Hepatocyte Growth Factor/Scatter Factor Differentially Regulates Expression of Proangiogenic Factors through Egr-1 in Head and Neck Squamous Cell Carcinoma

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Abstract

Hepatocyte growth factor/scatter factor (HGF) and the angiogenesis factors platelet-derived growth factors (PDGF), vascular endothelial growth factor (VEGF), and interleukin-8 (IL-8) are found in elevated concentrations in serum or tumor tissue of patients with head and neck squamous cell carcinomas (HNSCC), suggesting these factors may be coregulated. A cDNA microarray analysis for HGF-inducible genes revealed that HGF also modulates PDGFA expression, a gene recently shown to be inducible by the transcription factor, early growth response-1 (Egr-1). In the present study, we investigated the potential role of HGF-induced Egr-1 in expression of PDGFA, VEGF, and IL-8. HGF induced expression of all three factors and Egr-1 expression and DNA-binding activity. The analysis of promoter sequences showed putative Egr-1 binding sites in the PDGFA or VEGF but not in the IL-8 promoter, and HGF-induced Egr-1–binding activity was confirmed by chromatin immunoprecipitation (ChIP) assay. The maximal basal and HGF-induced promoter activity for the PDGFA gene existed within −630 bp of the promoter region, and overexpression of Egr-1 significantly increased such activity. Consistent with this, expression of PDGFA and VEGF but not IL-8 showed corresponding differences with Egr-1 expression in HNSCC tumor specimens and were strongly suppressed by transfection of Egr-1–antisense or small interference RNA (siRNA) oligonucleotides. HGF-induced expression of Egr-1, PDGFA, and VEGF was suppressed by pharmacologic and siRNA inhibitors of mitogen-activated protein kinase kinase 1/2 (MEK1/2) and protein kinase C (PKC) pathways. We conclude that the HGF-induced activation of transcription factor Egr-1 by MEK1/2- and PKC-dependent mechanisms differentially contributes to expression of PDGFA and VEGF, which are important angiogenesis factors and targets for HNSCC therapy. (Cancer Res 2005; 65(16): 7071-80)

Introduction

Hepatocyte growth factor/scatter factor (HGF) is a soluble heterodimeric protein that elicits diverse responses in different tissues by binding to the tyrosine kinase receptor, c-Met (1). Activation of HGF/c-Met pathway has been shown to play a significant role in carcinogenesis, tumor-induced angiogenesis, tumor invasion, and metastasis (1). C-Met overexpression has been found in numerous human cancers including head and neck squamous cell carcinoma (HNSCC) and is associated with metastasis and poor prognosis (2). Although the oncogenic activity of HGF has been attributed primarily to its ability to promote tumor proliferation and invasion, much evidence now suggests that HGF may also play a prominent role in tumor-induced angiogenesis. HGF production and c-Met overexpression correlate with vessel density in human tumors and in tumor xenograft models (3). HGF can act directly on endothelial cells to stimulate growth, migration, and capillary formation (4) but may also act indirectly by stimulating the release of other proangiogenic factors.

We and others have reported that HGF and the angiogenesis factors platelet-derived growth factors (PDGF), vascular endothelial growth factor (VEGF), and interleukin-8 (IL-8) are often elevated in serum and tumor tissue of patients with HNSCC (5–9), suggesting that these factors may be coregulated. HGF/c-MET receptor–induced activation of the mitogen-activated protein kinase kinase (MEK) and phosphatidylinositol 3-kinase (PI3K) signal pathways was previously shown to contribute to expression of IL-8 and VEGF by HNSCC and other cells (9, 10). We also recently showed that enforced expression of HGF promoted expression of homologues Gro 1 and VEGF and angiogenesis, tumorigenesis, and metastasis of SCC overexpressing c-Met in a murine model (11). VEGF, IL-8, and Gro 1/Gro α (KC in mouse) have been shown to be key factors in mediating angiogenesis in SCC and other cancers (12–19). Recently, we completed a cDNA microarray analysis for other HGF-inducible genes, which provided evidence that PDGFA is an HGF-inducible gene in UM-SCC cells.1

The PDGF family of signaling molecules has been shown to promote mesenchymal cell proliferation, chemotaxis, cell survival, and transformation in vitro and direct stimulation of tumorigenesis of mesenchymal tumors such as gliomas in vivo (20). PDGFs have been shown to promote angiogenesis (20, 21). The regulation of PDGF expression is controlled at the transcriptional level and has been shown to be inducible through a transcription factor, early growth response-1 (Egr-1, refs. 22, 23). Egr-1 is a zinc-finger transcription factor that is rapidly and transiently induced in response to a number of stimuli including growth factors, cytokines, and mechanical stress (24). Egr-1 seems particularly important in the process of tumor angiogenesis. One study has shown that silencing of Egr-1 expression with DNAzymes significantly inhibited breast cancer growth and angiogenesis in vivo (25). However, the role of HGF in activation of Egr-1 and

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1 B. Worden, unpublished data.
their specific role in regulation of expression of PDGF and other proangiogenic cytokines in HN SCC and other tumor cells has not been elucidated.

