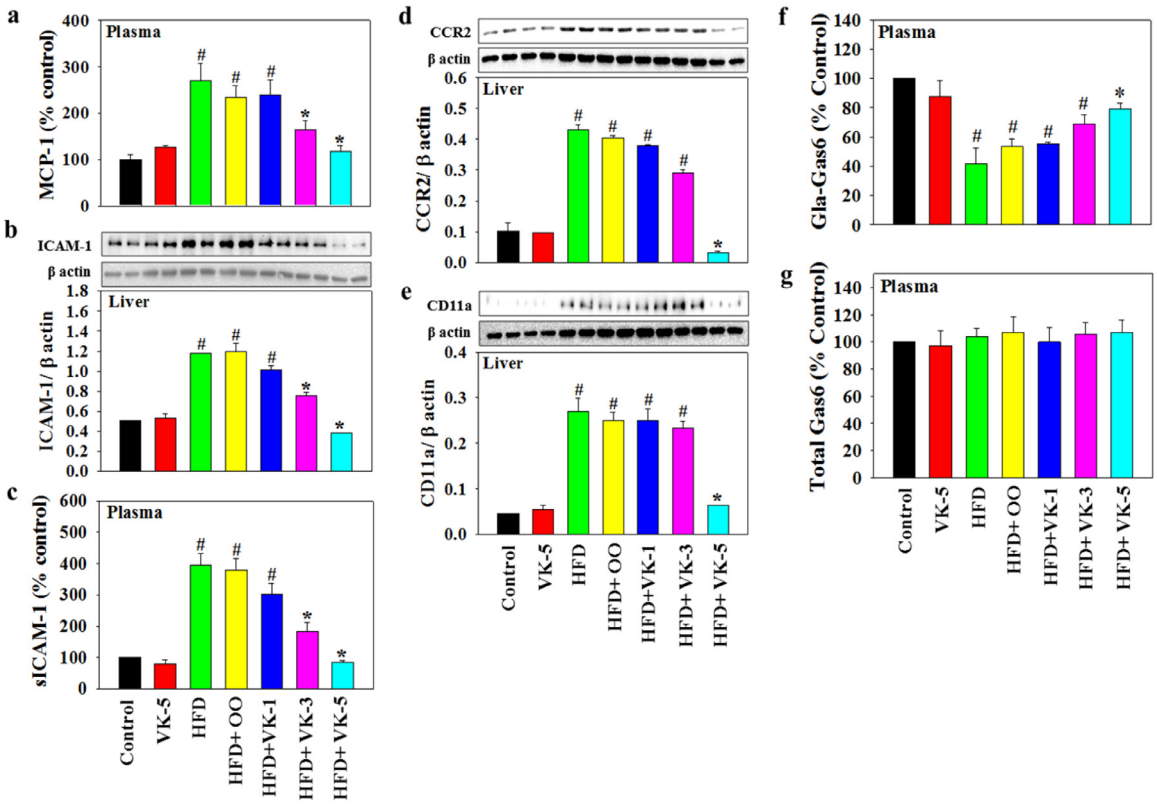

Fig. 2. Effect of vitamin K (VK, Phylloquinone) supplementation on the plasma levels of (a) MCP-1, (c) sICAM-1, (f) Gla-Gas6, and (g) total Gas6, and hepatic protein expressions of (b) ICAM-1, (d) CCR2, and (e) CD11a in high fed diet (HFD)-fed mice. Control mice were gavaged with VK at a dose of 5 μg/kg BW (VK-5) and high fat diet (HFD)-fed mice were gavaged with either olive oil (HFD+OO) or VK at a dose of 1 (HFD+VK-1), 3 (HFD+VK-3), or 5 (HFD+VK-5) μg/kg BW daily for 8 weeks. Each blot in Figures b, d, and e represents the hepatic protein expressions in any two random mice of each group of animals. Each bar in Figures a-g represents the average protein expression levels assessed from plasma as well as liver tissue samples of all the five mice of each group. Full-length blots are presented in supplementary figure. The details of animal experiments have been provided in materials and methods section. Data are expressed as mean ± SE (n=5). "#" denotes the significant difference from control group (#$P<.05$) and "*" denotes the significant difference from HFD-fed group (*$P<.05$).

Moreover, the protein expressions of both CCR2 and CD11a were also over-expressed in the liver tissues of HFD-fed mice suggesting an infiltration of the immune cells into liver tissues. Supplementation with different doses of VK in HFD-fed mice reduced the protein expressions of ICAM-1, CCR2, and CD11a in a dose-dependent manner.

The information regarding body weight gain, food intake, plasma lipid levels, and the liver and kidney enzymes among the experimental mice have been presented in Table 2. The percentage of body weight gain was observed to be significantly higher in HFD-fed mice compared to control mice. Moreover, plasma triglyceride and total cholesterol levels were observed to be significantly elevated in HFD-fed mice. Interestingly, VK supplementation dose-dependently lowered the body weight gain and lipid levels in HFD-fed mice. However, no significant difference in food intake and the levels of liver and kidney function enzymes were observed among the experimental animals.

To further investigate the direct role of Gla-Gas6 and the underlying molecular mechanism behind the beneficial effect of VK against hyperlipidemia associated inflammation *in vitro* study was performed by using both hepatocytes and monocyte cell lines.

