行政院國家科學委員會專題研究計畫
成果報告

介白素與動脈硬化的關係

計畫類別：個別型計畫
計畫編號：
執行期間：年月日至年月日
執行單位：國立成功大學生物化學暨分子生物學科（所）

計畫主持人：張明熙

計畫參與人員：張明熙、魏琪珍、李宜霖、謝美儀、許育祥、李幸慧、陳威宇

報告類型：精簡報告

處理方式：本計畫可公開查詢

中華民國年月日
行政院國家科學委員會補助專題研究計畫成果報告

（計畫名稱）

計畫類別：■ 個別型計畫 □ 整合型計畫
計畫編號：NSC－94－2320－B－006－093
執行期間：94年8月1日至95年7月31日

計畫主持人：張明熙
共同主持人：無
計畫參與人員：魏琪珍、李宜霖、謝美儀、許育祥、李幸慧、陳威宇

成果報告類型(依經費核定清單規定繳交)：■精簡報告 □完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：國立成功大學醫學院生物化學所

中華民國95年7月20日
Interleukin-20 promotes angiogenesis in a direct and indirect manner.

Ming-Shi Chang

Abstract:
IL-20 belongs to the IL-10 family and is involved in the pathogenesis of keratinocyte hyperproliferation in vivo. Endothelial cells express IL-20 receptors. To explore the function of IL-20 on endothelial cells, we treated human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells (HMECs) with human IL-20 and analyzed its effect on endothelial cells. IL-20 induced proliferation of endothelial cells and the activity was specifically blocked by anti-human-IL-20 monoclonal antibody and soluble (s)IL-20 receptor (R)1 and sIL-20R2. An alternatively spliced variant of IL-20 was isolated and also shown to induce proliferation of HUVECs and HMECs. Treatment of HUVECs with both IL-10 and IL-20 demonstrated that IL-10 antagonized the activity of IL-20 because it diminished IL-20-induced proliferation of HUVECs. IL-20 significantly induced HUVECs migration and vascular tube formation on Matrigel in vitro. In vivo, IL-20 also enhanced tumor angiogenesis. Incubation of IL-20 with HUVECs induced transcripts of bFGF, VEGF, MMP-2, MMP-9 and IL-8. Furthermore, incubation of HUVECs with IL-20 induced phosphorylation of ERK1/2, p38, and JNK. Thus, IL-20 is a pleiotropic cytokine and promotes angiogenesis.

Keywords: Cytokines, Inflammation, IL-20, Angiogenesis

Introduction

IL-20 is identified as a member of the IL-10 family, which includes IL-10, -19, -20, -22, -24 (MDA-7), and -26 (AK155). Overexpression of IL-20 in transgenic mice causes neonatal death and aberrant epidermal differentiation. IL-20 is preferentially expressed in monocytes, and selectively enhances multipotential hematopoietic progenitors in vitro and in vivo. IL-20 induces STAT3 activation on keratinocytes through binding to two types of IL-20 receptor (R) complexes, either IL-20R1 and IL-20R2 or IL-20R2 and IL-22R.

Angiogenesis is complex in both physiological and pathophysiological processes, and is regulated through the production of several pro-angiogenic and anti-angiogenic factors. Normally, inhibitory factors, such as endostatin, predominate, but various signals can tip the finely tuned balance in favor of angiogenesis — the ‘angiogenic switch’. Angiogenic factors — in particular, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) — activate endothelial cells, which leads to the secretion and activation of matrix metalloproteinases (MMPs) and plasminogen activators. This results in the degradation of the basement membrane, which allows the endothelial cells to invade the surrounding matrix.

Little is known about the in vitro biological function of IL-20. Endothelial cells expressed IL-20 receptors, IL-20R1 and IL-20R2. Our aims, therefore, were to explore whether IL-20 involves in the process of angiogenesis. We treated human umbilical vein endothelial cells (HUVECs) with human (h)IL-20 and monitored the proliferation, migration and differentiation of the cells. We also treated the endothelial cells with both IL-10 and IL-20 and demonstrated that IL-10 antagonized the activity of IL-20. Signal transduction pathway of IL-20 on HUVECs was further analyzed and demonstrated to mediate through the molecules different from what was observed in keratinocytes.

