Hypoxia-Induced Activation of p38 Mitogen-Activated Protein Kinase and Phosphatidylinositol 3’-Kinase Signaling Pathways Contributes to Expression of Interleukin 8 in Human Ovarian Carcinoma Cells

Lei Xu, Pooja S. Pathak, and Dai Fukumura
Edwin L. Steele Laboratory, Department of Radiation Oncology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts

ABSTRACT

Purpose: Overexpression of interleukin 8 (IL-8) is associated with disease progression in human ovarian cancer. Hypoxia, a common feature in solid tumors, induces IL-8 expression in human ovarian carcinoma cells through activation of nuclear factor-kappa B and activating protein-1. Here we show the upstream components of these signal transduction pathways that lead to IL-8 expression under hypoxia.

Experimental Design: We incubated Hey-A8 human ovarian carcinoma cells under hypoxic condition (1% O2) and determined hypoxia regulation of phosphatidylinositol 3’-kinase (PI3K)/Akt pathway, mitogen-activated protein kinases (MAPKs), and effects of ras and vascular endothelial growth factor by Western and Northern blots, the use of specific inhibitors, in vitro kinase assays, luciferase reporter genes, and ELISA.

Results: While investigating the upstream signaling pathways, we found that Akt kinase and p38 MAPK are activated by hypoxia. Both hypoxia-induced Akt and p38 MAPK functional activity, and IL-8 mRNA and protein expression were reduced with the inhibition of PI3K and p38 MAPK. Oncogenic ras overexpression resulted in an increase in the hypoxia-induced IL-8 expression, whereas the inhibition of ras by transfection of dominant-negative ras inhibited the hypoxia-induced IL-8 expression.

Conclusions: These results show that hypoxia activates ras, PI3K/Akt pathway, and p38 MAPK pathway to enhance IL-8 gene transcription under hypoxia, and suggest these signaling pathways as potential targets for controlling IL-8 expression and angiogenesis by human ovarian carcinoma cells.

INTRODUCTION

Interleukin (IL) 8 is a chemokine that elicits multiple effects on tumor growth and metastasis (1). It exerts various biological activities by inducing neutrophil activation (2), stimulating cell proliferation (3), and inducing angiogenesis (4). There is also evidence that IL-8 modulates collagenase secretion (5), suggesting that it could modulate invasiveness and/or extracellular matrix remodeling in the tumor environment. IL-8 has been shown to promote tumor growth (6, 7), and its expression has been shown to correlate with the metastatic potential of various cancer cells (8).

Hypoxia, a common feature of solid tumors (9), has been shown to up-regulate IL-8 expression in various tumor cells (10). The induction of IL-8 gene expression at the transcriptional levels involves nuclear factor κB (NFκB), activating protein-1 (AP-1), and nuclear factor (NF)-IL-6. The NFκB binding site is dispensable for IL-8 gene expression in all of the cell types. It combines with CAAT/enhancer binding protein/NF-IL-6 or AP-1 binding sites, depending on the cell type (11). A recent study showed that NFκB and AP-1 binding sites are dispensable for IL-8 gene expression during hypoxia (10). However, little is known about the upstream signaling events that are activated by hypoxia and mediate its effects.

NFκB is normally inactivated and sequestered in the cytoplasm with the inhibitory subunit, inhibitor of NFκB (IκB). Upon stimulation, IκB rapidly phosphorylated, ubiquitinated, and then degraded, resulting in the release and subsequent nuclear translocation of activate NFκB (12). Phosphatidylinositol 3’-kinase (PI3K) has been shown to activate NFκB regulation (13, 14). Akt, a serine/threonine kinase, regulated by PI3K, has been shown specifically to contribute to NFκB regulation through association with and activation of IκB kinase (15, 16). Furthermore, Akt has been shown to phosphorylate p65/RelA, an effect independent of IκB degradation (17). Therefore, the PI3K/Akt pathway provides multiple potential links in NFκB regulation.

Mitogen-activated protein kinases (MAPKs) are involved in the regulation of gene expression by activating transcription factors and through post-transcriptional mechanisms (18). Mammalian cells express several groups of MAPKs, including, extracellular signal-regulated kinase (ERK)-1/2, c-Jun NH2-terminal kinase (JNK), and p38 proteins. The best characterized function is their regulation of transcription factor AP-1 (19).

