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Cover Letter

The Editor
Prof B Halliwell
National University of Singapore (NUS),
Singapore.
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10th December 2009

Dear Editor,

Re: "*Identification of Chemerin Receptor (ChemR23) in Human Endothelial Cells: Chemerin-Induced Endothelial Angiogenesis*"

Jaspreet Kaur (BSc), Raghu Adya (MBBS, MSc), Bee K Tan (MBBS, MRCOG, PhD), Jing Chen (PhD), & Harpal S Randeva (MBChB, FRCP, MD, PhD)

Please find enclosed our above named manuscript. We would be grateful if you could consider it as a publication as a "*Basic Research Paper*" in your *Journal*.

We declare that the manuscript has not been submitted to/published by any other journal.

The increasing incidence of atherosclerotic cardiovascular disease is one of the leading causes of mortality and morbidity. Recently, there has been significant interest in bioactive molecules secreted from adipose tissue, and their interactions with vascular endothelium in cardiovascular diseases.

A recently identified adipokine, chemerin, has been shown to be elevated in pro-inflammatory states like obesity and metabolic syndrome. In our current manuscript, we have shown for the first time the presence and proinflammatory cytokine mediated regulation of chemerin receptor-ChemR23 in human endothelial cells. More importantly, we have shown that chemerin induces functional angiogenesis in endothelial cells and activates MAPKs and Akt pathways.
We feel that this manuscript would be of great interest to both the clinical and scientific readership of your *Journal*.

Finally, due to a conflict of interest, we would be very grateful if our above named manuscript was NOT reviewed by:

1. Moon Kyoung Bae and Su Ryun Kim, or any others, from the School of Dentistry, Pusan National University, Republic of Korea;
2. Berndt J, Klöting N, Kralisch S, Kovacs P, Fasshauer M, Schön MR, Stumvoll M, Blüher M, or any others from Leipzig, Germany;

Thank you.

Yours sincerely,

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*Manuscript

Identification of Chemerin Receptor (ChemR23) in Human Endothelial Cells: Chemerin-Induced Endothelial Angiogenesis

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Keywords: Chemerin, CMKLR1/ChemR23, inflammatory cytokines, angiogenesis, migration, proliferation, MAP Kinase

Abstract

Chemerin acting *via* its distinct G protein-coupled receptor CMKLR1 (ChemR23), is a novel adipokine, circulating levels of which are raised in inflammatory states. Chemerin shows strong correlation with various facets of the metabolic syndrome; these states are associated with an increased incidence of cardiovascular disease (CVD) and dysregulated angiogenesis. We therefore investigated the regulation of ChemR23 by pro-inflammatory cytokines and assessed the angiogenic potential of chemerin in human endothelial cells (EC). We have demonstrated the novel presence of ChemR23 in human ECs and its significant up-regulation ($P<0.001$) by pro-inflammatory cytokines, TNF-α, IL-1β and IL-6. More importantly, chemerin was potently angiogenic, as assessed by conducting functional *in-vitro* angiogenic assays; chemerin also dose-dependently induced gelatinolytic (MMP-2 & MMP-9) activity of ECs ($P<0.001$). Furthermore, chemerin dose-dependently activated PI3K/Akt and MAPKs pathways ($P<0.01$), key angiogenic and cell survival cascades. Our data provide the first evidence of chemerin-induced endothelial angiogenesis and MMP production and activity.

Introduction

Dysregulated angiogenesis is the hallmark of cardiovascular diseases (CVD), with obesity and metabolic syndrome (MS) being significant contributors to CVD [1]. The metabolic syndrome is associated with excessive accumulation of central body fat. Adipose tissue produces several hormones and cytokines termed ‘adipokines’ having widespread metabolic effects on vascular endothelium [2,3]. Adipokines also appear to play important roles in the pathogenesis of insulin resistance, diabetes, and atherosclerosis [4]. Moreover, modulation of neo-angiogeneic responses of adipokines has been convincingly demonstrated within adipose tissue, further establishing the link between MS and CVD [5].

Chemerin is a recently discovered 16-kDa adipokine and chemoattractant protein that serves as a ligand for the G protein-coupled receptor, CMKLR1 (ChemR23), with a role in adaptive and innate immunity [6,7]. Furthermore, chemerin is elevated in obesity and shows strong correlation with various facets of the MS, including dyslipidaemia and hypertension; we have recently shown serum and adipose tissue chemerin levels to be increased in women with MS [8,9]. Others, have reported elevated levels of circulating chemerin in inflammatory states, such as subjects with rheumatoid arthritis who are reported to have increased CVD; inflammation being a key player in immune mediated atherosclerosis [10,11].

Recently, the chemerin/ChemR23 system has been implicated in mediating cellular migration under inflammatory conditions [12], a prerequisite of endothelial angiogenesis. This is of interest, as it is increasingly evident from the literature that adipokines play a significant role in the induction of atherogenesis and dysregulated angiogenesis [13,14]. However, no studies to date have described the presence of ChemR23 in human endothelial cells (ECs) and its role in endothelial angiogenesis.

With the aforementioned, we sought to investigate the possible interplay between chemerin/ChemR23 system and the human endothelium. In the present study, we found and report for the first time the presence of ChemR23 in human ECs, and its regulation by pro-inflammatory cytokines. More importantly, chemerin induced endothelial angiogenesis and induced multiple signalling cascades including MAPK and Akt pathways and activates endothelial gelatinases (MMP-2/9).

