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# Adrenomedullin Up-regulates the Expression of Vascular Endothelial Growth Factor in Epithelial Ovarian Carcinoma Cells via JNK/AP-1 Pathway

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**Objective:** Adrenomedullin (AM), a potent vasodilator peptide, presents in various kinds of tumors and promotes angiogenesis. We have previously reported that AM is expressed in epithelial ovarian carcinoma tissue. Here, we investigated the hypothesis that AM might regulate production of vascular endothelial growth factor (VEGF) in epithelial ovarian carcinoma and further promote angiogenic processes.

**Methods:** The messenger RNA expression of VEGF in human epithelial ovarian carcinoma cells (HO-8910) was examined by real-time polymerase chain reaction. Transcriptional control was analyzed by transient transfection assay of VEGF promoter-luciferase hybrid genes and chromatin immunoprecipitation assay. Activation of c-Jun N-terminal kinase (JNK) was detected by Western blotting. The formation of capillarylike structures by EA.hy926 cells cocultured with HO-8910 cells on Matrigel was also studied.

**Results:** We found that in HO-8910 cells, AM ( $10^{-10}$  to  $10^{-7}$  mol/L) enhanced VEGF messenger RNA expression in a time- and concentration-dependent manner, as well as promoter activity. Furthermore, JNK was activated by AM stimulation. The AM-induced increase in VEGF expression was significantly attenuated by SP600125, a specific JNK inhibitor. Chromatin immunoprecipitation assay and promoter activity analysis showed that VEGF expression induced by AM required the activator protein 1 motif on the VEGF promoter. In an in vitro angiogenesis system for endothelial cells (EA.hy926) cocultured with HO-8910 cells, we observed that the addition of AM stimulated endothelial cell tube formation, which could be abolished by VEGF neutralizing antibody.

**Conclusions:** Our findings suggest that the JNK/Activator protein 1 pathway is involved in AM-induced VEGF expression in HO-8910 cells.

**Key Words:** Adrenomedullin (AM), Vascular endothelial growth factor (VEGF), Epithelial ovarian carcinoma, c-Jun N-terminal kinase (JNK), Activator protein 1 (AP-1)

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Ovarian cancer is one of the most common cause of death from all cancers among women and the leading cause of death from gynecological malignancies. Epithelial ovarian carcinoma is a common malignant ovarian neoplasm with poor 5-year survival rate. Many factors regulate the rapid growth of epithelial ovarian carcinoma. The autocrine secretion hypothesis proposes that as a result of oncogene activation, neoplastic cells can produce certain growth factors to promote the tumor proliferation or angiogenesis.<sup>1</sup>

Dysregulated angiogenesis is involved in tumorigenesis and progress.<sup>2</sup> Tumor vessels develop by sprouting or intussusception from preexisting vessels. Circulating endothelial precursors, shed from the vessel wall or mobilized from the bone marrow, can also contribute to tumor angiogenesis.<sup>3,4</sup> Various signals trigger tumor angiogenesis, including the active peptides secreted by tumor itself. Adrenomedullin (AM) is a potent vasodilator peptide consisting of 52 amino acids, which was originally isolated from human pheochromocytoma based on its ability to increase cyclic adenosine monophosphate levels in platelets.<sup>5</sup> Adrenomedullin has been shown to be synthesized by a variety of tissues, including adrenal medulla, heart, lung and kidney.<sup>6</sup> Adrenomedullin seems to be ubiquitously expressed in many types of cells, such as vascular endothelial cells, vascular smooth muscle cells, fibroblasts, adipocytes, and neurons.<sup>6,7</sup> Adrenomedullin expression was observed in various kinds of tumors, such as breast, colon, and lung tumors.<sup>8</sup> We have previously reported that AM is expressed in epithelial ovarian carcinoma tissue.<sup>9</sup> Although AM is reported to participate in tumor angiogenesis,<sup>10</sup> however, the role and mechanism of AM in epithelial ovarian carcinoma angiogenesis still remains unclear.

