Activation of Epidermal Growth Factor Receptor/p38/Hypoxia-Inducible Factor-1α is Pivotal for Angiogenesis and Tumorigenesis of Malignantly Transformed Cells Induced by Hexavalent Chromium

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Hexavalent chromium (Cr(VI))-containing compounds are well established environmental carcinogens. Most mechanistic investigations of Cr(VI)-induced carcinogenesis focus on oxidative stress and various cellular responses, leading to malignant cell transformation or the first stage of metal-induced carcinogenesis. The development of malignantly transformed cells into tumors that require angiogenesis is the second stage. This study focuses on the second stage, in particular, the role of EGFR signaling in angiogenesis and tumorigenesis of Cr(VI)-transformed cells. Our preliminary studies have shown that EGFR is constitutively activated in Cr(VI)-transformed cells. The results show that Cr(VI)-transformed cells are angiogenic. Hypoxia-inducible factor-1α, pro-angiogenic protein matrix metalloproteinase 1, and VEGF are all highly expressed in Cr(VI)-transformed cells, in lung tissue from non-smoking worker occupationally exposed to Cr(VI) for 19 years. Using in vitro and in vivo models, the present study has investigated the role of EGFR in angiogenesis of Cr(VI)-transformed cells. The results show that Cr(VI)-transformed cells are angiogenic. Hypoxia-inducible factor-1α, pro-angiogenic protein matrix metalloproteinase 1, and VEGF are all highly expressed in Cr(VI)-transformed cells, in lung tissue from non-smoking worker occupationally exposed to Cr(VI) for 19 years. Inhibition of EGFR by its shRNA or kinase inhibitor reduced p38 MAPK, resulting in decreased expression of hypoxia-inducible factor-1α, metalloproteinase 1, and VEGF, leading to suppressions of angiogenesis and tumorigenesis. Overall, the present study has demonstrated that EGFR plays an important role in angiogenesis and tumorigenesis of Cr(VI)-transformed cells.

Hexavalent chromium (Cr(VI))-containing compounds are toxic and carcinogenic. Occupational and environmental exposure to Cr(VI) are associated with a high rate of lung cancer (1–4). The International Agency for Research on Cancer has classified Cr(VI) as group 1 human carcinogens in environmental and occupational settings (1). Most investigations of Cr(VI) carcinogenesis focus on the first stage of carcinogenesis, in which normal cells transform to malignant cells. There is a great need to investigate the second stage of carcinogenesis: transformed cells progress to tumor. Angiogenesis plays an important role in this second stage.

Angiogenesis, the formation of new blood vessels from existing vasculature, is essential for tumor initiation and progression (5, 6). A number of different growth factors and cytokines influence proliferation and migration of microvascular endothelial cells and regulate angiogenesis (7, 8). Previous studies have demonstrated that signaling through tyrosine kinase EGFR receptor (EGFR) regulates pro-angiogenic factors in tumor cells of different histological origin (9–11). Our previous study has observed that EGFR was constitutively activated in Cr(VI)-transformed human bronchial epithelial BEAS-2B cells (12). Inhibition of EGFR by its shRNA or kinase inhibitor reduced tumorigenesis of Cr(VI)-transformed cells, indicating the essential role of EGFR in Cr(VI)-induced tumorigenesis (12). It has been reported that activated EGFR phosphorylated p38 MAPK (13). The p38 MAPK pathway is an intracellular signaling pathway important in angiogenesis and cancer development (Ref. 14; see comment in PubMed Commons below).

Matrix metalloproteinases (MMPs) are highly expressed in many cancers, and their up-regulations are associated with invasive tumor growth and poor prognosis (15–17). MMP-1, a zinc-dependent matrix metalloproteinase, is an interstitial collagenase. It plays a major role in invasive growth of tumor cells (18). This protein is frequently overexpressed in a large number of human cancers (19–21) and is produced by various types of cells, including cancer cells, fibroblasts, and inflammatory cells.
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(22–24). It has been reported that high MMP-1 level in patient lung tumor tissue is associated with metastasis of non-small cell lung cancer (25, 26). MMP-1 knock-out mice exhibit reduced growth and angiogenesis of lung tumors (27). A previous study has observed that treatment of human U937 macrophages with Cr(III) elevates MMP-1 expression (28). Recent studies have indicated that EGFR mediates synthesis and function of MMPs in epithelial cells (29–31). However, the question concerning how EGFR regulates these pro-angiogenic proteins remains to be answered. The present study investigated the role of constitutive activation of EGFR in angiogenesis of Cr(VI)-transformed cells and mechanisms concerning how EGFR regulates angiogenesis by focusing on the p38 MAPK/hypoxia-inducible factor-1α (HIF-1α) pathway.

