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Autocrine role of Interleukin-8 in induction of endothelial cell proliferation, survival, migration and MMP-2 production and angiogenesis

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Abstract

Interleukin-8 (IL-8/CXCL8), a paracrine angiogenic factor, modulates multiple biologic functions in CXCR1 and CXCR2 expressing endothelial cells. Several reports suggest that inflammation, infection, cellular stress and tumor presence regulate IL-8 production in endothelial cells. In the present study, we test the hypothesis that IL-8 regulates multiple biological effects in endothelial cells in an autocrine manner. We examined the autocrine role of IL-8 in regulating angiogenesis by using a neutralizing antibody to IL-8, CXCR1 or CXCR2 in human vein umbilical endothelial cell (HUVEC) and human dermal microvascular endothelial cell (HMEC). Neutralizing antibody to IL-8, CXCR1 or CXCR2 inhibited endothelial cell proliferation, and MMP-2 production as compared to cells cultured with medium alone or control antibody. In addition, we observed that the number of apoptotic cells was significantly higher in anti-IL-8, anti-CXCR1 and anti-CXCR2 treated endothelial cells, which coincided with decreased survival-associated gene expression. We observed reduced migration of endothelial cells treated with anti-IL-8 and anti-CXCR2 antibody, but not anti-CXCR1 antibody as compared to controls. Further, we observed an inhibition of capillary tube formation and neovascularization following treatment with anti-IL-8, anti-CXCR1 and anti-CXCR2 antibodies. Together these data suggest that IL-8 functions as an important autocrine growth and angiogenic factor in regulating multiple biological activities in endothelial cells.

Abbreviations: IL-8 – interleukin-8; HUVEC – human umbilical vein endothelial cells; HMEC – human dermal microvascular endothelial cell; RT-PCR – reverse transcriptase-polymerase chain reaction; MMPs – matrix metalloproteinases

Introduction

Angiogenesis, the formation of new blood vessels from an existing capillary bed, is a crucial process involved in various physiological and pathophysiological conditions, including embryonic development, wound healing, chronic inflammation, and tumor growth. It comprises a cascade of events, emanating from endothelial cell proliferation, survival, migration, and maturation to form capillary tubes [1–4]. Interleukin-8 (IL-8, CXCL-8), a member of the ELR (Glu–Leu–Arg) motif positive (ELR^+) CXC chemokines, has been shown to play an important role in embryonic/adult vascular bed formation and tumor angiogenesis [1, 2, 5–8]. The receptors for IL-8 are CXCR1 and CXCR2, which bind to IL-8 with high affinity [9, 10]. IL-8 is expressed in normal cells such as leukocytes, fibroblasts, endothelial cells and various tumor cells [4, 6, 8, 11–16], and plays an important role in chemoattraction, inflammation, angiogenesis, tumor growth and metastasis [5, 15, 17–20]. Studies have demonstrated inhibition of endothelial cell migration by neutralizing antibody to CXCR2 [21, 22] and delayed vascularization in CXCR2 knock out mice [23].

Several reports have demonstrated that IL-8 directly modulates endothelial cell proliferation and migration and regulates angiogenesis *in vitro* and *in vivo* [24–27]. These studies were exclusively focused on the paracrine role of IL-8 in angiogenesis. However, several reports suggest that endothelial cells themselves are a major source of IL-8, and inflammation, infection, stress and interaction with tumors can regulate its

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expression [28–33]. Besides an indirect mechanism involving IL-8 released by activated leukocytes and tumor cells, we hypothesize that IL-8, produced by endothelial cells, may directly regulate endothelial cell function and activate the pro-angiogenic process in an autocrine manner. In the present study, we tested this hypothesis by using neutralizing antibodies to IL-8, CXCR1 and CXCR2 and examined the regulation of endothelial cell proliferation, survival, migration, matrix metalloproteinase (MMP)-2 production and angiogenic response. We observed an autocrine role for IL-8 in modulating endothelial cell proliferation, survival, migration, MMP-2 production, capillary tube formation and angiogenesis.

Materials and methods

Cell lines and reagents

Human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cell-1 (HMEC) were obtained from Clonetics (Biowhittaker, Walkersville, MD) and Center for Disease Control (Atlanta, GA). Cell lines were maintained in culture as adherent monolayers in endothelial cell growth media (EGM-2 MV, Clonetics, Biowhittaker, Walkersville, MD) containing fetal bovine serum (FBS), hydrocortisone, endothelial cell growth supplement, and gentamycin. Recombinant human IL-8 and monoclonal antibodies to IL-8 (Clone 6217), CXCR1 (Clone 42705) and CXCR2 (Clone 48311), FGF-2 and VEGF-A were obtained from R&D Systems Inc. (Minneapolis, MN). Normal mouse IgG was purchased from Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

In vitro *proliferation and apoptosis assay*

HUVEC and HMEC (1×10^5) were seeded onto triplicate wells of a 24-well plate coated with 2% gelatin in EGM-2 medium (without growth factor supplement) and containing 5% FBS. Following overnight incubation for adherence, the cultures were washed and cultured with fresh medium alone (EGM-2 medium without growth factor supplement and containing 5% FBS) or medium containing different concentrations of cytokines and/or antibodies, and incubated for 72 h. Cells were trypsinized and viable cells were counted using trypan blue (ICN Inc., Aurora, Ohio).