In this study, we report that HGF is able to induce production of the proangiogenic factor PDGF-AB by HN SCC cells, in addition to VEGF and IL-8, reported previously (9). Furthermore, we present the novel observation that HGF induces PDGFA and VEGF gene expression in HN SCC cells by rapidly inducing the expression and the DNA-binding activity of Egr-1 through a mechanism dependent, at least in part, on MEK1/2 and protein kinase C (PKC). Transfection of cells with antisense oligonucleotides or small interference RNA (siRNA) specific for Egr-1 abrogated the HGF-mediated induction of PDGFA and VEGF expression. These data indicate that Egr-1 is a downstream transcription factor differentially regulating HGF-induced PDGFA and VEGF expression in HN SCC.

Materials and Methods

Cell culture. UM-SCC-1, 6, 11A and 38 cells were obtained from Dr. Thomas Carey (University of Michigan) and cultured as previously described (9). Human normal keratinocytes (HNKC) were obtained from Cascade Biologics, Inc. (Portland, OR) and were maintained in keratinocyte serum-free medium (KGM) containing 0.08 mM of calcium chloride and supplemented with human keratinocyte growth supplements (HKGS). The final concentration of HKGS in the complete medium are 0.2% (v/v) of bovine pituitary extract, 5 μg/mL of bovine insulin, 0.18 μg/mL of hydrocortisone, 5 μg/mL of transferrin, and 0.2 ng/mL of human recombinant EGF. All HNKC were used within five passages.

Cytokine quantitation by ELISA. Cells were plated in 24-well tissue culture plates at a density of 5 × 10^4 cells per well for 24 hours and followed with serum starvation for 48 hours with EMEM plus 1% bovine serum albumin. Cells were treated with recombinant human HGF (R&D Systems, Minneapolis, MN), and the supernatants were collected 24 hours after treatment. VEGF and PDGF-AB concentrations were determined using Quantikine ELISA kits from R&D Systems (Minneapolis, MN), and the supernatants were collected 24 hours after treatment. VEGF and PDGF-AB concentrations were determined using Quantikine ELISA kits from R&D Systems. IL-8 concentrations were determined using human ELISA IL-8 kits from Pierce Endogen (Rockford, IL).

Real-time reverse transcription-PCR. UM-SCC cells were cultured until 40% to 50% confluent and serum starved for 48 hours. Cells were then treated with 40 ng/mL HGF, and total RNA was isolated using the Trizol method (Invitrogen, Carlsbad, CA). Reverse transcription and real-time reverse transcription-PCR (RT-PCR) was done using the High-Capacity cDNA Archive Kit and gene-specific primers and probes from the Assays-on-Demand product line (ABI Prism 7700, Applied Biosystems, Foster City, CA). Relative expression was calculated using the ΔΔCt method with the expression of 18S rRNA or peptidylprolyl isomerase A (cyclophilin A, PPIA) as the references. For inhibitor experiments, cells were pretreated with 30 μM/L of the PI3K inhibitor LY294002 (Promega, Madison, WI), 30 μM/L of the MEK inhibitor U0126 (Promega), or 1 μM/L of the PKC inhibitor RO 31-8220 (Upstate Biotechnology, Lake Placid, NY) for 30 minutes and treated with 40 ng/mL HGF for 60 minutes. RNA was isolated and analyzed by real-time RT-PCR.

DNA-binding assay. UM-SCC-11A cells were cultured and serum starved as described and treated with 40 ng/mL HGF for 1 hour. Nuclear extracts were collected using the Transfactor Extraction Kit from BD Biosciences (Palo Alto, CA). Egr-1 DNA-binding activity was determined using BD Mercury TransFactor assays from BD Biosciences (Palo Alto, CA). Nuclear extracts from phorbol-treated K562 cells (Santa Cruz Biotechnology, Santa Cruz, CA) were used as a positive control. The absorbance of the colorimetric reaction at 650 nm is read using a microplate reader.

