Hypoxia-induced Elevation in Interleukin-8 Expression by Human Ovarian Carcinoma Cells

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ABSTRACT

The expression level of interleukin-8 (IL-8) directly correlates with the progression of human ovarian carcinomas implanted into the peritoneal cavity of nude mice, but the mechanism of induction is unknown. Because hypoxia induces expression of vascular endothelial growth factor/vascular permeability factor, which, like IL-8, is an angiogenesis-regulating molecule, we determined whether hypoxic conditions could regulate the expression of IL-8. Surgical specimens of human ovarian carcinomas were prepared for immunohistochinal and in situ hybridization analysis. Elevated levels of IL-8 mRNA and protein were found in tumor cells adjacent to necrotic zones. In vitro exposure of human ovarian carcinoma cell lines SKOV3 i.p.l and Hey-A8 to hypoxia resulted in a time-dependent increase in steady-state levels of IL-8 mRNA (Northern blot) and in increased production and secretion of IL-8 protein. Hypoxia-mediated transient induction of IL-8 expression could be ascribed to both an increase in IL-8 mRNA stability and transcriptional activation of the IL-8 gene promoter. Detailed functional analysis of the IL-8 promoter revealed that the sequence between −133 and −98 bp relative to the transcription initiation site was primarily responsible for IL-8 gene transcriptional activation by hypoxia. Point-mutated luciferase reporter studies indicated that AP-1 and NF-κB-like factor binding elements were mainly responsible for hypoxia-induced increase in IL-8 gene expression in human ovarian cancer cells, and that IL-8 transcription activation by hypoxia required the cooperation of NF-κB and AP-1 binding sites.

INTRODUCTION

The major cause of death from ovarian cancer is due to the growth of tumor cells in lesions throughout the peritoneal cavity (1). As is true for other cancers, the growth and spread of ovarian cancer is dependent on the formation of adequate vascular support, i.e., angiogenesis (2). Indeed, the extent of angiogenesis inversely correlates with survival in patients with ovarian cancer (3). During the last decade, many angiogenesis-regulating molecules have been identified, including basic fibroblast growth factor, VEGF/VPF (22), and IL-8 (2, 4, 5), whereas survival of the mice inversely correlated with expression of IL-8 by the human ovarian carcinoma cells (6).

IL-8, which belongs to the superfamily of CXC chemokines, has a wide range of proinflammatory effects and was initially described as a neutrophil chemoattractant (7). IL-8 promotes proliferation of tumor cells (8), induces angiogenesis (9, 10), and modulates secretion of collagenase (11). Because cell proliferation, angiogenesis, migration, and invasion are all important components of the metastatic process, the expression of IL-8 by tumor cells can influence their metastatic capacities (6, 12–14).

Human tumors are known to be heterogeneously oxygenated, and hypoxia is a common feature of solid tumors (15). Hypoxia has the potential to promote malignant progression by altering gene expression related to several important tumor phenotypic activities (16), including genes encoding cell cycle-regulatory proteins, angiogenesis regulatory proteins, metastasis-promoting proteins, metabolic enzymes, and transcription factors (17–19). Moreover, clinical observations suggest that tumors with low oxygen tension may have a higher metastatic potential than tumors with high oxygen tension (20, 21).

How IL-8 gene expression is regulated in human ovarian cancer cells is unclear, but a local decrease in oxygen tension and nutrients due to inadequate vasculature and heterogeneous perfusion has been considered a major cause for the induction of such angiogenic molecules as VEGF/VPF (22). We, therefore, determined the kinetics and molecular regulation of IL-8 expression in human ovarian cancer cell lines exposed to hypoxic environments. We report that hypoxia up-regulated the expression of IL-8, and that the induction of IL-8 expression was mediated by transactivation of the IL-8 promoter by the transcription factors AP-1 and NF-κB and the prolongation of IL-8 mRNA stability.

MATERIALS AND METHODS

Reagents. Eagle’s MEM and FBS were purchased from M. A. Bioproducts (Walkersville, MD). Actinomycin D (Sigma Chemical Co., St. Louis, MO) was used at a final concentration of 5 μg/ml. All of the reagents used in tissue culture were free of endotoxin as determined by Limulus amebocyte lysate assay (sensitivity limit of 0.125 ng/ml) purchased from Associates of Cape Cod (Falmouth, MA).