Results

Increased Angiogenesis of Cr(VI)-transformed Cells—Our previous study has shown that chronic exposure of human bronchial epithelial BEAS-2B cells to Cr(VI) is able to cause malignant cell transformation. Those transformed cells are tumorigenic (12). Angiogenesis is a vital pathological process during tumorigenesis. To investigate whether Cr(VI)-transformed cells exhibit elevated angiogenesis, three approaches were used: in vitro tube formation of HMLECs, ex ovo chicken chorioallantoic membrane (CAM) blood vessel formation, and in vivo Matrigel plug angiogenesis assay. As shown in Fig. 1A, tube formation was observed in Cr(VI)-transformed cells (middle panel) but not in passage-matched normal cells (left panel). The results from quantitation of tube length show that tube formation in Cr(VI)-transformed cells increased by 100% compared with that in passage-matched normal cells (Fig. 1A, right panel). The results from CAM assay show that there were abundant blood vessels and vessel branches in Cr(VI)-transformed cells but not in the passage-matched normal ones (Fig. 1B, left and middle panels). Quantitative analysis revealed that the number of newly formed blood vessels in Cr(VI)-transformed cells increased 3-fold compared with that in passage-matched normal cells (Fig. 1B, right panel). To further verify elevated angiogenesis in Cr(VI)-transformed cells, in vivo Matrigel plug assay was performed. The plugs containing Cr(VI)-transformed cells exhibit a red color, indicating new blood vessel formation (angiogenesis) in the plugs (Fig. 1C, middle panel). The plugs containing passage-matched normal BEAS-2B cells were light red or pale pink, indicating less blood vessel formation (Fig. 1C, left panel). The extent of angiogenesis was also quantified by measuring hemoglobin content in the plugs. The results show that the hemoglobin concentration in the plugs containing Cr(VI)-transformed cells was 16.9 μg/mg Matrigel (Fig. 1C, right panel). It was 1.2 μg/mg Matrigel in the plugs containing passage-matched normal cells (Fig. 1C, right panel). These results demonstrate increased angiogenesis in Cr(VI)-transformed cells.

Up-regulation of Pro-angiogenic Genes in Cr(VI)-transformed Cells—Given the observation of increased angiogenesis in Cr(VI)-transformed cells, a total of 43 angiogenic proteins were examined using angiogenesis antibody array. Fig. 2A shows that expression of angiogenin (dot 3), IL-6 (dot 4), and MMP-1 (dot 2) was increased in Cr(VI)-transformed cells compared those in passage-matched normal cells. mRNA levels of these genes were also increased in these cells (Fig. 2B). Angiogenic factors are responsible for increased angiogenesis. Among those factors, VEGF and MMP-1 are key proteins to induce angiogenesis. Our results show that expression of VEGF and MMP-1 was markedly increased in Cr(VI)-transformed cells (Fig. 2C). VEGF concentrations secreted from BEAS-2B and Cr(VI)-transformed cells were also measured. The results show that VEGF concentration was increased 4-fold in Cr(VI)-transformed cells compared that in normal ones (Fig. 2D). The HIF-1α level was dramatically increased in Cr(VI)-transformed cells (Fig. 2E). Expression of these angiogenic proteins in lung tissues from animals exposed to Cr(VI) for 12 weeks was examined. High expressions of MMP-1, HIF-1α, and VEGF were observed in Cr(VI)-exposed lung, but not in the control without Cr(VI) exposure (Fig. 2, F–H). Expression of MMP-1, HIF-1α, and VEGF in normal lung tissue from a non-smoking healthy patient and in lung tumor and its adjacent normal tissues from a non-smoking worker exposed to Cr(VI) for 19 years with diagnosis of stage 1 squamous lung carcinoma were detected. The results show that MMP-1 was highly expressed in lung tumor tissue and that it was much less expressed in adjacent normal tissue (Fig. 2F). This protein was not detectable in normal tissue from a healthy patient (Fig. 2F). Similarly, HIF-1α (green fluorescence) and VEGF (red fluorescence) were highly expressed.
in parenchyma of tumor lung tissue, and these proteins were slightly expressed in the adjacent normal tissue (Fig. 2). These proteins were not expressed in normal lung tissue from a healthy patient (Fig. 2).