Head and neck squamous cell carcinoma tissue and laser capture microdissection. Fresh frozen tumor from UM-SCC-11A xenografts and from HN SCC specimens of two anonymous patients were obtained under an exemption from the Institutional Review Board review by the Office of Human Subjects Research, NIH. The human HN SCC were obtained through the Cooperative Human Tissue Network, Midwestern Division (Columbus, OH). The specimens were from larynxes of 75-year-old (HN SCC1) and 67-year-old (HN SCC2) white male patients with pathologic diagnosis of moderately differentiated squamous cell carcinoma. Sections of 12 μm were made using a cryostat and stored at −80°C until use. Before laser capture microdissection (LCM), sections were stained with Histogene LCM Frozen Section Staining Kit (Arcturus Engineering, Mountain View, CA) according to manufacturer’s protocol with minor modification. LCM of cancer cells was done using a PixCell II System (Arcturus Engineering) with the following variables: 15-μm laser diameter, 4.5-millisecond pulse duration, and 7.5-mW pulse power. Cancer cells were collected from three sections of each specimen after 2,500 to 3,500 bursts of laser shots. Total RNA was extracted from laser-captured cells using PicoPure RNA Isolation Kit (Arcturus Engineering) according to manufacturer’s protocol. Removal of genomic DNA was done by on-column DNase digestion during RNA purification (RNase-free DNase Set; Qiagen, Valencia, CA). The quality and quantity of total RNA was assessed by Agilent 2100 Bioanalyzer using RNA 6000 Pico Assay kit (Agilent Technologies, Wilmington, DE) according to manufacturer’s protocol.

Chromatin immunoprecipitation assay. The chromatin immunoprecipitation (ChIP) assay was done using the ChIPs assay kit (Upstate Biotechnology, Waltham, MA) following the manufacturer’s directions. Briefly, cultured UM-SCC-11A cells at 70% to 80% confluence were adapted to serum-free medium for 24 hours and then treated with HGF (40 ng/mL) for 1 hour. DNA and proteins were cross-linked by 1% formaldehyde and sonicated using SONICATOR XL2020 (Misonix, Inc., Farmingdale, NY). The DNA/protein complex was immunoprecipitated, and genomic DNA was recovered by phenol/chloroform extraction. Primers designed for the PDGFA promoter (~15 to ~222 bp) were 5′-CGGGGCTTTGTATGATTAG-3 (forward) and 5′-GGCGGAGAGGTTATAGTAC-3 (backward); for the VEGF promoter region (+5 to +224 bp) were 5′-TTTCCAGGTCTT-GAACCCCT-3 (forward) and 5′-GATCCTCCCCGCCTACCG-3 (backward); and for the IL-8 promoter were 5′-GGGCCCATCGATGCAAATC-3 (forward) and 5′-TTCCCTCCCGGTTCTTCTC-3 (backward).

Reporter gene assay. UM-SCC-11A cells were cultured to 5.0 × 10^4 per well in a 24-well plate (Costar, Corning, Inc., Corning, NY) 24 hours before transfection. Plasmids (0.3 μg per well) containing serially deleted PDGFA promoters (i.e., −900, −630, −260, −77, and −55) conjugated to luciferase were kindly provided by Dr. Toru Suzuki (Tokyo, Japan; ref. 26) using Qiagen Effectene Transfection Reagents (3.2 μl per well of Enhancer and 4.0 μl per well of Effectene for 3 hours; Qiagen). Transfection reagents were then aspirated and cells were treated with HGF (40 ng/mL) in complete culture medium for 48 hours. Whole cell lysates were then collected using the reagents from Tropix Dual Light System (Applied Biosystems), and chemoluminescence of luciferase or β-galactosidase activity was measured by the luminometer (Monolight 2010, BD Biosciences, San Jose, CA). For Egr-1 expression experiments, 0.2 μg per well of Egr-1 expression or control vector (GeneCopeia, Frederick, MD) was cotransfected with 0.2 μg per well of PDGFA promoter plasmid using the same experimental conditions as described above.

Antisense experiments. The Egr-1 sense oligonucleotide is agTGTCC-CCGCCCCCGCA or the Egr-1 antisense oligonucleotide: tgcGGGGGCG-GGGGAACacT (where the bases in lowercase are phosphorothioated) were synthesized and HPLC purified by Integrated DNA Technologies (Coralville, IA). UM-SCC-11A cells (5 × 10^4 per well) were plated in 6-well tissue culture plates for 24 hours and transfected with Egr-1 antisense or sense oligos using Cytotectin transfection reagent following manufacturer’s suggestions (Gene Therapy Systems, Inc., San Diego, CA). Cells as well as supernatants were harvested for cell count and ELISA analysis, respectively. For analysis of cytokine gene expression, cells were harvested 24 hours after transfection and treated with HGF treatment (40 ng/mL) for 4 hours.

