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

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ABSTRACT

The expression of interleukin 8 (IL-8) by human ovarian cancer cells correlates directly with disease progression, but the exact mechanism of IL-8 induction is not clear. The extracellular pH in solid tumors is generally acidic because of elevated acid production and impaired clearance of acidic metabolic wastes. We determined whether acidic conditions also regulate the expression of IL-8 in human ovarian cancer cells. Culturing SKOV3 ip1 ovarian cancer cells in acidic medium (pH 6.6) significantly increased IL-8 mRNA (Northern blot) and protein (ELISA). The acidosis-mediated transient increase in IL-8 expression involved both transcriptional activation of the IL-8 gene and enhanced stability of the IL-8 mRNA. 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 acidosis. Point-mutated luciferase reporter studies indicated that activator protein-1 (AP-1) and nuclear factor-κB (NF-κB)-like factor were responsible for acidic pH-induced transcriptional activation of the IL-8 gene, and EMSA demonstrated that both NF-κB and AP-1 bound to these sites on the IL-8 promoter. These results indicate that acidic pH activates NF-κB and AP-1 in human ovarian cancer cells and in doing so increases IL-8 gene expression.

INTRODUCTION

To grow beyond 1 mm in diameter, tumors must induce the development of a new vascular bed (1, 2). Despite the exuberant angiogenesis characteristic of the edge of some tumors, the overall tumor vasculature is poorly organized and only marginally functional (3–5). Because of structural abnormalities in tumor vasculature, perfusion of the tumor tissue is heterogeneous in regard to region and time (6–8). Cells farther than 100–200 μm from the nearest functional blood vessel experience low influx of metabolites and a low efflux of potentially toxic metabolic wastes (5, 9). This elevated anaerobic glycolysis and impaired clearance of acidic metabolic wastes lead to a low extracellular pH (10–12). Indeed, microelectrode measurements in human and rodent solid tumors demonstrated that pHt has a broader distribution and is on average 0.5 pH units more acidic than normal tissue pHt (13, 14).

Low environmental pH has been shown to inhibit cell proliferation, survival, and activity under in vitro conditions (15–18). Low tumor pH has been shown to determine the degree of cell response to radiation and chemotherapy, with cells in an acidic microenvironment being less sensitive to drugs that are active against cycling cells (19). It also increases the invasive potential of murine and human melanoma cells (20) and has been implicated in the induction of metastasis (19). Hypoxia, another common feature in solid tumors (5, 21), has been implicated in the induction of metastasis (22) and increases the invasive potential of murine and human melanoma cells (20). Hypoxia, another common feature in solid tumors (5, 21), has been implicated in the induction of metastasis (22) and increases the invasive potential of murine and human melanoma cells. It also increases the invasive potential of murine and human melanoma cells. Hypoxia, another common feature in solid tumors (5, 21), has been implicated in the induction of metastasis (22) and increases the invasive potential of murine and human melanoma cells.

How IL-8 gene expression is regulated in human ovarian cancer cells is unclear. A local decrease in oxygen tension and nutrients attributable to inadequate vasculature can induce expression of VEGF/VPF (38), and recent data show that hypoxia can also induce expression of the IL-8 gene (39, 40). Because hypoxia in neoplasms is often associated with low pH, we wished to determine whether an acidic pH environment could also regulate IL-8 expression in human ovarian cancer cells. Our exposure of human ovarian cancer cells to acidic medium (pH 6.6) rapidly induced transcription of the IL-8 gene (by transactivation of the IL-8 promoter) and increased stability of the IL-8 message.

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 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, established from ascitic fluids, was obtained from the American Type Tissue Culture Collection (Rockville, MD; Ref. 41). The SKOV3 ip1 variant was derived from ascites arising in a nude mouse given an i.p. injection of SKOV3 cells (42).

The tumor cell lines were maintained as monolayer cultures in RPMI 1640 supplemented with 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. To vary the medium’s pH, we added 20 mM 2-(N-morpholino)ethane-sulfonic acid and 20 mM Tris (hydroxymethyl) aminomethane.

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.