Results

Isolation of hIL-20 splice variant

We used a pair of hIL-20-specific primers to amplify hIL-20 transcript on a panel of human cDNA libraries including normal tissues of kidney, lung, spleen, lymph node, thymus, bone marrow, brain, fetal liver, placenta, heart, testis, liver, and small intestine. PCR analysis showed that hIL-20 has two different transcripts. One is identical to what was reported (accession number AF224266) and named "wild-type" (hIL-20W). The other, a shorter transcript variant, was named "short-form". The wild-type is expressed in kidney, lung, and placenta tissues. The alternatively-spliced variant (short-form, hIL-20S) was found in lung tissue only (Fig. 1A). The hIL-20 gene was predicted to contain five exons and four introns. Exon 4...
was deleted in the hIL-20 short-form (Fig. 1B).

**IL-20 induced proliferation of HUVECs and HMECs**

Endothelial cells expressed IL-20 receptors, IL-20R1 and IL-20R2. Therefore, we expressed and purified recombinant proteins of hIL-20W and hIL-20S from *E. coli* and explored whether hIL-20 targeted on endothelial cells. We treated HUVECs with either hIL-20W or hIL-20S and analyzed their effect on endothelial cell proliferation. We used bFGF (10 ng/ml) as a positive control. Human IL-20W induced proliferation of HUVECs at concentrations between 100 and 200 ng/ml, while hIL-20S induced proliferation of HUVECs at concentrations between 25 and 100 ng/ml (Figs. 2A-B). To confirm the specificity of IL-20-induced proliferation of HUVECs, we used the anti-hIL-20 monoclonal antibody, 7E, to neutralize hIL-20. While 7E per se did not affect the proliferation of HUVECs, it blocked the proliferation of HUVECs induced by hIL-20W and hIL-20S (Figs. 2C-D). In addition to HUVECs, we also treated human microvascular endothelial cells (HMECs) with IL-20 and analyzed the proliferation response. IL-20W and IL-20S also induced proliferation of HMECs, and the activity of IL-20 was neutralized by 7E (Figs. 2E-F). The concentration required for hIL-20S was lower than that for hIL-20W, suggesting that hIL-20S may be more potent.

**sIL-20R1 and sIL-20R2 inhibited IL-20-induced proliferation of HUVECs and HMECs**

IL-20 transduces its signal through the heterodimer receptor complex IL-20R1 and IL-20R2. We constructed sIL-20R1 and sIL-20R2 cDNA by cloning the extracellular domain of the receptors and expressed the soluble receptors to analyze whether the soluble receptors block IL-20 activity. hIL-20W or hIL-20S at their optimal concentrations was incubated with sIL-20R1 or sIL-20R2 at 4°C for 30 minutes before being used to treat the HUVECs and HMECs. Soluble IL-20R1 or IL-20R2 alone had no effect on HUVEC proliferation. The IL-20-induced proliferation was attenuated by hIL-20W or hIL-20S pre-incubated with sIL-20R1 (Fig. 3A and 3C) and was completely abolished by hIL-20W or hIL-20S pre-incubated with sIL-20R2 (Fig. 3B and 3D).

**Interaction of IL-20 and IL-10**

IL-10 is a potent anti-inflammatory cytokine that inhibits the release of TNF-α, IL-1, IL-6, and IL-8 from monocytes, macrophages, and neutrophils.**10,11,12** IL-10 also blocks VEGF- and FGF-2-induced proliferation of microvascular endothelial cells in vitro.**13** These observations suggest a potent role for IL-10 in the prevention of angiogenesis. With the paradigm of the inflammatory nature of angiogenesis, we tested the hypothesis that IL-10 may antagonize the IL-20-induced proliferation. Various concentrations of IL-10 were added to the optimal concentration of hIL-20W (200 ng/ml) or hIL-20S (50 ng/ml) and co-incubated with HUVECs for 60 hours. IL-10 at concentrations between 12.5 and 200 ng/ml did not affect the HUVEC proliferation per se (Fig. 4A); it antagonized the hIL-20W- and hIL-20S-induced proliferation (Figs. 4B and 4C).