3.4. Role of Gla-Gas6 in mediating the beneficial effect of VK against high PA-induced inflammation

Firstly, the effect of VK supplementation on high PA-induced MCP-1/ICAM-1 mediated inflammatory pathophysiology has been examined by using both hepatocyte and monocyte cell culture models and the results have been represented in Figures 3 and 4. Treatment with high PA significantly elevated the extracellular secretion of both MCP-1 and sICAM-1 from hepatocytes compared to the control group. Moreover, the protein expression of ICAM-1 in hepatocytes and both CCR2 and CD11a in monocytes were up-regulated upon PA-exposure. We also investigated the cell surface expressions of ICAM-1, CCR2, and CD11a by using flow cytometry and an increase in surface expressions of ICAM-1 in hepatocytes and both CD11a and CCR2 in monocytes were also observed under high PA-exposure. These events altogether resulted in adhesion of monocyte to the hepatocyte monolayer under the condition of high PA treatment. Interestingly, VK supplementation in PA-treated cells dose-dependently (1, 5, and 10 nM) reduced the secretion of both MCP-1 and sICAM-1 from hepatocytes, down-regulated the protein expression as well as cell surface expression of ICAM-1 in hepatocytes and both CCR2 and CD11a in monocytes followed by lowering the monocyte-hepatocyte adhesion.

Using siRNA mediated GGCX silencing in both hepatocyte and monocyte cell culture models, the present study further investigated the role of VK-dependent Gla-proteins in regulating the beneficial effect of VK against high PA-induced inflammatory pathophysiology (Figs. 5 and 6). Results showed that VK supplementation could not inhibit the increased secretion of both the pro-inflammatory mediators MCP-1 and sICAM-1 and the intracellular as well as cell surface expression of ICAM-1 in GGCX knockdown hepatocytes against high PA exposure. Moreover, VK supplementa-

Table 2
Body weight gain, food intake, liver (ALT) and kidney (creatinine) function test, triglyceride (TG), and total cholesterol (TC) levels in the experimental animals.

Parameters	Control	VK-5	HFD	HFD+OO	HFD+ VK-1	HFD+ VK-3	HFD+ VK-5
Body weight gain (g)	1.66±0.3	1.62±0.28	3.44±0.25*	2.94±0.33	2.86±0.33	2.34±0.29†	2.12±0.09†
Food intake (g/mouse/day)	2.54±0.029	2.53±0.017	2.49±0.007	2.49±0.014	2.52±0.01	2.52±0.005	2.53±0.01
ALT (U/L)	7.65±0.85	7.85±0.43	9.17±1.15	8.15±0.44	7.68±0.86	6.05±0.78	5.39±0.66†
Creatinine (mg/dL)	0.042±0.0035	0.052±0.0037	0.049±0.0075	0.048±0.0051	0.049±0.0019	0.043±0.0014	0.039±0.0018†
Triglyceride (mM)	0.58±0.015	0.64±0.031	1.68±0.054*	1.69±0.06	1.54±0.086	1.24±0.07	0.83±0.13†
Total Cholesterol (mM)	0.29±0.019	0.33±0.045	0.54±0.077*	0.49±0.047	0.41±0.017	0.34±0.055†	0.24±0.031†

Note: Control mice were gavaged with VK at a dose of 5 μg/kg BW (VK-5) and high fat diet (HFD)-fed mice were gavaged with either olive oil (HFD+OO) or VK at a dose of 1 (HFD+VK-1), 3 (HFD+VK-3), or 5 (HFD+VK-5) μg/kg BW daily for 8 weeks. Values are mean±SE (n=5).
* denotes the significant difference from control ($P<0.05$).
† denotes the significant difference from HFD ($P<0.05$).