In this study, we investigated the role of the MAPK cascade and PI3K in hypoxia-induced IL-8 expression using a human ovarian carcinoma cell line, Hey-A8. Our study shows...
that hypoxia activates p38 MAPK and Akt kinase, and the inhibition of p38 MAPK and PI3K with specific inhibitors prevents hypoxic induction of IL-8. Oncogenic ras (V12) overexpression resulted in an increase in hypoxia-induced IL-8 expression. These results suggest potential therapeutic use of PI3K or p38 MAPK inhibitors to block the effect of hypoxia on tumor progression.

MATERIALS AND METHODS

Reagents. The PI3K inhibitor LY294002 (Cell Signaling Technology, Beverly, MA) and the p38 MAPK inhibitor SB203580 (Sigma, St. Louis, MO) were dissolved in DMSO and used at a final concentration of 10 μM and 20 μM, respectively. There was no cytotoxicity observed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at the above concentrations. Rabbit polyclonal antibodies to phospho-Akt, Akt and phospho-p38, were purchased from Cell Signaling. RasV12 and RasN17 were obtained from Clontech (Palo Alto, CA).

Cell Culture. The Hey-A8 cell line obtained from Dr. Isaiah J. Fidler (M.D. Anderson Cancer Center, Houston, TX) was established in culture from a peritoneal deposit of a patient with moderately differentiated papillary cystadenocarcinoma (20). This cell line was chosen for this study because of its constitutive expression of IL-8 and its increased expression of IL-8 under hypoxia (10). The tumor cell line was maintained as an adherent monolayer in Eagle’s MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, 1-glutamine, and 2-fold vitamin solution (CMEM; Flow Laboratories, Rockville, MD).

Hypoxic Treatment. One million tumor cells in 10 ml of culture medium were plated in 10-cm dishes and cultured at 37°C overnight. Then, they were incubated in a hypoxia chamber (Precision Scientific, Winchester, VA) and incubated with 1% O2, 5% CO2, and balance nitrogen. Cells were plated in culture dishes 48 h before incubation in hypoxic conditions. After 12-h exposure to hypoxia, the cells excluded trypan blue dye (95%) and exhibited no morphological changes by light microscopy.

Northern Blot Analysis. Northern blot was performed as described previously (10). IL-8 and β-actin cDNA probes were synthesized by PCR using primers for IL-8: 5’-GTC TGC TAG CCA GGA TCC AC-3’; 5’-ACA CAG CGT GCA ATG ACA AG-3’; and for β-actin: 5’-TGG ATG CCT CTG GTC GTA CC-3’; and 5’-CAA CGT CAC ACT TCA TGA TGG-3’.

Western Blot Analysis. Western blot was performed as described previously (10). Hey-A8 cells were incubated in serum-free medium overnight and then incubated under either normoxia or hypoxia for different time points. The cells were scraped from plates, pelleted, and resuspended in lysis buffer. Thirty μg of protein/sample was separated on 10% SDS-polyacrylamide gels.

Protein Kinase Assay. Subconfluent cultures of Hey-A8 cells were grown in DMEM containing 0.5% fetal bovine serum for 24 h at 37°C. Hey-A8 cells were then incubated in normoxic or hypoxic conditions for different time periods. Cells were then washed rapidly with ice-cold PBS and immediately lysed in lysis buffer. Two hundred μl of total cellular extract was immunoprecipitated with an immobilized phospho-Akt antibody, washed, and incubated with glycosyn synthase kinase (GSK) fusion protein as a substrate. This allows immunoprecipitated active Akt to phosphorylate GSK. Phosphorylation of GSK was measured by Western blotting using a phospho-GSK antibody.

Luciferase Reporter Gene Assay. NFκB-Luc and AP-1-luc reporter genes, containing four tandem copies of the NFκB or AP-1 consensus sequence fused to a TATA-like promoter region from the herpes simplex virus thymidine kinase promoter were obtained from Clontech.

The cells were cotransfected with 20 μg of the luciferase reporter gene and 2 μg of pRL-TK/plate using Lipofectamine2000. pRL-TK, obtained from Promega (Madison, WI), contains the herpes simplex virus thymidine kinase promoter region upstream of Renilla luciferase and was used as an internal control for transfection efficiency. The transfected cells were then exposed for 6 h to normoxic or hypoxic conditions. Cell lysates were prepared using the Dual Luciferase Assay System (Promega). The light intensity was measured on 20 μl of cell lysates using a luminometer.