Human Ovarian Cell Lines. The SKOV3 cell line was established from ascitic fluids and obtained from the American Type Culture Collection (Rockville, MD; Ref. 23). The highly malignant SKOV3 i.p.l variant was derived from ascites arising in a nude mouse given an i.p. injection of SKOV3 cells (24). The HEY-A8 cell line (25), which originated from a xenograft of a peritoneal deposit of a cystadenocarcinoma of the ovary was the gift of Dr. G. B. Mills (Division of Medicine, The University of Texas M. D. Anderson Cancer Center, Houston, TX).

The tumor cell lines were maintained as monolayer cultures in DMEM supplemented with 5% FBS, 1× sodium pyruvate, 1× nonessential amino acids, 1× l-glutamine, and 2× vitamin solution (Life Technologies, Inc., Gaithersburg, MD). The cell cultures were maintained as monolayers on plastic Petri dishes and were incubated in 5% CO2–95% air at 37°C. The cultures were free of Mycoplasma and pathogenic murine viruses. The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

Hypoxic Treatment. Tumor cells were incubated in a hypoxia incubator (Precision Scientific, Winchester, VA) and incubated with 1% O2 balanced with CO2 and nitrogen. Cells were plated in culture dishes 4 h before incubation in hypoxic conditions. When the cultures reached 80% confluency, fresh medium was added, and the dishes were incubated in normoxic or hypoxic environment for different times. After 12-h exposure to hypoxia, all of the cells excluded trypan blue dye (>95%) and exhibited no morphological changes by light microscopy. To prevent changes in pH, the medium was buffered to pH 7.35 with 20 mM 2-(N-morpholino)ethylsulfonic acid and 20 mM Tris (hydroxymethyl) aminomethane. Reoxygenation was achieved by replenishing the medium and returning the cell cultures to a normoxic environment.
**Immunohistochemistry.** Tissue sections (5 μm thick) of formalin-fixed, paraffin-embedded human ovarian cancer surgical specimens were deparaffinized in xylene and rehydrated in graded alcohol. The endogenous peroxidase was blocked by use of 3% hydrogen peroxide in PBS for 12 min. The samples were incubated for 20 min at room temperature with a protein-blocking solution consisting of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum and were incubated at 4°C with a 1:50 dilution of rabbit polyclonal anti-IL-8 antibody (Biosource International, Camarillo, CA). The samples were then rinsed and incubated for 1 h at room temperature with a peroxidase-conjugated antirabbit IgG. The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics). The sections were then washed three times with distilled water, counterstained with Mayer’s hematoxylin (Biogenex Laboratories, San Ramon, CA), washed once with distilled water and once with PBS. The slides were mounted with a universal mount (Research Genetics) and examined with a bright-field microscope. A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm.

**ISH.**ISH was performed as described previously (26). ISH was carried out according to the Microprobe manual staining system (Fisher Scientific, Pittsburgh, PA). The sequence of the antisense oligonucleotide DNA probes complementary to the IL-8 mRNA transcripts was 5’-CTG-CAC-CC-3’. A poly d(T)20 oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample; hybridization of the probes were carried out for 45 min at 45°C, and the samples were then washed three times for 2 min each time with 2x SSC at 45°C. The samples were then incubated with alkaline phosphatase-labeled avidin for 30 min at 45°C, rinsed in 50 mmol/L Tris buffer (pH 7.6), rinsed with alkaline phosphatase enhancer for 1 min, and incubated with a chromogen substrate for 15 min at 45°C. A positive reaction in this assay stained red. Controls for endogenous alkaline phosphatase included treatment of the sample in the absence of the biotinylated probe and use of chromogen alone.