**Effects of IL-20 on endothelial cell migration**

Angiogenesis is a biological process whereby endothelial cells divide and migrate to form new blood vessels. To investigate whether IL-20 plays a role in angiogenesis, we treated HUVECs with either hIL-20W or hIL-20S for 4 hours and analyzed their effects on the migration of endothelial cell by using the Boyden chamber. hIL-20W (200 ng/ml) or hIL-20S (50 ng/ml) was added to the lower chamber. M199 medium with 0.1% FBS was used as a negative control and bFGF was used as a positive control. The number of HUVEC on the lower surface of the filter was determined microscopically. As shown in Fig. 5, hIL-20W and hIL-20S induced significant migration of HUVEC cells compared to the negative control. The result indicated that IL-20 can be a potent angiogenesis factor.

**IL-20 induced vascular tube formation on Matrigel**

IL-20 induced the proliferation and migration of endothelial cells. To evaluate whether IL-20 could promote the differentiation of endothelial cells, we further performed an in vitro vascular tube formation assay on Matrigel. Treatment of HUVECs with hIL-20W and hIL-20S for 12 hours promoted
endothelial tube formation compared to the control. Quantitative analysis of the number of endothelial tubes per well showed significant ($P < .05$) induction of tube formation by hIL-20W and hIL-20S (Figs. 6A and 6B).

**IL-20 enhanced tumor angiogenesis in vivo**

IL-20 exhibited angiogenic activity in vitro. To further exam whether IL-20 enhanced angiogenesis in vivo, mouse hepatoma cells, ML-1, were dorsally co-injected with Matrigel containing saline, IL-20W, IL-20S or VEGF into BALB/c mice. After 8 days, tumors were excised and immuno-histochemical stained with CD31 for microvessel density analysis (Fig 7). IL-20W and IL-20S, similar to VEGF, enhanced vascularization around the solid tumors compared with saline treated control (Fig. 7A). CD31 staining also showed higher microvessel density in IL-20W and IL-20S treated tumors than that in saline treated tumors (Fig. 7B, C). These results provided evidences of the angiogenic activity of IL-20 in vivo.

**Effects of IL-20 on induction of angiogenesis factors and matrix metalloproteinases**

IL-10 can inhibit the generation of new vessels within a tumor both directly on tumor cells and indirectly by influencing infiltrating immune cells. IL-10 reduced the secretion of MMP-2 and MMP-9 from prostate cancer cells and consequently inhibited microvessel formation. Because IL-10 downregulates several factors associated with angiogenesis, and IL-20 increases the proliferation and migration of endothelial cells, we tested the possibility that IL-20 may upregulate angiogenesis factors. Therefore, we treated HUVECs with hIL-20 and analyzed the transcripts of angiogenesis factors and matrix metalloproteinases, viz., bFGF, VEGF, MMP-2, MMP-9 and IL-8. The primers specific for human angiogenesis factors were used in semi-quantitative RT-PCR analysis. Human β-actin transcript was used as an internal control. Human IL-20W upregulated the transcription of bFGF, VEGF, MMP-2 and IL-8, and human IL-20S potently induced MMP-9 in addition to bFGF, VEGF, MMP-2 and IL-8 (Fig. 8). MMP-9 transcript was not detected in HUVECs treated with human IL-20W. Similar results were obtained from HMECs except that MMP-9 was detected from both IL-20W and IL-20S treated cells (data not shown). Thus, IL-20 in addition to being an angiogenesis factor by itself, also induces other angiogenesis factors from HUVECs and HMECs.

**VEGF antibody partially diminished IL-20-induced proliferation of HUVECs**

Human IL-20W and IL-20S upregulated the transcription of VEGF in HUVECs. To investigate whether VEGF mediated IL-20 induced proliferation of HUVECs, we used VEGF antibody to analyze its effect on IL-20 induced proliferation of HUVECs. While VEGF antibody per se did not affect the proliferation of HUVECs, it partially blocked the proliferation of HUVECs induced by hIL-20W and hIL-20S (Figs. 9A-B), suggesting that VEGF partially contributed to IL-20-induced proliferation of HUVECs.