Northern Blot Analysis. mRNA was extracted by using the FastTrack mRNA isolation kit (Invitrogen, San Diego, CA). mRNA (2 μg/lane) was fractionated on a 1% denaturing formaldehyde/agarose gel, electrophoresed at 0.6 amp to GeneScreen nylon membrane (DuPont, Boston, MA), and 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 corre-
sponding to human IL-8 (a generous gift of Dr. K. Matsuhashi, Kanazawa University, Kanazawa, Japan). 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). In preliminary studies, we incubated the ovarian cancer cells in media with pH ranging from 7.3 to 6.3. Northern blot analysis for expression of IL-8 revealed induction at pH 7.0 and that the level of expression inversely correlated with pH. We chose pH 6.6 for all experiments because of the significant enhancement of IL-8 expression and its relevance to physiological conditions (15–21).

**Measurement of IL-8 Production by ELISA.** Culture supernatants of human ovarian carcinoma cells cultured in normal medium (pH 7.3) or in acidic medium (pH 6.6) 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 to the standard curve.

**mRNA Stability Assay.** The half-life of IL-8 mRNA was determined by treating SKOV3 ip1 cells with actinomycin D as described by Lindholm et al. (43). SKOV3 ip1 cells were cultured in normal pH medium or in acidic pH medium for 6 h, and then actinomycin D (Sigma, St. Louis, MO) was added into the medium (5 µg/ml) to block transcription. Immediately after the addition of actinomycin D, the cells were returned to the same culture conditions. mRNA was prepared at 0, 1, 2, 4, and 8 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 ip1 cells (1 x 10^5) were seeded into each 150-mm tissue culture dish and incubated overnight at 37°C. The cells were incubated in normal medium (pH 7.3) or in acidic medium (pH 6.6) for 3 h. The nuclei were isolated, aliquoted, and stored in a −80°C freezer. For each in vitro transcription reaction, 100 µl of nuclei from each sample were thawed and mixed with equal volumes of 2× reaction buffer containing 100 mM MgCl2, 10 mM DTT, 300 mM KCl, 100 mM HEPES (pH 7.4), 1 µM each of ATP, CTP, and GTP, and [α-32P]UTP (200 µCi, 3000 Ci/mmol; Amersham Corp., Arlington Heights, IL). The reaction mixture was incubated at 30°C for 30 min, and the 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 binding reaction. Where indicated, anti-p65, anti-p50, anti-c-Rel (Calbiochem, San Diego, CA) and anti-RelA, anti-c-Fos, anti-Jun B, and anti-Rel antibody (Santa Cruz Biotechnology, 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.

**Immunoblot Analysis.** Nuclear extracts were prepared as described above. Protein extracts were electrophoresed through SDS-polyacrylamide gels and transferred to nitrocellulose membranes using standard procedures. Proteins were detected with anti-HIF-1α (Transduction Laboratory, Lexington, KY).

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

**RESULTS**

**Induction of IL-8 Production in Human Ovarian Carcinoma Cells by Acidic pH.** In the first set of experiments, SKOV3 ip1 cells were incubated under normal (pH 7.3) or acidic (pH 6.6) conditions for 1, 3, or 6 h. Culture supernatants were collected for ELISA analysis. The exposure of SKOV3 ip1 cells to the acidic environment increased the steady-state levels of IL-8 mRNA in a time-dependent manner (Fig. 1A). IL-8 mRNA increased by 3 h of incubation in acidic culture and reached an 11-fold increase by 6 h. No discernible differences in amount were found among the samples of β-actin. Consistent with the increase in mRNA expression, SKOV3 ip1 cells released an increased amount of IL-8 protein into the culture medium by 12 h of incubation in acidic medium, reaching a maximum of 2332 ± 36 pg/ml (Fig. 1B).

Collectively, these data indicate that acidic pH induced and enhanced expression of IL-8 in human ovarian cancer cells.

**Acidic pH-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 ip1 cells were first incubated under normal pH (7.3) or acidic pH (6.6) conditions for 6 h; the medium was then replaced, and the cells were incubated in pH 7.3 medium for 0, 0.5, 1, and 3 h. IL-8 expression was determined by Northern blot analysis (Fig. 2A). The initial incubation (6 h) in acidic pH
pH medium induced a 14-fold increase in IL-8 mRNA. However, once the cells were returned to normal pH medium for 0.5 h, the IL-8 transcript decreased, and within 3 h, IL-8 mRNA reached basal levels (Fig. 2). Thus, the up-regulation of IL-8 by acidic pH was transient.