**Northern Blot Analysis.** mRNAs was extracted by using the FastTrack mRNA isolation kit (Invitrogen Co., San Diego, CA). mRNA (2 μg/lane) was fractionated on a 1.0% denaturing formaldehyde/agarose gel, electrophoresed at 0.6 amp to GeneScreen nylon membrane (DuPont Co., Boston, MA), an UV-cross-linked with a UV-Stratalinker 1800 (Stratagene, La Jolla, CA). The cDNA probe used in this study was a 0.5-kb EcoRI cDNA fragment corresponding to human IL-8 (a generous gift from Dr. K. Matsushima, Kanazawa University, Kanazawa, Japan). DNA fragments were purified by agarose gel electrophoresis, recovered by use of GeneClean (column to separate DNA; BIO 101, Inc., La Jolla, CA), and radiolabeled with the use of the random primer technique with [α-32P]deoxyribonucleotide triphosphates.

The steady-state expression of IL-8 mRNA transcripts was quantified by densitometry of autoradiographs with the use of the Image Quant software program (Molecular Dynamics, Sunnyvale, CA); each sample measurement was calculated from the ratio of the average areas between the specific mRNA transcripts and the β-actin mRNA transcript in the linear range of the film.

**Measurement of IL-8 Production by ELISA.** Culture supernatants of human ovarian carcinoma cells cultured under normoxic or hypoxic conditions were collected after 12 h 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.

**mRNA Stability Assay.** The half-life of IL-8 mRNA was determined by treating SKOV3 i.p.1 cells with actinomycin D as described by Lindholm (27). SKOV3 i.p.1 cells were cultured under normoxic or hypoxic conditions for 12 h and actinomycin D (Sigma, St. Louis, MO) was then added to the medium (5 μg/ml) to block transcription. Immediately after adding actinomycin D, the cells were returned to the same cultured conditions. mRNA was prepared at 0, 1, 3, 6, and 9 h, and Northern blot analysis was performed as described above. The amount of IL-8 mRNA and β-actin mRNA was quantified by densitometry. The half-life of IL-8 mRNA was calculated by drawing the best fit linear curve on a plot of the IL-8-β-actin ratio.

**Nuclear Run-On Assay.** SKOV3 i.p.1 cells (1×10⁷) were seeded into each 150-mm tissue culture dish and incubated overnight at 37°C. The cells were exposed to normoxia or hypoxia for 6 h. The nuclei were isolated, aliquoted, and kept at −80°C. For each in vitro transcription reaction, 100 μl of nuclei from each sample were thawed and mixed with an equal volume of 2× reaction buffer containing 100 mM MgCl₂; 10 mM DTT; 300 mM KCl; 100 mM HEPEs (pH 7.4); 1 mM each ATP, CTP, and GTP; and [α-32P]UTP (200 μCi, 3000 Ci/mmol; Amersham Corp., Arlington Height, IL). The reaction was incubated at 30°C for 30 min, and 32P-labeled RNA was then isolated and precipitated with ethanol. Labeled nuclear RNA was hybridized with dot blots containing the IL-8 and β-actin inserts at 65°C for 72 h. The filters were washed twice with 2× SSC and then exposed to X-ray film at −80°C for 1 day. Quantitative results were obtained by densitometry and standardized to β-actin.

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**Fig. 1.** Localization of IL-8 expression in a surgical specimen of human ovarian cancer. Human ovarian cancer specimen was prepared for immunohistochemistry and ISH analyses as described in the “Materials and Methods” section. A, H&E staining; B, anti-IL-8 antibody staining; C, oligo d(T) staining; D, IL-8 antisense oligo staining. T, tumor cells.
HYPOXIA AND IL-8

Results

IL-8 Expression in Surgical Specimens of Human Ovarian Cancer. In the first set of experiments, we determined the pattern of IL-8 expression in serial sections of human ovarian cancer specimens. Expression of IL-8 mRNA and protein was elevated in tumor cells (T) immediately surrounding necrotic areas (Fig. 1). Since Desbaillets et al. (28) reported the up-regulation of IL-8 in glioblastoma cells deprived of oxygen by necrosis, the following in vitro studies were designed to determine whether hypoxia can directly induce overexpression of IL-8 in ovarian carcinoma cells.