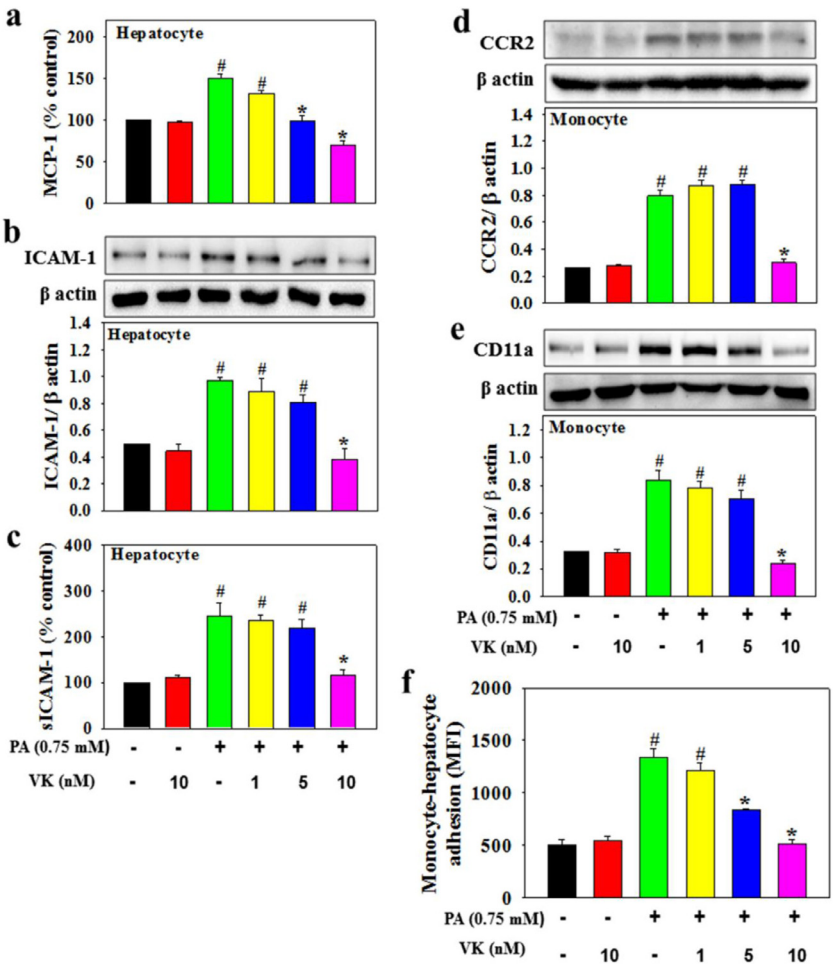

Fig. 3. Effect of vitamin K (VK, Phylloquinone) supplementation on the secretions of (a) MCP-1 and (c) sICAM-1, protein expressions of (b) ICAM-1, (d) CCR2, and (e) CD11a, and (f) monocyte-hepatocyte adhesion under high palmitic acid (PA)-treatment condition. The graphs are representatives of three independent experiments. Full-length blots are presented in supplementary figure. Data are expressed as mean ± SE. "#" denotes the significant difference from control group ($\#P<.05$) and "*" denotes the significant difference from PA-treated group ($^{*}P<.05$).

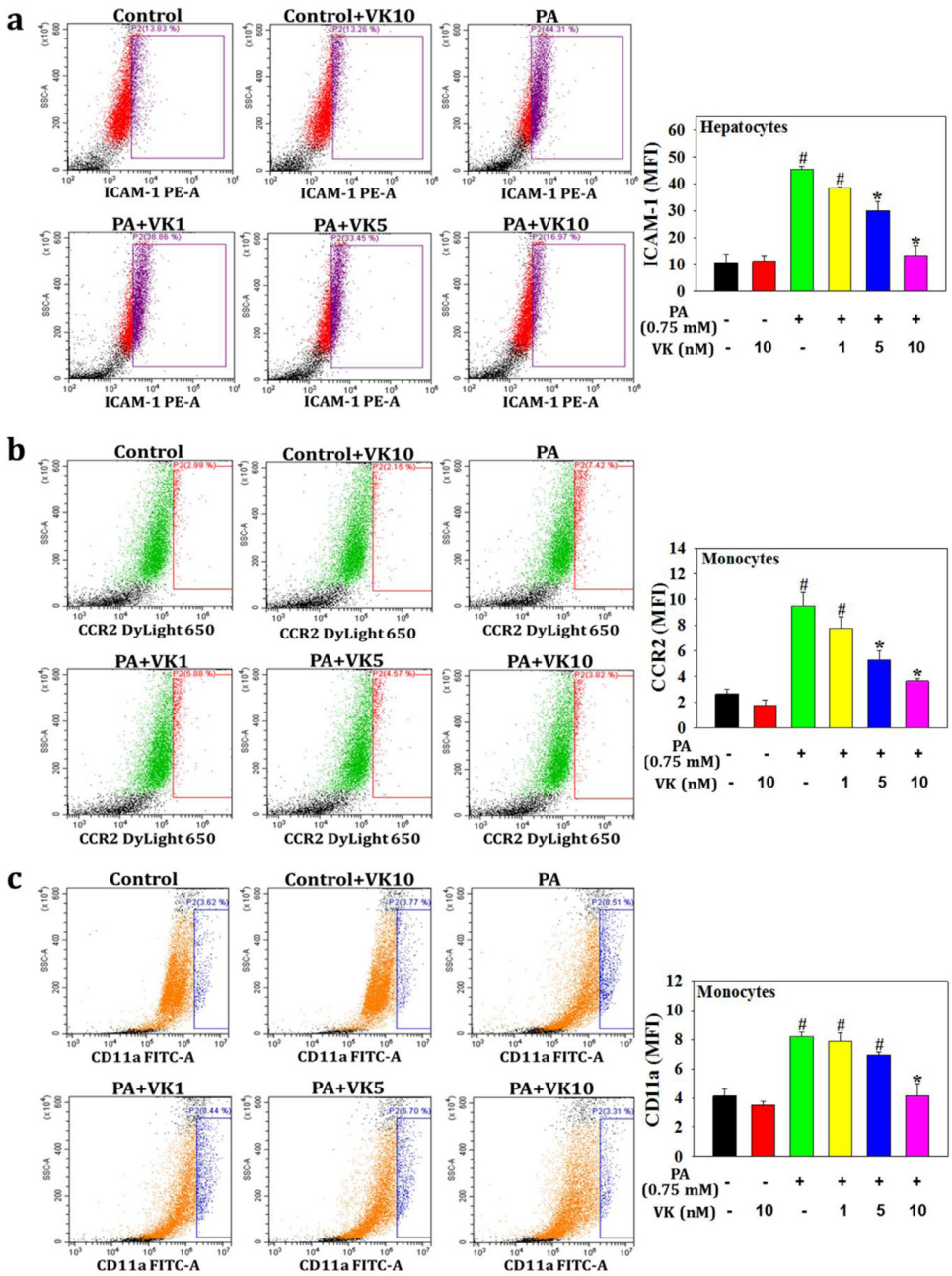

Fig. 4. Effect of vitamin K (VK, Phylloquinone) supplementation on the surface expressions of (a) ICAM-1, (b) CCR2, and (c) CD11a under high PA-treatment condition. The quantification has been performed based on three independent experiments which is expressed as mean fluorescent intensity (MFI). Data are expressed as mean ± SE. "#" denotes the significant difference from control group (#P<.05) and "*" denotes the significant difference from PA-treated group (*P<.05).

tion was unable to prevent the high PA-induced up-regulation of intracellular and cell surface expressions of both CCR2 and CD11a in GGCX knockdown monocytes. Furthermore, under GGCX knockdown condition, VK treatment was unable to inhibit the adhesion of monocytes to hepatocytes against high PA-exposure. These findings suggest an important role of Gla-proteins in mediating the beneficial effect of VK supplementation against hyperlipidemia associated inflammatory pathophysiology.