Transcriptional Regulation of IL-8 Expression by Acidic pH. Next, we determined, using a nuclear run-on assay, whether acidic pH increases the transcription rate of IL-8 in SKOV3 ip1 cells. Consistent with Northern blot analysis data, the transcription rate of IL-8 (but not β-actin) in SKOV3 ip1 cells increased by 9-fold in cells incubated for 3 h in acidic pH conditions (Fig. 3).

Regulation of IL-8 mRNA Stability by Acidic pH. To evaluate the effect of acidic pH on IL-8 mRNA stability, we measured the half-life of IL-8 mRNA in SKOV3 ip1 cells incubated in normal pH medium and in acidic pH medium for 6 h. Cells were then treated with actinomycin D to stop transcription. mRNA was collected at 0, 1, 2, 4, and 8 h, and Northern blot analysis was performed. As shown in Fig. 4A, in 7.3 pH medium, IL-8 mRNA decreased rapidly with a half-life of 1 h or less. In contrast, in acidic conditions, a high level of IL-8 mRNA was still evident even after 9 h. The half-life was longer than 9 h (the longest time point taken; Fig. 4B), indicating that acidic pH increases IL-8 mRNA stability. These data and the nuclear run-on studies indicate the induction of IL-8 mRNA in SKOV3 ip1 cells by low pH is attributable to both transcriptional activation and mRNA stabilization.

Identification of Acidic pH-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 acidic pH. To characterize the DNA sequences involved in IL-8 gene transcriptional activation by acidic pH, we used luciferase reporter plasmids in which the IL-8 5′-flanking sequences were fused to the firefly luciferase coding sequences (Fig. 5).
The region from −133 to −70 contains the binding site for AP-1, NF-IL-6, and NF-κB (44, 45). To determine which one of these cis elements is essential for the responsiveness to acidic pH, we examined the effects of mutation of each cis element on IL-8 promoter-driven luciferase activity in SKOV3 ip1 cells during acidosis. Luciferase reporter plasmids for these regions were transfected into SKOV3 ip1 cells. pRL-TK was cotransfected into SKOV3 ip1 cells as an internal control to monitor transfection efficiency. The transfected SKOV3 ip1 cells were incubated under normal pH (pH 7.3) or acidic pH (pH 6.6) for 6 h, and the induced luciferase activity was measured using a dual luciferase assay kit. The mutation of NF-κB and AP-1 binding sites abolished the responsiveness to acidic pH, whereas that of the NF-IL-6 binding site did not (Fig. 5). 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 acidic pH and that IL-8 transcription activation by acidic pH required both NF-κB and AP-1 binding sites.

Acidic pH-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 acidic pH-induced protein factors that bind to the identified promoter regions of the IL-8 gene. Radiolabeled NF-κB and AP-1 probes were incubated with nuclear protein extract from SKOV3 ip1 cells that had been incubated in either normal medium (pH 7.3) or acidic medium (pH 6.6). Nuclear proteins extracted from cells incubated in normal medium did not form any complexes (Fig. 6, A and B), whereas exposure of the

Fig. 6. Gel mobility shift assay. A, nuclear proteins were extracted from SKOV3 ip1 cells incubated in normal or acidic conditions for 1, 3, and 6 h. Binding reactions were performed as described in Materials and Methods. All lanes contain the labeled NF-κB probe. Arrow, acidic pH-enhanced species. B, binding reactions were performed with the same nuclear extracts as described above. All lanes contain the labeled AP-1 probe. Arrow, acidic pH-enhanced species. C, supershift assay. Antibodies against p65, p50, and c-rel were incubated with the gel shift binding reaction. All lanes contain the 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 lanes contain the labeled AP-1 probe. Arrow, supershifted band by anti-c-Jun antibody.
SKOV3 ip1 cells to acidic conditions for 1 h or more substantially increased the binding activity of NF-κB (Fig. 6A) and AP-1 (Fig. 6B), indicated as the acidic pH-enhanced species. The binding of this protein to the probe was sequence specific, because it was blocked by competition with an unlabeled NF-κB or AP-1 oligomer but not by an unrelated SP-1 oligomer.

