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**Interleukin-6 Triggers Human Cerebral Endothelial Cells Proliferation and Migration:  
The Role for KDR and MMP-9**

**Short Title:** IL-6 triggers angiogenesis

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## ABSTRACT

Interleukin-6 (IL-6) is involved in angiogenesis. However, the underlying mechanisms are unknown. Using human cerebral endothelial cell (HCEC), we report for the first time that IL-6 triggers HCEC proliferation and migration in a dose-dependent manner, specifically associated with enhancement of VEGF expression, up-regulated and phosphorylated VEGF receptor-2 (KDR), and stimulated MMP-9 secretion. We investigated the signaling pathway of IL-6/IL-6R responsible for KDR's regulation. Pharmacological inhibitor of PI3K failed to inhibit IL-6-mediated VEGF overexpression, while blocking ERK1/2 with PD98059 could abolish IL-6-induced KDR overexpression. Further, neutralizing endogenous VEGF attenuated KDR expression and phosphorylation, suggesting that IL-6-induced KDR activation is independent of VEGF stimulation. MMP-9 inhibitor GM6001 significantly decreases HCEC proliferation and migration ( $p < 0.05$ ), indicating the crucial function of MMP-9 in promoting angiogenic changes in HCECs. We conclude that IL-6 triggers VEGF-induced angiogenic activity through increasing VEGF release, up-regulates KDR expression and phosphorylation through activating ERK1/2 signaling, and stimulates MMP-9 overexpression.

**Key Words:** angiogenesis, interleukin-6, matrix metalloproteinase; proliferation; human cerebral endothelial cell; vascular endothelial growth factor.

## INTRODUCTION

Interleukin-6 (IL-6) is a proinflammatory cytokine produced by multiple cell types including endothelial cells (ECs) in response to diverse stimuli [1]. IL-6 plays an important role in inflammatory responses, immunological regulation and hemopoietic responses in many organs including human brain tissue [2, 3]. IL-6 also plays a crucial role in the pathology of several CNS diseases regarding vascular remodeling through influencing cell growth and differentiation [3]. IL-6 stimulates vascular endothelial growth factor (VEGF) synthesis and release, and promote angiogenesis occurrence *in vivo* and *in vitro* [4-8].

Vascular endothelial growth factor (VEGF) is a potent angiogenic agent acting as specific mitogen for endothelial cells through specific membrane receptors, kinase domain-containing receptor (KDR), and the fin-like tyrosine kinase 1(Flt-1) [9]. When VEGF binds to its receptors, it initiates auto-phosphorylation, induces tyrosine kinase activity, and subsequently stimulates cellular responses in normal and abnormal angiogenesis [10]. There is no evidence that other cytokines or growth factors can react with VEGF receptors in human cerebral endothelial cells (HCECs).

ERK1/2 and PI3K regulate many different cell responses in the endothelial cells, and have a particularly well-documented role in cell growth and survival [11]. Recent studies reported that these two signaling pathways are major contributors in the IL-6 signaling [12]. However, how these signaling pathways are involved in IL-6 induced angiogenic changes in the HCECs is unknown.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidase actively participating in the angiogenic process. MMP-9 in particular plays a major role in regulating cell attachment, proliferation, migration, and growth in many cell types including

HCECs. Growth factors and cytokines can stimulate MMP-9 expression during angiogenesis [13]. HCECs are characterized by a unique pattern of gene expression in vascular and angiogenic factors, neurotrophic and growth supporting factors, as well as immunoregulatory factors [14]. It plays a key role in determining how IL-6, VEGF, KDR and MMP-9 influence HCECs' proliferation and migration.

To explore the role of IL-6 on HCEC proliferation and migration, we: 1) investigated the changes of VEGF and its receptor expression following IL-6 stimulation in HCEC culture; 2) evaluated the functional activity of VEGF receptors by monitoring receptor phosphorylation; 3) elucidated the specific signal pathways in up-regulation of KDR by IL-6; and 4) further explored MMP-9 expression and activity under the influence of IL-6.

