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Research Article

Human papillomavirus 16 E5 up-regulates the expression of vascular endothelial growth factor through the activation of epidermal growth factor receptor, MEK/ERK1,2 and PI3K/Akt

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Abstract. The E5 oncoprotein of human papillomavirus (HPV) 16 plays an important role in early cervical carcinogenesis. Vascular endothelial growth factor (VEGF) plays a central role in switching on the angiogenic phenotype during early cervical carcinogenesis. However, the relationship between E5 and VEGF has not previously been examined. To clarify the regulatory role of E5 in VEGF expression, we transferred the E5 gene into various cell types. E5 increased VEGF expression. The addition of epidermal growth factor receptor (EGFR) inhibitor significantly suppressed VEGF expression, demonstrating that E5 stimulates VEGF expression through the activation of EGFR. E5-mediated EGFR activation was accompanied by phosphorylation of Akt and ERK1/2, which are also involved in VEGF expression. Furthermore, the mRNA stability of VEGF was not affected by E5, but VEGF promoter activity could be modulated by inhibitors of the EGFR, MEK-ERK1/2 and PI3K/Akt pathways in E5-expressing cells. Collectively, these novel results suggest that HPV 16 E5 increases VEGF expression by activating EGFR, MEK/ERK1/2 and PI3K/Akt.

Keywords. Human papillomavirus, E5 oncoprotein, vascular endothelial growth factor, epidermal growth factor receptor.

Introduction

Infection with human papillomavirus (HPV) is considered to be necessary for cervical cancer to develop [1]. HPV infection results in expression of viral oncogenes such as E5, E6 and E7, which modify the functions and expression patterns of various cellular genes [2, 3]. These oncoproteins have specific complementary and synergistic effects in the development and progression of cervical cancer. E6 and E7 have been shown to play important

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roles throughout the whole disease process; E6 mediates degradation of p53, while E7 inactivates the phosphorylated retinoblastoma (pRb), a tumor suppressor protein [4, 5].

E5 is often deleted during the progression to cervical cancer [6, 7], suggesting that this protein may play an important role in the early stage of cervical carcinogenesis. The HPV 16 E5 protein is a hydrophobic, 83-amino acid polypeptide that associates with the Golgi apparatus, endoplasmic reticulum, and perinuclear membrane [8, 9]. E5 activates the epidermal growth factor receptor (EGFR) in a ligand-dependent fashion. Studies have shown that expression of E5 increases EGFR activity via the interaction of E5 with the 16- kDa subunit of vacuolar ATPase, blocking endosome acidification and subsequent degradation of substrates such as EGFR and other proteins in endosomes [10–13]. These interactions are thought to be important in cellular transformation induced by E5.

The acquisition of an angiogenic phenotype, which plays a major role in tumor progression and metastasis, has been examined in a number of tumor types, including cervical cancer. Studies have shown that the angiogenic factor vascular endothelial growth factor (VEGF) is involved in carcinogenesis and progression in various cell types [14–17]. VEGF was found to be up-regulated from the early stage of cervical cancer [18, 19], and HPV 16 E6 and E7 have been identified as inducers of proangiogenic factors such as VEGF and IL-8 in cervical cancer [20]. However, the relationship between E5 and VEGF expression has not been identified.

The present study was therefore designed to determine whether and how HPV 16 E5 up-regulates VEGF expression.

Materials and methods

Cell lines and reagents. All the cell lines used in this study (C33A and HT3 human cervical cancer, human embryonic kidney 293 and human immortalizing keratinocyte HaCaT cell lines) were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin (all from Invitrogen, Carlsbard, CA).

The inhibitors acting against EGFR (tyrphostin AG1478), MEK (PD98059, U0126) and phosphatidylinositol 3-kinase (PI3K) (Wortmannin, LY294002), JNK inhibitor (SP600125) and p38 mitogen-activated protein kinase (MAPK) inhibitor (SB202190) were purchased from Calbiochem (La Jolla, CA). Stock solutions were freshly prepared in dimethyl sulfoxide (DMSO) and added to the cell cultures to obtain the indicated final inhibitor concentrations. The DMSO concentration was 0.001% and the same concentration was used as vehicle. DMSO alone (0.001%) was found to have no significant effect on cell function compared to untreated cells.

Antibodies specific for phosphorylated (p-) ERK1/2, total ERK1/2 (ERK) and EGFR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylated, total types of Akt (p-Akt and Akt), p38 (p-p38, p38), and JNK (p-JNK, JNK) and phospho-EGFR, were obtained from Cell Signaling (Beverly, MA). The anti-α-tubulin antibody (used as a loading control) was obtained from Sigma Chemical (St. Louis, MO), and the Lipofectamine 2000 transfection reagents were purchased from Invitrogen.