Materials and Methods

Cell Culture and Treatments

Human Microvascular Endothelial Cells (HMECs) were obtained from the Centre for Disease Control (CDC) in Atlanta, Georgia, USA. Briefly HMECs were cultured in MCDB medium (Sigma-Aldrich, Dorset, UK) as described previously [15].

For ChemR23 protein expression studies, optimization experiments were carried out by treating serum-starved ECs with or without human recombinant TNF-α (0-20ng/mL) (NBS biologicals, Cambridgeshire, UK), IL-1β (0-100ng/mL) (ABCAM, Cambridgeshire, UK) and IL-6 (0-100ng/mL) (NBS biologicals, UK) in time-dependent manner (1 – 24 hours). ChemR23 blocking peptide was purchased from Sigma, UK.

RNA Isolation and Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen Ltd, UK) according to the manufacturer's protocol, followed by reverse transcription into cDNA, by using 5IU/ RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas, York, UK) as described previously [15], as was Quantitative PCR [15].

Protocol conditions consisted of denaturation at 94 °C for 1 min, then 38 cycles of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 30 s, followed by a 7 min extension at 72 °C.

Gene	Forward primer	Reverse primer	Product size
ChemR23	5'-CAACCTGGCAGTGGCAGATT-3	5'AGCAGGAAGACGCTGGTGAA-3	153 (bp)
β-actin	5'-AAGAGAGGCATCCTCACCCT-3	5'-TACATGGCTGGGGTCTTGAA-3'	216 (bp)

Table-1. Primer sequences

PCR products were analyzed using Blast-Nucleic-Acid-Database Searches, confirming the identity of our products

MTS Proliferation Assay

Cell proliferation was determined with CellTiter-96 AQueous One Solution Cell Proliferation Assay (MTS) kit (Promega, UK) according to the manufacturer's instructions. Briefly, serum-starved cells were dose-dependently treated with human recombinant chemerin (0 - 30 nM) (R and D systems, Abingdon, UK) for 4 - 72 hours. Following chemerin treatment, 20µl MTS reagent was added to 100µl of culture medium per well. The absorbance at 490 nm was recorded using an ELISA plate reader (EL800, Bio-Tek Instruments, Inc., Winooski, VT, USA). The percentage of the absorbance was calculated against untreated cells.

In-Vitro Angiogenesis Assay

Angiogenesis was assessed by studying the formation of capillary-like structures by ECs on a Matrigel (BD Biosciences, San Jose, CA, USA) as described previously [15]. Briefly, serum-starved ECs were pre-treated with or without chemerin (0-30 nM) and VEGF (10ng/mL) for 24hrs. Cells were then seeded onto Matrigel coated plates at a density of 4-5 × 10^3cells/well and incubated at 37 °C for 4, 9 or 18 hours. Capillary tube formation images were captured with a digital microscope camera system (Olympus, Tokyo, Japan). Tube lengths were quantified using Image-Pro Plus software; the length of tubes in 3-4 randomly selected fields in each of the wells was measured with the untreated groups.

Migration Assay

EC migration assay was performed according to the BD BioCoat Angiogenesis System (BD Biosciences) protocol. The assay was performed using a modified Boyden chamber as described previously [15]. Briefly, trypsinised, EC suspension of 4.0 x 10^5 cells/ml was prepared and 250 µl of which was into the transwell inserts, followed by addition of 750µl of starvation media to the lower chamber. ECs were labelled by incubating with 50 nM Calcein-AM in HBSS, for 90 minutes. The cells were treated with chemerin (0-30 nM) for 4, 8, 12 and 24 hours, VEGF served as a positive control. ECs were the fixed by 2% formaldehyde. The migrated cells were quantified by using fluorescence plate reader.

Gelatin Zymography

The gelatinolytic activity of secreted MMP-2 and MMP-9 in the conditioned media was measured by gelatin zymography as described previously [15]. White bands were observed following de-staining indicating gelatinolytic activity of the expressed MMPs and the band intensities were measured [Gel Pro image analysis (Gel Pro 4.5, Media Cybernetics, USA)].

Western Blot Analysis

For the analyses and regulation of ChemR23 protein, serum-starved ECs were treated with or without human recombinant TNF-α (0-20ng/mL), IL-1β (0-100ng/mL) and IL-6 (0-100ng/mL) in a time-dependent (1 – 24 hours) manner. The protocol for protein lysates preparation and western-blot analysis was as previously described [15], using primary anti-ChemR23 antibody (dilution 1:1000) (Santa Cruz biotechnology, Middlesex, UK), and secondary anti-goat horseradish-peroxidase-conjugated Ig (1:2000) (Dako Ltd, Cambridge, UK).

Membranes were also re-probed with the β-actin antibody (Cell Signalling Technology Inc., Beverly, MA, USA; 1 in 8,000 dilution) to determine equal protein loading. Likewise, for MAPK ($ERK_{1/2}$ and p38 MAPK) and Akt activation, immunoblotting was carried out as described previously [15].

Statistical analysis

All of the data in the present study are expressed as mean ± SEM. Comparisons among groups were made by ANOVA (non-parametric). When significance ($P < 0.05$) was detected, a post hoc Dunns multiple-comparison test was performed [Graph Pad software (version 4.0)].

Results

ChemR23 Expression in Human Endothelium

RT-PCR analysis revealed the presence of ChemR23 mRNA in both micro (HMECs) and macro-vascular human ECs (HUVECs and EA.hy926) (Fig. 1A). Western blotting, using specific chemerin receptor antibody, confirmed its expression in both these cell types as a 42 kDa band (predicted molecular weight) (Fig. 1B). The specificity of which was further confirmed by employing a ChemR23 blocking peptide (data not shown). Additionally, immunocytochemical analysis established the presence and distribution of ChemR23 in ECs (Fig. 1C).