**Signal transduction pathway of IL-20 in HUVECs**

IL-20 was shown to act on keratinocytes through the STAT3 signal transduction pathway. However, we did not detect significant phosphorylation of STAT3 in HUVECs (data not shown). The ERK pathway, which is activated by many growth factors, may mediate endothelial cell proliferation and migration. It has also been reported that p38 MAPK activation by VEGF mediates actin reorganization and cell migration in HUVECs and thus may be an important regulator of angiogenesis. JNK, one of the MAPK members, is also involved in the regulation of cell migration and cytoskeletal integrity. To assess whether ERK1/2, p38, and JNK activities were involved in the IL-20-induced HUVEC proliferation and migration, we studied the effects of IL-20 on ERK1/2, p38, and JNK phosphorylation using Western blotting. Both IL-20W and IL-20S induced ERK1/2, p38, and JNK phosphorylation in HUVECs, whereas total ERK, p38, and JNK proteins remained constant (Figure 10). Induction of phosphorylation by IL-20 was time dependent, with maximal phosphorylation occurring at 2 minutes (ERK and JNK) and 5 minutes (p38) (Figure 10).
Discussion

The present study demonstrated that hIL-20 at concentrations between 25 and 500 ng/ml induced proliferation of HUVECs. The concentration required to induce proliferation of HUVECs or HMECs to 175% was 200ng/ml for hIL-20W, and 50ng/ml for hIL-20S. Thus, hIL-20S seems to be a more potent inducer of proliferation than hIL-20W. hIL-20W protein produced from mammalian cells also showed lower activity than hIL-20S at the same dose (data not shown). Our E. coli-derived hIL-20W protein also showed the same potency as commercial hIL-20W protein (R&D Systems Inc.) derived from E. coli. Thus, the lower potency of hIL-20W is not due to the expression system or refolding of the recombinant proteins. Exon 4 was spliced out in hIL-20S. The detailed crystallographic structure of IL-20 remains unidentified to date, but the structures of the genes that encode members of the IL-10 family are expected to be highly conserved. Exon 4 encodes the DE loop and the E helix is spliced out in the short-form of hIL-20. Further analysis of the ligand/receptor interaction of these two forms is necessary to clarify their different activities.

Monoclonal antibody 7E, which was raised against hIL-20W, completely neutralized the proliferation activity induced by hIL-20. This finding indicates that the activity is specific. In addition, sIL-20R1 and sIL-20R2 blocked the proliferation activity of both hIL-20W and hIL-20S, further demonstrating that hIL-20-induced proliferation was specific. IL-20 shares the same receptor complexes with IL-24; IL-20R1 /IL-20R2 or IL22R1/IL20R2. Our results demonstrated that IL-20 promotes angiogenesis. However, IL-24 exhibits its anti-angiogenic effect through IL-22R1. Thus, IL-20 probably promotes angiogenesis through IL-20R1/IL-20R2 instead of IL-22R1/IL-20R2.

Recently, IL-20 was reported to be an anti-angiogenesis cytokine that inhibited COX-2 expression. It was shown that IL-20 downregulates COX-2 and PGE2 in human bronchial epithelial and endothelial cells. IL-20-dependent inhibition of COX-2/PGE2 occurs through the IL-22R1/IL-20R2 dimers and exerts anti-angiogenic effects, inhibiting experimental angiogenesis. Our results showed IL-20 promote angiogenesis in vitro and in vivo. The different results may be attributed to different assay systems. They treated cells with PMA and showed IL-20 inhibited PMA-induced angiogenesis through the COX-2 regulatory pathway. Thus, it is an indirect assay and other different pathways may be involved. It is worth exploring the different downstream signaling of IL-20 through different receptor complexes. It is possible that IL-20 regulate angiogenesis process via its pro- and anti-angiogenic activities in different tissues according to the microenvironment.