Construction and transfection of the E5 expression vector. We used all the HPV-negative cell lines including C33A cervical cancer cell lines that had been stably transfected with HPV 16 E5 or the empty vector pcDNA3 as controls. A DNA sequence containing the E5 open reading frame (nucleotides 3863–4099) flanked by *Bam*HI-*Xho*I restriction sites was PCR amplified (5′-GGG GAT CCA CCA TGG TAC TGC ATC CAC AAC ATT ACT and 5′-CGC TCG AGT TAT GTA ATT AAA AAG CGT GC; Kozak consequence is underlined) in which Kozak sequence was introduced to increase the potential translational level [21]. The resulting fragment was inserted into *Bam*HI/ *Xho*I-precut pcDNA3 (Invitrogen) to generate pcDNA3-E5. The desired sequence was confirmed by direct DNA sequencing.

For transfection, C33A cells were grown to 70% confluency and transfected in serum-free medium for 6 h with 3.5 µl Lipofectamine2000 and 2 µg of either pcDNA3-E5 or pcDNA3. Cells were selected with 250 µg/ml G418 (Life Technologies, Inc., Grand Island, NY), and resistant clones were screened for E5 expression. The presence of E5 RNA in the cells was detected by real-time RT-PCR analysis.

Enzyme immunoassay. The VEGF levels of the cell culture supernatants were determined by ELISA using a commercially available kit (R&D Systems). This VEGF immunoassay kit can determine relative mass values for natural human VEGF, especially $VEGF_{165}$ and $VEGF_{121}$, the expression of which was detected by RT-PCR done in this study. Each measurement was repeated in triplicate, and the average value was recorded as pg/ml.

Total RNA isolation and RT-PCR analysis. Total RNA was isolated with TRIzol reagent (Life Technologies, Inc.) and cDNA were synthesized from 1 µg total RNA using M-MLV reverse transcriptase (Invitrogen) with random hexamer priming. The primers for RT-PCR were derived from external exons shared by all differentially spliced VEGF mRNA species [22]. PCR was performed with specific primers (VEGF sense 5′-CCA TGA ACT TTC TGC CTT CTT-GG-3′, antisense 5′-CTC ACC GCC

TCG GCT TGT CAC-3′, β-actin sense 5′-ACA CTG CCA TCT ACG AGC-3′, antisense 5′-AGG GGC CGG ACT CGT CAT ACT-3′) using the following amplification conditions: 95 °C for 2 min, followed by 28 cycles of denaturation step at 95 °C for 45 s, and annealing step at 60 °C for 1 min, and the extension step at 72 °C for 1 min.

Real-time RT-PCR. Real-time quantitative PCR was performed in an iCycler IQ (Bio Rad Laboratories) using cDNA Master SYBR Green I dye (Roche Molecular Biochemicals). Measurements of the threshold cycles were made at the end of each extension step, using the second-derivative method offered by the iCycler IQ optical system software (version 3.0a, Bio-Rad Laboratories). Melting curve analysis was performed to confirm the identifies of peaks of interest in each samples. Results were normalized compared to the amount of β-actin mRNA.

Transient transfections. Cells [human embryonic kidney 293 (HEK 293), spontaneously immortalized human keratinocyte cell (HaCaT) and HPV-negative cervical cancer cells (HT-3)] were transfected with 2 μg of either pcDNA3-E5 or pcDNA3 using Lipofectamine2000 (3.5 μl) in serum-free medium. After transfection for 6 h, normal medium was added, with further incubation for 48 h.

Western blotting. Protein extraction was performed as described previously [23]. The protein concentration of the lysate was measured using the bicinchoninic acid method (Pierce). Cell lysate (50 μg) were separated by SDS-PAGE on an 8% gel, transferred onto a nitrocellulose membrane, and immunoblotted with the indicated antibodies. Primary antibodies for p-ERK, ERK, p-Akt, Akt, EGFR and p-EGFR were used at 1:1000 dilutions and incubated at 4 °C for overnight. The membrane was then incubated with the appropriate secondary antibody (1:3000 dilution) in 5% skimmed milk/Tris-based saline with 0.05% Tween-20 (TBST) for 2 h at room temperature.

In some case, the membrane was stripped by incubation in stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM 2-mercaptoethanol) at 65 °C for 30 min, washed for 1 h (three times for 20 min) with TBST, reblocked with TBST containing 5% skimmed milk, and then re-probed with α-tubulin antibody (1:3000).

mRNA stability assay. The half-life of VEGF mRNA was assayed as a measure of VEGF mRNA stability. The transcription inhibitor, actinomycin D (Sigma) was added to the media of E5, control transfected cells at 5 μg/ml. Total mRNA was isolated at the indicated times and the VEGF mRNA levels were quantified by RT-PCR and normalized against β-actin. The half-life of VEGF mRNA was calculated by drawing the best-fit linear curve on a plot of VEGF mRNA level *versus* time.