Regulation of Endothelial ChemR23 by Proinflammatory Cytokines

Studies have elucidated the involvement of ChemR23 in chemerin induced inflammatory response/chemotaxis and specific recruitment of antigen-presenting cells to inflammatory sites [10]. Moreover, since these states are marked with increased circulating pro-inflammatory cytokines, we hypothesised that; TNF-α, IL-1β, and IL-6 may have a regulatory effect on ChemR23 expression in ECs. We incubated serum-starved ECs with dose-dependent TNF-α (0-20 ng/mL), IL-1β (0-100 ng/mL) and IL-6 (0-100 ng/mL) for various time points (1-24 hours). TNF-α significantly and dose-dependently increased ChemR23 protein levels at both 12 and 24 hours (Fig 1D; ChemR23; $P<0.001$). IL-1β significantly and dose-dependently increased ChemR23 protein levels at 12 hours, with maximal response at 100ng/mL. However at 24 hours, a dose-dependent decrease in ChemR23 protein expression, with maximal response at 1ng/mL, was observed (Fig 1E; $P<0.001$). IL-6 like IL-1β induced a significant dose-dependent increase in ChemR23 protein expression at 12 hours with maximal response at 100ng/mL (Fig 1F; $P<0.001$).

Chemerin Induced *In-Vitro* Angiogenesis

Chemerin increases EC proliferation

Proliferation assay was performed in a time-dependent manner (4-48 hours) with maximal response being noted at 24 hours (data not shown). Serum starved ECs were treated with dose-dependent chemerin (0-30nM) and VEGF (10ng/mL) (positive control). At 24 hours, a significant increase in proliferation was observed at 0.1nM and 1.0 nM of chemerin and VEGF treatments [Fig 2A; 1.77 fold - VEGF ($P < 0.001$), and 1.41 fold-chemerin (0.1 nM) ($P < 0.01$) compared to basal (untreated); n=6 experiments]. These were additionally confirmed by a colorimetric Alamar-blue proliferation assay (data not shown).

Chemerin induced capillary tube formation and migration of ECs

Endothelial migration and capillary tube formation, like EC proliferation, are critical steps in angiogenesis, we performed Matrigel based capillary-like tube formation assay. Treatment with chemerin promoted angiogenesis, as evidenced by capillary-like tube formation, in a time (0-24 hours) and dose (0-30 nM) dependent manner. Quantitative analyses revealed a significant increase in tube length induced by chemerin at 24 hours (Fig-2B1/B2; $P<0.001$, compared to basal; $n = 6$ experiments).

Serum-starved, calcein labelled ECs were subjected to migration assay, treated with dose-dependent chemerin (0-30nM) and VEGF (positive control) for 4, 8 12, and 24 hours. Chemerin increased migration in a dose-dependent and time-dependent manner, with a maximal effect at 30 nM after 24 hours (Fig-2C1/C2; $P<0.001$ chemerin treated vs. basal); confirming the migratory potential of chemerin in ECs. VEGF used a positive control, also significantly increased endothelial migration.

Chemerin induced MAPKinase and Akt Signalling

p38 MAPKinase and $ERK_{1/2}$ activition

MAPK signalling pathways are involved in EC proliferation and specifically, p38 MAPK signalling has been documented to be critically involved in endothelial angiogenesis [16]. We investigated transient phosphorylation of p38 MAPK in EC lysates treated with both time (0-30 minutes) and dose (0-30nM) dependent chemerin. Chemerin significantly increased p38 MAPK phosphorylation, maximally at 15 minutes (Fig-3A1: 3-fold increase compared to controls $P<0.001$) and at 0.1 nM (Fig-3A2: 3-fold compared to controls $P<0.001$). Interestingly, higher doses of chemerin (10nM and 30nM) failed to induce any significant changes in p38 MAPK phosphorylation.

$ERK_{1/2}$ signalling pathways are also involved in endothelial proliferation. Interestingly, time-dependent stimulation of ECs with chemerin induced a biphasic response in $ERK_{1/2}$ phosphorylation, with maximal response at 5min, followed by a decline at 10 min, and a significant increase at 15min (Fig-3B1: $P<0.001$). Additionally, chemerin (0.1 nM) significantly increased phosphorylated $ERK_{1/2}$ (Fig-3B2: 6-fold compared to controls; $P<0.01$). However, higher doses of chemerin (10nM and 30nM) failed to induce any change in phosphorylation of $ERK_{1/2}$. This dose specific mediated $ERK_{1/2}$ phosphorylation is particularly mirrored in chemerin induced EC proliferation, perhaps due to the vital involvement of $ERK_{1/2}$ in EC proliferation. Interestingly, chemerin had no effect on JNK activity (data not shown).

Chemerin Induced Akt signalling

As for the MAPK signalling cascades, the Akt pathway plays a critical role in angiogenesis [17]. In order to address whether chemerin activates this pathway in ECs, we treated serum starved cells with dose (0-30 nM) and time-dependent (0-30 minutes) chemerin. A significant increase in Akt phosphorylation was observed at 5 minutes (Fig-2C1: 4-fold compared to controls; *P*<0.001), which decreased at 10 minutes (Fig-2C1: 1.5-fold compared to controls; *P*<0.05). Interestingly, unlike MAPK pathways, chemerin also induced a significant dose-dependent increase in Akt phosphorylation, with maximum response at 10nM (Fig-2C2: 4-fold compared to controls; *P*<0.001).