To determine whether treatment of HUVEC with neutralizing antibodies to IL-8, CXCR1 and CXCR2 induced apoptosis, cells were incubated in EGM-2 medium with 5% FBS overnight. The cultures were washed and incubated with medium alone or medium containing IL-8 (10 ng/ml), or anti-IL-8 (5 μg/ml), anti-CXCR1 (5 μg/ml), anti-CXCR2 (5 μg/ml) antibodies for 24 h. HUVEC cells were stained for apoptosis using the CaspACE FITC-VAD-FMK *in situ* marker kit (Promega Corporation, Madison, WI) and counterstained with DAPI (Molecular Probes, Eugene OR) according to the manufacturer's instructions. The number of apoptotic cells was determined by enumerating immunostained cells using a Nikon fluorescence microscope in five independent high-power fields (×200) with each field containing 50–100 cells.

Enzyme-linked immunosorbent assay (ELISA) for IL-8

HUVEC and HMEC cells (3000 cell/well in 96 well plate) were cultured in TC199 medium with serum for 48 h. Cell free culture supernatants were harvested and IL-8 levels were determined using an ELISA method as described earlier [13]. This assay employs the quantitative immunometric 'sandwich' enzyme immunoassay technique. A curve is prepared, plotting the optical density vs the concentration of IL-8 in the standard wells. By comparing the optical density of the samples to the standard curve, the concentration of IL-8 in the unknown samples was determined.

Migration assay

HUVEC migration in response to neutralizing antibodies to IL-8, CXCR1 and CXCR2 was determined using the method described earlier [27, 34]. Briefly, HUVEC (1×10^4) were plated onto transwell chambers with serum-free medium alone or medium containing antibodies in duplicate and incubated at 37 °C in CO_2 for 2.5 h. MTT was added and cells were incubated for an additional 2 h. Cells from the top of the transwell chambers were removed using a cotton swab (residual cells). Cotton swabs containing residual cells and the transwell chamber (migrated) cells were placed in separate wells of a 24-well plate containing 400 μl of dimethyl sulfoxide (DMSO). Following 1 h of gentle shaking, 100 μl samples were removed and absorbance was determined at 570 nm using an ELISA plate reader. The percent migratory activity was calculated as: percent migration $= A/[A+B] \times 100$, where A is the absorbance of migrated cells, and B is the absorbance of residual cells.

In vitro *capillary tube formation assay*

An *in vitro* capillary tube formation assay was performed as described earlier [35]. Briefly, 2×10^4 HUVEC cells were plated in wells of a Matrigel-coated 48-well plate with medium alone or medium containing IgG, or neutralizing antibody to IL-8, CXCR1 or CXCR2. After 3 h of incubation, the plate was examined for capillary tube formation and photographed. Each assay was done in duplicate and each experiment was repeated three times. The qualitative difference in tube formation was examined in treated or untreated cultures.

Chick chorioallantoic membrane (CAM) Assay

Fertilized eggs were incubated with the upper blunt pole at 70% relative humidity at 39 °C. After 3 days of incubation, anti-IL-8, anti-CXCR1, anti-CXCR2 and control IgG (100 μg/egg) were applied to the CAM and incubated for an additional 96 h. Chick embryos were visualized and CAM were examined under a stereo microscope attached to a digital camera [36].

mRNA analysis

Total cellular RNA was isolated from HUVEC (1×10^5 cells) using Trizol® reagent (Invitrogen, Carlsbad, CA). RT-PCR was performed [27]. Briefly, cDNA was synthesized using total RNA (2 μg), oligo $(dT)_{12-18}$ primer and superscript RT (Gibco BRL, Gaithersburg, MD). Two μl of first strand cDNA (1:10 dilutions) were amplified using PCR primer sets (Table 1) and a DNA thermal cycler (Perkin Elmer, Foster City, CA) [27]. PCR fragments were separated on a 2% agarose (3:1) gel containing ethidium bromide (0.5 μg/ml), visualized and photographed using a gel documentation system (Alpha-Innotech, San Leandro, CA). Relative intensity of specific gene expression was determined using ImageQuant 5.1 software (Molecular Dynamics, Inc., Sunnyvale, CA). mRNA expression is presented as expression index, the ratio of each signal to the signal from the housekeeping gene, β-actin. The expression index for cells cultured with medium alone was arbitrarily set to 1 in order to represent the difference in expression levels among HUVEC from different treatment groups.

Gelatin zymography

HUVEC cells 2×10^4 per well in a 48-well plate were plated in EGM-2 medium containing 5% FBS overnight for adherence. The cultures were washed and cells were incubated in RPMI-1640 (phenol free medium containing 0.1% BSA) alone or medium containing different treatments for 24 h. Culture supernatants were used for gelatin zymography as described previously [27, 37]. Briefly, the gelatin-degrading activity in the culture supernatants was analyzed by electrophoresis on an 8% SDS-PAGE containing gelatin. After electrophoresis, the gel was washed in 2.5% Triton-X 100 to remove SDS, incubated in 50 mM Tris–HCl buffer, pH 7.5, containing 0.15 M NaCl, 10 mM $CaCl_2$, and 0.02% (w/v) NaN_3 for 16 h at 37 °C and stained with 0.1% Coomassie brilliant blue R250 (Sigma Chemical Co., St. Louis, MO). Gelatinolytic activity was visualized by negative staining. Zymograms were analyzed using gel documentation system and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The expression of MMP-2 activity for cells cultured with medium alone was arbitrarily set to 1 in order to represent the difference in the expression level among HUVEC from different treatment groups.

Statistical analysis

Significance between means was determined by the Student's *t*-test (two-tailed) using SPSS software (SPSS Inc., Chicago, IL). A *P*-value of < 0.05 was deemed significant.