Various molecules are involved in the different mechanisms of vascular growth.<sup>11</sup> Among these, vascular endothelial growth factor (VEGF) has a predominant role. The angiogenic activity of VEGF is tightly regulated by gene dosage.<sup>12</sup> Vascular endothelial growth factor promotes growth, proliferation, migration, formation of new blood vessels,<sup>13</sup> and survival of endothelial cells. Vascular endothelial growth factor regulation can occur at both transcriptional and posttranscriptional levels in a cell-specific manner. However, whether VEGF expression could be regulated by AM in epithelial ovarian carcinoma is currently unknown.

In the present study, we investigated whether VEGF expression could be induced by AM in human epithelial ovarian carcinoma cells; we also explored the mediating mechanism and possible significance.

## MATERIALS AND METHODS

### Chemicals and Reagents

Human epithelial ovarian carcinoma cell line (HO-8910 cells) and human umbilical vein cell line (EA.hy926 cells) were purchased from American type culture collection (ATCC, Manassas, VA). RPMI1640 medium was purchased from Hyclone Co (Logan, UT). Recombinant human AM and its antagonist AM22-52 were purchased from Phoenix Pharmaceuticals (Belmont, CA). Rabbit-antiphosphorylated c-Jun N-terminal kinase (JNK) and rabbit-anti-JNK were purchased from Cell Signaling Technology (Beverly, MA).

Mouse-anti-c-Jun antibody and purified rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antihuman VEGF neutralizing antibody was purchased from Oncogene Science (Cambridge, MA). SP600125 was purchased from CalBiochem (La Jolla, CA). SR11302 was purchased from Tocris Bioscience (R&D Systems, Minneapolis, MN). JetPEI reagent was purchased from Polyplus-transfection (France, BP). The pAP-1-luciferase reporter ( $7 \times$  AP-1) was purchased from Stratagene (La Jolla, CA). All other chemicals and drugs were purchased from Sigma Chemical (St. Louis, MO).

### Cell Culture

HO-8910 cells, a human epithelial ovarian carcinoma cell line, were cultured in RPMI1640 medium containing 10% fetal bovine serum and penicillin/streptomycin (100 U/mL) in a humidified 37°C incubator. When confluent, cells were treated with AM ( $10^{-11}$  to  $10^{-7}$  mol/L) for 4 to 24 hours. For the inhibition experiments, cells were pretreated with AM22-52, SP600125, or SR11302 for 1 hour before stimulation with AM at  $10^{-8}$  mol/L for 24 hours.

### RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNAs were isolated using Trizol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Total RNA (2  $\mu$ g) was reverse-transcribed using reverse transcription system (Promega, Madison, WI). One microliter of the reaction mixture was subjected to polymerase chain reaction (PCR). The amount of PCR products formed in each cycle was evaluated on the basis of SYBR Green I fluorescence. The forward and reverse PCR primers were: human VEGF 5'-CTT GCC TTG CTG CTC TAC C-3' and 5'-CAC ACA GGA TGG CTT GAA G-3' (NM\_001171623.1); human  $\beta$ -actin 5'-ATC TGG CAC CAC ACC TTC -3' and 5'-AGC CAG GTC CAG ACG CA-3' (NM\_001101.3). All amplification reactions were performed using the Mx3000 multiplex quantitative PCR system (Stratagene, La Jolla, CA) under the following conditions: 95°C for 5 minutes, followed by 40 cycles at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Results were analyzed with Stratagene Mx3000 software, and VEGF mRNA levels were normalized with respect to the levels of  $\beta$ -actin in each sample. Polymerase chain reactions were performed in duplicates, and each experiment was repeated for 3 to 5 times.