Analysis of VEGF promoter activities. The VEGF luciferase reporter gene construct was kindly gift by Dr. Tae-you Kim (Seoul National University, Korea) [24]. C33A cells expressing E5 were seeded at 5×10 cells/well in 12-well culture plates, incubated overnight, and then transfected with a VEGF luciferase reporter gene construct (0.4 μg/well) and pSV-β-galactosidase (0.1 μg/well) using 3.5 μl Lipofectamine2000. At 6 h post transfection, cells were incubated in complete medium containing 10% FBS. At 48 h post transfection, cells were washed twice with PBS, and luciferase activities were measured using a Bioluminescent Reporter Gene Assay System (Tropix Inc.) according to the manufacturer's instructions. The luciferase activities were normalized against that of β-galactosidase.

Results

HPV 16 E5 induces up-regulation of VEGF expression in various cell types. We established E5-expressing cells derived from the human cervical carcinoma C33A cells. After subcloning the G418-resistant cells, two clones of E5-expressing cells (E5-1 and E5-3) were selected. We confirmed that E5-1 and E5-3 cells expressed significantly high levels of E5 mRNA using real-time RT-PCR, but control (vector transfected) cells did not (Fig. 1a). To examine our primary hypothesis that E5 oncoprotein contributes to inducing angiogenic factors, we examined the expression of VEGF, which is a well-known potent inducer of angiogenesis. First, using RT-PCR, we assessed the expression level of VEGF mRNA in E5-expressing and control cells (Fig. 1b). The major amplified species on RT-PCR were 580- and 450-bp fragments (Fig 1b, d). The two major products correspond in size to the products expected for the 165-amino acid isoform of VEGF ($VEGF_{165}$: upper band) and the 121-amino acid isoform of VEGF ($VEGF_{121}$: lower band), respectively [22]. E5-expressing cells enhanced the expression level of VEGF mRNA in parallel with the E5 expression level. Consistent with the mRNA levels, the increase in secreted VEGF protein was verified in E5-expressing cells by enzyme-linked immunosorbent assay (ELISA, Fig. 1c). Thus, we found that HPV 16 E5 oncoprotein enhanced the expression of VEGF mRNA and protein in cervical cancer cells. This enhancement of VEGF expression by E5 was demonstrated in other cell types, such as HEK 293, HaCaT and HT-3, using transient transfection (Fig. 1d).

EGFR kinase activity is involved in E5-induced VEGF expression. Many studies have demonstrated that E5 activates EGFR [11, 25]. The activation of EGFR can initiate biochemical cascades associated with several malignant processes, including angiogenesis. We therefore examined whether phosphorylation of EGFR is increased in