Chemerin- induced Gelatinolytic Activity in Human ECs

The angiogenic potential of ECs is greatly enhanced by extra-cellular matrix degradation, where gelatinases MMP-2/-9 play vital roles. To assess their involvement in chemerin induced angiogenesis, we performed gelatin zymography with the condition media of the aforementioned proliferation, migration and capillary tube formation assay (data not shown). Chemerin dose-dependently increased both MMP-2 (Fig-4A; *P*<0.001 chemerin treated vs. Basal) and MMP-9 (Fig-4B; *P*<0.001 chemerin treated vs. Basal) gelatinolytic activity in ECs.

Discussion

We describe novel findings, of the presence and regulation of endothelial ChemR23 by pro-inflammatory cytokines. More importantly, we report that chemerin, whose circulating concentrations are altered in obesity and obesity-related disorders, activates key survival and angiogenic signalling cascades like MAPK and Akt pathways. Additionally, we demonstrate for the first time chemerin induced functional angiogenesis in human ECs, by promoting migration and capillary tube formation; and activation of endothelial gelatinases (MMP-2/-9).

Altered expression of chemokines and their receptors during inflammatory processes may modify the equilibrium between angiostatic and angiogenic processes resulting in dysregulated angiogenesis leading to the development of CVD [18]. Of interest, chemerin was reported to strongly correlate with components of metabolic syndrome and pivotal inflammatory markers of CVD like TNF-α, IL-6 and CRP [19].

Studies have previously shown that up-regulation of chemokine receptors results in amplification of immunological responses by making the cells more responsive to stimuli. Furthermore, cytokine mediated synergistic inflammatory cascades are enhanced by modulation of receptor expression for one cytokine by the other [20-22]. In our present study, we observed a significant up-regulation of the chemokine receptor ChemR23 protein expression by pro-inflammatory cytokines (TNF-α, IL-1β and IL-6). Hence, given the existing pro-inflammatory environment and raised circulating cytokines in metabolic syndrome, it is tempting to propose a critical role mediated by chemerin in these inflammatory states.

At a functional level, we report chemerin induced activation of key angiogenic and cell survival cascades, namely the MAPKs and Akt pathways, in humans ECs. A significant finding in our study is the highly reproducible biphasic pattern of activation of $ERK_{1/2}$

occurring within 2 minutes, peaking at 5minutes, followed by a rapid decline at 10 minutes, and then a subsequent increase at 15 minutes. The precise explanation for this observation remains to be determined; however, similar findings in other GPCR models that trigger a biphasic feed into Ras/Raf/MEK/ERK cascade have been reported but are poorly understood [23]. The plausible pathways implicated in the biphasic response include MMP-mediated shedding of heparin-sensitive EGF receptor ligands, EGF receptor auto-phosphorylation, and MEK1 activity [23]. In this context, it is interesting to note our robust data on chemerin induced gelatinolytic activity (MMP-2/-9) in ECs. Alternatively, this time mediated termination and reappearance of signalling cascade may involve feedback loops, consisting of degradation, inactivation of proteins and differential protein trafficking [24]. Future studies are therefore required to study and elucidate these interesting chemerin induced effects on ERK1/2 and p38 MAPK pathways.

In addition, the dose-dependent effects of ERK phosphorylation were mirrored by chemerin induced EC proliferation. However, and of interest, unlike EC proliferation, both chemerin induced migration and capillary tube formation seemed to follow a dose-dependent response similar to Akt activation. Of note, activation of Akt kinase has been implicated in orchestrating a number of signalling pathways potentially involved in angiogenic processes and survival pathways [17].

Enhanced production of MMPs, in particular gelatinases (MMP-2/-9), is an early feature of vascular remodelling and dysregulated angiogenesis, contributing to endothelial barrier dysfunction [25,26]. Our observations of chemerin inducing functional angiogenesis, with concurrent increases in gelatinolytic activity, suggest a potential causal relationship between chemerin induced MMP activity and angiogenesis. However, future studies are required to elucidate the precise role of gelatinases in chemerin induced angiogenesis.

In conclusion, we demonstrate for the first time, presence of ChemR23 and chemerin induced *in-vitro* angiogenesis in human ECs. Our findings also confirm that chemerin activates key survival and angiogenic pathways including MAPK and Akt kinases. Finally, our data add to the diverse effects of chemerin, but more importantly reveal novel insights into the potential role(s) of chemerin in human EC angiogenesis.

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Disclosures

Authors have no conflict of interest

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Figure Legends

Figure-1. ***Identification of Chemerin receptor, ChemR23, and its regulation by proinflammatory cytokines; TNF-α, IL-1β, and IL-6 in ECs***

Using PCR, western blot and immunocytochemical studies, ChemR23 mRNA and protein expressions were identified in human ECs. Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described [15]. (**A**)/(**B**) represent mRNA and protein expression levels of ChemR23 in HMECs, EA.hy926 and HUVECs using PCR and western blot analyses respectively. Figure (**C**) shows immunostaining of ChemR23 protein in ECs. In the next set, serum starved HMECs were treated with TNF-α [0-20 ng/mL], IL-1β [0-100 ng/mL] and IL-6 [0-100 ng/mL] for 12 and 24 hours. Protein expression levels of ChemR23 were measured by western blot analyses. Figures (**D**), (**E**) and (**F**) denote representative western-blot analysis of ChemR23 protein expression following TNF-α, IL-1β and IL-6 treatments respectively. ***P<0.001, **P<0.01 vs. basal, Results are means ± S.E.M n = 6 experiments per group.