### Preparation of Cytosolic Proteins and Western Blot Analysis

After treatment, the cells were packed by centrifuging the cells for 3 minutes at 200g and homogenized in ice-cold fractionation buffer (50-mmol/L Tris-HCl, pH 7.4, 1-mmol/L EDTA, 150-mmol/L NaCl, 1% Triton X-100, 1-mmol/L phenylmethylsulfonyl fluoride, 10- $\mu$ g/mL leupeptin, 10- $\mu$ g/mL pepstatin A, 10- $\mu$ g/mL aprotinin, 1-mmol/L sodium orthovanadate [ $\text{Na}_3\text{VO}_4$ ], 10-mmol/L sodium pyrophosphate [ $\text{Na}_4\text{P}_2\text{O}_7$ ], and 50-mmol/L sodium fluoride [ $\text{NaF}$ ]). The cell lysate was incubated on ice for 15 minutes and then centrifuged at 20,000g for 30 minutes at 4°C. The cytosolic fraction was collected and subjected to sodium dodecyl sulfate polyacrylamide gel

electrophoresis with a 10% running gel. Protein concentrations were determined by bicinchoninic acid protein assay kit (PIERCE, Rockford, IL). The proteins were transferred to a polyvinylidene fluoride membrane. The membrane was incubated successively with 5% bovine serum albumin in Tris-Tween-buffered saline (TTBS) at room temperature for 1 hour, with different first antibodies at 4°C for 12 hours and then with horseradish peroxidase-labeled second antibody for 1 hour. After each incubation, the membrane was washed extensively with Tris-Tween-buffered saline, and the immunoreactive band was detected with Electro-Chemi-Luminescence-detecting reagents (PIERCE).

### Plasmid Construction and Transient Transfection Assays

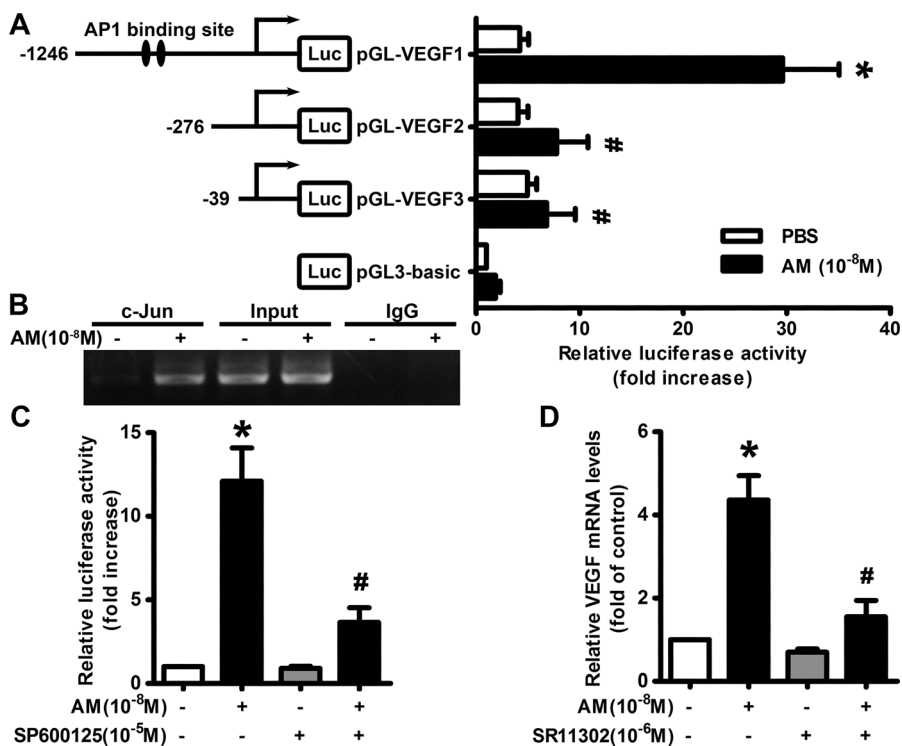
The human VEGF promoter-luciferase hybrid genes were constructed as previously reported.<sup>14</sup> A series of fragments (positions -1246, -276, and -39 to +316) were amplified

from peripheral blood mononuclear human genomic DNA and subcloned into the pGL3-basic luciferase reporter vector (Promega) termed pGL-VEGF1-3 (Fig. 1A).