## **MATERIALS AND METHODS**

### ***Human cerebral endothelial cell culture***

HCECs were purchased from Cell systems (St. Katharinen, Germany) and their homogenous nature was confirmed by immunostaining with CD-31 antibody. Cultured cells were maintained at 37°C in 5% CO<sub>2</sub>/95% ambient mixed air and the culture media were changed every two days. All experiments were performed on HCEC 2 to 4 passages. In IL-6 treatment experiments, HCECs were grown in the EC growth medium (Clonetics, Walkersville, MD) in 35 mm polystyrene plates to 90% confluent state, and rinsed 3 times with a serum-free medium before arresting with serum-free medium containing 0.2% bovine albumin. The cells were then incubated with IL-6 and other antibodies in specified conditions.

### ***HCEC proliferation and migration assays***

HCECs proliferation was performed by a combination of BrdU incorporation and counting cell numbers. HCECs grew to 90% confluence in T-25 flask, and were harvested using 0.025% Trypsin. The cells ( $1 \times 10^4$ ) were seeded onto 96 well gelatin coated plate overnight, and treated with different antibodies for 24 hours, followed by incubation with BrdU for two hours (BrdU cell proliferation ELISA Kit, Roche, Penzberg, Germany) and measured absorbance at 450 nm (reference wavelength 690 nm) using an ELISA reader (E max)

HCEC migration was evaluated using 24-well Transwell cell culture chambers with 8.0  $\mu\text{m}$  pore polycarbonate filter inserts. The filters were coated with matrigel, which was suitable for EC invasion assay (BD Biosciences, Bedford, MA). The stock solution of matrigel was diluted to 300  $\mu\text{g}/\text{ml}$  using serum-free DME medium. An aliquot of 75  $\mu\text{l}$  matrigel was added into each filter insert and incubated overnight at room temperature under a laminar flow hood. The next day, the coated insert was rehydrated with 0.5 ml of serum free DME medium for 2 hours. Cultured HCECs were trypsinized and suspended in 0.2% BSA/DMEM at a concentration of  $2.5 \times 10^5/\text{ml}$ . A total of 500  $\mu\text{l}$  of 0.2% BSA/DMEM was added to the lower chamber and 100  $\mu\text{l}$  cell suspension was applied to coated insert filters. The chamber was incubated at  $37^\circ\text{C}/5\% \text{CO}_2$  for 1 hour to allow cell attachment. It was then incubated for 18 hours at  $37^\circ\text{C}/5\% \text{CO}_2$  to allow cell migration; the insert was removed and the membrane washed with 0.1M PBS. No migrated cells on the upper side of filter were scraped; migrated cells on the lower side of chamber were fixed and stained with hematoxylin. Membrane was mounted on a slide and then examined under a microscope. Migration was quantified by measuring the stained cells in five random areas per membrane. The experiments were performed three times.

### ***MMP-9 activity assay using zymograms***

The method of Zymographic assay was performed as described previously [15]. A 70 µg sample protein was separated under non-reducing conditions in a 10% zymogram gel (Invitrogen, Carlsbad, CA) containing 0.1% gelatin as a substrate. Following electrophoresis, gels were washed and incubated in developing buffer overnight at 37°C. The gels were then stained with 0.5% Coomassie Blue R-250. MMP-9 activity can be detected as white bands of lysis against the Coomassie blue staining gel. Protein activity bands in zymography were semi-quantitated through scanning densitometry.