Using γ-glutamyl carboxylated Gas6 (Gla-Gas6) protein and GGCX knockdown in both hepatocyte and monocyte cell culture models, this study finally examined the direct role of Gla-Gas6 against high PA-induced inflammatory pathophysiology (Fig. 7). It has been observed that in GGCX knockdown hepatocytes treatment with Gla-Gas6 to the high PA-treated cells significantly lowered the secretion of both MCP-1 and sICAM-1 and the total protein expression of ICAM-1 compared to those seen in high PA-treated GGCX silenced hepatocytes. Likewise, high PA-induced upregulation in the protein expressions of both CCR2 and CD11a were also inhibited in Gla-Gas6 treated GGCX knockdown monocytes. Furthermore, treatment with Gla-Gas6 was found to inhibit the monocyte-hepatocyte adhesion against high PA exposure in GGCX knockdown cells. These observations suggest a direct role of Gla-Gas6 in preventing high PA-induced inflammatory event.

Combining all, cell culture studies suggest a direct role of Gla-Gas6 in mediating the beneficial effect of VK against high PA-induced inflammatory pathophysiology.

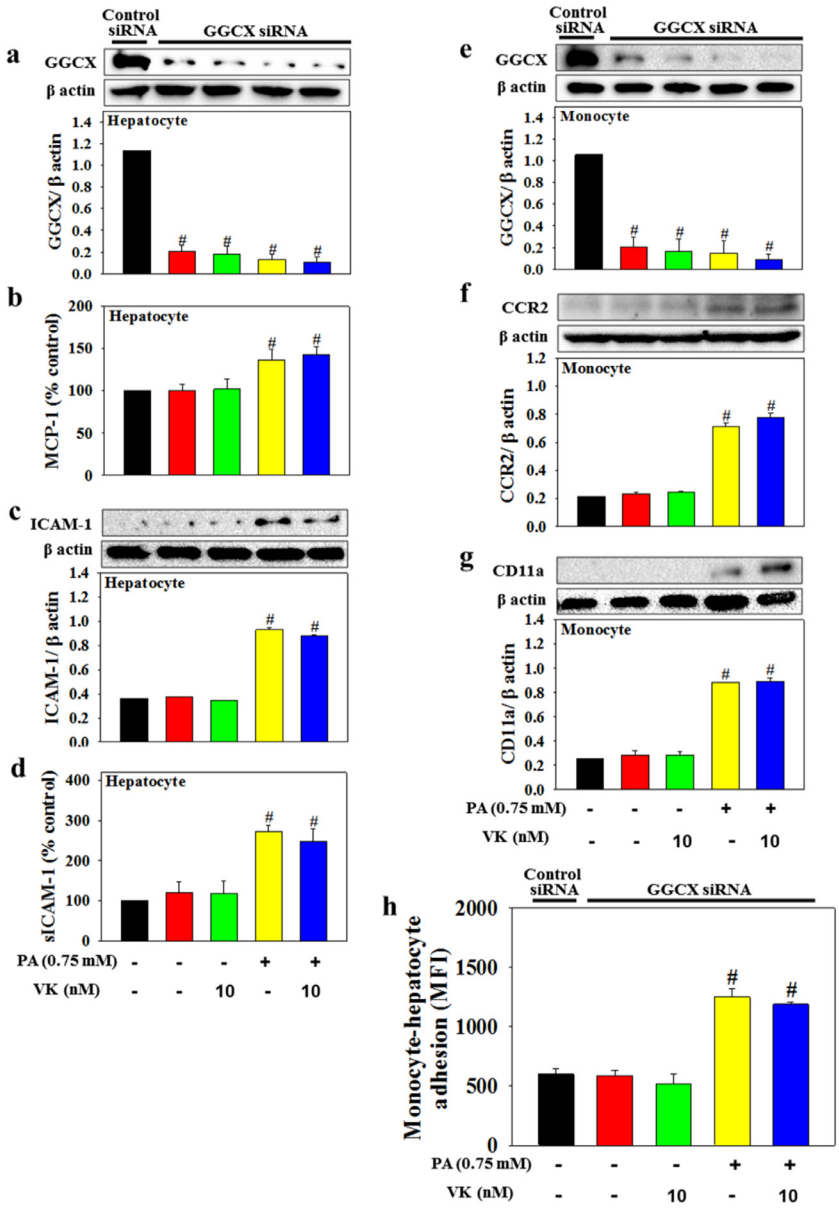

Fig. 5. Effect of vitamin K (VK, Phylloquinone) supplementation on the secretions of (b) MCP-1 and (d) sICAM-1, protein expressions of (c) ICAM-1, (f) CCR2, and (g) CD11a, and (h) monocyte-hepatocyte adhesion in GGCX siRNA transfected cells exposed to high PA. (a) and (e) represent the GGCX protein expression in hepatocytes and monocytes respectively. The graphs are representatives of three independent experiments. Full-length blots are presented in supplementary figure. Data are expressed as mean ± SE. "#" denotes the significant difference from control group (#P<.05) and "*" denotes the significant difference from PA-treated group (*P<.05).