Results

Neutralization of IL-8 and CXCR2 inhibits endothelial cell proliferation and apoptosis

We examined the autocrine role of IL-8 in the regulation of endothelial cell growth, and the levels of endogenous IL-8 secreted by HUVEC and HMEC cells by ELISA. We observed constitutive expression of IL-8 in HUVEC (356 + 12 pg/ml) and HMEC (238 + 21 pg/ml). Further, we treated HUVEC and HMEC (1×10^5) cells with IL-8 (10 ng/ml) or neutralizing antibody to IL-8, CXCR1 or CXCR2. FGF-2 (10 ng/ml) and VEGF-A (10 ng/ml) were used as positive controls in these assays. After 72 h of culture, the number of viable cells significantly increased in HUVEC and HMEC cells treated with IL-8, FGF-2 and VEGF-A (Figures 1a and b). We observed a significant decrease in HUVEC and HMEC cell number following treatment with anti-IL-8, anti-CXCR1 or anti-CXCR2 treated cells as compared to cells treated with medium alone or control IgG (Figures 1a and b).

Next, we examined the autocrine role of IL-8, CXCR1 and CXCR2 in the regulation of endothelial cell apoptosis. HUVEC (2×10^4) were plated onto 4-well chamber slides and allowed to adhere overnight

Table 1. Primers used for RT-PCR analysis.

Gene	Orientation	Sequence	Product size
Bcl-x	Sense	5′- GACGAGTTT-GAACTGCGGTA-3′	Bcl-X_L 378 bp; Bcl-Xs 190 bp
	Antisense	5′- CACAGTCATG-CCCGTCAG-3′	
Bcl-2	Sense	5′- TCCATGTCTTTG-GACAACCA-3′	203 bp
	Antisense	5′- CTCCACCAGT-GTTCCCATCT-3′	
Bax	Sense	5′- TCTGACGGCA-ACTTCAACTG-3′	188 bp
	Antisense	5′- TTGAGGAGTCT-CACCCAACC-3′	
GAPDH	Sense	5′- ACGCATTTGGT-CGTATTGGG-3′	230 bp
	Antisense	5′- TGATTTTGGA-GGGATCTCGC-3′	

in EGM-2 medium containing 5% FBS. The cultures were washed and incubated in EGM-2 medium alone or medium containing IL-8 (10 ng/ml), control IgG, anti-IL-8, anti-CXCR1 or anti-CXCR2 for 24 h. Apoptotic cells showing positive CaspACE-FITC stain in the cytoplasm were counted using fluorescence microscopy. The levels of apoptotic cells decreased in HUVEC cells incubated with IL-8 (10 ng/ml) (Figure 2). We observed a significant increase in apoptotic cell number in anti-IL-8, anti-CXCR1 or anti-CXCR2 treated HUVEC (Figure 2) compared to medium alone or control IgG treated HUVEC. These data suggest that IL-8 directly regulates endothelial cell survival and apoptosis.

To determine downstream regulators of IL-8-, CXCR1- and CXCR2-mediated endothelial cell survival, we analyzed expression of survival-associated genes in untreated and antibody-treated HUVEC. Total RNA was isolated from HUVEC cultured with medium alone or medium containing different treatments for 4 h and bcl-2, bcl-x and bax mRNA expression was examined by RT-PCR (Figure 3). The expression index is shown under specific bands to demonstrate the quantitative differences in the expression levels. In HUVEC treated with anti-IL-8, anti-CXCR1 or anti-CXCR2, we observed decreased mRNA levels for the anti-apoptotic genes bcl-2 and bcl-x_L compared to medium alone or control antibody treated HUVEC. In contrast, the bax mRNA level showed no obvious changes in anti-IL-8 and anti-CXCR2 treated but was decreased in anti-CXCR1 treated cells. In addition, the ratio of bcl-2 to bax decreased in anti-CXCR1 and anti-CXCR2 treated cells tilting the balance in favor of apoptosis (Figure 3). Taken together, these data suggest that treatment of HUVEC with neutralizing antibodies to IL-8, CXCR1 or CXCR2 inhibit expression of survival-associated genes bcl-2 and bcl-x_L.