### ***MMPs and VEGF mRNA measurement using real time PCR***

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen). Reverse-transcription was performed using a RETROscript kit (Ambion, Austin, TX). Oligonucleotide primers are listed in **Table 1**. Real-time polymerase chain reaction was determined using 2.5 µl (5 ng) cDNA, 12.5 µl SYBR green PCR Master Mix (2X) for MMP-9, or TagMan PCR Master Mix (2X) for VEGF, 2.5 µl primer pair mix (5 pmol each primer), and water to a 25 µl final volume (ABI-prism 7000, Foster city, CA). Thermocycling conditions consisted of an initial holding at 50°C for 2 minutes, and then 95°C for 3 minutes. This was followed by 94°C for 15 seconds, 56°C for 45 seconds, and 72°C for 30 seconds for 45 cycles for MMP-9.

Thermocycling conditions for VEGF were an initial holding at 50°C for 2 minutes, and then 95°C for 10 minutes. This was followed by a two-step Tag Man PCR program consisting of 95°C for 15 seconds, and 60°C for 60 seconds for 40 cycles. Results were analyzed using an ABI sequence detection software; MMP-9 and VEGF mRNA levels were normalized to GAPDH.

### ***VEGF, KDR, Flt-1 and phosphor-KDR protein determination using Western blot analysis***

HCECs were harvested and homogenized using a lysis buffer. The protein concentration was determined using the *Bio-Rad* system. An equal amount of protein was loaded and electrophoresed on 4-12% gradient gel. Subsequently, proteins were electroblotted onto a polyvinylidene difluoride membrane (PVDF, Bio-Rad, Richmond, CA) in a transfer buffer (ICN biomedical). The membrane was placed in 5% dry milk TBS with 0.1% Tween 20 for 1 hour to block non-specific binding. The membrane was immunoprobed with anti-VEGF antibody (1:200 dilution, Santa Cruz Biotech, Santa Cruz, CA), anti-KDR antibody (1:200 dilution), anti-Flt-1 antibody (1:300 dilution), or phosphor-KDR (1:100 dilution) at 4<sup>0</sup>C overnight. After washing, the membrane was incubated with HSP-conjugated anti-rabbit or anti-mouse antibodies (1:2000 dilution, Amersham) at room temperature for 1 hour and developed with an enhanced ECL Kit (Amersham). Protein bands were scanned and semi-quantified by densitometry using KODAK image analysis software (Eastman Kodak Co., Rochester, NY).

### ***Statistical analysis***

Results of the dose-dependent proliferation and inhibition experiments were analyzed using an ANOVA with post-hoc multiple comparison test. All data are represented as mean±standard deviation (mean ± SD). Statistical significances were determined with an ANOVA. A random probability value less than 0.05 is considered statistically significant for the comparisons.

## **RESULTS**



### ***Effect of IL-6 on VEGF expression in HCECs***

Involvement of IL-6 in angiogenesis is related to VEGF production [16]. Because mitogenic activity of HCECs could be stimulated by VEGF or IL-6, we therefore investigated the interaction between IL-6 and VEGF expression. The data showed that IL-6 stimulates VEGF expression by two-fold in both mRNA and protein levels as compared to the control group (**Fig. 1A** and **1B**). Dose response results indicated that this effect was maximal at 100 ng/ml of IL-6. The maximal increase in the mRNA expression was at 16 hours, and in the protein expression, at 24 hours following IL-6 treatment. Interestingly, VEGF stimulation could not increase IL-6 expression in the HCECs under the experimental condition *in vitro* (**Fig. 2**,  $p>0.05$ ). To verify whether the increase of VEGF was specially induced by IL-6, we used anti-IL-6 antibody neutralizing the IL-6 action. We found that at the concentration of 10  $\mu\text{g/ml}$ , IL-6 induced VEGF expression was completely abolished in both mRNA and protein levels (**Fig. 1C** and **1D**), while the same concentration of nonimmune goat IgG could not reduce the expression of VEGF (data not shown). MTT assay shows no detectable cytotoxicity of tested antibodies in the experiment, indicating that the changes in cellular events were not due to non-specific IgG or cytotoxicity.