Figure-2. ***Chemerin induced proliferation, capillary tube formation and migration of ECs in vitro.***

MTS Proliferation Assay: - Serum starved HMECs were treated with/without chemerin [0–30 nM] and VEGF [10ng/mL] (positive control) for 24 hours and EC proliferation was assessed by MTS assay **(A)**. Results were expressed as percentage of cells in relation to basal (untreated) and represents the mean of triplicates. ***P<0.001, **P < 0.01, *P < 0.05 vs. control, n = 6 experiments per group.

Capillary Tube Formation Assay: **-** Chemerin [0-30nM] induced capillary tube formation in HMECs, and VEGF [10ng/mL] was used as a positive control. Figure (**B1**) shows graphical representation of capillary tube length (expressed as a percentage difference relative to basal), and (**B2**) represent images of capillary tube formation. Results are means ± S.E.M, n = 6 experiments. ***P < 0.001, **P < 0.01, *P < 0.05 vs. basal.

EC Migration Assay: - Serum starved Calcein-AM labelled HMECs were treated with chemerin [0-30 nM] for 4, 8, 12 and 24 hours. VEGF [10ng/ml] was used as a positive control. Migrated cells were quantified using a fluorescence plate reader. The migrated cells were expressed as the ratio of the fluorescence compared to the control. Figure (**C1**) shows graphical representation of migratory distance (expressed as a percentage difference relative to basal), and (**C2**) represent images of wound scratch assay. Results are means ± S.E.M., n = 6 experiments. ***P < 0.001, **P < 0.01, *P < 0.05 vs. basal.

Figure-3. ***Chemerin-induced activation of p38 MAPKinase, $ERK_{1/2}$ and Akt signalling pathways*.**

Serum starved HMECs were treated with chemerin [0.1nM] in a time (0-30 minutes) and dose-dependent manner [0-30nM chemerin for 10 minutes]. Using western-blot analysis, phosphorylation of p38 MAPKinase, $ERK_{1/2}$ and Akt were measured in above protein lysates. Figures (**A1**), (**B1**) and (**C1**) represent time, (**A2**), (**B2**) and **(C2)** denote dose-dependent effects of chemerin on p38 MAPKinase, $ERK_{1/2}$ and Akt protein phosphorylation respectively. The results were represented as a ratio of phosphorylated/total protein, expressed as fold changes over basal. Results are means ± SEM., n = 6 experiments. ***P<0.001, **P < 0.01, *P < 0.05 vs. basal.

Figure-4. ***Chemerin enhances MMP-2 and MMP-9 gelatinolytic activity in ECs***

Chemerin enhanced gelatinolytic activity of both MMP-2/-9, when the condition media of the aforementioned capillary tube formation assay was subjected to gelatine zymography. Figures(**A) and (B**) denotes representative zymograms and densitometric analysis of MMP-2 and MMP-9 protein activity in conditioned media for 24hours respectively. Results are means ± S.E.M of six independent experiments. n = 6 per group ***P<0.001, **P < 0.01, *P < 0.05 vs. basal.