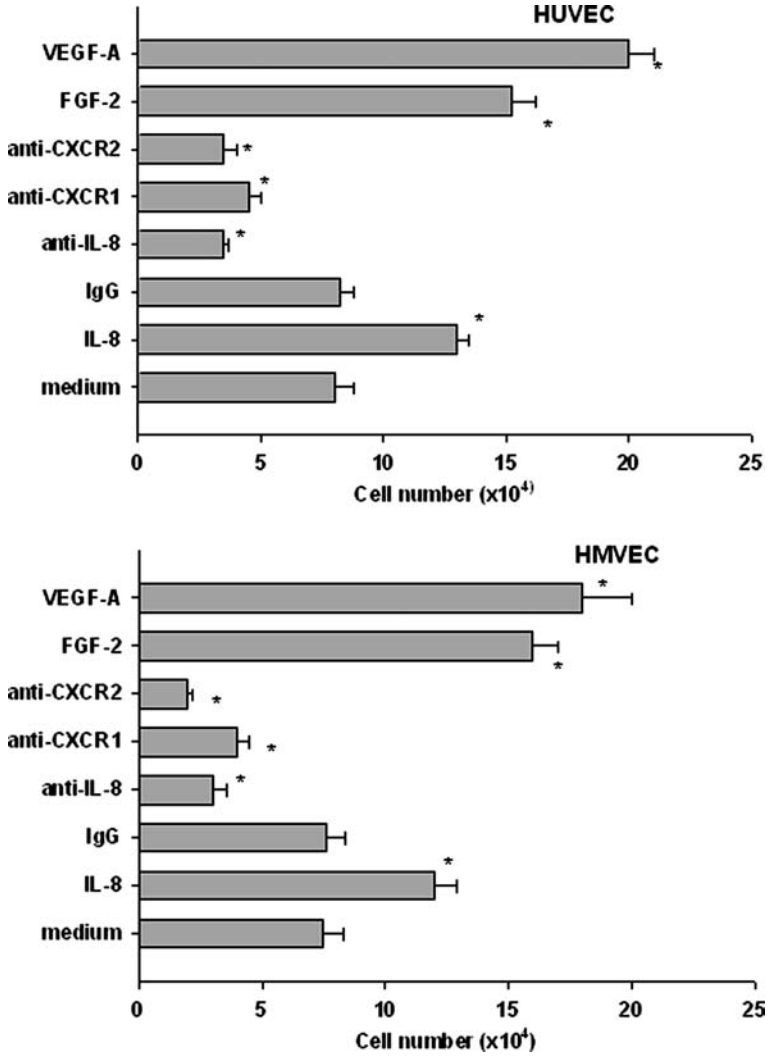

Figure 1. Neutralizing antibody to IL-8, CXCR1 or CXCR2 inhibited endothelial cell proliferation. HUVEC (a) or HMEC (b) were plated at 1×10^5 per well in 24-well culture plates and cultured with medium alone or medium containing IL-8 (10 ng/ml), FGF-2 (10 ng/ml) or VEGF-A (10 ng/ml), control IgG (5 μg/ml), anti-IL-8 (5 μg/ml), anti-CXCR1 (5 μg/ml) or anti-CXCR2 (5 μg/ml). After 72 h of culture, number of viable cells was determined. The values are mean viable cell number ±SEM. This is one representative of three experiments performed in triplicate. *Significantly different from medium alone culture, $P < 0.05$.

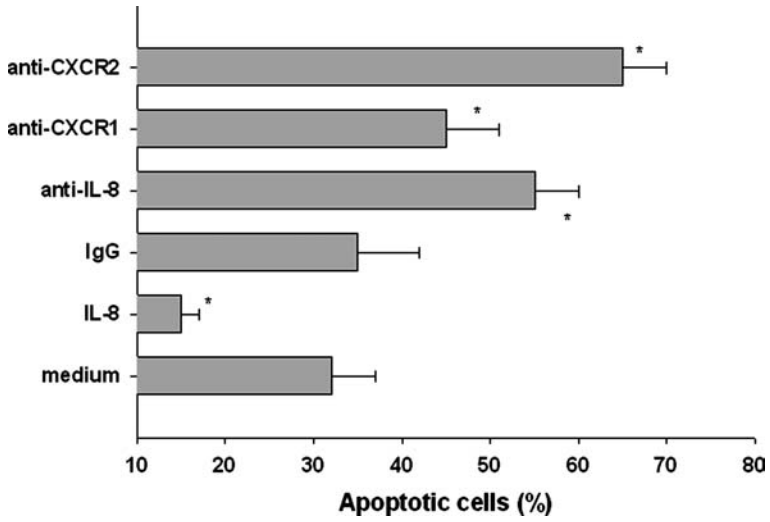

Figure 2. Neutralizing antibody to IL-8, CXCR1 or CXCR2 inhibited endothelial cell survival and enhanced apoptosis. HUVEC were incubated with medium alone or medium containing 5 μg/ml control antibody, anti-IL-8, anti-CXCR1 or anti-CXCR2 for 24 h. Cells were stained with CaspACE FITC-VAD-FMK *in situ* marker kit and counterstained with DAPI. The frequencies of CaspAce positive endothelial cells in antibody treated and untreated cultures were determined by counting five fields (×200) for each treatment. The values are mean percent apoptotic cells ±SEM. This is one representative experiment of three performed in duplicate.

Neutralizing antibody to IL-8 and CXCR2 inhibited HUVEC migration

HUVEC were plated on Matrigel-coated transwells. After 3 h culture with control medium or medium containing different concentrations of anti-IL-8, anti-CXCR1 or anti-CXCR2, percent migration was determined. The percent migration was significantly decreased in anti-IL-8 and anti-CXCR2 treated HUVEC (Figure 4a). We did not observe any significant effect of anti-CXCR1 treatment in HUVEC migration. This data suggests that CXCR2, not CXCR1 may play a critical role in endothelial cell migration.

Down regulation of MMP-2 expression and activity in HUVEC treated with neutralizing antibody to IL-8, CXCR1 and CXCR2

Supernatants from HUVEC incubated with medium alone or medium containing antibody to IL-8, CXCR1 or CXCR2 for 4 h were examined for gelatinase activity. We observed a 24–42% decrease in pro-MMP-2 as well as active-MMP-2 in HUVEC treated with anti-IL-8, anti-CXCR1 or anti-CXCR2 compared to medium alone or control antibody treated culture (Figure 4b) suggesting that IL-8 and its receptors regulate MMP-2 production and activity in endothelial cells in an autocrine manner.

Inhibition of angiogenesis by antibodies to IL-8, CXCR1 and CXCR2 using angiogenesis assays

Next, we determined the autocrine role of IL-8 in angiogenesis by examining the effect of neutralization of IL-8, CXCR1 or CXCR2 on the organization of endothelial cells into capillary tube structures. Matrigel-coated wells were plated with 1×10^4 HUVEC and cultured with medium alone, medium containing control IgG (5 μg/ml) or neutralizing antibody (5 μg/ml each) to IL-8, CXCR1 or CXCR2. After 4 h of incubation, plates were examined for capillary tube formation under an inverted microscope. HUVEC formed capillary tube-like structures in medium alone, control antibody and IL-8-treated cultures (Figure 5). We observed no capillary tube formation in HUVEC incubated with anti-IL-8, anti-CXCR1 or anti-CXCR2 antibodies (Figure 5).

Further, we examined the effect of CXCR1, CXCR2 or IL-8 neutralization on neovascularization using the CAM assay. Anti-IL-8, anti-CXCR1 but not anti-CXCR2 antibodies significantly inhibited neovascularization in the chicken embryos as compared to control IgG treated fertilized eggs (Figure 6).