Our data demonstrated that IL-10 antagonized IL-20-induced proliferation. IL-10 and IL-20 do not share common receptors, but they may compete for intracellular molecules for signal transduction. Alternatively, they may antagonize each other by promoting or inhibiting specific angiogenesis factors. IL-10 downregulates the production of VEGF, MMP-2, and MMP-9. In contrast, hIL-20 upregulates the production of VEGF, bFGF, MMP-2 and MMP-9 (IL-20S only). Thus, IL-10 may inhibit the proliferation effects of IL-20 through downregulation of VEGF or other angiogenic mediators.

Even though IL-20 induced transcripts of bFGF and VEGF, the angiogenic activities of IL-20 demonstrated in this study may not be completely mediated through either bFGF or VEGF. Induction of transcripts was detected after incubation of HUVECs with IL-20 for 4 hours, while cell migration and vascular tube formation in HUVECs were observed in 4 hours and 12 hours respectively. The de novo protein synthesis of bFGF or VEGF would not occur during either 4 or 12 hour period because protein synthesis generally takes place 12 to 24 hours after incubation. Thus, IL-20 induced migration of endothelial cells is compelling for a direct effect.

IL-20 induced proliferation of HUVECs was partially diminished at the presence of anti-VEGF antibody. In addition, a potent angiogenic CXC chemokine, IL-8, was induced by IL-20 in HUVECs and HMECs, suggesting that VEGF or IL-8 may play partial role in IL-20 induced proliferation of endothelial cells. Therefore, IL-20 may promote angiogenesis by itself or by inducing other angiogenesis factors.

Matrix metalloproteinases (MMP-9 and MMP-2) have been extensively studied for its contribution to angiogenesis. In vitro studies have shown that MMP-2 is important for the
differentiation of endothelial cells into tube-like structures. The role of MMP-9 in in vivo angiogenesis has also been supported by data obtained from mice with targeted disruption of these MMP genes. Mice lacking MMP-9 are viable, but show abnormal development of growth plates in long bones associated with delayed and aberrant vascularization. Our data demonstrated that hIL-20W induced MMP-2 transcript in HUVECs, and that hIL-20S induced both MMP-2 and MMP-9, indicating that hIL-20 may play a crucial role in angiogenic process. We did not detect any MMP-9 transcript from cells treated with human IL-20W, even after two runs of PCR amplification. However, we detected MMP-9 transcript from HMECs treated with either human IL-20W or IL-20S. The difference in molecular mechanisms of these two forms of IL-20 on MMP-9 induction is worth exploring. The tertiary structures of the proteins may shed light on this aspect.

Previous study showed that IL-20 signaled through STAT3 phosphorylation in keratinocytes. Our results on HUVECs demonstrated that IL-20 did not induce phosphorylation of STAT3; instead, it induced phosphorylation of p38, ERK, and JNK. These three signal transduction pathways are involved in various cellular responses to extracellular signals and are associated with cell proliferation, migration, and differentiation. This novel discovery of signaling provides new insight on the molecular mechanism of IL-20.

In summary, we demonstrated that IL-20 is an angiogenesis factor because it induced proliferation, migration, and vascular tube formation on endothelial cells. It also induced the expression of other angiogenesis factors and matrix metalloproteinases. In vivo, IL-20 enhanced tumor vascularization and increased microvessel density. Thus, IL-20 promotes angiogenesis by itself or through induction of other angiogenesis factors.

Materials and Methods

**Isolation of hIL-20 spliced variants**

We used a pair of primers on exon 1 (sense primer: 5'-CTCCAGATTTCAGGCCTAAGATG-3') and exon 5 (antisense primer: 5'-ATTGAAGACTGGAGCTTTGACC-3') in PCR amplification to detect the short-form transcript on a panel of human cDNA libraries (Clontech, Inc., Palo Alto, CA).

**Expression and purification of recombinant hIL-20**

Both wild-type (W) and short-form (S) hIL-20 were expressed in E. coli. A cDNA-clone coded for the hIL-20 sequences from leucine to leucine (aa 25-176) was inserted into pET43a (Novagen, Madison, WI). A taq of six histidine residues was placed at the C-terminus of the recombinant proteins. The protein was found mostly in the cytosol and was purified to more than 95% using affinity chromatography.