Next, we performed immunological assays to identify the protein factors that are induced by acidic pH and bind to the NF-κB and AP-1 site, 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 supershifted band (Fig. 6C). These results indicate that p65 and p50 were induced by acidic pH and bound to the NF-κB site of the IL-8 gene in SKOV3 ip1 cells. We next performed supershift assays using antibodies against the AP-1 family members c-Jun, c-Fos, Jun B, and Jun D. A supershifted band was only detectable with antibody against c-Jun (Fig. 6D), indicating that binding to the AP-1 complex was increased in acidic pH environment and that this DNA-protein binding complex contained c-Jun.

Expression of HIF-1α Protein in Human Ovarian Cancer Cells. In the last set of experiments, we determined whether HIF-1α, which mediates induction of many genes in hypoxic cells, is also involved in pH-enhanced expression of IL-8 in SKOV3 ip1 cells. Previous data from our laboratory demonstrated that hypoxia can induce expression of IL-8 in human ovarian cancer cells. We measured the effect of pH on HIF-1 expression, because exposure of mammalian cells to hypoxia is known to up-regulate expression of HIF-1, which binds to hypoxia enhancer regions of many genes (46). HIF-1α was not detected in nuclear proteins extracted from SKOV3 ip1 cells incubated under normoxic conditions. In contrast, HIF-1α expression was highly elevated in SKOV3 ip1 cells incubated under hypoxic conditions but not in SKOV3 ip1 cells incubated in acid medium under normoxic conditions (Fig. 7). Because cells incubated in acid medium express high levels of IL-8, the expression of HIF-1α per se is not a prerequisite for the up-regulation of IL-8 expression.

**DISCUSSION**

The present results demonstrate that acidic pH, which is often found in solid neoplasms, up-regulates the expression of IL-8 mRNA and protein in human ovarian carcinoma cells. Acidic conditions (pH 6.6) induced the binding activity of transcription factors AP-1 and NF-κB and the transcriptional activation of the IL-8 promoter. Acidic pH also increased the stability of IL-8 mRNA. Thus, both transcriptional and posttranscriptional mechanisms were responsible for the increase in IL-8.

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 (26). IL-8 promotes proliferation of tumor cells (28–30), induces angiogenesis (27), and modulates secretion of collagenase (31). 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 capabilities (32–37).

IL-8 is expressed in normal ovarian cells (47) and in human ovarian cancer cells (34, 48). We have found that the expression of IL-8 by human ovarian cancer cells directly correlates with their aggressive nature (34), and high expression of IL-8 has been detected in malignant ascites (49) and in tumors treated with chemotherapy (50). How IL-8 expression is regulated in ovarian cancer cells remained unclear. We hypothesized that one cause might be acidic pH, which is associated with tumor tissues. Low pH (<6.8) has been shown to induce the synthesis of metalloproteinases (51), transcription of both glucose-regulated and heat shock proteins (52, 53), and expression of inducible nitric oxide synthase (54). Acidic pH has recently been shown to enhance expression of VEGF and basic fibroblast growth factor as well as secretion of basic fibroblast growth factor (55). Our study shows for the first time that an acidic environment can also increase the expression of IL-8 in human cancer cells by transcriptional activation of the gene and stabilization of the message.

One h after incubation of human ovarian carcinoma cells in acid medium (pH 6.6), the steady-state level of IL-8 mRNA increased 2-fold, suggesting transcriptional activation of the gene. After a 3-h incubation in acid medium, IL-8 gene transcription increased 9-fold (determined by a nuclear run-on assay). The increased transcription was mediated by specific sequences located in the 5′ flanking region in the IL-8 gene. We base this conclusion on the following. IL-8 promoter activity appears to be regulated by the differential activation and binding of the AP-1, NF-IL-6, and NF-κB families of transcription factors (56–58). The NF-κB binding site is indispensable for IL-8 gene expression in every cell type examined thus far (44, 45), albeit cooperation with either AP-1 or NF-IL-6 is required for optimal IL-8 gene activation in several cell types (58). Previous reports concluded that the region from −126 to −120 bp relative to the transcription start site contains the binding site for AP-1, the region from −80 to −71 bp, the binding site for NF-κB-like factor, and the region from −94 to −81 bp, the binding site for CCAAT/enhancer binding protein-like factor NF-IL-6 (44, 45). In our transient transfection experiments, we used serial deletion constructs to demonstrate that mutation of the AP-1 and NF-κB binding sites abolished increased transcriptional activation of the IL-8 gene expression at acidic pH, suggesting that NF-κB and AP-1 are indispensable to the induction of IL-8 expression by an acidic microenvironment. Moreover, EMSA revealed that acidic pH induced the formation of NF-κB and AP-1 complexes and that the NF-κB complexes were supershifted by antibodies against either p65 or p50 but not other members of the NF-κB family. The AP-1 complexes were supershifted by an antibody against c-Jun but not other AP-1 family members. Collectively, the data show that acidic pH induced the binding of p65/p50 heterodimers and c-Jun homodimers to the corresponding cis elements, enhancing the activation of the IL-8 gene.