4. Discussion

Gas6, a member of coagulation unrelated VKDP family is considered as a major player in various pathological conditions such as atherosclerosis, cancer, hyperglycemia, and cardiovascular disease. Gas6 acts as a ligand to the members of TAM receptor tyrosine kinase subfamily by interacting through its C-terminal sex hormone-binding globulin (SHBG) domain [22]. Gas6/TAM signaling cascade is involved in the regulation of diverse cellular activities such as vascular smooth muscle cell (VSMC) proliferation [1], cellular homeostasis [1], leukocyte sequestration and migration [23,24], hematopoiesis [3], and apoptosis [1]. Moreover, several scientific reports have also shown that Gas6 functions as an important anti-inflammatory agent in different pathophysiological conditions [4–8]. It has been reported by various studies that, receptor binding as well as biological activities of Gas6 requires the VK-dependent post-translational carboxylation of Glu residues present in the N-terminal Gla-domain of Gas6 into Gla by the enzyme GGCX [11,12]. Our previous study demonstrated that Gla-Gas6 plays a major role in the management of hyperlipidemia associated impaired hepatic lipid metabolism among the hyperlipidemic subjects [9]. Although a lot of research has been performed regarding the association of Gas6 with diverse pathophysiological conditions, the association between Gla-Gas6 and hyperlipidemia associated inflammation remains lacking.

Increased levels of pro-inflammatory mediators, namely cytokines, chemokines, and soluble adhesion molecules have been reported previously in subjects with hyperlipidemia [25]. Plasma level of Gla-Gas6 and total Gas6 have been determined by using affinity chromatography and ELISA respectively in both hyperlipi-