After treatment, HO-8910 cells in 24-well plates were transfected by jetPEI reagent with each plasmid DNA, followed by 24-hour incubation, and harvested. Cells were then lysed, and luciferase activity was measured. The amounts of DNA used for transfection were 0.5  $\mu$ g of a test fusion gene and 10 ng of an internal control, Renilla Luciferase Reporter Vector (PRL-TK) (Promega). Expression of reporter genes and PRL-TK were determined with the dual-luciferase reporter assay system (Promega).

### Chromatin Immunoprecipitation and PCR Analysis

After treatment, cells underwent immunoprecipitation with anti-c-Jun. Immunoprecipitated chromatin fragments were amplified by PCR. The primers used for amplification



**FIGURE 1.** Activator protein 1 acted on the motif of VEGF promoter. A, Relative luciferase activity in transient transfection assays using the series of plasmid constructs containing dissected fragments of VEGF promoter. The basal promoter activity of each test plasmid is indicated as luciferase activity normalized by each internal control activity (PRL-TK). Relative luciferase activity was normalized with respect to the activity of PGL3-basic in untreated cells. B, Chromatin immunoprecipitation of VEGF promoter complexes. HO-8910 cells underwent immunoprecipitation with anti-c-Jun antibody or control IgG. Immunoprecipitated chromatin fragments were amplified by PCR. C, Attenuation of AM-induced pAP-1-luciferase reporter ( $7 \times$  AP-1) activation by SP600125 in HO-8910 cells. Cells were transfected with  $7 \times$  AP-1 together with control plasmid with use of jetPEI reagent. After transfection for 24 hours, cells were pretreated with (+) or without (-) SP600125 ( $10^{-5}$  mol/L) for 1 hour, then incubated in the presence (+) or in the absence (-) of AM ( $10^{-8}$  mol/L) for 24 hours. (D) Attenuation of AM-induced VEGF mRNA expression by SR11302, the AP-1 inhibitor, in HO-8910 cells. Cells were pretreated with (+) or without (-) SR11302 ( $10^{-6}$  mol/L) for 1 hour, then incubated in the presence (+) or in the absence (-) of AM ( $10^{-8}$  mol/L) for 24 hours. Relative VEGF mRNA levels were normalized with respect to the levels for untreated cells. The results are representative of 4 independent experiments performed in triplicate. Data are means  $\pm$  SEM. \* $P < 0.05$  versus untreated cells; # $P < 0.05$  versus AM treatment alone.

of the VEGF promoter were: sense, 5'- CCT GCC CCC TTC AAT ATT CCT -3; and antisense, 5'- ATA TCA AAT TCC AGC ACC GAG C -3'.<sup>15</sup>

### Capillarylike Tube Formation Assay

The formation of capillarylike structures by EA.hy926 cells on Matrigel (Becton Dickinson, Bedford, MA) was studied. HO-8910 cells were cultured in lower chambers of 24-well transwell culture plates and were pretreated with or without AM ( $10^{-8}$  mol/L) for 24 hours. Upper chambers were coated with Matrigel according to the manufacturer's instructions. EA.hy926 cells were then seeded onto the coated chambers at  $10^4$  per well in the fresh assay medium and incubated for 12 hours. The formation of capillary tubes in Matrigel was examined by use of an inverted microscope equipped with a digital camera (Olympus, Tokyo, Japan). The level of the tube formation was quantified by measuring the length of tubes in 5 randomly chosen fields from each well using an Image-Pro Plus software.

### Statistical Analysis

Quantitative data are presented as mean  $\pm$  SEM determined from the indicated number of experiments. Statistical analysis was based on the Student *t* test for comparison of 2 groups or one-way analysis of variance for multiple comparisons.  $P < 0.05$  was used to determine statistical significance.