**EGFR Is a Positive Regulator of Angiogenesis in Cr(VI)-transformed Cells**—To determine whether EGFR plays an important role in angiogenesis of Cr(VI)-transformed cells, expression of EGFR was inhibited by its shRNA (Fig. 3A). Results from tube formation assay show that knockdown of EGFR markedly reduced tube formation compared with that in scramble Cr(VI)-transformed cells (Fig. 3B). Knockdown of EGFR in Cr(VI)-transformed cells significantly reduced numbers of new blood vessels (Fig. 3C). Similarly, results from *in vivo* Matrigel plug angiogenesis assay show that both new blood vessel formation and hemoglobin concentration in the plugs were suppressed in Cr(VI)-transformed cells with EGFR knockdown (Fig. 3D). To determine whether elevated angiogenic proteins in Cr(VI)-transformed cells are regulated by EGFR, expression of these angiogenic proteins was measured. The results show that knockdown of EGFR in Cr(VI)-transformed cells decreased MMP-1 expression at both mRNA and protein levels (Fig. 4, A, C, and D). Knockdown of EGFR also reduced expression and secretion of VEGF (Fig. 4, A–C). However, inhibition of EGFR did not influence mRNA level of angiogenin, IL-6, or GM-CSF (Fig. 4D), indicating that the increased level of angiogenin, IL-6, or GM-CSF is independent of EGFR. The above results demonstrate that EGFR plays an important role in angiogenesis of Cr(VI)-transformed cells and that MMP-1 and VEGF are downstream targets of EGFR.

**Inhibition of MMP-1 Reduces Angiogenesis and Tumorigenesis of Cr(VI)-transformed Cells**—Next, to investigate whether activation of MMP-1 contributes to elevated angiogenesis of...
Cr(VI)-transformed cells, MMP-1 stable knockdown cells were established (Fig. 5A). Knockdown of MMP-1 markedly reduced tube formation (Fig. 5B), new blood vessel formation, and hemoglobin concentration in the plugs (Fig. 5C) compared with those in scramble cells. Thus, it is expected that MMP-1 plays a positive role in tumorigenesis of Cr(VI)-transformed cells.

Results from xenograft tumor growth assay show that all animals injected with Cr(VI)-transformed cells grew tumors and that none of animals grew any tumor in MMP-1 knockdown cells (Fig. 5D). These results indicate that MMP-1 plays a major role in angiogenesis and tumorigenesis of Cr(VI)-transformed cells.

**EGFR-dependent Activation of p38/HIF-1α Signaling and Its Role in Angiogenesis of Cr(VI)-transformed Cells**—To understand mechanism of angiogenesis of Cr(VI)-transformed cells, p38/HIF-1α signaling was examined. The results show that phos-
phosphorylation of p38 (p-p38) was elevated in Cr(VI)-transformed cells compared with that in passage-matched normal cells (Fig. 6A). An increased level of p-p38 was also observed in lung squamous carcinoma tissue from a non-smoking worker occupationally exposed to Cr(VI) for 19 years (Fig. 6B). Phosphorylation of p42/p44, another member of the MAPK family, was examined in the present study. The results show that phosphorylation of p42/p44 in Cr(VI)-transformed cells was similar to that in passage-matched normal cells (Fig. 6A). To test whether elevated VEGF production is due to increased binding to HIF-1α, a ChIP assay was performed. The results show that binding of HIF-1α to hypoxia response element region of VEGF promoter increased ~6-fold in Cr(VI)-transformed cells compared with that in passage-matched normal ones (Fig. 6C). Knockdown of EGFR in Cr(VI)-transformed cells reduced binding of HIF-1α to VEGF (Fig. 6C). The activation of HIF-1α in Cr(VI)-transformed cells is through prolyl hydroxylation as evidenced by increased prolyl hydroxylase 2/EGL-9 expression (Fig. 6A).