ELISA. The supernatant of human ovarian carcinoma cells cultured in the presence and absence of recombinant human VEGF was collected at different time point and stored at −70°C. The production of IL-8 protein was analyzed by ELISA using the Quantikine IL-8 ELISA kit (R&D systems). The concentration of IL-8 in unknown samples was determined by comparing the absorbance of the samples with the standard curve.

Statistical Analysis. The significance of the data was analyzed by Student’s t test (two-tailed).

RESULTS

Hypoxia Activates Akt Kinase and p38 MAPK. A recent study showed that NFκB and AP-1 binding sites are indispensable for IL-8 gene expression during hypoxia (10). It has also been shown that PI3K/Akt is activated by hypoxia in various cells (21, 22). In addition, it has been suggested that this pathway is critical for the activation of NFκB pathway. Thus, we determined whether activation of this signaling pathway contributes to the hypoxic induction of IL-8 expression. Hey-A8 cells were incubated in normoxic or hypoxic condition for different time period, and the activation of the PI3K/Akt was monitored by the phosphorylation of Akt. Thirty min of hypoxia treatment resulted in the induction of phosphorylated Akt, whereas the total Akt level remains the same, indicating the hypoxic activation of PI3K/Akt (Fig. 1, A and B).

Many cellular stresses can stimulate MAPK activity leading to the activation of AP-1 (18). We next investigated whether hypoxia activates ERK1/2, JNK, and p38 MAPK. Phosphorylation of the MAPK was determined by Western blot analysis using anti-phosphorylated ERK1/2, JNK, and p38 MAP kinase antibody. Fig. 1, C and D, showed phosphorylation of p38 MAPK in Hey-A8 cells treated with hypoxia. The highest level of p38 MAPK phosphorylation was observed between 60 and 90 min, and declined thereafter. Western blot for phospho-JNK/
stress-activating protein kinase, and ERK1/2 MAPK did not reveal any detectable phosphorylated kinases (data not shown).

To investigate whether this hyperphosphorylated Akt and p38 MAPK correlated with functional activation, in vitro kinase assays were performed. Hey-A8 cells were incubated in serum-free medium overnight and then incubated in normoxic or hypoxic condition with or without the specific inhibitors against PI3K and p38 MAPK for 60 min. Phosphorylation of GSK by immunoprecipitated phospho-Akt was detected by a phosphospecific anti-GSK antibody. A significant induction in the level of phosphorylated GSK was detected after 60-min incubation in hypoxic condition and LY294002 abolished the GSK phosphorylation by hypoxia (Fig. 2A), indicating that phosphorylation of Akt is accompanied by its functional activation. Phosphorylation of activating transcription factor (ATF) by immunoprecipitated phospho-p38 MAPK was detected by a phosphospecific anti-ATF antibody. A significant induction in the level of phosphorylated ATF was detected 60 min after incubation in hypoxic environment (Fig. 2B); and SB203580 abolished the ATF phosphorylation induced by hypoxia, indicating phosphorylation of p38 MAPK is accompanied by its functional activation.

Hypoxia-Induced NFκB and AP-1 Activity Is Inhibited by PI3K Inhibitor and p38 MAPK Inhibitor. To confirm the involvement of PI3K and p38 MAPK in IL-8 regulation under hypoxia, we tested the influence of the PI3K inhibitor LY294002 and p38 MAPK inhibitor SB203580 on IL-8 expression. Hey-A8 cell showed a reduction in the hypoxia-induced IL-8 mRNA level after 6 h of treatment with LY294002 (Fig. 4A) or SB203580 (Fig. 4B). The constitutive expression of IL-8 protein did not change significantly after treatment with the PI3K and p38 MAPK inhibitors, but the induction of IL-8 protein by hypoxia was reduced after 24 h of treatment with inhibitors of either PI3K or p38 MAPK (Fig. 4C).

Effect of Oncogenic ras Overexpression on Hypoxia-Induced IL-8 Expression. Next, we investigated the role of oncogenic ras on the expression of IL-8. Using Hey-A8 cells stably transfected with V12-ras, a constitutively active ras, we showed that overexpression of ras does not affect the basal level of IL-8 mRNA expression, but enhances hypoxia-induced IL-8 expression. With the transfection of inactivated ras, N17-ras, hypoxia-induced IL-8 mRNA expression is decreased (Fig. 5A).