Small interference RNA. Cultured cells were seeded in 6-well plates to reach 60% to 80% confluency after 24- to 48-hour incubation, washed with Opti-MEM I Reduced Serum Medium (Invitrogen), and transfected with a total of 50 nmol/L of a mixture of four double-stranded RNA oligonucleotides directed against human EGR-1, MAP2K2, MAP2K1, PKRCA, PKRCD,
HGF induces angiogenic cytokines through Egr-1

Results

Hepatocyte growth factor/scatter factor induces expression of PDGF, VEGF, IL-8, and Egr-1 in UM-SCC cells. We previously showed that HGF induces expression of angiogenesis factors VEGF and IL-8 by multiple UM-SCC cell lines but not normal keratinocytes (9). Using cDNA microarray to identify additional HGF-inducible genes in UM-SCC-11A cells, we consistently observed up-regulation of PDGFA by HGF (data not shown). To confirm whether HGF-induced expression of PDGF, UM-SCC-11A and keratinocytes were exposed to HGF over the same concentration range found previously to induce IL-8 and VEGF, and expression of PDGF-AB protein was quantified by ELISA. As shown in Fig. 1A, HGF induced a dose-dependent increase in PDGF-AB production in the culture supernatants of UM-SCC-11A cells. However, in cultured normal human keratinocytes, a minimal basal level of PDGF-AB was detected, and no increase in PDGF-AB was observed after HGF treatment (Fig. 1B). We also tested HGF-induced PDGF-AB production in a broader panel of UM-SCC cell lines. As shown in Fig. 1C, HGF induced PDGF-AB protein production in all cell lines tested, with the highest induction observed in UM-SCC-11A cells. Thus, HGF-induced PDGF-AB expression in several different UM-SCC cells but not in normal keratinocytes, a pattern similar to that observed previously for VEGF and IL-8 (9).

To compare the effects of HGF on the magnitude and kinetics of expression of PDGF-AB, VEGF, and IL-8, we determined the cumulative protein expression by ELISA and kinetics of mRNA induction by real-time RT-PCR using samples from UM-SCC-11A cells treated with an optimal stimulatory concentration of exogenous HGF. As shown in Fig. 2A, HGF induced a 2- to 3-fold increase in production of all three factors by 24 hours. The absolute concentrations of these cytokines in untreated UM-SCC-11A culture supernatants were PDGF-AB (61.8 pg/mL), IL-8 (11,080 pg/mL), VEGF (1,896 pg/mL). No significant induction of PDGF-AA or PDGF-BB was observed (data not shown). Figure 2B-D shows the kinetics of HGF-induced expression of PDGFA, VEGF, and IL-8 mRNA. After HGF stimulation, the induction of both PDGFA and VEGF gene expression peaked at the 4-hour time point (Fig. 2B-C) and IL-8 expression peaked at the 8-hour time point (Fig. 2D).

Based on previous studies indicating a role of the transcription factor Egr-1 in the regulation of PDGFA gene expression, we hypothesized that this transcription factor might be a downstream target of c-Met signaling in HNSCC. Using real-time RT-PCR, we examined Egr-1 expression in UM-SCC-11A cells treated with HGF. Fig. 2E shows that HGF induces >20-fold induction of Egr-1 expression, which peaks at 60 minutes and returns to the baseline level within 4 hours. Next we evaluated the effect of HGF on the DNA-binding activity of Egr-1 protein. As shown in Fig. 2F, HGF treatment significantly increased Egr-1–binding activity to Egr-1 consensus oligonucleotide, and such binding was completely blocked by excess Egr-1 oligonucleotide, confirming the specificity of the interaction. We included nuclear extracts from K562 cells which have elevated Egr-1 in the binding assay as a positive control. Thus, HGF strongly induced expression of Egr-1, before the
peak of HGF-inducible expression of PDGF, VEGF, and IL-8 mRNA and protein.

Hepatocyte growth factor/scatter factor–induced Egr-1 binding at the proximal promoter regions of the PDGFA and VEGF genes and role in functional activation of the PDGFA gene. We analyzed the promoter sequences of the PDGFA, VEGF, and IL-8 genes to identify putative Egr-1–binding sequences. The TFSEARCH program (27) was used to screen the sequences for potential binding sites. Any identified site was rejected if the sites showed mismatches >2 bp for the recognized Egr-1 consensus binding sequence (28, 29). Ten putative Egr-1–binding sites were identified within the PDGFA promoter, three sites within the VEGF promoter, whereas no binding sites were identified within the IL-8 promoter (Fig. 3A). The majority of these sites are located within the proximal promoter regions of these genes (Fig. 3A).