### ***Effect of IL-6 on KDR expression in HCECs***

Since VEGF mediates its function through binding to its receptors, we next investigated the expression of VEGF receptors in HCECs. IL-6 did not influence Flt-1 expression. However, IL-6 significantly up-regulated KDR expression in a dose-dependent manner in the cultured HCECs ( $p<0.05$ , **Fig. 3A** and **3B**). To examine whether up-regulation of KDR was not due to increased VEGF, we further neutralized VEGF action using VEGF antibody. Anti-VEGF

antibody at 2  $\mu\text{g/ml}$  produced only 40% inhibition of KDR expression compared to IL-6 alone, and the increasing concentration of VEGF antibody did not cause further inhibitory effect on the KDR expression (**Fig. 3C and 3D**). There was no change of KDR levels after treatment with nonimmune goat IgG at 2  $\mu\text{g/ml}$  (data not shown). Interestingly, exogenous VEGF at a concentration of 20 ng/ml also up-regulated KDR expression; however, this effect was weaker compared to the IL-6. These findings suggest that IL-6 could directly up-regulate KDR expression without depending on endogenous VEGF in HCECs. To clarify whether IL-6-stimulated KDR was functional, we examined phospho-KDR expression in cell extracts from IL-6-stimulated HCECs. The result demonstrated that IL-6 could directly induce KDR phosphorylation, which peaked at 15 minutes of IL-6 stimulation (**Fig. 3E**).

#### ***Effect of IL-6 on activation of ERK1/2 and PI3K/Akt signaling pathways***

ERK1/2 MAP kinase and PI3K/Akt activation were investigated to assess the specificity of IL-6 receptor engagement by ligand. An increase of phospho-ERK expression was detected after 5 minutes of IL-6 stimulation (**Fig. 4A**). This response was rapid, suggesting that it may not be indirect activation through a VEGF-dependent signaling pathway. No detectable changes were observed for the phospho-Akt. We then evaluated the role of ERK1/2 MAP kinase signaling in IL-6-induced KDR expression. ERK1/2 inhibitor PD98059 significantly reduced IL-6-induced KDR up-regulation ( $P < 0.05$ , **Fig. 4B**), but did not influence IL-6-induced up-regulation of VEGF (**Fig. 4C**).

#### ***Effect of IL-6 on MMP expression in HCECs***

Many studies revealed that MMP-9 could be necessary for the endothelial cell proliferation and migration *in vitro*. Therefore, we explored the nature of IL-6 in the MMP-9 expression as well as its relation to HCEC proliferation and migration. Real time RT-PCR demonstrated that MMP-9 mRNA was significantly increased in a dose-dependent manner following 12 hours of IL-6 treatment ( $P < 0.05$ , **Fig. 5C**). We also measured MMP-9 activity using a zymographic assay. Parallel to the mRNA changes, IL-6 also increases MMP-9 activity in a dose-dependent manner following 24 hours of IL-6 treatment (**Fig. 5A** and **5B**). However, IL-6 did not change MMP-2 expression in both mRNA and protein levels. To determine whether IL-6 induced KDR expression mediates MMP-9 response, we used neutralizing antibodies against IL-6, VEGF, and phospho-KDR. We found that anti-IL-6 antibody completely abolished MMP-9 activity. Inactivating VEGF and KDR only reduced MMP-9 by 30% and 42% in both mRNA and protein levels (**Fig. 5C, 5D** and **5F**), which suggests that there is a direct effect of IL-6 and KDR on MMP-9 activation. No changes were detected in the control cells treated with goat or mouse IgG at the same concentration, and additional administration of these inactivated antibodies failed to induce MMP-9 inhibition (data not shown)

### ***Effect of IL-6 on HCEC proliferation and migration***

We used BrdU proliferation assay to assess the HCECs proliferation, and Boyden chamber to examine HCEC migration as described in the method section. We found that IL-6 greatly stimulated HCEC proliferation and migration in a dose-dependent manner ( $p < 0.05$ , **Fig. 6A Fig. 6C**). The responses of HCEC to IL-6 in proliferation and migration were partially inhibited after incubation with MMP-inhibitor GM6001 (**Fig. 6B** and **6D**), confirming the role of MMP-9 in HCEC proliferation and migration. Inactivated VEGF and KDR function abolished

IL-6-induced HCEC proliferation and migration by 31% and 45%, and the same dose of IgG had no effect on the control group, indicating the specific action of VEGF and KDR in IL-6-induced cellular responses.