Induction of IL-8 Production in Human Ovarian Carcinoma Cell Lines by Hypoxia. In the next set of experiments, SKOV3 i.p.1 (Fig. 2A) and Hey-A8 (Fig. 2B) were incubated under normoxic or hypoxic conditions for 3, 6, 9, and 12 h. Supernatants were collected for ELISA, and cellular mRNA was isolated for Northern blot analysis. The exposure of SKOV3 i.p.1 and Hey-A8 cells to hypoxia increased the steady state levels of IL-8 mRNA in a time-dependent manner. IL-8 mRNA increased by 3 h of hypoxia and reached a 13-fold increase by 12 h. No discernible differences were found among the samples of β-actin. Consistent with the increase in mRNA expression, SKOV3 i.p.1 and Hey-A8 cells released an increased amount of IL-8 protein into the culture medium by 12 h of hypoxia; up to 1480.74 ± 23.18 μg/ml and 1635.42 ± 15.45 pg/ml in SKOV3 and Hey-A8, respectively.

EMSA. EMSA was performed using nuclear extracts prepared from SKOV3 i.p.1 cells cultured for various time periods under normoxic or hypoxic conditions. For EMSA experiments, the following double-stranded oligonucleotides were used: (a) NF-κB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; (b) AP-1, 5′-CGC TTG ATG AGT CAG CCG AAA-3′; and (c) nonspecific oligonucleotide (SP-1), 5′-ATT CGA TCG GGG GGC GAC C-3′. The oligonucleotide was annealed and 5′-end-labeled with [32P]ATP (3000 Ci/mmol) with T4 polynucleotide kinase using standard procedures. Binding reaction was carried out by preincubating nuclear extract protein (5 μg) in 20 mM HEPES (pH 7.9), 50 mM NaCl, 5% glycerol, 0.1 mM DTT, 1 μg poly(dI-dC) at room temperature for 15 min, followed by the addition of the double-stranded 32P-labeled oligonucleotide and a second incubation at room temperature for 15 min. For competition assays, 50-fold molar excess of unlabeled oligonucleotide was added to the binding reaction. Where indicated, anti-p65, anti-p50, anti-c-Rel (Oncogene, Cambridge, MA) or anti-c-Jun, anti-c-Fos, anti-c-Jun B and anti-c-Jun D antibody (Santa Cruz, Santa Cruz, CA) were added to the binding reaction for 45 min on ice. Samples were loaded on a 5% polyacrylamide gel. Electrophoresis was performed at room temperature for 3 h at 100 V. The gel was then dried at 80°C for 1 h and exposed to Kodak film at −70°C. Statistical Analysis. The significance of the data were analyzed by Student’s t test (two-tailed).

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ip1 and Hey-A8 cells, respectively (Fig. 2C). Collectively, these data indicate that hypoxia induced and enhanced expression of IL-8 in human ovarian cancer cells.

**Hypoxia-mediated Expression of IL-8 is Reversible.** In the next set of experiments, we determined whether the change in IL-8 gene expression was permanent or transient. SKOV3 i.p.1 cells were first incubated under normoxic or hypoxic conditions for 12 h and then shifted to normoxic conditions (reoxygenation) for 0, 0.5, 1, and 3 h. IL-8 expression was determined by Northern blot analysis. The initial exposure to hypoxia for 12 h induced a 16-fold increase in IL-8 mRNA. When the cells were reoxygenated for 0.5 h, the IL-8 transcript degraded, and, within 3 h, IL-8 mRNA reached basal levels (Fig. 3, A and B). Thus, the up-regulation of IL-8 by hypoxia is transient.

**Transcriptional Regulation of IL-8 Expression by Hypoxia.**

Next, we used the nuclear run-on assay to determine whether hypoxia increased the transcription rate of IL-8 in SKOV3 i.p.1 cells. Consistent with the Northern blot analysis data, the transcription rate of IL-8 (but not β-actin) increased by 7–fold in SKOV3 i.p.1 cells incubated for 6 h under hypoxic conditions (Fig. 4).