Figure

Fig. 1

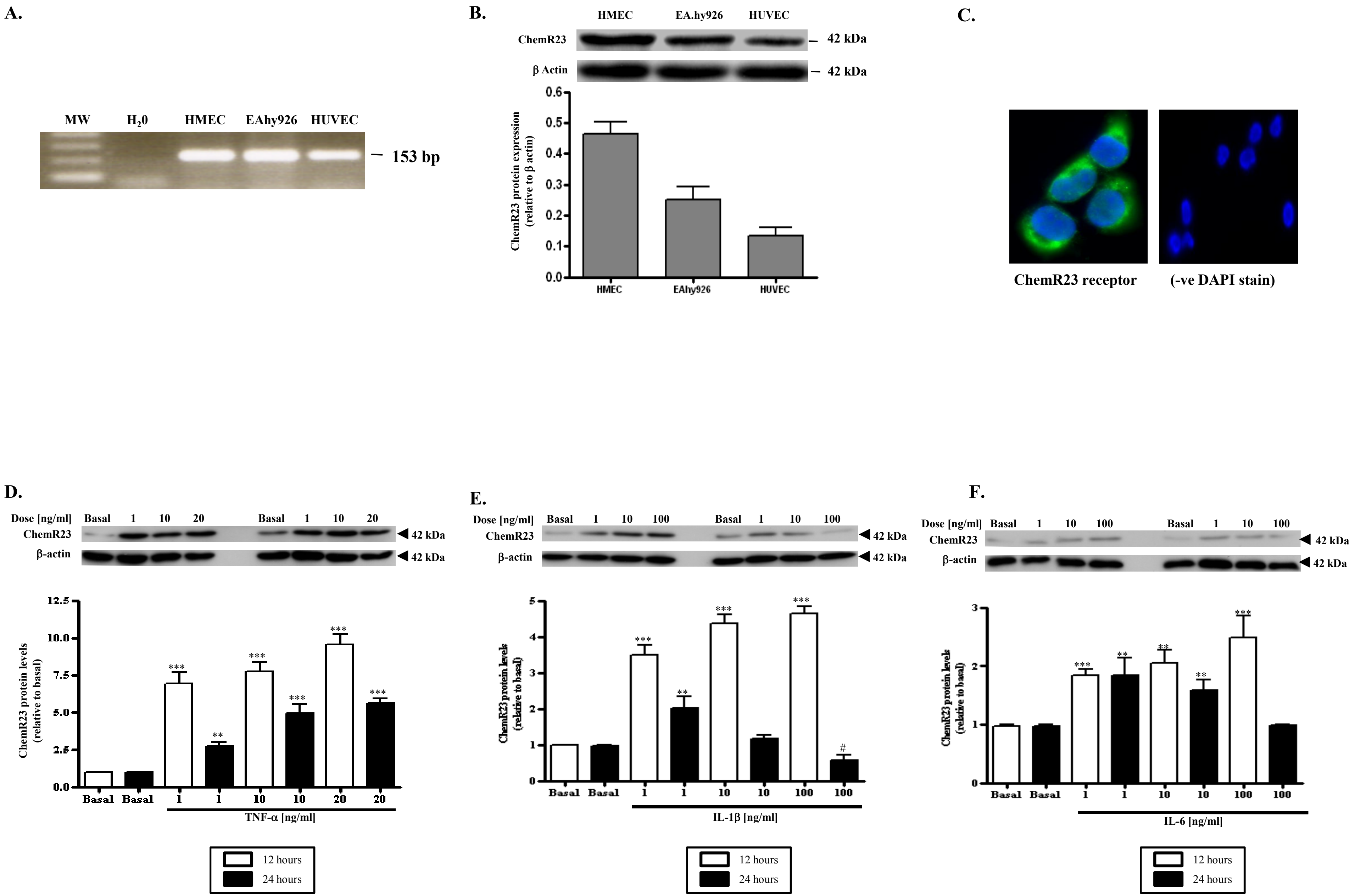

A.
MW
H_20
HMEC
EAhy926
HUVEC
153 bp
B.
HMEC
EA.hy926
HUVEC
ChemR23
42 kDa
β Actin
42 kDa
ChemR23 protein expression (relative to β actin)
HMEC
EAhy926
HUVEC
C.
ChemR23 receptor
(-ve DAPI stain)
D.
Dose [ng/ml]
Basal
ChemR23
β-actin
42 kDa
ChemR23 protein levels (relative to basal)
TNF-α [ng/ml]
12 hours
24 hours
E.
Dose [ng/ml]
ChemR23
β-actin
42 kDa
ChemR23 protein levels (relative to basal)
IL-1β [ng/ml]
12 hours
24 hours
F.
Dose [ng/ml]
ChemR23
β-actin
42 kDa
ChemR23 protein levels (relative to basal)
IL-6 [ng/ml]
12 hours
24 hours