Furthermore, knockdown of EGFR abolished p-p38 and HIF-1α in Cr(VI)-transformed cells (Fig. 6D, left panels). Similar results were observed in the tumor tissues from animals injected with Cr(VI)-transformed cells with or without EGFR knockdown (Fig. 6D, right panels). To determine whether p38/HIF-1α signaling regulates angiogenesis in Cr(VI)-transformed cells, the cells were pretreated with p38 inhibitor, SB203580, or HIF-1α inhibitor, PX-478. The results show that inhibition of p-p38 decreased HIF-1α expression, but not vice versa (Fig. 6E), indicating that p38 is an upstream regulator of HIF-1α. Inhibition of either p38 or HIF-1α in Cr(VI)-transformed cells abolished MMP-1 expression (Fig. 6E), suggesting that p38/HIF-1α are positive regulators of MMP-1. As expected, inhibition of p38/HIF-1α in Cr(VI)-transformed cells decreased tube formation (Fig. 6F). Further study shows that knockdown of HIF-1α in Cr(VI)-transformed cells markedly reduced expression of MMP-1 and VEGF (Fig. 6G), indicating the essential role of HIF-1α in up-regulations of MMP-1 and VEGF. These results demonstrate that activation of p38/HIF-1α/MMP-1-VEGF signaling is pivotal for angiogenesis of Cr(VI)-transformed cells. To further confirm that p38, HIF-1α, MMP-1, and VEGF are targets of EGFR, we have performed rescue experiments by examining expression of these proteins in Cr(VI)-transformed cells transfected with EGFR expression plasmid and/or EGFR shRNA. The results show that enforced expression of EGFR in Cr(VI)-transformed cells elevated levels of p-p38, HIF-1α, MMP-1, and VEGF compared with the scramble cells (Fig. 6H).

Co-transfections of EGFR expression plasmid with EGFR shRNA reduced these proteins to levels similar to those in Cr(VI)-transformed cells (Fig. 6H). The results above demonstrated that p38, HIF-1α, MMP-1, and VEGF are targets of EGFR in Cr(VI)-transformed cells.

Discussion

Changes from normal cells to malignantly transformed cells are considered the first stage of metal-induced carcinogenesis (12, 13). The development of malignantly transformed cells into tumor is the second stage. Angiogenesis is required for tumor growth and is very important in this second stage of metal or Cr(VI) carcinogenesis (Fig. 7). Angiogenesis occurs by sprouting new vessels from pre-existing blood vessels or by inserting interstitial tissue columns into the lumen of pre-existing vessels (14–16, 19). Tumor angiogenesis is a key mechanism for tumor growth and metastasis. Thus, studying the mechanism of
angiogenesis of Cr(VI)-transformed cells is very much needed for understanding the progression of Cr(VI)-transformed cells to tumor. EGFR is frequently overexpressed and mutated in various types of cancer, including lung (32). Anti-EGFR therapeutic approaches, specifically receptor blocking monoclonal antibodies and small molecule tyrosine kinase inhibitors, prolong tumor stabilization (33, 34). EGFR signaling is involved in blood vessel development and angiogenesis by directly or indirectly regulating pro-angiogenic factors (35–37). Ligand-induced EGFR dimerization triggers downstream signaling cascades including MAPK, PI3K-AKT, STAT, and PKC pathways. Our previous study has shown that constitutive activation of EGFR in Cr(VI)-transformed BEAS-2B cells is dependent on its ligand amphiregulin (12). Knockdown of EGFR reduced both tumor incidence and volume of Cr(VI)-transformed cells (12). The present study has found that knockdown of EGFR in Cr(VI)-transformed cells inhibited angiogenesis in vitro and in vivo, suggesting the essential role of EGFR in angiogenesis of Cr(VI)-transformed cells. Our previous study has also demonstrated angiogenesis of Cr(VI)-transformed cells is very much needed for understanding the progression of Cr(VI)-transformed cells to tumor.