## RESULTS

### AM Induced VEGF mRNA Expression in Epithelial Ovarian Carcinoma Cells

Exposure of HO-8910 cells, the human epithelial ovarian carcinoma cell line, to AM ( $10^{-8}$  mol/L) induced VEGF mRNA expression in a time-dependent manner. Elevation in VEGF mRNA level occurred as early as 8 hours and remained increased for up to 24 hours (Fig. 2A). The AM treatment

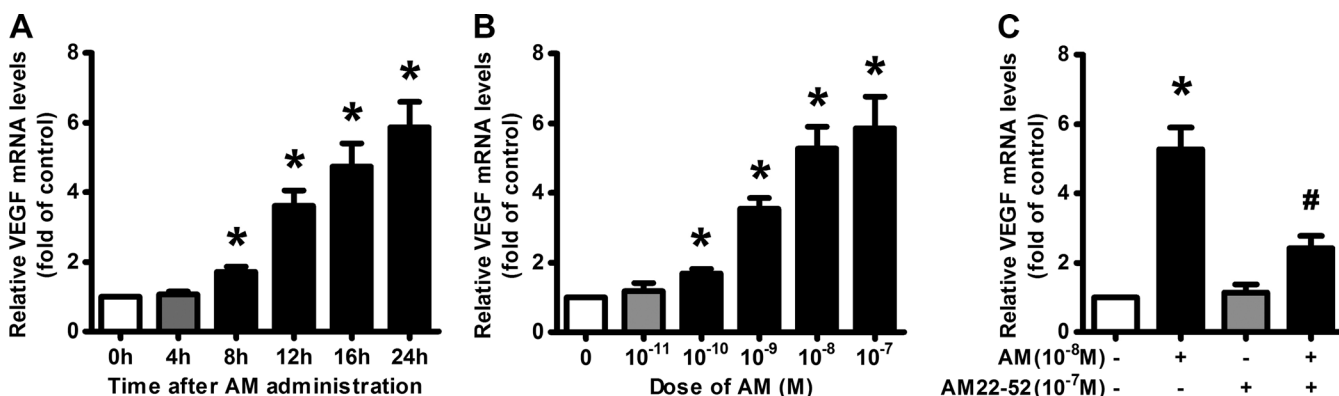
( $10^{-10}$  to  $10^{-7}$  mol/L) for 24 hours also caused a concentration-dependent increase in VEGF mRNA expression (Fig. 2B), with maximal induction of 5.9-fold found with  $10^{-7}$  mol/L of AM. The AM receptor antagonist, AM22-52, significantly attenuated the AM-induced VEGF mRNA expression (Fig. 2C), which indicated that AM-stimulated VEGF expression was receptor dependent.

### AM Up-regulated VEGF Expression Through a Transcriptional Mechanism

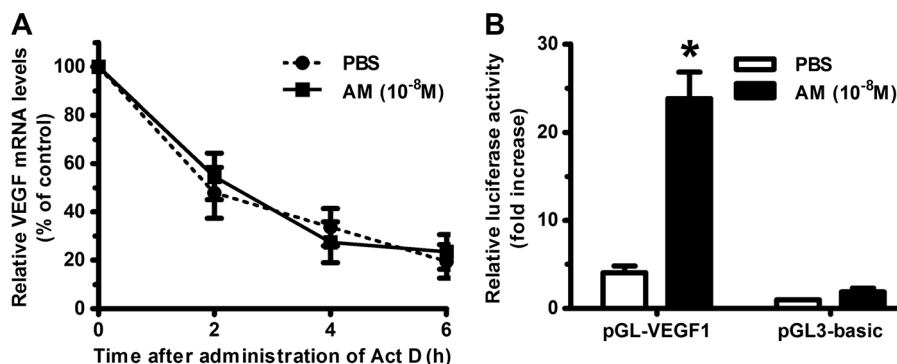
An initial step in dissecting the mechanism by which AM regulates VEGF expression was to determine whether the up-regulation of VEGF by AM is transcriptional or posttranscriptional. For this purpose, HO-8910 cells were treated with or without AM in the presence of 5  $\mu$ g/mL of the transcriptional inhibitor actinomycin D (Act D). Adrenomedullin treatment did not increase the half-life of VEGF mRNA as would have been expected if AM increased VEGF mRNA expression by a posttranscriptional mechanism (Fig. 3A). Furthermore, we transfected the VEGF promoter fragment (pGL-VEGF1) into HO-8910 cells and found that AM induced luciferase activity significantly (Fig. 3B). Therefore, AM up-regulated VEGF expression in epithelial ovarian carcinoma cells through transcription pathway.