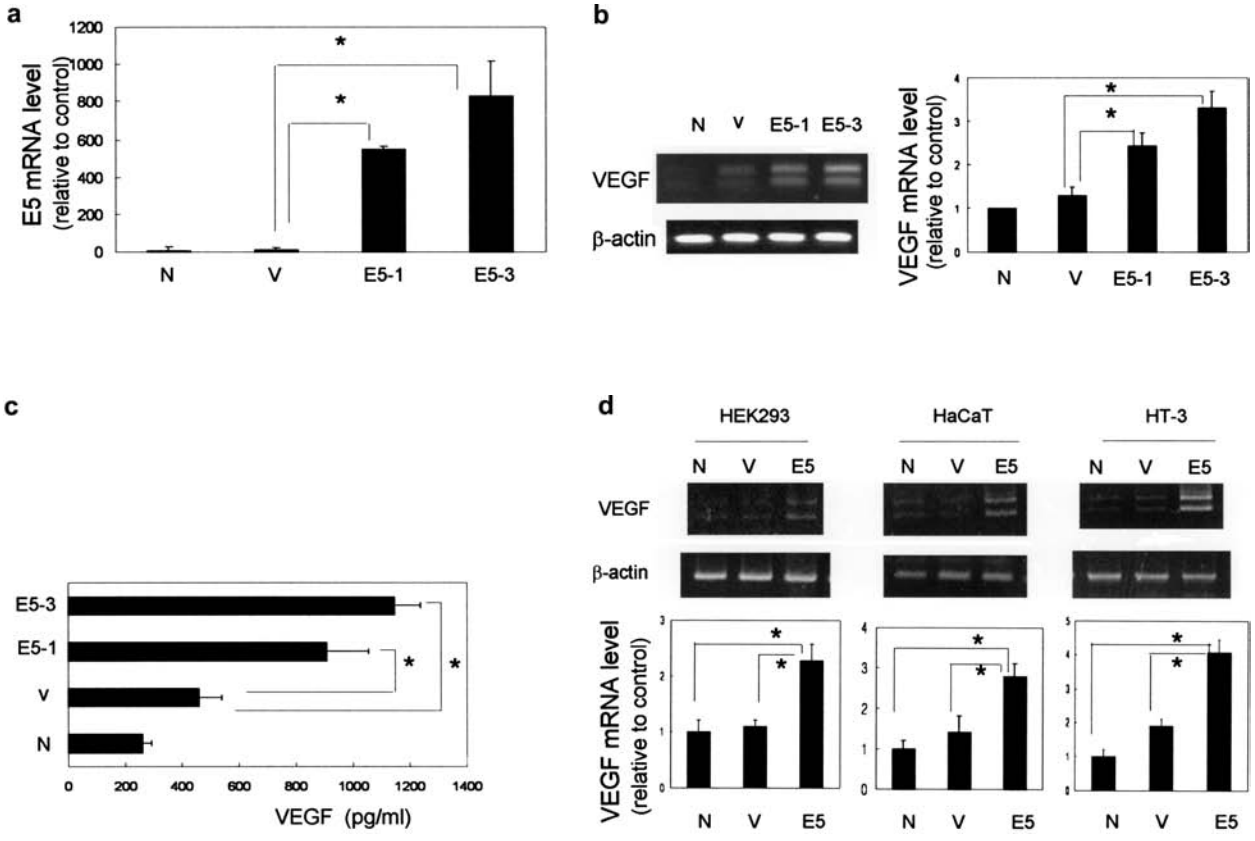

Figure 1. Effect of E5 on VEGF expression in various cell types. Total RNA (1 µg) from mock- (N), control- (V, pcDNA3), and E5-1/-3 (pcDNA3-E5)-transfected cells was used for real-time RT-PCR. RT-PCR was done using specific primers for E5 and VEGF as described in Materials and methods. The primers for VEGF recognize two isoforms [22]. The expression of mRNA of E5 and VEGF was normalized to the expression of β-actin, given arbitrarily equal to one in control cells. (*a*) Detection and quantification of E5 expression in pcDNA3-E5 transfected cells were done by real-time RT PCR. E5-1 and E5-3 clones were selected from three independent transfections of two different pools of C33A cell lines to evaluate the dose relationship between E5 and VEGF expression. (*b*) Increased expression of VEGF in parallel with the level of E5 expression, using RT-PCR. (*c*) Culture supernatants were harvested and analyzed for VEGF protein secretion using ELISA, and the VEGF levels were normalized against the total amount of protein determined by the bicinchoninic acid method. (*d*) Effect of transient expression of E5 on VEGF expression in various cell types (HEK293, HaCaT and HT3). The graph represents the mean ± SD of triplicate samples from three independent experiments ($n = 9$). The analysis of variance test between groups was determined by post-hoc comparison (*, $p < 0.05$ *versus* pcDNA3-transfected control).

E5-expressing cells. E5-expressing cells were grown for 24 h, and Western blotting of cell lysates validated the presence of the 170- kDa EGFR and increased EGFR phosphorylation without a change of EGFR expression level (Fig. 2a). To examine the regulatory role of EGFR activation on VEGF expression, we treated E5-expressing cells with AG1478, a selective inhibitor of EGFR tyrosine phosphorylation. The treatment with AG1478 significantly inhibited E5-mediated EGFR phosphorylation (Fig. 2b). In parallel with the inhibition of EGFR phosphorylation, expression of VEGF mRNA (Fig. 2c) and protein (Fig. 2d) was also suppressed in E5-expressing cells. These findings support the hypothesis that EGFR activation plays a key role in VEGF expression induced by E5.

MEK-ERK and PI3K/Akt pathways are both involved in E5-induced VEGF expression. To gain further insight into the roles of EGFR signaling in VEGF expression, we examined activation of the MAPK and PI3K pathways, which represent the main downstream targets of the EGFR signaling pathway [26, 27]. PI3K has been reported to modulate phosphorylation of Akt and GSK3-β [28, 29], while MAPK kinase was shown to modulate phosphorylation of ERK, JNK and p38 MAPK [30]. Since these kinases are well-characterized regulators of angiogenesis in various cancer cells, we determined the phosphorylation levels of ERK1/2, JNK, p38 MAPK and Akt in E5-expressing cells. As shown in Fig. 3a, higher activation of ERK1/2 and Akt was found in E5-expressing cells than in control cells. In contrast, the level of p-p38 and p-JNK, two other representative MAPKs, was not affected by E5 (Fig. 3a). Treatment with AG1478 completely blocked the E5-induced activation of ERK1/2 and Akt in E5-expressing and control cells (Fig. 3b), implicating that activation of EGFR plays an important role in E5-induced signaling cascade. We then checked