**Expression and purification of the extracellular domains of hIL-20R1 and hIL-20R2 recombinant protein**

RNA was isolated from HaCaT cells and reverse transcribed into cDNA. The extracellular domain of IL-20R1 (shIL-20R1) was amplified with PCR using the sense primer and the antisense primer. The amplified PCR fragment coding from Val to Lys (aa 30-250) was inserted into the E. coli expression vector of pMAL-c2X (NEB, Beverly, MA). The extracellular domain of IL-20R2 (shIL-20R2) was constructed as previously described. A taq of six histidine residues was placed at the C-terminus of the recombinant proteins. Both proteins were purified using metal affinity chromatography.

**IL-20 antibody**

Human IL-20 monoclonal antibody, 7E, was generated and its specificity was determined as described previously.

**Cell proliferation assay**

HUVECs and HMECs were purchased from Cascade Biologics (Cascade Biologics, Inc., Portland, OR) and plated in 24-well plates at a density of 3 × 10⁴ cells per well. Cells were incubated with various concentrations of hIL-20W or hIL-20S for 48 hours in HUVECs and for 96 hours in HMECs. Cells were then incubated with a 1-mg/ml solution of 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) for 4 hours. Two hundred µl of DMSO (Sigma) was added to the culture. Absorbance of 550 nm was determined. Basic fibroblast growth factor (bFGF) (Peprotech Ec Ltd., London, UK) was used as the positive control in the
cell proliferation assay. For neutralization of IL-20 activity, anti-hIL-20 mAb, 7E or sIL-20R1 or sIL-20R2 proteins were incubated with an optimal concentration of hIL-20S (50 ng/ml) or hIL-20W (200 ng/ml) for 30 minutes at 4°C before treatment.

Cell migration assay
Migration was measured with modified Boyden chamber housing a polycarbonate filter with 8µm pores (Nucleopore, Cabin John, MD) as described previously. 1.5 × 10^4 cells were placed in the upper compartment of the Boyden chamber. Lower chambers were filled with each chemotactant, hIL-20W (200ng/ml), hIL-20S (50ng/ml), or bFGF (10ng/ml) in M199 medium with 0.1% FBS. M199 medium with 0.1% FBS was used as a negative control and bFGF was used as a positive control. The chambers were incubated for 4hr at 37°C to allow cell migration. The number of HUVECs on the lower surface of the filter was determined microscopically by counting six randomly picked fields (original magnification × 100). Experiment was performed twice using quadruplicate wells and migration was expressed as the average number ± SE of total cells counted per field.

Analysis of angiogenesis in vitro and in vivo with Matrigel
Two hundred microliters of Matrigel (BD Biosciences) containing hIL-20W or hIL-20S was added to a 96-well plate and allowed to polymerize for 30 minutes at 37°C. Cell suspensions of HUVECs were plated on Matrigel-coated wells and incubated for 12 hours to allow tube formation. The number of tubes was calculated by taking the average tube numbers of triplicate wells. For in vivo tumor angiogenesis assay, mouse hepatoma cells, ML-1 (1 × 10^6 cells), were dorsally co-injected with Matrigel containing IL-20W (100ng/ml), IL-20S (100ng/ml), VEGF (10ng/ml) or saline. Tumors were excised after 8 days and frozen sectioned for anti-CD31 (PharMingen, San Diego, CA, USA) staining. Slides were incubated with the AEC substrate kit (Vector Laboratories, Burlingame, CA, USA) and counterstained with Mayer’s hematoxylin (ThermoShandon, Pittsburgh, PA, USA). Mean microvessel density was counted in 6 randomly chosen fields (0.16 mm^2) at ×40 magnification as described previously.