EGFR is frequently overexpressed and mutated in various types of cancer, including lung (32). Anti-EGFR therapeutic approaches, specifically receptor blocking monoclonal antibodies and small molecule tyrosine kinase inhibitors, prolong tumor stabilization (33, 34). EGFR signaling is involved in blood vessel development and angiogenesis by directly or indirectly regulating pro-angiogenic factors (35–37). Ligand-induced EGFR dimerization triggers downstream signaling cascades including MAPK, PI3K-AKT, STAT, and PKC pathways. Our previous study has shown that constitutive activation of EGFR in Cr(VI)-transformed BEAS-2B cells is dependent on its ligand amphiregulin (12). Knockdown of EGFR reduced both tumor incidence and volume of Cr(VI)-transformed cells (12). The present study has found that knockdown of EGFR in Cr(VI)-transformed cells inhibited angiogenesis in vitro and in vivo, suggesting the essential role of EGFR in angiogenesis of Cr(VI)-transformed cells. Our previous study has also demonstrated
that activation of PI3K-AKT is dependent on EGFR in Cr(VI)-transformed cells (12). Inhibition of EGFR by its tyrosine kinase inhibitor or shRNA abolished expression of PI3K and AKT, leading to an increase in apoptosis and a decrease in tumorigenesis of Cr(VI)-transformed cells (12). It has been reported that EGFR also activates JNK and p38 (38). The observations in the present study indicate that activation of p38 is EGFR-dependent in Cr(VI)-transformed cells. Activation of p38 has been shown to correlate with increased tumor malignancy and poor prognosis of cancer patients (39, 40). Although hypoxia up-regulates HIF-1α through p38 in various cells (41), it has been reported that activation of p38 by hypoxia is mediated by HIF-1α in human hepatoma BEL-7405 cells (41). Our previous study has shown that short term exposure of Cr(VI) to DU145 human prostate cancer cells activated p38, which in turn up-regulated HIF-1α (42). Our previous study has also demonstrated that reactive oxygen species are responsible for Cr(VI)-induced malignant cell transformation (43). p38 is an oxidative stress-sensitive MAPK. It appears that Cr(VI)-induced ROS generation caused phosphorylation of p38 and subsequently activation of HIF-1α. The present study has found that inhibition of p38 decreased expression of HIF-1α and MMP-1, resulting in reduction of tube formation of human umbilical vein endothelial cells (HUVECs) stimulated by Cr(VI)-transformed cells, demonstrating that p38 is an upstream regulator of HIF-1α and that p38 is indeed involved in angiogenesis of Cr(VI)-transformed cells.

In the angiogenesis process, MMPs degrade basement membrane and other ECM components by facilitating endothelial cell migration and by releasing ECM-bound pro-angiogenic factors such as basic FGF, VEGF, and TGF-β (44). Inhibitors of MMPs reduced angiogenesis in vivo and in vitro (45, 46). MMP-1 is one of the essential components for angiogenic mesh. MMP-1 deficiency causes decreased growth and angiogenesis of lung tumors (27). It has been reported that Cr(VI)-treated cells exhibited an increased MMP-1 expression via tyrosine kinases (28). MMP-1 coordinates a paracrine signaling cascade for bone metastasis by proteolytic release of membrane and other ECM components by facilitating endothelial cell migration and by releasing ECM-bound pro-angiogenic factors such as basic FGF, VEGF, and TGF-β (47). EGFR ligand epiregulin, COX2, and MMP-1 collectively facilitate sequential steps of pulmonary metastasis (48). The present study has shown that MMP-1 expression was elevated in Cr(VI)-transformed cells, in lung tissue from Cr(VI)-exposed animals, and in lung tumor tissue from Cr(VI)-exposed worker. Knockdown of EGFR suppressed MMP-1 expression, suggesting that activation of MMP-1 in Cr(VI)-transformed cells is dependent on EGFR. Importantly, knockdown of MMP-1 reduced angiogenesis and tumorigenesis of Cr(VI)-transformed cells, indicating an important role of MMP-1 in up-regulating angiogenesis and tumorigenesis induced by Cr(VI).