To study the effect of ras expression on NFκB and/or AP-1 activation, we transiently transfected NFκB and AP-1 luciferase reporter genes into Hey-A8 cells that stably express V12-ras or N17-ras. Overexpression of active ras enhanced NFκB and AP-1 activity under hypoxia (Fig. 5B), whereas dominant-negative ras reduced their activity under hypoxia.

Effect of VEGF on IL-8 Expression. Because hypoxia up-regulates VEGF expression and a previous study has shown...
Mechanism of Hypoxia-Induced Interleukin 8

In vitro

60 min. kinase inhibitor, or 20 μM specific p38 MAPK inhibitor, SB203580 for 60 min. In vitro kinase assays were performed. Pictures shown are representative of at least three independent experiments.

DISCUSSION

In this study, we investigated the upstream signaling events involved in hypoxia-induced IL-8 expression in the human ovarian carcinoma cell line, Hey-A8. We obtained evidence that the activation of PI3K and p38 MAPK contributes to hypoxia-induced IL-8 expression.

IL-8 is expressed in normal human ovarian cells (23) and in human ovarian cancer cells (24). High expression of IL-8 mRNA has been detected in clinical specimens of late-stage ovarian carcinomas (25). Hypoxia has been shown to up-regulate the expression of IL-8 in human endothelial cells (26), glioblastoma (27), and ovarian carcinoma cells (10). Furthermore, it has been shown previously that hypoxia induces IL-8 expression in human ovarian carcinoma cells by activating gene transcription via AP-1 and NFκB (10). To additionally define the upstream signaling pathway leading to the transcriptional activation of the IL-8 by hypoxia, we investigated the role of PI3K and MAPK pathways.

AP-1 may be activated by signal transduction events and cellular responses through various MAPK signal transduction pathways (18). MAPKs are serine-threonine protein kinases that are activated by diverse stimuli including cytokines, growth factors, neurotransmitters, hormones, cellular stress, and cell adhesion (18). Mammalian cells express several groups of MAPKs, including ERK1/2, JNK, and p38 proteins. The MAPK activity is regulated through three-tiered cascades: MAPK/ERK1/2 kinase activates ERK1/2, MAPK kinase 3/6 activates p38, and MAPK kinase 4/7 (JNK1/2) activates JNK. Each MAPK kinase can be activated by more than one MAPK kinase pathway, increasing the complexity and diversity of MAPK signaling (18). We have shown that p38 MAPK, but not ERK1/2 and JNK/stress-activating protein kinase, are activated upon hypoxic treatment in human ovarian carcinoma cells in a time-dependent manner. Although it is not yet known which components of the signal transduction cascades lead to hypoxic activation of p38 MAPK, hypoxia activation of p38 has been shown in various types of cells (28, 29). Hypoxia selectively activates p38α and p38γ isoforms of the p38 pathway in PC12 pheochromocytoma cell (28). Mitochondrial reactive oxygen species initiates phosphorylation of p38 MAPK in cardiomyocytes (29).

Several growth factor and cytokine signal transduction pathways have been reported to contribute to the activation of NFκB (30, 31). Inhibitor of NFκB kinase mediates the convergence of signals from the pathways that activate NFκB by phosphorylation of IκB (30) and by direct phosphorylation of the RelA p65 subunit of the NFκB/RelA (p50/p65) heterodimer (32). Akt/protein kinase B has been shown to activate NFκB

Fig. 2 In vitro Akt (A) and p38 mitogen-activated protein kinase (MAPK; B) kinase assays. Subconfluent cultures of Hey-A8 cells were grown in 0.5% fetal bovine serum medium for 24 h at 37°C. Hey-A8 cells were incubated in normal or hypoxic environment in the presence of absence of 10 μM LY294002, a specific phosphatidylinositol 3′-kinase inhibitor, or 20 μM specific p38 MAPK inhibitor, SB203580 for 60 min. In vitro kinase assays were performed. Pictures shown are representative of at least three independent experiments.