Discussion

Several reports demonstrate that IL-8 directly modulates proliferation and migration of endothelial cells, and regulates angiogenesis measured *in vitro* and *in vivo* [24–27]. These studies were exclusively focused on the paracrine role of IL-8 in angiogenesis. However, endothelial cells themselves are a major source of IL-8, which is significantly enhanced during inflammation, infection, stress and interaction with tumors [28–33]. In the present study, our data demonstrate that IL-8 functions as an autocrine growth factor regulating endothelial cell growth, survival, migration and MMP-2 production. Neutralization of IL-8 activity resulted in inhibition of capillary tube organization and neovascularization in a CAM assay.

The expression and paracrine function of IL-8 has been demonstrated in HUVEC [8, 38] and HMEC [8, 21, 27]. Our previous study suggests that constitutive expression of CXCR1 and CXCR2 in both HUVEC and HMEC is involved in endothelial cell growth, migration and regulation of endothelial cell survival and MMP expression [27]. In the present study, we demonstrate that inhibition of IL-8, CXCR1 or CXCR2 differentially modulates endothelial cell proliferation, survival and migration, suggesting an autocrine role for IL-8. Both anti-CXCR1 and anti-CXCR2 inhibited HUVEC and HMEC cell proliferation. Furthermore, treatment of HUVEC with IL-8 decreased endothelial cell apoptosis. Anti-IL-8, anti-CXCR1 or anti-CXCR2 treatment of HUVEC enhanced endothelial cell survival significantly and enhanced apoptosis. The underlying mechanism(s) for differences in the inhibition of HUVEC (large vessel endothelial cells) and HMEC (capillary endothelial cells) cell proliferation following treatment with anti-IL-8, anti-CXCR1 or anti-CXCR2 antibody is not clear. We have observed constitutive expression of IL-8 in HUVEC and HMEC. Earlier reports have demonstrated that endothelial cells themselves are a major source of IL-8, which is significantly enhanced by inflammatory signals [28–33]. Our data suggest that blocking IL-8 activity (anti-IL-8, anti-CXCR1 or anti-CXCR2) inhibits endothelial cell proliferation and induces apoptosis, suggesting that IL-8 may function as an autocrine growth factor. However, the role of other angiogenic chemokines which bind to

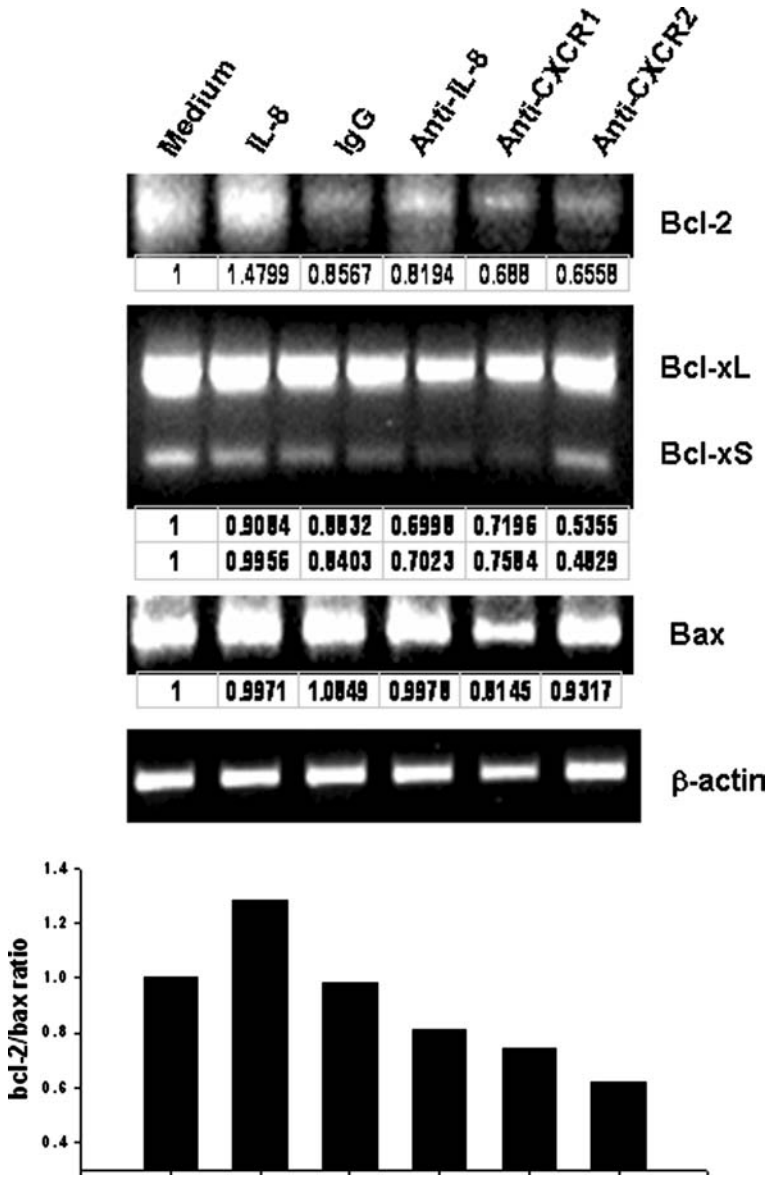

Figure 3. Neutralizing antibody to IL-8, CXCR1 or CXCR2 modulated endothelial cell expression of apoptosis-associated genes. Total RNA from untreated and antibody treated HUVEC were analyzed by RT-PCR. To facilitate the comparison between untreated and antibody treated groups, expression levels of apoptosis-associated genes and mRNA production was normalized to untreated controls. For mRNA, expression index was calculated as the ratio of the optical densities for specific mRNA transcripts and the housekeeping gene, β-actin, prior to normalization to untreated controls. The values shown are fold increase/decrease in protein or mRNA levels compared to untreated controls. This is representative of three experiments with similar results.