HIF-1α signaling plays a critical role in tumor metastasis, invasion, metabolism, and angiogenesis (49, 50). HIF-1α activation in tumor cells is one of the key elements orchestrating their adaptation mechanisms to the hypoxia environment. Activated HIF-1α is crucial in adaptive responses of tumor cells to changes in oxygen concentration through transcriptional activation of over 100 downstream genes that regulate biological processes required for tumor survival and progression. During hypoxia, activation of HIF-1α/VEGF plays a critical role in initiating angiogenesis. In addition to hypoxia, HIF-1α protein synthesis, stability, and activity can also be regulated by other mechanisms, such as growth factor-induced signaling (51). Signaling via the HER2/neu or IGF-1 receptor tyrosine kinase induces HIF-1α expression by an oxygen-independent mechanism that increases the rate of HIF-1α protein synthesis (52, 53). HIF-1α overexpression is associated with increased microvessel density and/or VEGF expression in non-small cell lung cancers (54). HIF-1α protein is subjected to rapid degradation at normoxia via the pVHL-mediated ubiquitin-proteasome pathway, whereas hypoxia blocks degradation of HIF-1α protein, leading to its accumulation. The association of HIF-1α with pVHL is triggered by post-translational hydroxylation of a proline residue that is mediated by prolyl hydroxylase. In the present study, reduced prolyl hydroxylation in Cr(VI)-transformed cells led to stabilization of HIF-1α. HIF-1α activates transcription of genes encoding proteases that degrade the extracellular matrix including several MMPs, such as MMP-2, MMP-9, and MMP-14 (55). MMP-1 is also up-regulated when HIF-1α is induced by hypoxia in A549 epithelial cell line (56). HIF-1α promotes tumor metastasis into distant and more oxygenated tissues through the transcriptional activation of oncogenic growth factors such as TGF-β3, EGF, and others. The increase in HIF-1α levels in response to hypoxia is due to decreased degradation of HIF-1α, whereas growth factor-induced HIF-1α synthesis is through activation of PI3K or MAPK pathways (51). Our results show that knockdown of EGFR down-regulated p38, resulting in inhibition of HIF-1α, indicating that activation of HIF-1α in Cr(VI)-transformed cells is at least partially through EGFR/p38 signaling. HIF-1α transcriptionally induces several pro-angiogenic factors such as VEGF, which in turn stimulates the development of new blood vessels to enrich tumor cells with oxygen for their growth. In the tumor angiogenic process, HIF-1α-driven VEGF expression occurs at the acute phase (57). Our results indicate that HIF-1α bound to promoter region of VEGF, resulting in its activation in Cr(VI)-transformed cells. In conclusion, constitutive activation of EGFR up-regulates p38 MAPK, resulting in activation of HIF-1α and further up-regulation of MMP-1 and VEGF, leading to an increase in angiogenesis and tumorigenesis of Cr(VI)-transformed cells.

Experimental Procedures

Chemicals and Reagents—Sodium chromate (Na2Cr2O7) and Drabkin reagent were from Sigma. DMEM, FBS, gentamicin, l-glutamine, oligo(dT)20, Lipofectamine 2000, and antibodies against Alexa Fluor® 488 goat anti-mouse IgG1 (γ1) and Alexa Fluor® 568 goat anti-rabbit IgG (H+L) were from Invitrogen. Endothelial cell basal medium-2 was from Clonetics (San Diego, CA). Endothelial cell growth medium kit (EGM-2 Single-Quot) was from Lonza (Walkersville, MD). HIF-1α inhibitor PX-478 was from MedKoo Biosciences (Chapel Hill, NC). The RNase easy mini kit, RNA extraction kit, and plasmid prep kit were from Qiagen. Moloney murine leukemia virus reverse transcriptase was from Promega (Madison, WI). Human angiogenesis array C1000 was from RayBiotech (Norcross, GA).
qScript cDNA synthesis kit and Perfecta Sybr Green Fastmix were from Quanta Biosciences (Gaithersburg, MD). shRNAs of EGFR, MMP-1, and HIF-1α were from Origene (Rockville, MD). p3XFLAG-CMV/EGFR and its control plasmids were kindly provided by Dr. Qiou Wei (Department of Toxicology and Cancer Biology, University of Kentucky) (58). Bradford protein assay reagent was from Bio-Rad. Enhanced chemiluminescence reagent was from GE Healthcare. Human VEGF Quantikine ELISA kit was from R&D Systems. Collagen type I was from BD Biosciences. Antibodies against VEGF, GAPDH, and β-actin were from Santa Cruz. Antibody against HIF-1α was from Millipore (Billerica, MA). Antibodies against EGFR, p-EGFR, MMP-1, p38, p-p38, p44/42, p-p44/42, and PH2/ EGL-9 and p38 inhibitor SB203580 were from Cell Signaling Technology (Danvers, MA). Matrigel basement membrane matrix was from BD Biosciences. Vectashield mounting medium containing DAPI was from Vector Laboratories (Burlingame, CA).