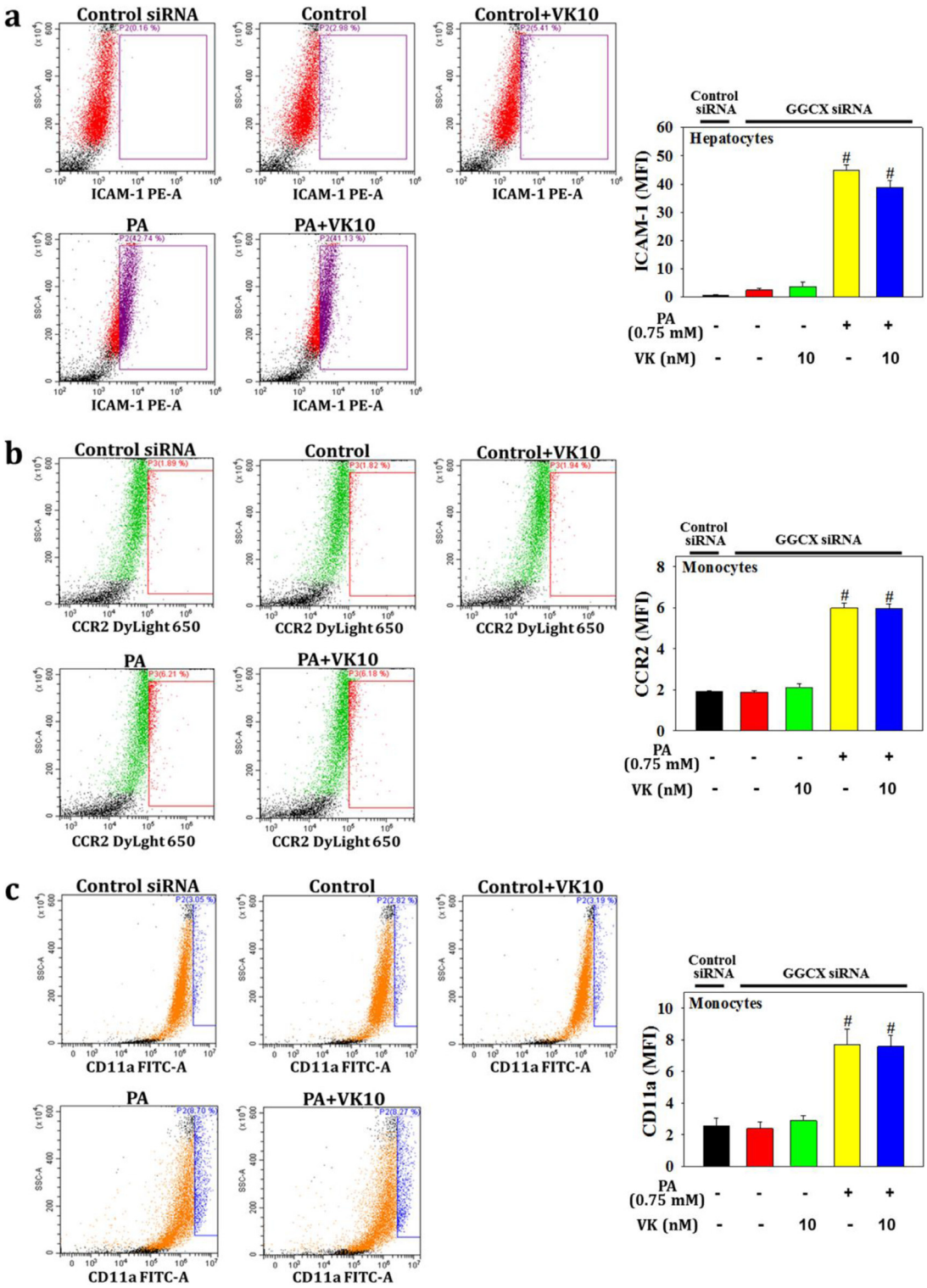

Fig. 6. Effect of vitamin K (VK, Phylloquinone) supplementation on the surface expressions of (a) ICAM-1, (b) CCR2, and (c) CD11a in GGCX siRNA transfected cells exposed to high PA. The quantification has been performed based on three independent experiments which is expressed as MFI. Data are expressed as mean ± SE. "#" denotes the significant difference from control group (#P<.05) and "*" denotes the significant difference from PA-treated group (*P<.05).

demic subjects and healthy control population. Phylloquinone is the major dietary source and the prime circulatory form of VK among both the dietary forms of VK. It has been successfully measured in various studies to assess the plasma VK status [26]. In the present study, it has been observed that the circulating levels of MCP-1 and sICAM-1 were significantly elevated among subjects with hyperlipidemia compared to age-matched healthy control. Moreover, plasma levels of Gla-Gas6 but not total Gas6 and VK were significantly lower among the hyperlipidemic subjects compared to healthy individuals. In the present study, among human

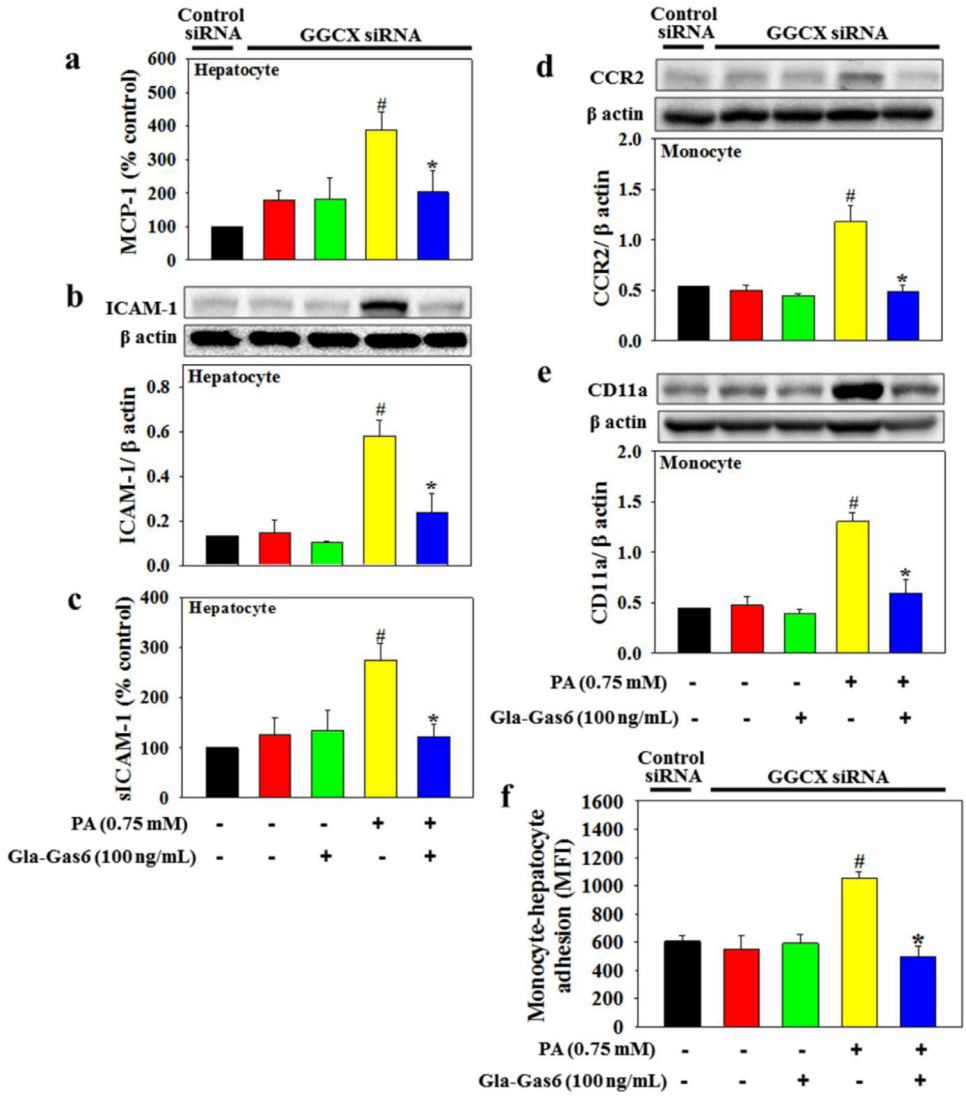

Fig. 7. Effect of Gla-Gas6 (100 ng/mL) supplementation on the secretions of (a) MCP-1, and (c) sICAM-1, protein expressions of (b) ICAM-1, (d) CCR2, and (e) CD11a, and (f) monocyte-hepatocyte adhesion in GGCX siRNA transfected cells exposed to high PA. The graphs are representatives of three independent experiments. Full-length blots are presented in supplementary figure. Data are expressed as mean ± SE. "#" denotes the significant difference from control group (#$P<.05$) and "*" denotes the significant difference from PA-treated group (*$P<.05$).