Fig. 2

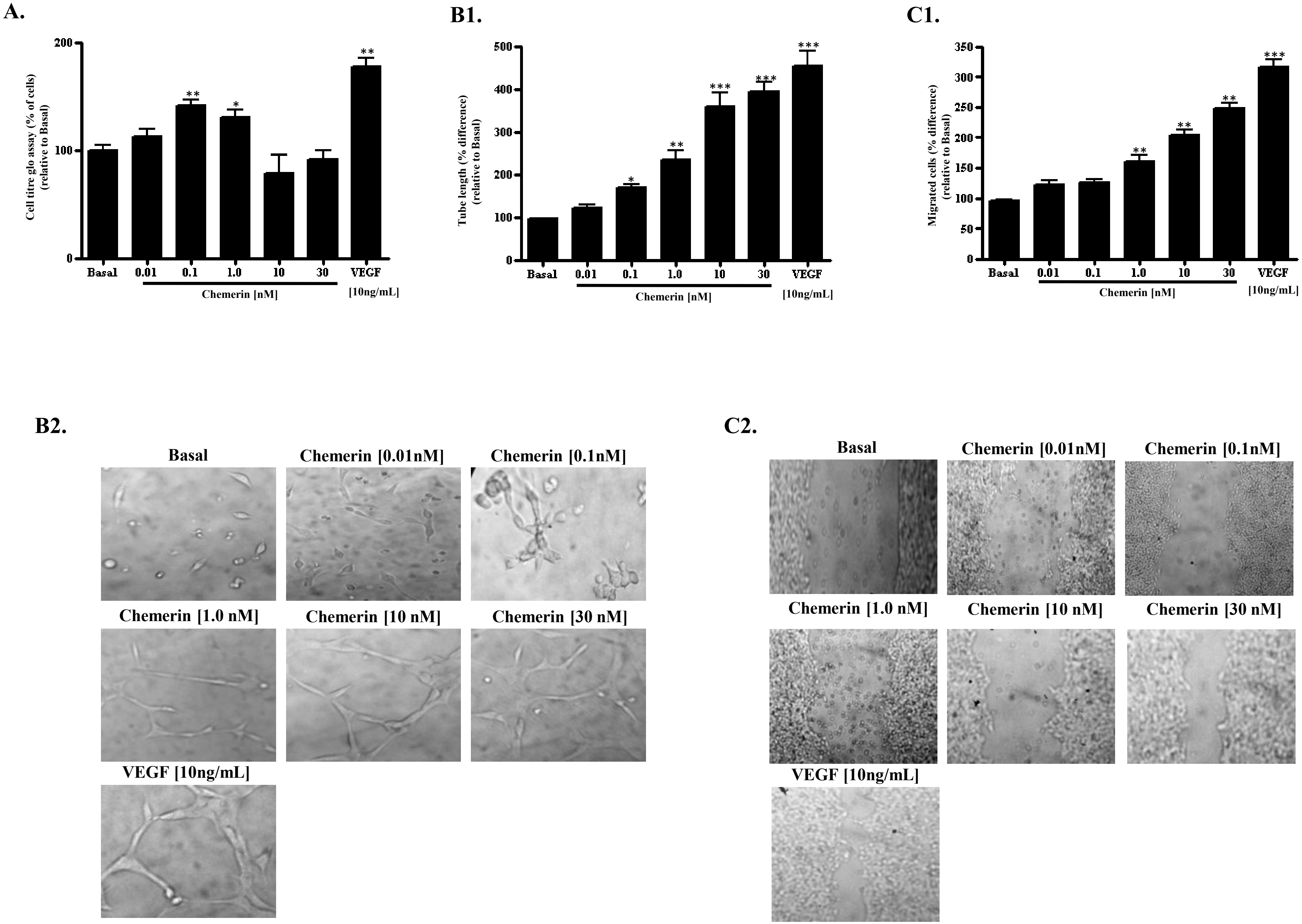

A.
Cell titre glo assay (% of cells) (relative to Basal)
200
100
0
**
*
**
Basal
0.01
0.1
1.0
10
30
VEGF
Chemerin [nM]
[10ng/mL]
B1.
Tube length (% difference) (relative to Basal)
500
400
300
200
100
0
*
**
***
***
***
Basal
0.01
0.1
1.0
10
30
VEGF
Chemerin [nM]
[10ng/mL]
C1.
Migrated cells (% difference) (relative to Basal)
350
300
250
200
150
100
50
0
**
**
**
***
Basal
0.01
0.1
1.0
10
30
VEGF
Chemerin [nM]
[10ng/mL]
B2.
Basal
Chemerin [0.01nM]
Chemerin [0.1nM]
Chemerin [1.0 nM]
Chemerin [10 nM]
Chemerin [30 nM]
VEGF [10ng/mL]
C2.
Basal
Chemerin [0.01nM]
Chemerin [0.1nM]
Chemerin [1.0 nM]
Chemerin [10 nM]
Chemerin [30 nM]
VEGF [10ng/mL]