Cell Culture—Both human bronchial epithelial BEAS-2B cells and HUVECs were from American Type Culture Collection (Rockville, MD). BEAS-2B cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin. HUVECs were maintained in endothelial cell basal medium-2 supplemented with 20% FBS and 1% penicillin-streptomycin.

Cell Transformation Assay—BEAS-2B cells were chronically exposed to 0.25 μM Cr(VI). Fresh medium containing Cr(VI) was added every 3 days. After 6 months of Cr(VI) exposure, these cells were subjected for soft agar assay. A single colony was picked up and continued to grow. Cells from the single colony were considered as Cr(VI)-transformed cells. Passage-matched cells without Cr(VI) exposure was used as control.

Chronic Exposure of Animals to Cr(VI) Particles—The 6–8-week-old, female BALB/c mice were from The Jackson Laboratory. Endotoxin-free basic zinc chromate particles were crushed using mortar and pestle followed by washing with distilled water and acetone to make a size of 4.7 μm and a purity of 99–100%. Cr(VI) particles were suspended in sterile 0.9% sodium chloride solution at a concentration of 0.6 mg/ml as described previously (59). Animals under a light anesthesia (isoflurane) were intranasally exposed to a 50–99–100%. Cr(VI) particles were suspended in sterile 0.9% saline春秋. A 6–8-month-old, female BALB/c mouse was intranasally exposed to a 99–100% Cr(VI) particles for 19 years. The worker was diagnosed with stage 1 squamous lung carcinoma.

Plasmid Transfection—Briefly, the cells were seeded in 6-well culture plates. After 70% confluency, the cells were transfected with 4 μg of plasmid using Lipofectamine. To establish stable transfected cells, the cells were selected using puromycin for 3 months followed by immunoblotting analysis for verification of expression.

Human Angiogenesis Array—Cr(VI)-transformed cells and passage-matched normal cells were cultured in serum-free medium for 24 h. Conditioned medium was collected. Proteins in the conditioned medium were hybridized with a human angiogenesis antibody array dotted with 43 human angiogenesis-related antibodies. The assay was performed according to the manufacturer’s manual. Briefly, conditioned medium was incubated with human angiogenesis array membranes. The membranes were washed with specific buffer and treated with biotin-conjugated antibodies followed by treatment with diluted horseradish peroxidase-conjugated streptavidin. X-ray film and a chemiluminescence imaging system were used to detect an angiogenesis array signal.

Real Time PCR—RNA was extracted and purified using a Qiagen RNeasy mini kit. 0.5 μg of RNA was reversely transcribed using qScript cDNA synthesis kit. Primers were designed using Primer-Blast yielding: angiogenin, 5′-CTG CCG TGT ACA CAC ACT CA-3′ and 5′-GCA AGA CCA ACA AA-3′; IL-6, 5′-AAA TGC CAG CCT GCT GAC GAA C-3′ and 5′-AAC AAT CTG AGG TGC CCA CCA TAC-3′; MMP-1, 5′-CTG GCC ACA ACT GCC AAA TG-3′ and 5′-CTG TCC CTG AAC AGC CCA GTA A-3′; GM-CSF, 5′-CAC TGC TGA GAT GAA TGA AA-3′ and 5′-GTC TGT AGG CAC GTC GGC TTC-3′; HIF-1α, 5′-CAT AAT CTG TTC GCG AAG TAA AG-3′ and 5′-TCA CAG AGG CCT TAT CAA GAT ATG-3′; and 5′-beta, 5′-CTG GAA CTG TGA AGG TGA TGA ATG C-3′ and 5′-AAG GAA CTT CCT GGT TCA ACA ATG-3′. All primers were tested using standard curves in 10-fold serial dilutions. Quantitative PCR was performed using Perfecta Sybr Green Fastmix in the CFX96 real time PCR detection system (Bio-Rad). The data were analyzed using CFX manager software (Bio-Rad).