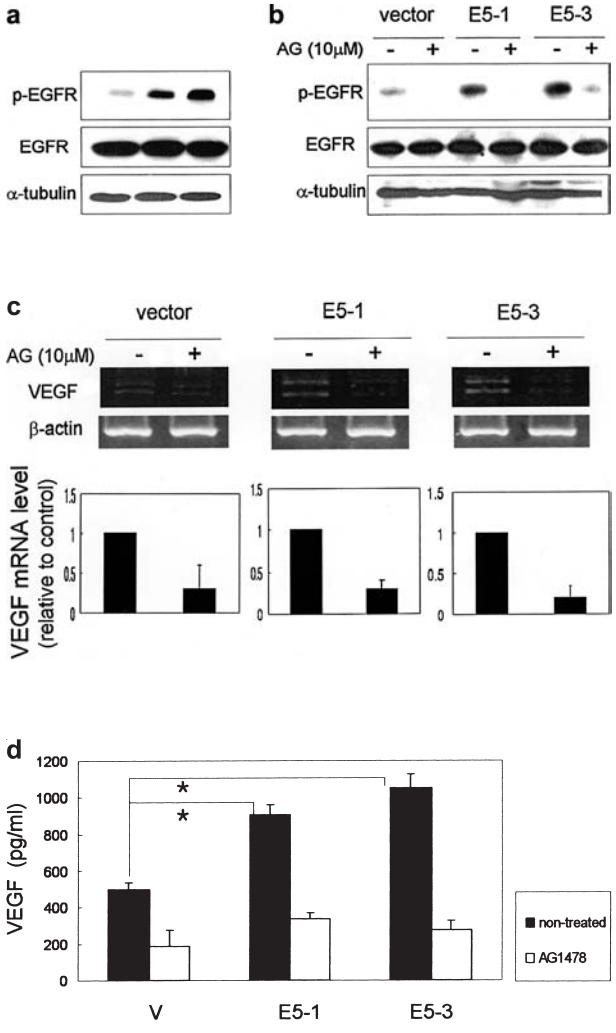

Figure 2. Relationship between EGFR activation and increased VEGF expression by E5. (*a*) Whole cell lysate (50 μg) from pcDNA3 (V) or pcDNA3-E5 (E5-1, E5-3) transfected cells was separated on a 8% polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with antibodies to EGFR and phosphorylated EGFR (1:1000). The blot was stripped and reprobed with antibody to α-tubulin to verify equal loading. (*b*) Effect of AG1478 (EGFR kinase inhibitor) on inhibition of EGFR phosphorylation. Cells were treated with 10 μM AG178 for 1 h and then cell lysates were analyzed for EGFR phosphorylation by Western blot analysis using a specific antibody to phosphorylated EGFR. (*c, d*) Transfected cells were pretreated with 10 μM AG1478 for 1 h as previously described. VEGF mRNA (*c*) and secreted protein levels (*d*) were analyzed as described in Fig. 1b and c. The blots were representative of three independent experiments showing similar trends. The graph represents the mean ± SD of triplicate samples from three independent experiments ($n = 9$) (*$p < 0.05$ *versus* pcDNA3-transfected control).

whether these signaling pathways are involved in up-regulation of VEGF by E5. We added the PI3K/Akt inhibitor (Wortmannin) and MEK-ERK1/2 inhibitor (PD98059) to E5-expressing cells. The dose of inhibitors used in this study was determined from the literature and from preliminary experiments [31, 32]. We checked the specificity of these inhibitors (Wortmannin, PD98059) on Akt and ERK1/2, respectively (Fig. 3c). MEK-ERK1/2 and PI3K/Akt inhibitors partially suppressed the expression of VEGF mRNA (Fig. 3d) and protein (Fig. 3e). Other pharmacological inhibitors that block the same signaling pathways (LY294002 for PI3K and U0126 for MEK) showed similar results (data not shown). In addition, when we added the JNK inhibitor (SP600125) and p38 MAPK inhibitor (SB202190) to E5-expressing cells, there was no changes in VEGF expression by E5 (data not shown). These results indicate that PI3K/Akt and MEK-ERK1/2 signaling pathways are involved in the E5-induced VEGF regulatory mechanism.

E5 up-regulates VEGF expression through increased VEGF promoter activity, but not through mRNA stability. Regulation of VEGF by various stimuli has been shown to occur both at the level of transcription and mRNA stability. To determine whether the E5-induced regulation of VEGF expression is associated with mRNA stability, we inhibited mRNA transcription using actinomycin D. The values of estimated VEGF mRNA half-life ($t_{1/2}$) of vector-, E5-1- and E5-3-transfected cells were 1.6, 2.1 and 2.6 h, respectively (Fig. 4a). However, there was no statistical significant difference between the half-lives of VEGF mRNA for each group. The VEGF mRNA degraded completely in the order of increasing mRNA abundance, that is, in the order of vector-, E5-1- and E5-3-transfected cells, between 4 and 6 h. This result implies that VEGF mRNA stability seems not to be involved in the enhancement of VEGF expression by E5. Next, the transcriptional activity of VEGF promoter assessed by luciferase reporter gene assay was increased in E5-expressing cells (Fig. 4b). Figure 4c shows that EGFR inhibitor significantly decreased VEGF promoter activity, as it did expression of VEGF mRNA, and treatment with other inhibitors (MEK-ERK, PI3K/Akt) partially decreased the activity of VEGF promoter in parallel with the expression level of VEGF mRNA shown earlier in this study.