To verify the prediction, ChIP assays were done. Sp1 binding was also included because other studies have suggested that Sp1 may play a prominent role in transactivation of PDGF and VEGF (30, 31). Strong constitutive and HGF-enhanced binding activity of Egr-1 to VEGF promoter was observed when compared with a relatively weaker Sp1-binding activity (Fig. 3B). Constitutive and HGF-enhanced binding activity of Egr-1 to VEGF promoter was also observed, along with relatively strong constitutive but not binding activity.
HGF-inducible Sp1-binding activity. We detected very weak constitutive and no inducible Egr-1- or Sp1-binding activity to the IL-8 promoter. Consistent with the differences in frequency of Egr-1 sites in the promoters of the factors, these data suggest that the differentially regulated Egr-1- and Sp1-binding activities contribute to constitutive and HGF-induced expression of PDGFA and VEGF but not IL-8.

To further confirm the functional role of HGF in activation of the promoter of one of the genes identified by sequence prediction and ChIP analysis, we examined a set of PDGFA promoter constructs with serial deletions spanning the portion of the promoter which includes the multiple Egr-1 sites in the PDGFA gene. UM-SCC-11A cells were transfected with the promoter constructs and incubated with HGF. As shown in Fig. 4A, the highest basal or HGF-induced PDGFA promoter activity was identified in the promoter region consisting of −630 bp of the DNA sequence. A serial deletion of the promoter sequence, from −260 to −55 bp, gradually decreased both basal and HGF-induced PDGFA promoter activity (Fig. 4A). To determine if overexpression and HGF-induced activation of Egr-1 specifically contributed to the promoter activity, UM-SCC-11A cells were cotransfected with the −630 or −260 bp PDGFA promoter constructs, with a vector containing Egr-1 gene under a constitutive promoter, or empty vector control, cultured without or with additional HGF. Fig. 4B shows that overexpression of Egr-1 gene further increased the basal and HGF-induced PDGFA promoter activity in UM-SCC-11A cells when tested with the promoter containing −630 bp sequence. No significant induction was observed with the promoter containing only −260 bp of the sequence (data not shown). These findings provide direct evidence that the portion of the PDGFA promoter containing Egr-1 sites (to −630) is optimal and activated by constitutive or HGF-induced overexpression of Egr-1.

**Correspondence among Egr-1, PDGFA, and VEGF gene expression in laser capture microdissected head and neck squamous cell carcinoma from xenograft and human tumor tissues.** To investigate if relative variation in Egr-1 expression is associated with corresponding differences in expression of HGF- and Egr-1-induced factors in tumors in situ, Egr-1, PDGFA, VEGF, and IL-8 expression was compared in UM-SCC-11A xenograft tumors and two human HNSCC specimens that differed markedly in Egr-1 expression. RNA was obtained from HNSCC epithelia isolated by laser capture microdissection. We observed a correspondence of gene expression among Egr-1, PDGFA, and VEGF but not IL-8 in UM-SCC-11A xenograft and both tumors with differing Egr-1 expression (Fig. 5A). Thus, in the limited sample tested, Egr-1 expression corresponded with differences in PDGFA and VEGF, consistent with evidence for role of Egr-1 in mediating HGF-induced expression of these genes found above.

Egr-1 differentially regulates PDGFA and VEGF gene expression and protein production induced by hepatocyte growth factor/scatter factor. To further verify the role of Egr-1 in the HGF-mediated induction of PDGF and VEGF, we transfected UM-SCC-11A cells with either antisense oligonucleotides or siRNA inhibitors of Egr-1 and then treated the cells with HGF. The Western blot in Fig. 5B shows that anti-Egr-1 oligonucleotides significantly decreased constitutive and HGF-induced Egr-1 protein

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**Figure 3.** HGF enhances Egr-1 binding at the promoter sequences of both PDGFA and VEGF genes. A, sequence of the proximal PDGFA, VEGF, and IL-8 promoters. Boxes mark the locations of Egr-1 binding sites. The vertical lines demarcate the span of the fragment amplified during the ChIP assays. The arrow marks the transcription initiation site. B, ChIP assay results show the amplification of a fragment of the proximal PDGFA, VEGF, and IL-8 promoters containing Egr-1 and Sp1 binding sites. Immunoprecipitation was carried out using either an anti-Egr-1 or an anti-Sp1 antibody. The experiment has been repeated twice.