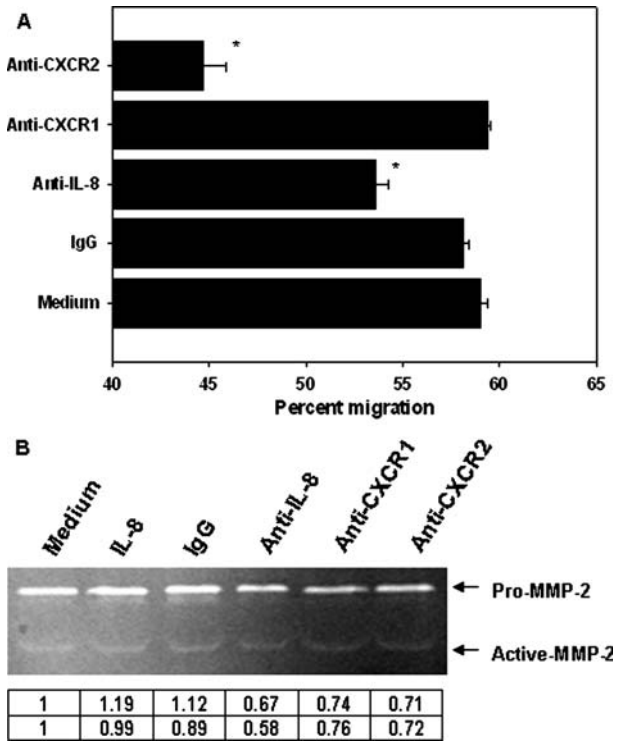

Figure 4. (a) Effect of anti-IL-8, anti-CXCR1 and anti-CXCR2 in endothelial cell migration. Migration of endothelial in response to anti-IL-8, anti-CXCR1 and anti-CXCR2 was examined using modified Boyden chambers as described in Materials and methods. The values are percent of migrated cells ±SEM. *Compared to medium alone, $P < 0.05$. This is a representative experiment of three performed in duplicate and yielding similar results. (b) Supernatants were collected from untreated and antibody treated HUVEC cultures. MMP-2 activity in the culture supernatants were determined by zymography. The values are arbitrary units (AU) for each treatment group. This is a representative of three experiments with similar results.

CXCR2 cannot be ruled out in endothelial cell proliferation and survival.

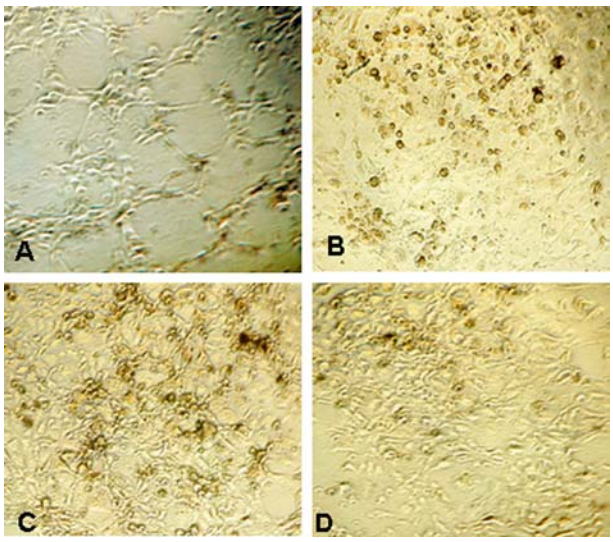

Figure 5. Capillary tube organization disrupted by anti-IL-8, anti-CXCR1 or anti-CXCR2. HUVEC were plated on Matrigel-coated 48-well plates and incubated with medium containing control antibody (a), anti-IL-8 (b), anti-CXCR1 (c) anti-CXCR2 (d) for 4 h. Capillary tube formation was examined by inverted microscope and photographed. This is a representative of three experiments with similar results.

Recent reports indicate that CXCR1 and CXCR2 activation and function are involved in Rho, Rac [22] and mitogen-activated protein (MAP) kinase signaling pathways [39], which are linked to cell growth [40] and migration [41–43]. Endothelial cell survival and programmed cell death are critical for maintenance of vascular structure in angiogenesis [44]. Differential expression of bcl-2 family members, which include anti-apoptotic (bcl-2 and bcl-x_L) and pro-apoptotic (bax and bcl-x_S) proteins, regulates apoptosis [45, 46]. The bcl-2 and bcl-x_L proteins interact with bax to suppress apoptosis, while bcl-x_S promotes cell death by inhibiting bcl-2 and bcl-x_L, [45] and constitutive expression of bcl-x has been shown in HUVEC [47]. In