Discussion

Altered angiogenesis is an important phenotype of high-grade cervical lesions and invasive cervical carcinomas [17, 18], and some studies have explored HPV oncoprotein-induced regulation of angiogenesis in this context. E6 and E7 oncoproteins of HPV 16 have been shown to induce VEGF expression in a p53-independent manner [20, 33]. However, the functional role of E5 oncoprotein in this process has not been studied to date. This study shows that E5 increases VEGF expression irrespective of cell type. Its effects on VEGF expression appears to be dependent upon E5 expression level. The evidence is that the expression level of E5 paralleled that of VEGF (Fig. 1b, c). The enhancement of VEGF expression by E5

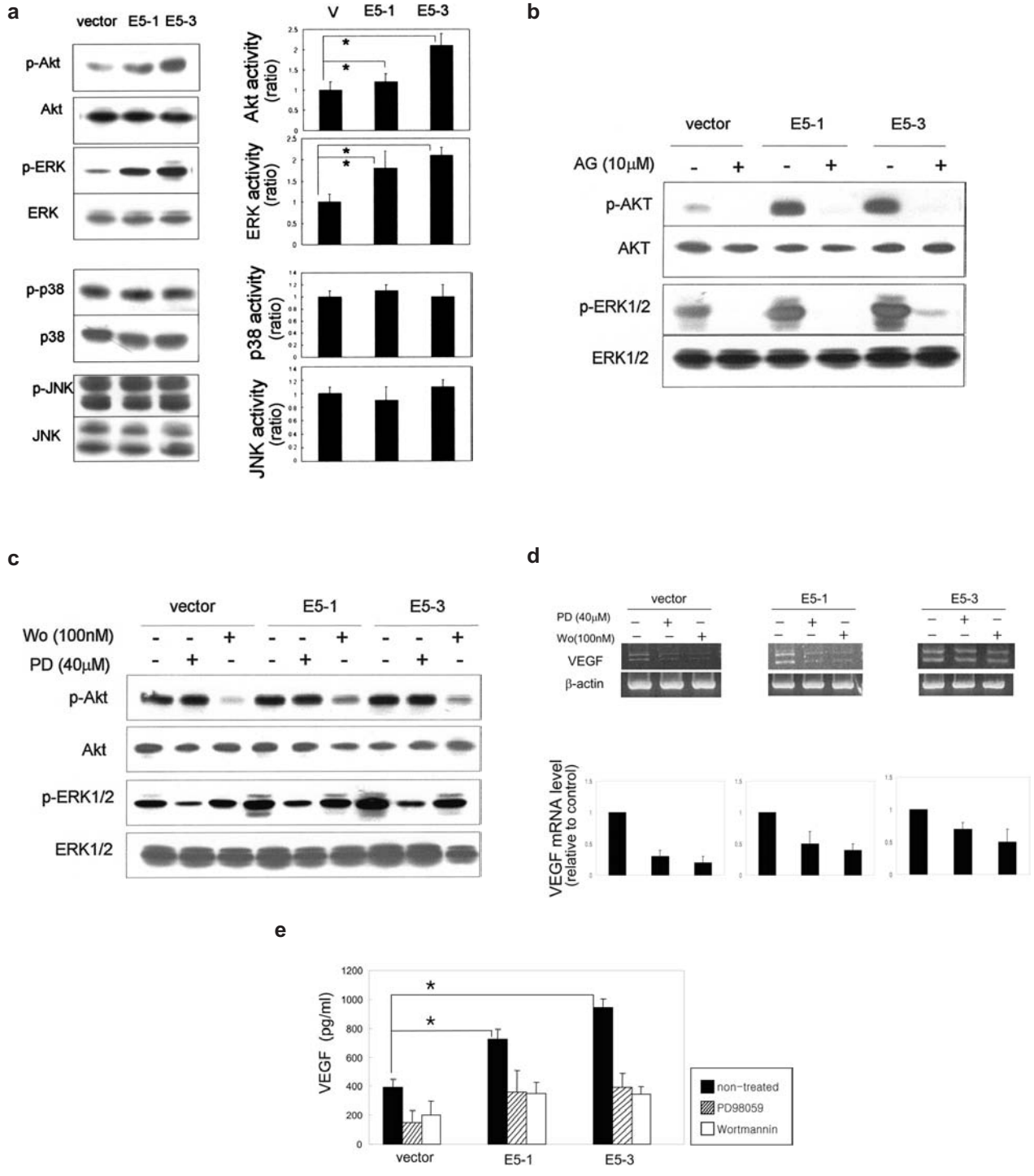

Figure 3. Involvement of MEK-ERK and PI3K/Akt increased VEGF expression by E5. (*a*) Whole cell lysate (50 μg) from pcDNA3 (V) or pcDNA3-E5 (E5-1, E5-3) transfected cells was separated on a 8% polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with antibodies to phosphorylated and non-phosphorylated Akt, ERK, p38 and JNK (1:1000) in transfected cells. Each activity was expressed as a ratio of the band density of pcDNA3-E5 transfected cells to that of pcDNA3-transfected cells. (*b*) Effect of AG1478 on p-Akt and p-ERK1/2. Cells were treated with 10 μM AG1478 for 1 h. Protein isolated after 1-h treatment was analyzed for phosphorylated and non-phosphorylated Akt and ERK (1:1000) by immunoblotting. (*c*) Specificity of inhibitors targeting MEK-ERK1/2, and P13K/Akt was checked using PD98059 (PD, 40 μM) and Wortmannin (100 nM), respectively. Cells were treated for 1 h with each drug. Then protein isolated after 1-h treatment was analyzed for phosphorylated and non-phosphorylated Akt and ERK (1:1000) by immunoblotting. (*d, e*) Treatment with PD98059 (PD, 40 μM), and Wortmannin (100 nM) for 1 h reduced VEGF expression increased by E5. VEGF mRNA (*d*) and secreted VEGF protein levels (*e*) were analyzed as described in Fig. 1b and c. The blots were representative of three independent experiments showing similar trends. The graph represents the mean ± SD of triplicate samples from three independent experiments ($n = 9$) (*$p < 0.05$ *versus* pcDNA3-tansfected control).

was also demonstrated in other cell types, such as HEK 293, HaCaT and HT-3 (Fig. 1d). This finding implies that the enhancing effect of E5 on VEGF expression seems to be general, not cell specific.