**Regulation of IL-8 mRNA Stability by Hypoxia.** To evaluate the effect of hypoxia on IL-8 mRNA stability, we measured the half-life of IL-8 mRNA in SKOV3 i.p.1 cells cultured for 12 h under normoxia or hypoxia. To block transcription, the cells were then treated with actinomycin D, and mRNA was collected at 0, 1, 3, 6, and 9 h for Northern blot analysis. As shown in Fig. 5A, under normoxia, IL-8 mRNA decreased rapidly with a half-life of 1.5 h. In contrast, under hypoxia, a high level of IL-8 mRNA was still evident even after 9 h, and the half-life was longer than 9 h (the longest time point taken; Fig. 5B). These results and the results from the nuclear run-on studies indicate that the increased steady-state IL-8 mRNA in SKOV3 i.p.1 cells was due to both transcriptional activation and mRNA stabilization.

**Identification of Hypoxia-responsive Elements of IL-8 Gene.**

On the basis of the data obtained from nuclear run-on assays, we expected cis-acting elements to control the transcriptional activation of the IL-8 gene in response to hypoxia. To characterize the DNA sequences involved in the IL-8 gene transcriptional activation by hypoxia, we used luciferase reporter plasmids in which the IL-8 5′-flanking sequences were fused to the firefly luciferase coding sequences (Fig. 6A). The series of deletion mutants were cotransfected into SKOV3 i.p.1 cells with pRL-TK, used as an internal control to monitor transfection efficiency. The transfected SKOV3 i.p.1 cells were incubated under normoxia or hypoxia for 6 h, and the induced luciferase activity was measured using a dual luciferase assay kit. As shown in Fig. 6B, the −133-luc reporter mediated the highest luciferase expression in cells exposed to hypoxia for 6 h. Deletion of sequence up to −98 bp abolished the inducibility of luciferase activity by hypoxia. These results show that the sequence between −133 bp and −98 bp is involved in the IL-8 gene activation by hypoxia.

It has been documented that −126 to −120 is the possible AP-1 binding site and the region from −94 to −71 bp shows sequence similarity with potential binding sites for NF-κB-like factor (−80 to −71) and C/EBP-like factor NF-IL-6 (−94 to −81 bp; Refs. 29–31). This region is a minimal essential element in conferring the responsiveness to IL-1, tumor necrosis factor α, and phorbol 12-myristate 13-acetate in a human fibrosarcoma cell line (29–31). To determine whether these cis-elements are essential for responsiveness to hypoxia, point-mutated luciferase reporter plasmid (Fig. 6C) for these regions were transfected into SKOV3 i.p.1 cells. The mutation of NF-κB and AP-1 binding sites abolished the responsiveness to hypoxia, whereas that of NF-IL-6 binding site did not (Fig. 6D). These results indicate that AP-1 and NF-κB-like factor binding elements were mainly responsible for the induction of the IL-8 gene in human ovarian cancer cells by hypoxia and that IL-8 transcription activation by hypoxia required both NF-κB and AP-1 binding sites.

**Hypoxia-mediated Activation of DNA-binding Proteins NF-κB and AP-1.** In the next set of experiments, we used gel mobility shift assay to identify the hypoxia-induced factors that bind to the identiﬁed promoter regions of the IL-8 gene. Radioactively labeled NF-κB and AP-1 probes were incubated with nuclear protein extracts from SKOV3 i.p.1 cells that had been exposed to either normoxia or hypoxia. Nuclear proteins extracted from unstimulated normoxic cells did not form any complexes (Fig. 7A and B). Exposure of the SKOV3 i.p.1 cells to hypoxic conditions for 1 h or more resulted in a substantial increase in the binding activity of NF-κB (Fig. 7A) and AP-1 (Fig. 7B), indicated as the hypoxia-enhanced species. The binding of this protein to the probe was sequence-specific because it was blocked by preincubation with an unlabeled NF-κB or AP-1 oligonucleotide.
by competition with an unlabeled NF-κB or AP-1 oligomer but not by an unrelated SP-1 oligomer.

Next, we carried out immunological assays to identify the protein factors that are induced by hypoxia and bind to the NF-κB and AP-1 sites using antibodies against several members of the NF-κB and Fos/Jun family of transcription factors. The addition of either anti-p65 antibody or anti-p50 antibody induced a super-shifted band (Fig. 7C), which indicated that p65 and p50 are induced by hypoxia and can bind to the NF-κB site of the IL-8 gene in SKOV3 i.p.1 cells. We next performed supershift assays using antibodies against c-Jun, c-Fos, Jun-B, and Jun-D. A supershifted band was detectable with antibody only against c-Jun (Fig. 7D), indicating that binding to the AP-1 complex is increased during hypoxia and that this DNA-protein binding complex contains c-Jun.