Fig. 3 Phosphatidylinositol 3′-kinase (PI3K) and p38 mitogen-activated protein kinase (MAPK) inhibitors inhibit nuclear factor κB (NFκB) and activating protein-1 (AP-1) activity under hypoxia. Twenty μg NFκB-luc and AP-1-luc were cotransfected with 2 μg of pRl-TK into Hey-A8 cells. After transfection (48 h), cells were incubated in normoxia or hypoxia in the presence or absence of PI3K or p38 MAPK inhibitors. The NFκB and AP-1 activity under hypoxia in the presence or absence of PI3K or p38 MAPK were assessed by normalizing the luciferase intensity to that of normoxia control. *, p < 0.05 as compared with corresponding hypoxia without inhibitors; bars, ±SE.
(17), and it is now known to be activated by the PDK1 after PI3K activation. Several studies have shown that hypoxia activates the PI3K/Akt signaling pathway (21, 22), and PI3K/Akt mediates hypoxia-induced hypoxia-inducible factor (HIF)-1α stabilization in Hep3B hepatoma cells (33), apoptosis in PC12 cell (21), and VEGF expression in ras-transformed cells (34). Hypoxia may activate PI3K/Akt by inducing the tyrosine phosphorylation of growth factor receptors (22) or by reactive oxygen species (33). Our current study shows that hypoxia activates Akt kinase in a time-dependent manner, and blocking of PI3K with a specific inhibitor abolishes hypoxia-induced IL-8 expression, indicating that PI3K/Akt signaling pathway is critical in hypoxia induced IL-8 expression.

Ras mediates Raf activation and, thus, initiates MAPK activation. This leads to an increase in the expression of c-Jun and c-Fos and, therefore, to increased AP-1 activity (35). Ras has been shown to translocate to the cell membrane upon activation and activates PI3K (36), which lead to the activation of Akt and downstream signaling pathway of inhibitor of NFκB kinase. Ras activation has also been shown to be involved with hypoxia in up-regulating VEGF (35). We analyzed the effect of change in ras expression on the induction of IL-8 by hypoxia. Our results showed that dominant-negative forms of ras abolished the inducible expression of IL-8, whereas overexpression of the oncogenic ras enhances the inducible expression of IL-8.

HIF-1 is widely expressed in mammalian cells (37). Transcriptional up-regulation of the VEGF gene during hypoxia is dependent on transactivation by the transcription factor HIF-1 (38). HIF-1, however, is not likely to play a direct role in hypoxic regulation of IL-8, because no HIF-1 binding motif appears in the published sequence of the IL-8 promoter region (39). Recent findings show that p300, a general transcriptional activator, specifically binds HIF-1, and thus, may play a critical role in transducing the signal from the HIF-1 to the apparatus responsible for the initiation of transcription (40). Whether p300 participates in the regulation of IL-8 is not known.

Because the previous study demonstrated that VEGF up-regulates IL-8 expression in brain microvascular endothelial
cells (41), and it is well known that hypoxia up-regulates VEGF expression in a variety of cell types, we investigated the effect of VEGF on IL-8 expression in Hey-A8 cells. However, our study showed that recombinant human VEGF does not induce IL-8 expression in Hey-A8 ovarian carcinoma cells even at a very high concentration. This indicates that hypoxia-induced IL-8 is not caused by any indirect effect through VEGF.

Our results have strong implications for the understanding of the pathophysiological mechanisms involved in the acquisition of the angiogenic phenotype. The upstream activation of NFκB and AP-1, regulated by PI3K and p38 MAPK, provides potential targets for inhibiting the coactivation of these signal pathways and expression of angiogenesis factors in human ovarian carcinoma cells. Combined inhibition of both MAPK and PI3K pathways may be needed for suppression of the signal transduction mechanisms regulating IL-8 expression and angiogenesis by human ovarian carcinoma cells. Therefore, it will be important to determine whether such inhibitors can suppress angiogenesis and the growth of human ovarian carcinoma cells in vivo.

**ACKNOWLEDGMENTS**

We thank Dr. Rakesh K. Jain for insightful input and support, Dr. Leo Gerweck for helpful comments, and Chelsea J. Swandal for excellent technical assistance.

**REFERENCES**

5. Luca, M., Huang, S., Gershovenwald, J. E., Singh, R. K., Reich, R., and Bar-Eli, M. Expression of interleukin-8 by human melanoma cells...


Hypoxia-Induced Activation of p38 Mitogen-Activated Protein Kinase and Phosphatidylinositol 3′-Kinase Signaling Pathways Contributes to Expression of Interleukin 8 in Human Ovarian Carcinoma Cells

Lei Xu, Pooja S. Pathak and Dai Fukumura


Updated version  Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/10/2/701

Cited articles  This article cites 39 articles, 22 of which you can access for free at: http://clincancerres.aacrjournals.org/content/10/2/701.full#ref-list-1

Citing articles  This article has been cited by 12 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/10/2/701.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.