subjects the proportions of ucGas6 vs. Gla-Gas6 have been found to be 98.8% vs. 1.2% (control population) and 99.1% vs. 0.9% (hyperlipidemic population). However, we found that the values of different parameters like circulating lipid profile, VK, Gla-Gas6, and total Gas6 investigated under present study were consistent with our previous literature [9]. Multiple linear regression analyses after adjustment of body weight showed that Gla-Gas6 levels or Gla-Gas6 to total Gas6 ratio was significantly and inversely associated with MCP-1 and sICAM-1 among hyperlipidemic subjects but not in healthy control. Interestingly, Gla-Gas6/total Gas6 ratio was found to be positively and significantly correlated with VK level among hyperlipidemic subjects. We observed that although Gla-Gas6 was significantly correlated with ICAM-1 (p= 0.0411), but, when Gla-Gas6 was normalized with total Gas6 the association was found to be more significant (p= 0.0293). Similar strong relationship of Gla-Gas6/total Gas6 ratio was also observed with MCP-1 and VK suggesting that although a small fraction of total Gas6 is carboxylated, it displays a significant association with pro-inflammatory markers and VK. In this study we have expressed the concentration of VK after normalizing with total plasma lipid levels since VK is a lipid soluble molecule and in circulation VK is transported on triglyceride-rich lipoproteins, with smaller fractions carried on HDL and LDL cholesterol [26]. Therefore, it is appropriate to express the concentration of circulating VK after normalization with lipid and the same has also been argued for other lipid soluble vitamins [27,28]. This study suggests that increasing the concentration of plasma VK may have a positive effect on up-regulating Gla-Gas6 level which may reduce the chronic low-grade inflammation among subjects with hyperlipidemia.

The liver, a unique immunological site, plays an important role in hyperlipidemia associated inflammatory pathophysiology [29]. Under hyperlipidemic condition, an increase in hepatic lipid accumulation causes the secretion of pro-inflammatory cytokine, MCP-1 which induces the increased expression of cell adhesion molecule, ICAM-1 leading to hepatocyte-monocyte adhesion and initiation of liver injury [30]. MCP-1 plays a pivotal role in recruitment of monocytes at the site of inflammation via binding to its ligand chemokine receptor type 2 (CCR2) [31–34]. ICAM-1, a mem-

ber of the immunoglobulin superfamily of adhesion molecules also plays a crucial role in mediating adhesion events leading to leucocyte infiltration via binding to its ligand CD11a, a subunit of Lymphocyte function-associated antigen-1 (LFA-1) [31].

Firstly, to explore the effect of VK on Gas6 carboxylation and hyperlipidemia associated inflammatory pathophysiology, an *in vivo* study has been conducted using swiss albino male mice which were fed with either control diet or HFD for a period of 16 weeks with or without VK supplementation. Results showed that dose-dependent supplementation with VK (1, 3, and 5 μg/kg BW) reduced the levels of pro-inflammatory mediators, MCP-1 and ICAM-1 and increased the levels of Gla-Gas6 in plasma of the HFD-fed mice compared to those seen in HFD-fed group supplemented with or without OO. However, it was observed that different treatments caused no changes in the plasma levels of total Gas6. The proportions of ucGas6 vs. Gla-Gas6 have been found to be 90.5% vs. 9.5% (control mice), 97.3% vs. 2.7% (HFD-fed mice and 91% vs. 9% (VK supplemented HFD-fed mice). Interestingly, the protein expression of ICAM-1 and monocyte/macrophages markers, namely CCR2 and CD11a were increased in the liver tissues of HFD-fed mice suggesting the infiltration of immune cells into liver tissues via MCP-1/CCR2 and ICAM-1/CD11a dependent pathway. Treatment with VK dose-dependently reduced the protein expressions of MCP-1, CD11a, and ICAM-1 in the liver tissues of the HFD-fed mice. Additionally, we have also observed that VK remarkably reduced the plasma levels of triglyceride and total cholesterol along with the body weight in HFD-fed mice. As demonstrated by our previous literature VK prevents hyperlipidemia associated impaired lipid metabolism via modulating the lipid metabolic pathways [9]. As studies have established that high plasma lipid profile is closely linked with occurrence of obesity, this might be reason behind upsurge in body weight in HFD-fed mice which was prevented upon supplementation with VK to the high fat diet. Altogether, outcomes from this *in vivo* study demonstrated that VK plays a significant role in the upregulation of plasma Gla-Gas6 level but not total Gas6 which might in turn mediate the beneficial effect of VK in the management of hyperlipidemia associated inflammation via down-regulating MCP-1/ICAM-1 signaling pathway.

Further studies have been conducted by using both hepatocyte and monocyte cell culture models to explore the direct role of Gla-Gas6 in mediating the beneficial effect of VK on inflammatory pathophysiology associated with hyperlipidemia. Our previous study demonstrated that high PA (0.75 mM) supplementation to the hepatocytes caused a significant increase in intracellular lipid deposition [9]. Following the same treatment condition, the present study showed an increased secretion of both MCP-1 and sICAM-1 from hepatocytes and up-regulation of total protein as well as cell surface expression of ICAM-1 in hepatocytes and both CCR2 and CD11a in monocytes leading to an increase in monocytes adhesion to hepatocytes compared to those seen in untreated cells. Interestingly, VK supplementation against high PA exposure was found to cause a significant reduction in the secretion of both MCP-1 and sICAM-1 from hepatocytes and down-regulation of total protein as well as cell surface expression of ICAM-1 in hepatocytes and both CCR2 and CD11a in monocytes. Likewise, VK treatment also prevented the increase in monocyte-hepatocyte adhesion upon high PA exposure. These results demonstrate an important and specific role of VK in preventing the hyperlipidemia-associated inflammatory pathophysiology.