Fig. 3

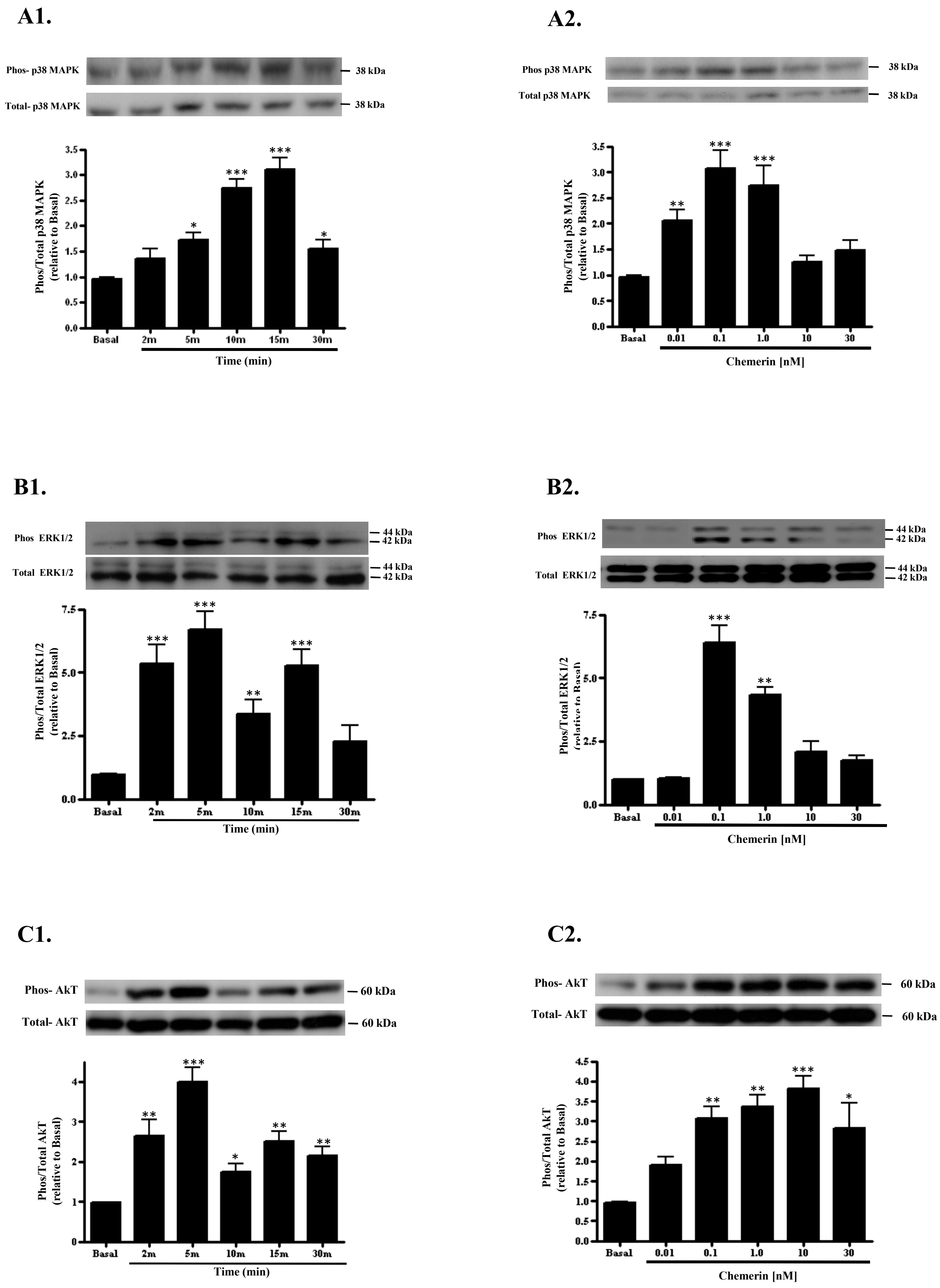
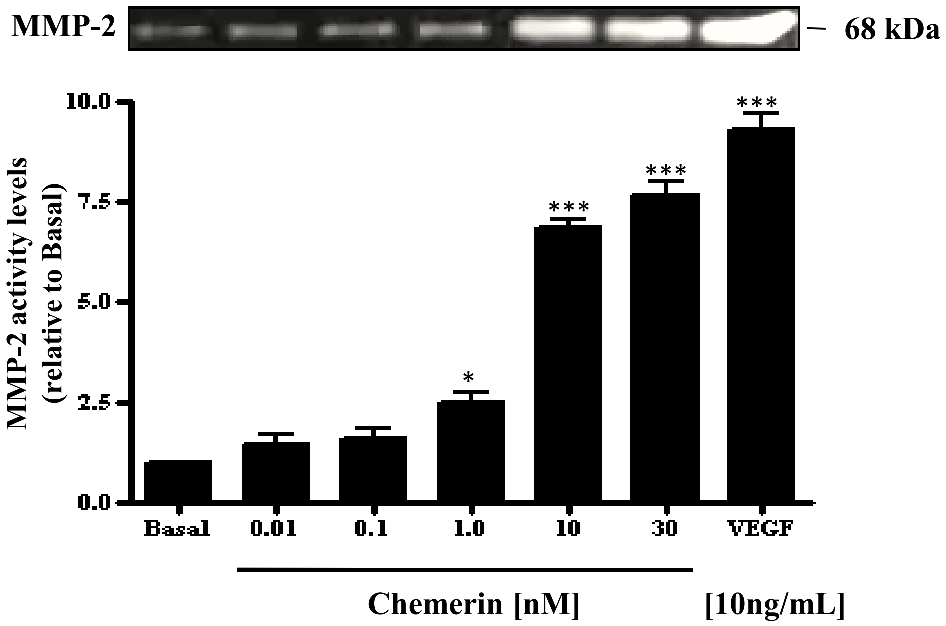
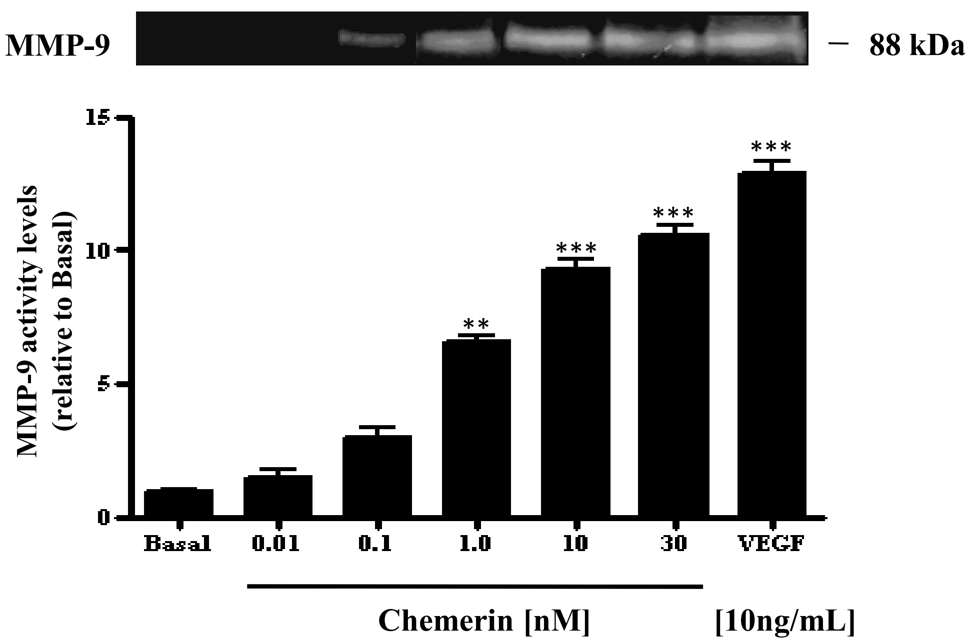

A1.
Phos- p38 MAPK
Total- p38 MAPK
38 kDa
Phos/Total p38 MAPK (relative to Basal)
Basal
2m
5m
10m
15m
30m
Time (min)
A2.
Phos p38 MAPK
Total p38 MAPK
38 kDa
Phos/Total p38 MAPK (relative to Basal)
Basal
0.01
0.1
1.0
10
30
Chemerin [nM]
B1.
Phos ERK1/2
Total ERK1/2
44 kDa
42 kDa
Phos/Total ERK1/2 (relative to Basal)
Time (min)
B2.
Phos ERK1/2
Total ERK1/2
Chemerin [nM]
C1.
Phos- AkT
Total- AkT
60 kDa
Phos/Total AkT (relative to Basal)
Time (min)
C2.
Phos- AkT
Total- AkT
60 kDa
Phos/Total AkT (relative to Basal)
Chemerin [nM]

Fig. 4

A.


MMP-2
68 kDa
MMP-2 activity levels (relative to Basal)
10.0
7.5
5.0
2.5
0.0
*
***
***
***
Basal
0.01
0.1
1.0
10
30
VEGF
Chemerin [nM]
[10ng/mL]

B.


MMP-9
88 kDa
MMP-9 activity levels (relative to Basal)
15
10
5
0
**
***
***
***
Basal
0.01
0.1
1.0
10
30
VEGF
Chemerin [nM]
[10ng/mL]