DISCUSSION

The present results demonstrate that hypoxia up-regulates expression of IL-8 mRNA and protein in human ovarian cancer SKOV3 i.p.1 and Hey-A8 cells. Hypoxia induced the binding activity of transcription factors AP-1 and NF-κB and the transcriptional activation of the IL-8 promoter, and it increased the stability of IL-8 mRNA. The relevance of hypoxia-mediated up-regulation was examined in surgical specimens of human ovarian carcinoma. Immunohistochemistry and ISH revealed that specific IL-8 mRNA and protein were predominantly expressed by cells surrounding necrotic areas.

IL-8 is expressed in normal human ovarian cells (32) and in human ovarian cancer cells (6). High expression of IL-8 mRNA has been detected in clinical specimens of late-stage ovarian carcinomas (Fig. 1) and in malignant ascites (33). Whether IL-8 acts as an autocrine growth factor or an angiogenic factor in ovarian cancer is unclear, but, in any case, several biological functions of IL-8 could be of significance to the pathology and treatment of ovarian cancer (34, 35).

Hypoxia has been shown to up-regulate expression of IL-8 in human endothelial cells (36), in glioblastoma cells (28), and in monocytes during reoxygenation (37). Our present results show that IL-8 is up-regulated in cells surrounding zones of necrosis in human ovarian carcinomas. Because cells surrounding necrotic areas are likely to undergo severe hypoxic stress, we hypothesized that hypoxia can induce IL-8 expression in ovarian carcinoma. IL-8 expression, however, can also be regulated by many proinflammatory cytokines (38); hence, it is unclear whether IL-8 up-regulation in ovarian cancers resulted from stimulation by proinflammatory cytokines (for example, IL-1, or tumor necrosis factor) that were secreted by leukocytes or result from hypoxia.

Hypoxia induced dramatic up-regulation of IL-8 mRNA and protein in the highly aggressive Hey-A8 and SKOV3 i.p.1 human ovarian cancer cells in the absence of inflammatory cells. The rapid induction and the subsequent decline of IL-8 mRNA to baseline levels on reoxygenation is highly suggestive of a transcriptional regulation and posttranscriptional mRNA stabilization, such as has been shown for...
VEGF/VPF (39, 40). A nuclear run-on assay showing that the transcriptional rate of IL-8 gene was increased 7-fold after 6 h of incubation in hypoxic condition indicated that up-regulation of IL-8 mRNA was mediated by transcriptional activation. Because the increase in transcription (7-fold) did not account for the total increase in IL-8 mRNA (13-fold), we studied the posttranscriptional regulation by measuring half-lives of IL-8 mRNA in SKOV3 i.p.1 cells under normoxia and hypoxia and found that the half-life of IL-8 mRNA in a hypoxic environment was significantly prolonged. Thus, the regulation of the IL-8 gene by hypoxia appeared to be similar to the regulation of the VEGF/VPF gene (39–41); that is, hypoxia increased the mRNA of both genes through both transcriptional activation and increased stability.