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expression when compared with sense or no oligonucleotide controls. As shown in Fig. 5C, Egr-1 antisense oligos completely blocked constitutive PDGF-AB production (100% inhibition), or nearly completely inhibited PDGF-AB induced by HGF (89% inhibition). A significant but partial inhibition of constitutive and HGF-induced VEGF production was observed (66% inhibition). In contrast, minimal effects of Egr-1 antisense on IL-8 expression were observed, consistent with the lack of Egr-1 promoter sites and binding found above. As shown in Fig. 5D, Egr-1 siRNAs suppressed basal and HGF stimulated Egr-1, PDGFA expression, and to a lesser extent, affected VEGF expression. We conclude that Egr-1 is an important modulator of expression of angiogenic cytokines, PDGF-AB and VEGF but not IL-8 in UM-SCC-11A cells.

Hepatocyte growth factor/scatter factor enhances expression of the transcription factor Egr-1 under the regulation of protein kinase C and mitogen-activated protein kinase kinase 1/2 but not phosphatidylinositol 3-kinase. Various studies have reported that Egr-1 expression is regulated by different signal molecules including PKC, MEK1/2, and PI3K in other cell types (32–37). We have previously shown that the phosphorylation of MEK1/2 and PI3K can be induced by HGF in HNSCC (9). To further elucidate the signal transduction pathways regulating Egr-1 induction by HGF in HNSCC, the specific pharmacologic inhibitors of MEK1/2 (U0126), PI3K (LY294002), and PKC (RO 31-8220) were used (Fig. 6A). The concentrations of the inhibitors have been previously titrated for the optimal inhibition of the specific kinases without apparent cytotoxicity (ref. 9; data not shown). Inhibitors of MEK1/2 and PKC but not PI3K significantly abrogated the HGF-mediated induction of Egr-1 gene expression (Fig. 6A).

To further confirm these observations, specific siRNA inhibitors were used to knock down the genes encoding several of the kinase isotypes [MAP2K1 and MAP2K2 (MEK1 and MEK2); PKC α, δ, ε, and ζ] potentially involved in HGF-induced activation of transcription factor Egr-1. The siRNAs consisted of a mixture of four double-stranded RNA oligonucleotides directed against target genes obtained commercially as described in methods. The efficiencies of gene knock down reached 60% to 90% among the various genes (Fig. 6B, top; data not shown). Simultaneous knock down of the genes MEK1 (MAP2K1) and MEK2 (MAP2K2) significantly suppressed HGF-induced Egr-1 gene expression and inhibited basal and/or HGF-induced PDGFA and VEGF gene expression to a lesser degree (Fig. 6B, left). Knocking down expression of the single gene PKC ε (PRKCE) significantly suppressed both basal and HGF-induced Egr-1, PDGFA, and VEGF gene expression (Fig. 6B, right). Knocking down other genes in PKC family, such as PKC α, PKC δ, and PKC ζ individually, or MEK1 and MEK2 separately, did not significantly suppress Egr-1-regulated gene expression (data not shown). Our data provide evidence that PKC ε and MEK1/2 are important components of signal pathways involved in basal and HGF-induced Egr-1, PDGFA, and VEGF gene expression.

Discussion

In this study, we showed that HGF is a strong inducer of the proangiogenic factors PDGF, VEGF, and IL-8 (Figs. 1 and 2), and the induction of PDGFA and VEGF gene is differentially regulated by Egr-1 in UM-SCC-11A cells (Figs. 3-6). The regulation of Egr-1 transcriptional activity has been reported to be controlled by Egr-1 gene transcription rather than by posttranslational modification (23). After HGF treatment, Egr-1 gene expression peaked by 60 minutes followed by the peak in PDGFA and VEGF gene expression 3 hours later (Fig. 2B-E), consistent with the need for Egr-1 protein expression for induction of these factors. Promoter sequences for PDGFA and VEGF but not IL-8 showed putative Egr-1 sites and HGF-induced Egr-1–binding activity by ChIP assay (Fig. 3). Consistent with this, expression of PDGFA and VEGF but not IL-8 showed corresponding differences with Egr-1 expression in HNSCC tumor specimens and were strongly suppressed by transfection of Egr-1 antisense or siRNA oligonucleotides (Fig. 5). Overexpression of Egr-1 significantly enhanced both basal and HGF-induced activation of the PDGFA promoter (Fig. 4), providing evidence that Egr-1 can directly modulate expression of PDGFA.