### JNK Pathway is Activated Upon AM Stimulation and Participated in VEGF mRNA Expression

Since the stress-responsive JNK pathway may account for tumor angiogenesis, we next examined the effect of AM on JNK phosphorylation in HO-8910 cells. As shown in Figure 4A, AM ( $10^{-8}$  mol/L) treatment for 2 hours significantly increased the phosphorylation of JNK. SP600125 ( $10^{-5}$  mol/L), an inhibitor of JNK, significantly blocked both the increase of VEGF mRNA expression (Fig. 4B) and VEGF promoter activity (Fig. 4C)



**FIGURE 2.** Adrenomedullin induced VEGF mRNA expression in epithelial ovarian carcinoma cells. A, Time course of AM ( $10^{-8}$  mol/L) treatment on VEGF mRNA levels in HO-8910 cells. B, Dose-response effect of 24-hour AM treatment on VEGF mRNA levels in HO-8910 cells. C, Attenuation of AM-induced VEGF expression by AM receptor antagonist in HO-8910 cells. Cells were pretreated with or without AM22-52 ( $10^{-7}$  mol/L) for 1 hour and then incubated in the presence of AM ( $10^{-8}$  mol/L) for 24 hours. Relative mRNA levels were normalized to that of untreated cells.  $\beta$ -actin was used as internal control. The results are representative of 4 independent experiments performed in triplicate. Data are mean  $\pm$  SEM. \* $P < 0.05$  versus untreated cells; # $P < 0.05$  versus AM treatment alone.



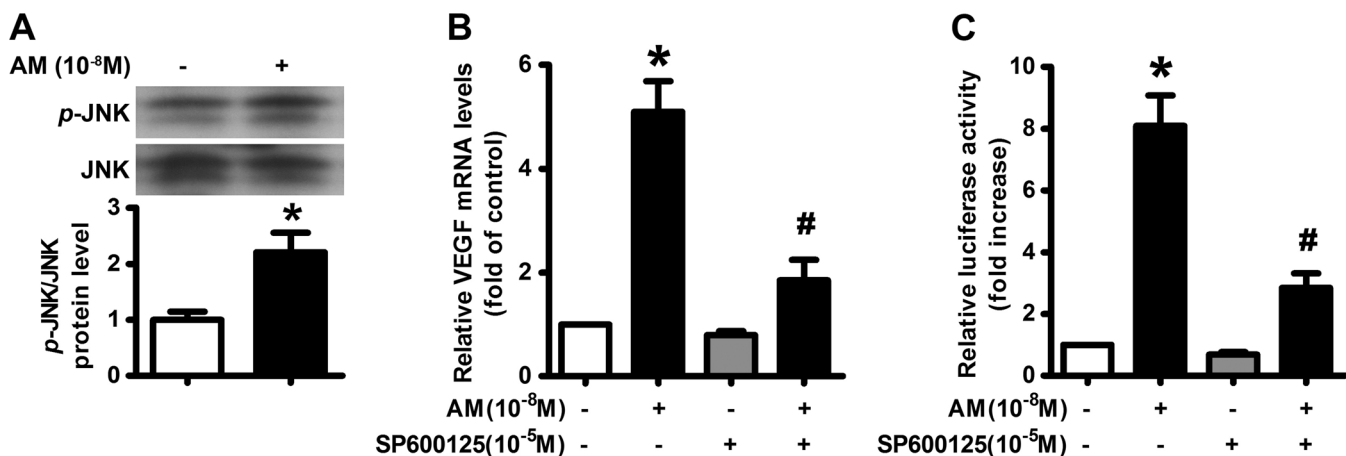
**FIGURE 3.** Adrenomedullin up-regulated VEGF expression through a transcriptional mechanism. A, Effect of AM ( $10^{-8}$  mol/L) treatment on the half-life of VEGF mRNA levels in HO-8910 cells in the presence of 5  $\mu$ g/mL of the transcriptional inhibitor actinomycin D. Relative mRNA levels were normalized to that of untreated cells in the same group.  $\beta$ -actin was used as internal control. B, Effects of AM on VEGF promoter activity in HO-8910 cells. Cells were transfected with pGL-VEGF1 together with control plasmid with use of jetPEI reagent. After transfection for 24 hours, cells were stimulated with AM ( $10^{-8}$  mol/L) or phosphate-buffered saline, then luciferase activity was measured. Relative luciferase activity was normalized with respect to the activity in untreated cells. The results are representative of 4 independent experiments performed in triplicate. \* $P < 0.05$  versus untreated cells.