## DISCUSSION

Our results provide evidence that IL-6 augments VEGF-induced HCEC proliferation and migration, up-regulates KDR phosphorylation and enhances MMP-9 activity. Our findings reveal a physiologically important feedback mechanism for the amplification of the angiogenic response *in vitro*, which may be relevant in many pathological situations.

IL-6 can be produced by HCECs and IL-6 receptors are expressed throughout CNS, including endothelial cells, astrocytes, microglia, and neurons [3, 4]. The function of IL-6 in triggering angiogenesis appears associated with VEGF [16]. In the presence of IL-6, VEGF mRNA could be detected in human epidermal carcinoma and C6 glioma cell line [4]. IL-6 significantly increases angiogenic activity in human cervical cancer cells through up-regulating VEGF expression [17]. In accordance with previous research [16], our study also demonstrated that VEGF expression is drastically enhanced in both mRNA and protein levels after IL-6 stimulation.

The most important finding in our study is that IL-6 directly up-regulates VEGF receptor KDR expression. Our experiments with anti-VEGF antibody provide evidence that induction of KDR is caused not mainly by endogenous VEGF. However, IL-6 does not influence Flt-1 expression and neutralizing Flt-1 activity does not change HCEC proliferation and migration, suggesting that IL-6 mediates its effects through activation of KDR, but not Flt-1.

We investigated the signaling pathways leading to IL-6-induced KDR expression. The binding of IL-6 and its receptor initiates homodimerization of gp130 and then triggers signaling cascades through the ERK1/2 and PI3-K/Akt pathways in many cell types [12]. PI3-K/Akt, a common signaling pathway for angiogenesis and growth factors, is not involved in the KDR activation by IL-6. However, we found that ERK1/2 dependent pathway is essential to the activation of KDR by IL-6 in the HCECs. Earlier studies have obtained similar results, showing that blocking ERK1/2 activity attenuated the growth of esophageal carcinoma cell line induced by IL-6 [18]. Several reports also observed that IL-6 modulates the function of androgen receptor, a key transcriptional factor in controlling prostate cancer cell growth via constitutively activation of ERK1/2 [19]. Our results provide further evidence that in HCECs, IL-6 directly regulates KDR, and that KDR-mediated cell growth is achieved through activation of ERK1/2 signaling.

KDR is a major receptor that mediates VEGF biological activities, including the induction of cell proliferation, migration and differentiation, and maintenance of vascular integrity [20-22]. It is well documented that VEGF up-regulates KDR expression and phosphorylation when angiogenesis is activated. However, recent studies demonstrated that other angiogenic mediators such as bFGF and thrombin also up-regulate KDR expression in promoting angiogenesis [23, 24]. For example, KDR can be detected only in the IL-6 overexpressed prostate cell line, and involved in IL-6 mediated proliferation response [25]. KDR inhibitor PTK787 greatly decreases IL-6-mediated multiple myeloma cell growth in the bone marrow milieu [26]. We provide additional evidence that IL-6 directly up-regulates KDR expression in HCECs, and that this KDR protein is functional; hence it can be phosphorylated

after exposure of the cells to IL-6. This finding further establishes the identity of KDR receptor protein.