The enzyme, GGCX causes the formation of carboxylated Gas6 (Gla-Gas6) by catalyzing the conversion of specific Glu residues present in the N-terminus region of Gas6 to Gla residues using VK as a co-factor [13]. By using both GGCX knock-down hepatocyte and monocyte cell culture models, the present study showed that VK supplementation could not prevent the increased expressions of both pro-inflammatory cytokines and adhesion molecules and hepatocyte-monocyte adhesion pathway in high PA treated cells. Gas6, a member of VK-dependent Gla-proteins family, has been reported to exhibit potential anti-inflammatory activity; however, the significance of Gas6 activation in mediating the biological functioning of VK against hyperlipidemia associated inflammation remains elusive. The present study showed that in GGCX knockdown cells direct treatment with Gla-Gas6, an activated form of Gas6 against high PA exposure significantly reduced the secretion of both MCP-1 and sICAM-1 form hepatocytes and the total protein and cell surface expression of ICAM-1 in hepatocytes and both CCR2 and CD11a in monocytes followed by the down-regulation of monocytes recruitment into hepatocytes. These observations suggest a direct role of Gla-Gas6 in mediating the beneficial effect of VK supplementation against high PA-induced inflammation. Figure 8 demonstrates the proposed mechanism underlying the positive effect of VK against hyperlipidemia associated inflammatory pathophysiology by impairing MCP-1/ICAM-1 mediated hepatocyte-monocyte adhesion pathway via augmentation of the gamma-glutamyl carboxylation of Gas6. Some studies have demonstrated that Gas6 promotes and accelerates the sequestration of monocytes on endothelial cell [23,24]. However, the effect of carboxylated form of Gas6 as the biological activity of Gas6 is chiefly dependent on the vitamin K-dependent carboxylation of N-terminal Glu residues into Gla residues. In our previous study we demonstrated that Gas6 is unable to impart its prophylactic activity in uncarboxylated form [9]. So, it is of crucial importance to focus on the aspect of carboxylation/uncarboxylation while studying the members of vitamin K-dependent protein family. This might be the most possible reason behind the contradictory results among the research works.

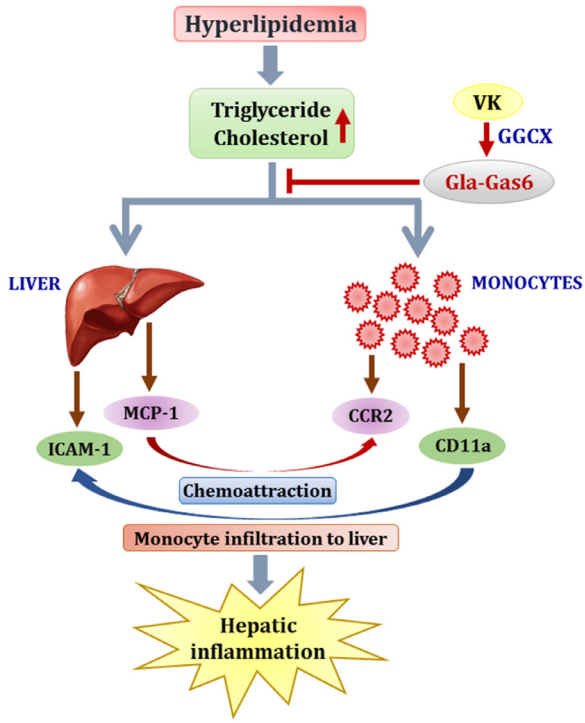

Fig. 8. Schematic diagram of proposed mechanism underlying the role of γ-glutamyl carboxylated Gas6 (Gla-Gas6) in mediating the beneficial effect of vitamin K (VK) against hyperlipidemia associated hepatic inflammation.

In conclusion, this is the first report to our knowledge to demonstrate the novel link between VK, Gla-Gas6, and hyperlipidemia associated inflammation, which will increase the under-

standing about the association of VK with the pathogenesis of hyperlipidemia associated inflammatory pathophysiology. The outcome of this study will be helpful for the development of a novel adjuvant therapy for improving the lives of the hyperlipidemic patient population.

Authors statement

Jijnasa Bordoloi: Conceptualization, data collection and analyses, original draft preparation, artwork preparation; Dibyajyoti Ozah: data collection and analyses; Jatin Kalita: Supervision, validation; Prasenjit Manna: Conceptualization, Supervision, original draft preparation, validation.

Declaration of competing interest

The authors have declared that no conflict of interest exists.

Acknowledgment

The authors are thankful to the Director, CSIR-NEIST, Jorhat for his support , DBT, Government of India for providing the junior research fellowship to Ms. Bordoloi, and Ramalingaswami Re-Entry Fellowship to Dr. Manna (BT/RLF/Re-Entry/34/2013). The authors thank Ms. Cassandra Warden (Vanderbilt University Medical Center, Department of Ophthalmology) for excellent editing of this manuscript.

Funding

The authors are thankful to DST, Government of India, for providing the financial support (ECR/2017/000297).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2021.108635.

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