Detection of induced transcripts for angiogenesis factors using RT-PCR
To investigate the induction of bFGF, VEGF, matrix metalloproteinase (MMP)-2, MMP-9, and IL-8 on hIL-20-stimulated HUVECs, cells were exposed to hIL-20W (200 ng/ml) or hIL-20S (50 ng/ml) for 4 hours in serum-free M199 medium. Total RNA was extracted and underwent RT-PCR. Amplification of β-actin was used as an internal control. The sequences of the human-specific PCR primers are given in Table 1. The relative quantity of PCR products was analyzed using the BIO-PROFIL program (Vilbert Lourmat, France).

Analysis of signal transduction
HUVECs (1 × 10^6) were pre-incubated with serum–free M199 medium for 6 hours and treated with serum–free M199 medium containing wild-type hIL-20 (150 ng/ml) or short form hIL-20 (50 ng/ml) for 2, 5, 10, 20 and 40 minutes. Cell lysates were separated using SDS-PAGE. The levels of phospho-ERK1/2 (Tyr 204), phospho-p38 (Thr-180/Tyr -182), and phospho-JNK were detected using Western blotting with specific antibodies (Cell Signaling Technology).

Statistical analysis
Significant difference was detected with Student’s t test using a statistical software package in Microsoft Excel. Results are given as mean ± SD (unless otherwise indicated), and statistical significance was set at P < .05.

References
4 Langer JA, Cutrone EC and Kotenko S. The Class II cytokine receptor (CRF2) family: overview and patterns of receptor-ligand interactions. Cytokine


19 Schnaper HW, Grant DS, Stetler-Stevenson WG, et al. Type IV collagenase(s) and TIMPs modulate endothelial cell morphogenesis in vitro. J Cell Physiol 1993; 156: 235-246.


**Figure Legends**

**Figure 1.** Tissue distribution of two alternative-spliced transcripts of the human (h)IL-20 gene. (A) Screening in human cDNA libraries, hIL-20 wild-type (W) (607 bp) is expressed in kidney (i), lung (ii), and placenta (iii) tissue; hIL-20 short-form (S) (532 bp) is expressed only in lung (ii) tissue. Tissues without IL-20 transcripts are not shown. (B) Comparison of amino acid sequences between hIL-20W and hIL-20S. Human IL-20W has 5 exons and 4 introns in the coding region. Exon 4 is deleted in hIL-20S. The locations of exon/intron junctions of the hIL-20 gene are indicated by ▼. Deleted exons in the short-forms are indicated by dashes.

**Figure 2.** Effect of human (h)IL-20 on proliferation of HUVECs and HMECs. Cell proliferation was monitored using MTT assay as described in Materials and Methods. hIL-20W (A) and hIL-20S (B) induced HUVECs proliferation in a dose-dependent manner. For neutralization of IL-20 activity, IL-20 specific antibody, mAb 7E, was added to the cells as a control or incubated with hIL-20W (200 ng/ml) (C) or hIL-20S (50 ng/ml) (D) before being added to HUVECs. hIL-20W (200 ng/ml) (E) and hIL-20S (12.5 ng/ml) (F) also induced HMECs proliferation and the activity was neutralized by mAb 7E. The ratio of IL-20 to 7E is 1:10. *P < .05 compared with control (medium alone) in (A) and (B). **P < .05 compared with hIL-20-stimulated HUVECs in (C) and (D). **P < .05 compared with hIL-20-stimulated HMECs in (E) and (F).

**Figure 3.** siIL-20R1 and siIL-20R2 inhibited IL-20-induced proliferation of HUVECs and HMECs. Neutralization activity of human soluble (s)IL-20R1(A) or sIL-20R2 (B) on hIL-20 in HUVECs. Neutralization activity of human soluble (s)IL-20R1(C) or sIL-20R2 (D) on hIL-20 in HMECs. The gray bar indicates treatment with siIL-20R1 or siIL-20R2 alone. The black bars indicate treatment with hIL-20W or hIL-20S alone. The hatched bars stand for treatment with both hIL-20 and soluble receptors together. Data represent means ± SD of triplicate experiments. The experiment was repeated five times with similar results. *P < .05 compared with hIL-20-stimulated HUVECs in (A) and (B). *P < .05 compared with hIL-20-stimulated HMECs in (C) and (D).