Our finding also demonstrates that IL-6 not only activates KDR, but also up-regulates MMP-9 activity in HCECs. This conclusion is supported by the inhibition of MMP-9 activity from the HCEC by inactivation of KDR function. Phospho-KDR contributes to MMP-9 gene regulation through activator protein-1 (AP-1) binding site, which exists in MMP-9 promoter [27]. In our experiment, blocking KDR activity greatly decreases MMP-9 mRNA expression, further supporting the hypothesis that phospho-KDR regulates MMP-9 transcriptional response. No change was detected for the MMP-2 activity, suggesting that alternative signaling pathway may be involved.

The potential role of enhanced MMP-9 activity is demonstrated by HCEC proliferation and migration. Gu et al. suggest a causal relationship between the MMP-9 activity and angiogenesis [28]. In the absence of MMP-9, epithelial cell proliferation is restricted, but when functional MMP-9 is added, cell proliferation is greatly increased [29, 30]. Increased MMP-9 activity leads to endothelial cell migration [31]. MMP-9 inhibitor TIMP-1 controls prostate cancer cell angiogenesis through MMP-9 inhibition [32]. We found IL-6 stimulating MMP-9 activity from HCEC provides an additional source of MMP-9 during the process of angiogenesis. HCEC-derived MMP-9 may permit its spatially controlled role in releasing matrix-bound cytokines, growth factors and degradation of subendothelial basement membrane. This is necessary for modulating the process of proliferation and migration. Inhibition of IL-6-stimulated HCEC proliferation and migration through Matrigel strongly suggests that IL-6-enhanced MMP-9 production is in an active form [30, 33-35]. The effect of IL-6 on the MMP-9 activation and HCEC proliferation and migration may also contribute to other pathologies in the

brain vessel wall. Since IL-6 enhancing MMP-9 activity from the HCECs is possibly via KDR activation, and since MMP-9 contributes to the HCEC proliferation and migration, therefore, it is possible that IL-6 is involved in brain vascular diseases, such as atherogenesis and arteriovenous malformation, by promoting HCEC angiogenesis. Moreover, increased IL-6 elicits pro-inflammatory responses, which induce additional angiogenic molecules and chemokines such as ICAM-1, MCP-1, IL-8, and PDGF overexpression [36-38]. These inflammatory mediators may also promote MMP activation and stimulate HCEC proliferation and migration.

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## FIGURE LEGENDS

### **Figure 1. *IL-6 up-regulates VEGF expression in the HCECs***

**A.** Photograph represents one experiment of Western blotting, which indicates VEGF expression after 24 hours of IL-6 stimulation in indicated doses. **B.** Bar graph shows semi-quantified densitometry from the Western blot analysis. **C.** Bar graph shows that IL-6-induced VEGF mRNA expression using real time PCR. Data are mean $\pm$ SD, N=3 in each group. \*p<0.05, vs. the control. The results demonstrated that IL-6 up-regulates VEGF levels in a dose-dependent manner in the HCECs. **D.** Photograph shows VEGF expression after being neutralized with IL-6 antibody by Western blot analysis. IL-6NA= IL-6 (10  $\mu$ g/ml) neutralization. **E.** Bar graph shows semi-quantitative VEGF expression after being neutralized with IL-6 antibody. **F.** Bar graph shows VEGF mRNA levels after being neutralized with IL-6 antibody using real time PCR. Data are mean $\pm$ SD, n=3 in each group. \*p<0.05, IL-6 stimulation vs. IL-6 neutralization. The results demonstrated that anti-IL-6 antibody completely abolishes IL-6 function in the HCECs.