Immunoblotting Analysis—The cell lysates were prepared in radioimmune precipitation assay buffer. Protein concentration was measured using Bradford protein assay. 30 μg of proteins were separated by SDS-PAGE followed by incubation with primary antibody for overnight. The blots were then reprobed with secondary antibody conjugated to horseradish peroxidase. Immunoreactive bands were detected using enhanced chemiluminescence reagent.

Immunofluorescence Assay—The cells were cultured on chamber slides. After washing with PBS, the cells were fixed with 4% paraformaldehyde for followed by incubation with PBS containing 1% Triton X-100. After washing with PBS-Tween 20 solution (containing 0.02% Tween 20), the cells were incubated with PBS containing 0.1% Triton X-100 and 10% normal serum. The cells were incubated with primary antibody for overnight followed by reprobed with secondary antibody. The slides were mounted with Vectashield mounting medium containing DAPI. Finally, the cells were visualized using Olympus BX53 fluorescence microscope (Pittsburgh, PA).
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**HUVEC Tube Formation Assay**—Tube formation assay was performed as previously described (60). HUVECs were seeded into wells coated with 50 μl of growth factor-reduced Matrigel matrix and then incubated with 100 μl of conditioned medium for 12 h to allow formation of tube-like structures. Tube formation was analyzed using Olympus IX-73 microscope (Pittsburgh, PA). The tube forming ability was determined by measuring the total tube length of HUVECs.

**Human VEGF Immunoassay**—Production of VEGF was detected using a human VEGF Quantikine ELISA kit according to the manufacturer’s manual. Briefly, 200 μl of cell supernatant was added in each well and incubated for 2 h followed by adding 200 μl of conjugate for 2 h. After aspirating and washing, 200 μl of substrate was added and incubated for 20 min. The absorbance was measured using Labsystems Multiskan MS plate reader (Thermo Scientific) at 450-nm wavelength.

**Ex Ovo Chick CAM Assay**—Pathogen-free fertile leghorn chicken eggs were from Poultry Breeding Farm at the University of Kentucky (Lexington, KY). A modified ex ovo (shell-less) culture method was used (61, 62). Briefly, in embryonic development day 3, the eggs were sprayed with 70% ethanol, air-dried in a laminar flow hood, and explanted into Petri dishes. The ex vivo cultures were maintained in a humidified incubator with 2% CO2 at 37 °C. In the day 9, 9 × 105 of cell suspension was mixed with 4.04 mg/ml of collagen type I. Matrigel grafts were placed on the top of CAMs, and eggs were incubated for 72 h until day 12. The images were captured using an Olympus SZ61 dissection microscope (Pittsburgh, PA).

**In Vivo Matrigel Plug Angiogenesis Assay**—A Matrigel plug assay was performed as described previously (63). 6-week-old female athymic nu/nu mice were from Charles River Laboratories (Wilmington, MA). The mice were housed in sterilized filter-topped cages and maintained in a pathogen-free animal facility at the Chandler Medical Center at the University of Kentucky (Lexington, KY). A modified ex ovo (shell-less) culture method was used (61, 62). Briefly, in embryonic development day 3, the eggs were sprayed with 70% ethanol, air-dried in a laminar flow hood, and explanted into Petri dishes. The ex vivo cultures were maintained in a humidified incubator with 2% CO2 at 37 °C. In the day 9, 9 × 105 of cell suspension was mixed with 4.04 mg/ml of collagen type I. Matrigel grafts were placed on the top of CAMs, and eggs were incubated for 72 h until day 12. The images were captured using an Olympus SZ61 dissection microscope (Pittsburgh, PA).

**Statistical Analysis**—The data were expressed as means ± S.D. Statistical significance of differences among treatment groups was determined by Student’s t test. A p < 0.05 was considered as statistical significance.

**Author Contributions**—Z. Z. and D. K. were responsible for conception and design; D. K., J. D., Y.-H. P., L. Y. F., L. W., P. P., Y.-O. S., M. X., K. K., and J. L. performed data acquisition, analysis, and interpretation; and D. K., X. S., and Z. Z. wrote the paper.

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EGFR/p38/HIF-1α in Cr(VI) Angiogenesis


Activation of Epidermal Growth Factor Receptor/p38/Hypoxia-inducible Factor-1 α Is Pivotal for Angiogenesis and Tumorigenesis of Malignantly Transformed Cells Induced by Hexavalent Chromium

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