Increased transcription of the IL-8 gene in cells exposed to hypoxia

Fig. 7. Gel mobility shift assay. In A, nuclear proteins were extracted from SKOV3 i.p.1 cells incubated in normoxia or in hypoxia for 1, 3, and 6 h. Binding reactions were performed as described in “Materials and Methods.” All of the lanes contain the labeled NF-κB probe. Arrow, hypoxia enhanced species. In B, binding reactions were performed with the same nuclear extracts as described above. All of the lanes contain the labeled AP-1 probe. Arrow, hypoxia enhanced species. C, supershift assay. Antibodies against p65, p50, and c-rel were incubated with the gel shift binding reaction. All of the lanes contain labeled NF-κB probe. Arrow, supershifted bands by anti-p65 or anti-p50 antibodies. D, supershift assays. Antibodies against c-Jun, c-Fos, Jun B, and Jun D were incubated with the gel shift binding reaction. All of the lanes contain labeled NF-κB probe. Arrow, supershifted band by anti-c-Jun antibody. FP, unbound free probe.
is mediated by specific sequences located in the 5’-flanking region in the IL-8 gene. Transient transfection experiments using serial deletion constructs identified a region from −133 to −98 relative to transcription start site, which is essential for transcriptional activation by hypoxia. The region from −133 to −70 contains the binding site for AP-1, NF-IL-6, and NF-kB. The relative importance of these three elements varies in a cell type-specific manner. The NF-kB binding site is indispensable for IL-8 gene expression; in all of the cell types, it combines with either NF-IL-6 or AP-1 binding sites, depending on the type of cells (42). Our data show that mutation of the AP-1 and NF-kB binding sites abolished transcriptional activation of the IL-8 gene during hypoxia, which suggests that the NF-kB and AP-1 binding sites are indispensable for IL-8 gene expression during hypoxia. Mobility shift assays performed with either AP-1 or NF-kB probes concluded that the binding activity of both AP-1 and NF-kB was increased during hypoxia.

Several transcription factors, including HIF-1 (43), AP-1 (44), and NF-kB (45), are known to be regulated by hypoxia. The AP-1-binding complex consists of either Jun-Fos heterodimers or Jun-Jun homodimers (46). In the SKOV3 i.p.1 cells exposed to hypoxia, the DNA-protein-binding complex contained c-Jun proteins. The activated form of NF-kB is heterodimer, which usually consists of two proteins, a p65 subunit and a p50 subunit. Exposure of SiHa squamous carcinoma cells to hypoxia increased NF-kB DNA-binding activity, consisting mainly of p65 and p50 subunits (47). Several studies have shown that AP-1 and NF-kB are differentially activated by oxygen tension. In HeLa cells, AP-1 was strongly induced during hypoxia, NF-kB was not activated under hypoxic conditions but rapidly and strongly activated during reoxygenation (48). A direct comparison in human squamous carcinoma cells of NF-kB and AP-1 induction showed a rapid increase in NF-kB activity, whereas AP-1 activation was detectable only after prolonged hypoxia (47). In contrast to these studies, we observed rapid activation of both AP-1 and NF-kB in SKOV3 i.p.1 cells during hypoxia. This suggests that the requirement and activation of transcription factors during hypoxia varies in a cell type-specific manner.

HIF-1 is widely expressed in mammalian cells (49). Transcriptional up-regulation of the VEGF/VPF gene during hypoxia is dependent on transactivation by the transcription factor HIF-1 (50), which binds to a HIF-1 consensus site located in the 5’-flanking region of the VEGF/VPF gene (51). HIF-1, however, is not likely to play a direct role in hypoxic regulation of IL-8 because no HIF-1-binding motif in the IL-8 promoter region (52), yet HIF-1 protein production is significantly induced in SKOV3 i.p.1 cells during hypoxia (data not shown). Recent findings show that p300, a general transcriptional activator, specifically binds HIF-1α. p300 may, therefore, play a critical role in transducing the signal from the HIF-1 to the apparatus responsible for the initiation of transcription (53). Whether p300 participates in the regulation of IL-8 is unclear.

Studies of the posttranscriptional regulatory mechanisms that modulate stability of VEGF/VPF mRNA during induction by hypoxia identified a 126-base HSR in VEGF/VPF 3’-untranslated region (54), which is critical for the stabilization of VEGF/VPF mRNA under hypoxia. Recently, a protein that interacts with the VEGF/VPF HSR in hypoxic cells has been identified (55). Whether IL-8 mRNA contains this HSR in the 3’-untranslated region remains unknown. In summary, we demonstrate that hypoxic human ovarian carcinoma cells overexpress IL-8 mRNA and protein. The hypoxia-mediated transient induction of IL-8 expression is due to an increase in IL-8 mRNA stability and transcriptional activation of the IL-8 gene promoter via cooperation of AP-1 and NF-kB. We are currently examining whether differential induction of IL-8 gene expression by a hypoxic environment can contribute to the growth, angiogenesis, and metastasis of human ovarian carcinomas.

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