Egr-1 is a critical transcription factor in mediating HGF-induced expression of genes that are important for the malignant progression. HGF-induced activation of Egr-1 contributes to expression of other molecules involved in metastasis, such as angiotsin-converting enzyme expression in bovine pulmonary artery endothelial cells (38), CD44v6 (32), and fibronectin matrix (33) in melanoma cells. The prominent role of Egr-1 in the regulation of angiogenesis has also been previously described. Lucerna et al. reported that adenovirus-mediated overexpression of NAB2, a coexpressor of Egr-1, significantly reduced tubule and sprout formation in vitro and in vivo (39). DNAzymes targeting
Egr-1 inhibited fibroblast growth factor–dependent endothelial proliferation, migration, and tubule formation in vitro, leading to suppressed tumor growth and decreased vessel density in xenograft models (25).

Khachigian et al. and Silverman et al. previously identified Egr-1 interaction with a GC-rich region of PDGFA proximal promoter located between −71 and −55 from the transcription starting site (30, 40, 41). Using promoter analysis software, we detected the cluster of multiple Egr-1–binding sites in PDGFA promoter, from −84 to −33 bp, as well as additional Egr-1–binding sites located upstream of the proximal PDGFA promoter region, from about −550 to −200 bp, and their functional activity was confirmed by ChIP assay (Fig. 3) and reporter gene assay (Fig. 4). Our data support the conclusion that the promoter region required for the maximal basal or HGF-induced PDGFA promoter activity is included within the −630-bp PDGFA construct. The previously identified proximal promoter region including −71 to −55 bp was not sufficient for the maximal levels of basal and HGF-induced PDGFA promoter activity in UM-SCC-11A cells.

In the current study, our data suggest Egr-1 and Sp1 transcription factors may play a differential role in controlling PDGF and VEGF expression. Inhibition of Egr-1 expression with Egr-1 antisense oligonucleotides completely blocked constitutive and HGF-mediated induction of PDGF-AB production but only partially suppressed VEGF production (Fig. 5C). In the PDGFA promoter region, Sp1-binding sites coexisted with Egr-1–binding sites; however, the relatively weaker Sp1-binding activity seen when compared with Sp1 binding of the VEGF promoter binding suggested that Sp1 may play a less important role in HGF-regulated PDGFA gene expression (Fig. 5B). In contrast, our and others’ data suggest that the regulation of VEGF expression is more complex and could possibly be controlled by multiple transcriptional and/or posttranscriptional mechanisms. Our ChIP assay showed strong constitutive binding but not much HGF-inducible binding of Sp1 in the VEGF promoter region (Fig. 3B), indicating that Sp-1 binding could be more important in constitutive VEGF expression in UM-SCC cells. A recent study by Reisinger et al. reports that HGF-induced Sp1 phosphorylation increases the transactivation capacity of this transcription factor leading to increased VEGF promoter activity (31). VEGF expression has also been reported to be modulated through a mechanism involving both promoter transactivation by hypoxia-inducible factor-1 (42) and stabilization of VEGF mRNA (43). Thus, HGF-regulated VEGF expression in UM-SCC-11A cells could be partially dependent on Egr-1 expression through MEK and PKC activation and partially dependent on other transcription factors or mechanisms.

Our experimental data using chemical inhibitors suggested that HGF-induced Egr-1 and PDGFA gene expression was mediated through MEK and PKC but not PI3K pathways (Fig. 6A). The experiments using siRNAs provided evidence supporting these conclusions, and specifically indicated that knocking down of PKC α, or a combination of MEK1 and MEK2, significantly suppressed Egr-1, PDGFA, and VEGF gene expression (Fig. 6B). Previous studies identified the involvement of PKC and MEK in regulating Egr-1 expression; however, most conclusions from the studies were limited to the use of chemical inducers or inhibitors (29, 30, 34–37). Conclusions using chemical inhibitors are often confounded by nonspecific or unknown inhibitory effects, particularly in terms of the involvement of the individual isoforms of kinases in the PKC.