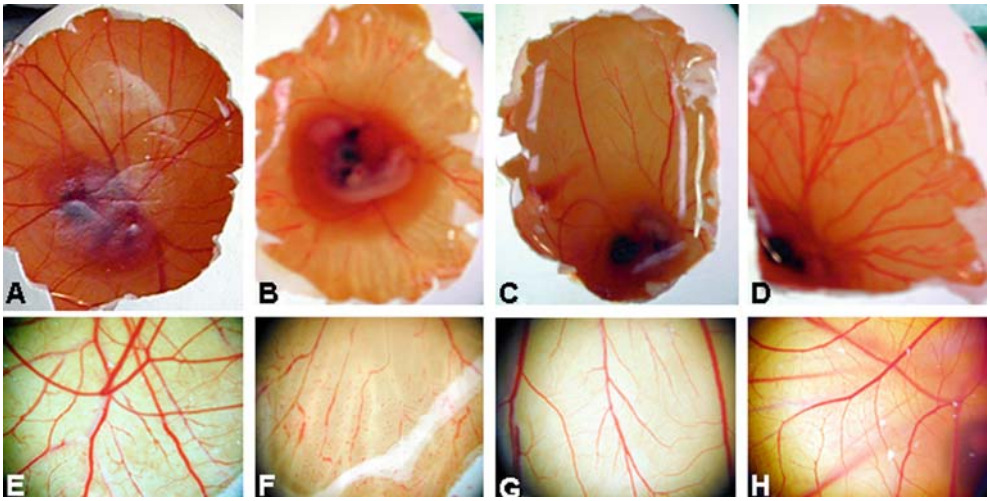

Figure 6. Inhibition of neovascularization by anti-IL-8, anti-CXCR1 or anti-CXCR2 in CAM assay. Fertilized eggs were inoculated with 100 μg/egg of control IgG (a, e) or anti-IL-8 (b, f), anti-CXCR1 (c, g) or anti-CXCR2 (d, h). After 4 days, embryos were examined for neovascularization. (a–d) 10× magnification; (e–h) 30× magnification. This is a representative of two experiments with similar results.

the present study, we observed significantly increased apoptotic cell numbers and decreased anti-apoptotic gene expression following treatment with neutralizing antibody to IL-8, CXCR1 or CXCR2.

Degradation of the extracellular matrix by MMPs is required in endothelial cell migration, organization and hence, angiogenesis [3, 4, 48, 49]. Recent reports suggest that IL-8 enhances migration and up-regulates MMPs expression in human melanoma cells [11, 12] and endothelial cells [27]. In the present study, decreased MMP-2 production was observed in endothelial cells treated with antibodies against IL-8, CXCR1 or CXCR2. We did not observe MMP-9 production in these cells. In addition, we observed inhibition of endothelial cell migration in anti-IL-8 and anti-CXCR2 treated cells, whereas anti-CXCR1 failed to inhibit endothelial cell migration. These data suggest a limited role for CXCR1 in the regulation of endothelial migration. Furthermore, we observed significant inhibition of capillary tube formation and neovascularization following treatment with anti-IL-8, anti-CXCR1 or anti-CXCR antibody. Since these studies were performed in the absence of any exogenous angiogenic factors, an autocrine mechanism for IL-8-mediated angiogenic responses is strongly suggested.

Taken together, our data demonstrates an autocrine role for IL-8 in the regulation of endothelial cell proliferation, migration, survival and MMP production. Thus, subsequent neovascularization, for which these cellular functions are required, was also modulated. Given the up-regulated expression of IL-8 and cognate receptors, CXCR1 and CXCR2, by endothelial cells during pathological conditions, and their multifunctional role in angiogenesis and tumor progression, this strongly suggests that targeting autocrine/paracrine pathways may emerge as therapeutic strategies for multiple diseases.

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References

1. Belperio JA, Keane MP, Arenberg DA et al. CXC chemokines in angiogenesis. J Leukoc Biol 2000; 68: 1–8.
2. Strieter RM. Chemokines: Not just leukocyte chemoattractants in the promotion of cancer. Nat Immunol 2001; 2: 285–6.
3. Fidler J, Singh RK, Yoneda J et al. Critical determinants of neoplastic angiogenesis. Cancer J Sci Am 2000; 3(6 Suppl): S225–S36.
4. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature 2000; 407: 249–57.
5. Baggiolini M, Dewald B, Moser B. Human chemokines: An update. Annu Rev Immunol 1997; 15: 675–705.
6. Murphy PM. Chemokines and the molecular basis of cancer metastasis. N Engl J Med 2001; 345: 833–5.
7. Ishiko T, Sakamoto K, Yamashita S-I et al. Carcinoma cells express IL-8 and the IL-8 receptor: Their inhibition attenuates the growth of carcinoma cells. Int J Oncol 1995; 6: 119–22.
8. Salcedo R, Resau JH, Halverson D et al. Differential expression and responsiveness of chemokine receptors (CXCR1–3) by human microvascular endothelial cells and umbilical vein endothelial cells. FASEB J 2000; 14: 2055–64.
9. Holmes WE, Lee J, Kuang WJ et al. Structure and functional expression of a human interleukin-8 receptor. Science 1991; 253: 1278–80.
10. Cerretti DP, Kozlosky CJ, Vanden Bos T et al. Molecular characterization of receptors for human interleukin-8, GRO/melanoma growth-stimulatory activity and neutrophil activating peptide-2. Mol Immunol 1993; 30: 359–67.
11. Wang JM, Taraboletti G, Matsushima K et al. Induction of haptotactic migration of melanoma cells by neutrophil activating protein/interleukin-8. Biochem Biophys Res Commun 1990; 169: 165–70.
12. Luca M, Huang S, Gershenwald JE et al. Expression of interleukin-8 by human melanoma cells up-regulates MMP-2 activity and increases tumor growth and metastasis. Am J Pathol 1997; 151: 1105–13.
13. Li A, Varney ML, Singh RK. Expression of interleukin 8 and its receptors in human colon carcinoma cells with different metastatic potentials. Clin Cancer Res 2001; 7: 3298–304.
14. Smith DR, Polverini PJ, Kunkel SL et al. Inhibition of interleukin 8 attenuates angiogenesis in bronchogenic carcinoma. J Exp Med 1994; 179: 1409–15.
15. Singh RK, Varney ML, Bucana CD, Johansson SL. Expression of interleukin-8 in primary and metastatic malignant melanoma of the skin. Melanoma Res 1999; 9: 383–7.
16. Inoue K, Slaton JW, Eve BY et al. Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. Clin Cancer Res 2000; 6: 2104–19.
17. Oppenheim JJ, Zachariae CO, Mukaida N, Matsushima K. Properties of the novel proinflammatory supergene 'intercrine' cytokine family. Annu Rev Immunol 1991; 9: 617–48.
18. Rossi D, Zlotnik A. The biology of chemokines and their receptors. Annu Rev Immunol 2000; 18: 217–42.
19. Koch AE, Polverini PJ, Kunkel SL et al. Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science 1992; 258: 1798–801.
20. Haghnegahdar H, Du J, Wang D et al. The tumorigenic and angiogenic effects of MGSA/GRO proteins in melanoma. J Leukoc Biol 2000; 67: 53–62.
21. Addison CL, Daniel TO, Burdick MD et al. The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. J Immunol 2000; 165: 5269–77.
22. Schraufstatter IU, Chung J, Burger M. IL-8 activates endothelial cell CXCR1 and CXCR2 through Rho and Rac signaling pathways. Am J Physiol Lung Cell Mol Physiol 2001; 280: L1094–L103.
23. Devalaraja RM, Nanney LB, Du J et al. Delayed wound healing in CXCR2 knockout mice. J Invest Dermatol 2000; 115: 234–44.
24. Koch AE, Polverini PJ, Kunkel SL et al. Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science 1992; 258: 1798–801.
25. Masood R, Cai J, Tulpule A et al. Interleukin 8 is an autocrine growth factor and a surrogate marker for Kaposi's sarcoma. Clin Cancer Res 2001; 7: 2693–702.
26. Strieter RM, Polverini PJ, Arenberg DA et al. Role of C-X-C chemokines as regulators of angiogenesis in lung cancer. J Leukoc Biol 1995; 57: 752–62.
27. Li A, Dubey S, Varney ML et al. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases

production and regulated angiogenesis. J Immunology 2003; 170: 3369–76.
28. Oude Nijhuis CS, Vellenga E, Daenen SM et al. Endothelial cells are main producers of interleukin 8 through toll-like receptor 2 and 4 signaling during bacterial infection in leukopenic cancer patients. Clin Diagn Lab Immunol 2003; 10: 558–63.
29. Liang F, Huang N, Wang B et al. Shear stress induces interleukin-8 mRNA expression and transcriptional activation in human vascular endothelial cells. Chin Med J (Engl) 2002; 115: 1838–42.
30. Vadeboncoeur N, Segura M, Al Numani D et al. Pro-inflammatory cytokine and chemokine release by human brain microvascular endothelial cells stimulated by Streptococcus suis serotype 2. FEMS Immunol Med Microbiol 2003; 35: 49–58.
31. Ramjeesingh R, Leung R, Siu CH. Interleukin-8 secreted by endothelial cells induces chemotaxis of melanoma cells through the chemokine receptor CXCR1. FASEB J 2003; 17: 1292–4.
32. Sica A, Matsushima K, Van Damme J et al. IL-1 transcriptionally activates the neutrophil chemotactic factor/IL-8 gene in endothelial cells. Immunology 1990; 69: 548–53.
33. Huber AR, Kunkel SL, Todd RF III, Weiss SJ. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. Science 1991; 254: 99–102.
34. Chu YW, Runyan RB, Oshima RG, Hendrix MJ. Expression of complete keratin filaments in mouse L cells augments cell migration and invasion. Proc Natl Acad Sci USA 1993; 90: 4261–5.
35. Montesano R, Orci L, Vassalli P. *In vitro* rapid organization of endothelial cells into capillary-like networks is promoted by collagen matrices. J Cell Biol 1983; 97: 1648–52.
36. West DC, Thompson WD, Sells PG, Burbridge MF. Angiogenesis assay using chick chorioallantoic membrane. In Murray JC (ed): Angiogenesis Protocols. Humana Press 2001; 107–30.
37. Rao VH, Singh RK, Delimont DC et al. Interleukin-1beta upregulates MMP-9 expression in stromal cells of human giant cell tumor of bone. J Interferon Cytokine Res 1999; 19: 1207–17.
38. Murdoch C, Monk PN, Finn A. Cxc chemokine receptor expression on human endothelial cells. Cytokine 1999; 11: 704–12.
39. Venkatakrishnan G, Salgia R, Groopman JE. Chemokine receptors CXCR-1/2 activate mitogen-activated protein kinase via the epidermal growth factor receptor in ovarian cancer cells. J Biol Chem 2000; 275: 6868–75.
40. Davis RJ. Transcriptional regulation by MAP kinases. Mol Reprod Dev 1995; 42: 459–67.
41. Keely PJ, Westwick JK, Whitehead IP et al. Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. Nature 1997; 390: 632–6.
42. Itoh K, Yoshioka K, Akedo H et al. An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. Nat Med 1999; 5: 221–5.
43. Schmitz AA, Govek EE, Bottner B, Van Aelst L. Rho GTPases: signaling, migration, and invasion. Exp Cell Res 2000; 261: 1–12.
44. Nor JE, Christensen J, Mooney DJ, Polverini PJ. Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. Am J Pathol 1999; 154: 375–84.
45. Antonsson B, Martinou JC. The Bcl-2 protein family. Exp Cell Res 2000; 256: 50–7.
46. Boise LH, Gonzalez-Garcia M, Postema CE et al. Bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 1993; 74: 597–608.
47. Ackermann EJ, Taylor JK, Narayana R, Bennett CF. The role of antiapoptotic Bcl-2 family members in endothelial apoptosis elucidated with antisense oligonucleotides. J Biol Chem 1999; 274: 11245–52.
48. McCawley LJ, Matrisian LM. Matrix metalloproteinases: Multifunctional contributors to tumor progression. Mol Med Today 2000; 6: 149–56.
49. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med 2000; 6: 389–95.