**Figure 4.** Interaction of IL-10 and IL-20 on proliferation of HUVECs. (A) Various concentrations of IL-10 (12.5 ng/ml-200 ng/ml) were added to HUVECs and proliferation was monitored. The optimal concentration of hIL-20W (200 ng/ml) (B) or hIL-20S (50 ng/ml) (C) was mixed with various concentrations of IL-10 (12.5 ng/ml-200 ng/ml) and incubated with
HUVECs. The effect of IL-10 on hIL-20W-induced proliferation of HUVECs was monitored using MTT assay. Data represent means ± SD of triplicate experiments. *P < .05 compared with hIL-20-stimulated HUVECs. **P < .05 compared with control (Medium alone).

Figure 5. Effect of hIL-20 in HUVECs on cell migration (A) hIL-20W (200 ng/ml), hIL-20S (50 ng/ml), or bFGF (10 ng/ml) were placed in the lower compartment. Migration assays were performed as described in Materials and Methods. Cells seeded in the upper compartment that migrated after 4 hours were stained. M199 medium with 0.1% FBS was used as a negative control and bFGF was used as a positive control. Quantitative analysis of the number of migrated cells showed significant (P < .05) induction of cell migration by hIL-20W and hIL-20S. *P < .05 compared with untreated control (Medium alone).

Figure 6. Effect of hIL-20 in HUVECs on tube formation. (A) HUVECs plated in Matrigel-coated 96-well plates containing PBS, hIL-20W (200 or 400 ng/ml) and hIL-20S (50 ng/ml) were screened for tube formation. (B) The average number of tubes per well was determined by counting under a microscope. All treatments were assayed in triplicate. The experiments were repeated twice with similar results. *P < .05 compared with untreated control (PBS alone).

Figure 7. hIL-20 enhanced tumor angiogenesis in vivo. (A) The hepatoma cells, ML-1 (1 × 10^6 cells), were dorsally co-injected into BALB/c mice (n=3) with Matrigel containing saline, hIL-20W (100ng/ml), hIL-20S (100ng/ml) or VEGF (10ng/ml). Tumors were observed after 8 days and the enhanced vasculature was observed. (B) Excised tumors were frozen sectioned and immunohistochemically stained with anti-CD31 monoclonal antibody for blood vessels. Slides were counterstained with hematoxylin. Representative sections showed enhanced microvessel density in hIL-20W and hIL-20S treated tumors than that in saline treated tumors. (C) Mean microvessel density was counted in 6 randomly chosen fields (0.16 mm²) at ×40 magnification. Bars represent 5mm (A) and 200µm (B). *P < .05 compared with saline-treatment.

Figure 8. Effect of hIL-20 in HUVECs on induction of angiogenic factors. (A) RT-PCR was performed as described in Materials and Methods. Equal amounts of cDNA and primers specific for bFGF, VEGF, MMP-2, MMP-9, and IL-8 were used in PCR to amplify the transcripts. Primer specific for
β-actin was used as an internal control. (B) The relative quantity of PCR products was analyzed using the BIO-PROFIL program and expressed as a fold-increase relative to untreated control cells. N/D, no detectable transcript.

Figure 9. VEGF antibody partially inhibited IL-20-induced proliferation of HUVECs.
Neutralizing VEGF antibody was co-incubated with an optimal concentration of (A) hIL-20W (200 ng/ml) or (B) hIL-20S (50 ng/ml) in HUVECs. Cell proliferation was monitored using MTT assay as described in Materials and Methods. *P < .05 compared with hIL-20-stimulated HUVECs.

Figure 10. Signal transduction pathway of IL-20 in HUVECs. Cells (1 x 10^6) were incubated with serum–free M199 medium for 6 hours followed by treatment with serum–free M199 medium containing wild-type hIL-20 (150 ng/ml) or short form hIL-20 (50 ng/ml) for 2, 5, 10, 20 and 40 minutes. Cell lysates were isolated and the levels of phospho-ERK1/2 (Tyr 204), phospho-p38 (Thr-180/Tyr -182), and phospho-JNK were detected using Western blotting with specific antibodies. Total ERK, p38 and JNK were also detected with specific antibodies.