The effect of E5 on VEGF expression was found to be closely related to the activation of EGFR. EGFR was activated with the introduction of the HPV 16 E5 gene into cells, which is consistent with a previous report [34]. The association of VEGF expression with EGFR activation was shown in two ways, by phosphorylation and pharmacological inhibition studies; firstly, E5 expression increased the phosphorylation of EGFR in parallel with expression level of E5 and, secondly, pharmacological inhibition of EGFR activation decreased VEGF expression at the level of mRNA and protein, in parallel with the inhibition of EGFR phosphorylation (Fig. 2b, c). Interestingly, pharmacological inhibition of EGFR reduced VEGF expression even in control cells. As shown in Fig. 2a, p-EGFR was detected in control cells. This can explain the inhibitory effect of EGFR inhibitor on VEGF expression in control cells. This result is consistent with the previous reports that EGFR is commonly overexpressed and activated in various types of malignancy, including cervical cancer [35, 36].

In this study, the enhancement of VEGF expression by E5 was found to be mediated through the activation of the MEK-ERK1/2 and PI3K/Akt, downstream of EGFR. This was shown by phosphorylation and pharmacological inhibition studies. First, the levels of active phosphorylated proteins of ERK1/2 and Akt, but not of JNK and p38 MAPK, increased in parallel with that of active p-EGFR (Fig. 3a). Second, inhibition of EGFR activation with AG1478 inhibited the phosphorylation of ERK1/2 and Akt (Fig. 3b). Furthermore, the inhibition of MEK-ERK1/2 and PI3K/Akt signaling pathways using pharmacological inhibitors also partially inhibited E5-induced VEGF expression at the level of mRNA and protein (Fig. 3d, e). Other pharmacological inhibitors to block the same signaling pathways (LY294002 for PI3K and U0126 for MEK) showed similar results (data not shown). In addition, the lack of the effect of pharmacologically relevant concentrations of inhibitors for JNK and p38 MAPK on VEGF expression supports the specificity of the effects seen with inhibitor of EGFR, MEK/ERK and PI3K (data not shown). Thus, we confirmed the dependence of VEGF expression on these signaling pathways. Our results are supported by reports that EGFR can induce the downstream activation of several important intermediate signal transduction kinases, including MEK/ERK and PI3K involved in tumor angiogenesis [26, 27].