Although the signaling events linking hypoxia to gene expression have received a great deal of attention (59, 60), little is known about the signaling events induced by an acidic environment. Most tumor cells can maintain an intracellular pH in or near the physiological range even in an acidic environment, suggesting that tumor cells are capable of regulating the level of intracellular pH (61–63). Our present results show that NF-κB and AP-1 are the pH response elements in human ovarian carcinoma cells. Both NF-κB and AP-1 have been shown to be redox-responsive transcription factors (64–66). Oxidants increase the transcription of the c-fos and c-jun genes (66), and both AP-1 and NF-κB DNA binding activity is regulated by...
the redox state of cysteine residue in the subunit (64, 66). Acidic pH induced expression of iNOS is also mediated by NF-kB (p50/p50 and p50/RelA; Ref. 54). Previous studies from our group have shown that hypoxia induces expression of IL-8 in human ovarian cancer cells by activating NF-kB and AP-1 (39). The present study reveals that AP-1 and NF-kB were also activated in acidic conditions. Sensing of pH and redox status are related through oxidative coupling between the respiratory chain and the electrochemical proton gradient (67), but the pathway that leads to the activation of transcription factors and eventually to gene regulation may not be the same and remains unknown.

HIF-1 plays a major role in mediating transcription of the VEGF/ VPF gene in response to hypoxia (46, 68). Because no HIF-1 binding motif has been detected in the published sequence of the IL-8 promoter region (69), HIF-1 may play an indirect role in hypoxic regulation of IL-8. Yet HIF-1 protein production is significantly induced in SKOV3 ip1 cells during hypoxia. Recent findings show that p300, a general transcriptional activator, binds specifically to HIF-1, suggesting that p300 may transduce the signal from HIF-1 to the apparatus responsible for the initiation of transcription (70, 71). In an acidic environment, however, HIF-1α was not induced, suggesting different mechanisms and pathways in regulating gene expression by hypoxia and acidic pH, and that HIF-1α is not crucial for expression of IL-8, agreeing with the recent data by D’Arcangelo et al. (55).

IL-8 expression is regulated at both the level of gene transcription (45) and the level of posttranscriptional control (72–74). In acidic environments, the increase in transcription alone does not account for all of the increase in IL-8 mRNA, and indeed, our data show that posttranscriptional regulation of IL-8 mRNA stability also plays a critical role (Fig. 4). The half-life of an mRNA can be determined by interactions of transacting factors with a specific cis element located within the 3′-untranslated regions (75, 76). A 126-base hypoxia stability region has been identified in human VEGF 3′-untranslated region that is critical for the stabilization of VEGF mRNA under hypoxia (77), and the protein that interacts with it has been identified (78). The cis and trans elements that stabilize the IL-8 mRNA in acidic tumor cells remain to be identified.

In summary, incubation of human ovarian cancer cells under acidic conditions (6.6 pH) increased expression of the IL-8 gene. The acidic pH-mediated transient induction of IL-8 gene expression was attributable to both transcriptional activation and enhanced mRNA stability. The transcriptional activation of the IL-8 gene by acidic pH was mediated by the activation of transcription factors NF-κB and AP-1. Because low pH, hypoxia are common characteristics of many solid neoplasms, their combined effect on expression of IL-8 by cancer cells is of obvious importance and a subject of an independent study.

Besides demonstrating a relation between pH and IL-8, our data suggest that pH be considered carefully in assessing tumor cell behavior. A large body of data demonstrate that tumor extracellular pH is more acidic than that of normal tissues. Nevertheless, most in vitro assays measuring tumor cell function are routinely performed at neutral-to-alkaline pH (79). This may lead to significant inaccuracy in understanding tumor cell behavior in vivo, because each pH unit shift represents a 10-fold change in H+ concentration, and slight changes in pH can have profound effects on cell phenotype and gene expression.

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