### **Figure 2. *VEGF does not up-regulates IL-6 expression***

Bar graph shows semi-quantified densitometry from the Western blot analysis, which indicates IL-6 expression after 24 hours of VEGF stimulation in indicated dose in the HCECs. Data are mean $\pm$ SD, N=3 in each group. The results demonstrated that VEGF did not stimulate IL-6 expression in the HCECs

### **Figure 3. *IL-6 up-regulates KDR expression and phosphorylation in HCECs***

**A.** Photographs show Western blot of KDR and flt-1 expression after 24 hours of IL-6 stimulation in indicated doses in the HCECs. **B.** Bar graph shows semi-quantified densitometry of KDR from Western blot analysis. Data are mean $\pm$ SD, N=3 in each group. \*p<0.05 vs. control. **C.** Photographs show Western blots of KDR expression in HCEC treated with IL-6 100 ng/ml, VEGF 20 ng/ml, IL-6 antibody (10  $\mu$ g/ml)-neutralized (IL-6NA), and VEGF antibody (10  $\mu$ g/ml)-neutralized (VEGFNA). **D.** Bar graph shows semi-quantified densitometry of KDR from Western blot analysis. Data are mean $\pm$ SD, N=3 in each group. ¶p<0.01 and \*p<0.05 IL-6 stimulation vs. antibody-neutralization. **E.** Phospho-KDR expression was detected by using Western blot after cells were treated with IL-6 100 ng/ml in the time indicated (2 to 45 minutes).

**Figure 4. IL-6 up-regulates KDR is through ERK1/2 signal pathway**

**A.** Phospho-ERK1/2 expression was detected after HCECs were treated with IL-6 100 ng/ml in the time indicated (0 to 30 minutes). **B.** Photographs show that Western blots of KDR and VEGF expression in HCEC treated with IL-6 100 ng/ml, IL-6 with IL-6 inhibitor PD98059 at 30  $\mu$ g/ml and 10  $\mu$ g/ml. **C.** Bar graph shows semi-quantified densitometry of KDR from Western blot analysis. Data are mean $\pm$ SD, n=3 in each group. \*p<0.05 IL-6 stimulation vs. IL-6 neutralization.

**Figure 5. IL-6 enhances MMP-9 secretion**

Left upper panel photograph shows MMP-9 activity in HCECs after 24 hours of IL-6 treatment in indicated doses. Sd= Standard marker. Bar graph shows semi-quantified densitometry of active (**A**) and latent (**B**) form of MMP-9 from zymograms. Data are mean $\pm$ SD, N=4 in each group; \*p<0.05 vs. control group. **C.** Bar graph shows that IL-6-induced MMP-9

mRNA expression in HCECs using real time PCR. Data are mean±SD, N=4 in each group; \*p<0.05 vs. the control. Right upper panel photograph shows MMP-9 activity in HCECs treated with IL-6 (100 ng/ml), IL-6 antibody 10 µg/ml (IL-6NA), anti-VEGF antibody 10 µg/ml (VNA), and anti-KDR antibody 5 µg/ml (KRNA). Supernatants were used for Gelatin zymographic analysis. Bar graph shows semi-quantified densitometry of active (**D**) and latent (**E**) form of MMP-9 from zymograms. Cont= Control group. Data are mean±SD, N=4 in each group; \*p<0.05 IL-6 stimulation vs. antibody-neutralized groups. **F**. Bar graph shows that MMP-9 mRNA expression in HCEC treated with IL-6, IL-6NA, VNA, and KRNA using real time PCR. Data are mean±SD, N=4 in each group; \*p<0.05 IL-6 vs. antibody-neutralized groups.

**Figure 6. *IL-6 stimulation causes HCEC proliferation through up-regulation of KDR***

Bar graphs show that IL-6 stimulates HCECs proliferation (**A**) and migration (**C**) in a dose-dependent manner. HCEC proliferation (**B**) and migration (**D**) in the IL-6 (100 ng/ml), VEGF (20 µg/ml), IL-6NA (10 µg/ml), VEGFNA (10 µg/ml), KDRNA (5 µg/ml), and GM6001 (10 ng/ml) treated HCECs. Data are mean ± SD, N=4; ¶p<0.01 and \*p<0.05 IL-6 stimulation vs. antibody-neutralized and GM6001 groups.