Finally, we explored the regulatory mechanism for E5-induced expression of VEGF. The expression of VEGF has been demonstrated to be regulated at multiple levels from the transcriptional to post-translational level. Several studies have reported that VEGF expression was modu-

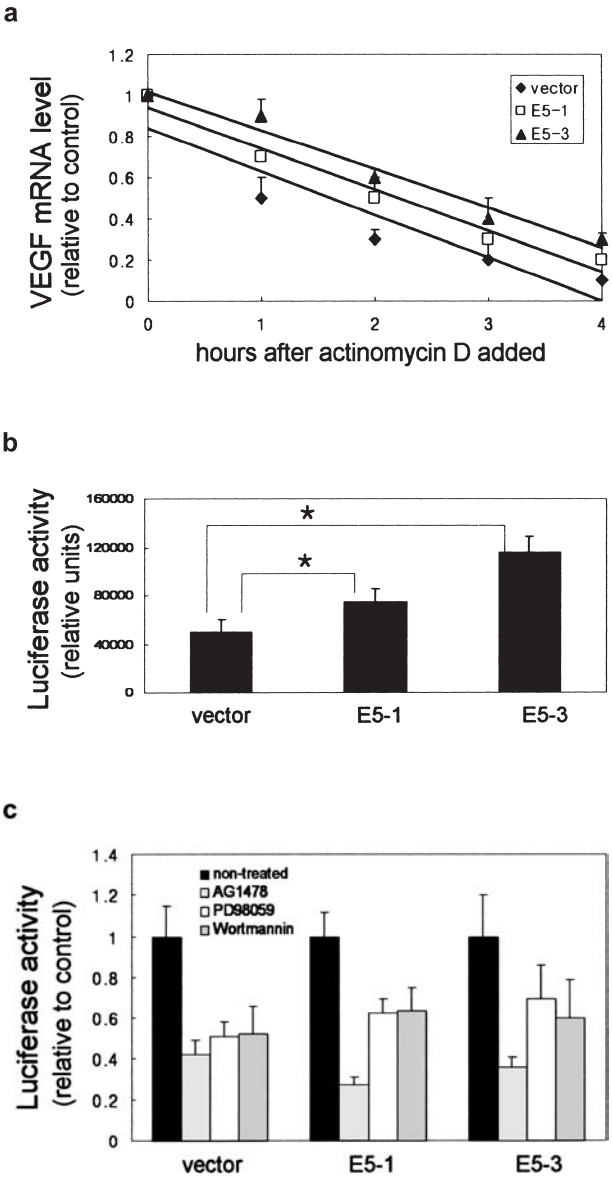

Figure 4. Effect of E5 on VEGF mRNA stability and promoter activity. (*a*) Transfected cells were treated with 5 µg/ml actinomycin D for the indicated times and analyzed for mRNA stability. Data are the relative level of mRNA (ratio of the value at indicated time *versus* that at time 0 of actinomycin D treatment) at the indicated times and normalized to the internal control β-actin level. (*b*) Transiently transfected pcDNA3 (V) and pcDNA3-E5 (E5-1, E5-3) cells were co-transfected with 400 ng VEGF promoter construct containing a 1.2-kb fragment and 100 ng pSV-β-galactosidase. After 48 h, cells were harvested and analyzed for luciferase and β-galactosidase activity. (*c*) Cells were co-transfected with 100 ng pSV-β-galactosidase and 400 ng VEGF promoter construct containing a 1.2-kb fragment. After 48 h, cells were either not treated or treated for 1 h with AG1478 (10 µM), PD98059 (40 µM), or Wortmannin (100 nM) and then analyzed as described in (*b*). The graph represents the mean ± SD of triplicate samples from three independent experiments ($n = 9$) (*$p < 0.05$ *versus* pcDNA3-tansfected control).

lated by mRNA stability under various conditions. Conditions such as hypoxia, ras, tyrosine kinase oncogene, UV irradiation, insulin, IGF and phorbol myristate acetate can increase mRNA stability of VEGF [37–41]. In this study, we found that E5-induced VEGF regulation occurred through change in VEGF transcriptional activity rather than alteration in mRNA stability (Fig. 4a, b). Although not significant, there was a trend towards slightly prolonged half-lives of VEGF mRNA in E5-expressing cells compared to cells transfected with empty vector (Fig. 4a). An explanation could be that the increasing expression of VEGF mRNA may saturate the mechanism for the elimination of VEGF mRNA such that mRNA stability was effectively increased.

In a further study using pharmacological inhibitors, treatment of E5-expressing cells with AG1478 led to a significant decrease in VEGF promoter activity, as did treatment with PI3K/Akt and MEK-ERK1/2 inhibitor (Fig. 4c). These data provide strong evidence that there is a correlation between EGFR activation and VEGF transcriptional activity via the MEK-ERK1/2 and PI3K/Akt pathways in E5-expressing cells.

In summary, we found that the HPV 16 E5 oncoprotein increased VEGF mRNA and protein expression in various cell types (human cervical carcinoma C33A and HT3, HEK 293 and HaCaT cells). The effect of E5 on the enhancement of VEGF expression is through increased transcriptional activity by the activation of EGFR signaling pathway. Our data may explain the important role of HPV 16 E5 in the angiogenesis of cervical cancer, and suggest that the EGFR signaling pathway could be a potential therapeutic target for cervical cancer.

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