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Figure 5. Egr-1, PDGFA, and VEGF gene expression in HNSCC tissues and Egr-1 siRNA or antisense oligos blocked Egr-1, PDGFA, and VEGF expression. A, relative gene expression of Egr-1, PDGFA, VEGF, and IL-8 in UM-SCC-11A xenografts and HNSCC tissues were detected by real-time RT-PCR after LCM. The gene expression levels in UM-SCC-11A xenograft tissues were used as controls. B, Egr-1 antisense oligos blocked Egr-1 protein expression by Western blot. β-Actin was used as the loading control. C, Egr-1 antisense oligos blocked PDGFA-AB and VEGF protein secretion. Cultured cells were transfected with Egr-1 antisense oligos for 6 hours and treated with HGF (40 ng/mL) for 48 hours. Cell culture supernatants were collected and the cytokines were measured by ELISA in triplicates. *, P < 0.05, statistically significant difference (Student’s t test). D, knocking down Egr-1 expression by siRNA suppressed basal and HGF-induced PDGFA and VEGF gene expression in UM-SCC-11A cells, detected by real-time RT-PCR. The experiments have been repeated three times.
or MEK pathways. Recently, Gaggioli et al. reported HGF-induced fibronectin matrix synthesis in melanoma cells through MEK induction of Egr-1 (33). They showed that HGF-induced Egr-1 promoter activity was abolished using chemical inhibitors of MEK or dominant-negative mutant plasmids of Ras, MEK1, and B-Raf (33). Our data from siRNA targeting MEK pathway support the role of MEK in Egr-1-induced expression of PDGFA and VEGF, and additionally, illustrate redundancy of MEK1 and MEK2 in basal and HGF-induced Egr-1, PDGF, and VEGF expression in UM-SCC cells (Fig. 6). There are even fewer reports regarding the specific components of PKC involved in HGF-induced Egr-1 induction. PKC is a phospholipid-dependent serine/threonine kinase family, consisting of at least 11 isoforms that exhibit related homologies in their structures (44–46). PKC ε belongs to the novel PKCs that do not require calcium for activation (44–46). PKC ε has been implicated in the promotion of the skin SCC progression, where overexpression of PKC ε in the mouse epidermis resulted in the rapid development of metastatic squamous cell carcinomas (47). In our study, we identified PKC ε as one of the important PKC components involved in HGF signal transduction (Fig. 6B), which has not been reported. Most of the previous observations regarding PKC-regulated Egr-1 expression were obtained with the use of phorbol 12-myristate 13-acetate–stimulated cells and chemical inhibitors, where the specific PKC components were not distinguished (28–30).

A significantly higher basal and HGF-induced PDGF protein production was observed in UM-SCC cells, when compared with cultured normal keratinocytes (Fig. 1). The minimal production of basal levels of PDGF and the lack of HGF induction in normal keratinocytes were not due to c-Met deficiency. Under the same
culture conditions, we previously showed that normal human keratinocytes expressed similar levels of c-Met protein as these UM-SCC cells by Western analysis (9). HGF (40 ng/mL) induced similar levels of c-Met and Erk phosphorylation in both normal keratinocytes and UM-SCC (9). In addition, we observed slightly higher levels of Egr-1 gene expression in normal keratinocytes when compared with UM-SCC-11A by real-time RT-PCR, and HGF was able to further induce Egr-1 expression in keratinocytes. HGF failed to induce PDGF (this study), as well as IL-8 and VEGF production (9) in normal keratinocytes, suggesting the possibility of important differences between normal keratinocytes and HNSCCs in downstream signal pathways, or other unidentified negative regulators.

Growth factors in the PDGF family have been implicated in angiogenesis and pathogenic effects in HNSCC and other malignancies (21). In HNSCC patients, we found an increase in serum PDGF growth factor levels when compared with the normal subjects, and such increase is associated with the elevated level of serum HGF.3 In UM-SCC-11A cells, constitutively higher levels of PDGF-AB (Fig. 1) and PDGF-AA (data not shown) were observed in the culture supernatants when compared with that from human normal keratinocytes. HGF is also able to induce PDGF-AB protein production in all HNSCC cells lines tested (Fig. 1C). In LCM-processed HNSCC tissues, a correspondence between differences in the levels of PDGFA and Egr-1 gene expression was found (Fig. 5A). Although we do not understand the mechanisms of the heterogeneous expression of Egr-1 and PDGF in HNSCC tissues, a recent report indicated that mutant p53 is able to up-regulate Egr-1 protein expression in tumors (48). Taken together, these observations support the notion that constitutive and HGF-induced PDGF growth factors are expressed in HNSCC cells and malignant epithelia and could affect the angiogenic and metastatic processes in tumor environment. The growth factors in the PDGF family also play crucial roles in the paracrine loop between the stroma cells and cancer cells. For instance, we have observed that IL-1 produced by UM-SCC cells in culture can induce HGF production by primary fibroblast cultures from HNSCC specimens, and HGF in turn was able to induce PDGF-AB production in the HNSCC cell lines. In addition, recombinant PDGF-AB and PDGF-AA from cultured condition medium of UM-SCC cells were able to induce HGF production by human fibroblasts.4

In summary, HGF stimulates HNSCC cells to produce proangiogenic factors like PDGF, VEGF, and IL-8. Our current study indicates that Egr-1 plays an important role in expression of PDGF and VEGF, which may explain how both HGF and Egr-1 contribute to the induction of factors that mediate angiogenesis. This transcription factor, PKC and MEK, together with the proangiogenic factors, may be promising targets for the development of future anti-angiogenic interventions.

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