evoked by AM ( $10^{-8}$  mol/L). Therefore, AM up-regulated VEGF expression through activation of JNK pathway.

### Localization of the AM Regulatory Element in the VEGF Gene Promoter

Activation of JNK triggers the phosphorylation of its downstream target protein c-Jun. c-Jun proteins can form stable homodimers or heterodimers with c-fos as a transcription factor, AP-1, and bind AP-1 DNA recognition elements. It was reported that promoter region of VEGF could bind the AP-1 transcription factor, which located in -1168 and -1015-bp

upstream of the transcriptional starting site.<sup>16</sup> To identify the AM-responsive region of the VEGF promoter, we examined a series of deletion constructs containing different lengths of the upstream VEGF gene fused to the reporter luciferase gene by transient transfection assay. As shown in Figure 1A, the deletion of a segment of the VEGF gene containing an AP-1 binding site (-1168 and -1015 bp) reduced the basal promoter activity by 70%, and the deletion of the more proximal segment without containing AP-1-binding site did not reduce the promoter activity further, indicating AP-1 bound to the VEGF gene promoter upon stimulation of AM ( $10^{-8}$  mol/L).



**FIGURE 4.** c-Jun N-terminal kinase pathway was activated upon AM stimulation. A, Effects of AM on JNK pathway activation in HO-8910 cells. Cells were incubated with AM ( $10^{-8}$  mol/L) for 2 hours, and whole-cell lysates underwent Western blotting to detect the phosphorylation of JNK. B, C, Attenuation of AM-induced VEGF mRNA expression (B) and promoter activity (C) by SP600125, the JNK inhibitor, in HO-8910 cells. Cells were pretreated with (+) or without (-) SP600125 ( $10^{-5}$  mol/L) for 1 hours, then incubated in the presence (+) or in the absence (-) of AM ( $10^{-8}$  mol/L) for 24 hours. Relative VEGF mRNA levels were normalized with respect to the levels for untreated cells. Relative luciferase activity was normalized with respect to the activity in untreated cells. The results are representative of 4 independent experiments performed in triplicate. Data are mean  $\pm$  SEM. \* $P < 0.05$  versus untreated cells; # $P < 0.05$  versus AM treatment alone.



Further study using the chromatin immunoprecipitation PCR analysis confirmed a bona fide interaction of AP-1 to these corresponding response elements within the VEGF promoter (Fig. 1B). Adrenomedullin also increased  $7 \times$  AP-1 promoter activity by 12 folds, which could be abolished by SP600125 (Fig. 1C). SR11302 ( $10^{-6}$  mol/L), an inhibitor of AP-1, significantly blocked the increase of VEGF mRNA expression evoked by AM (Fig. 1D). These data suggest that AP-1-binding sites are critical for the AM-induced VEGF gene expression in HO-8910 cells.

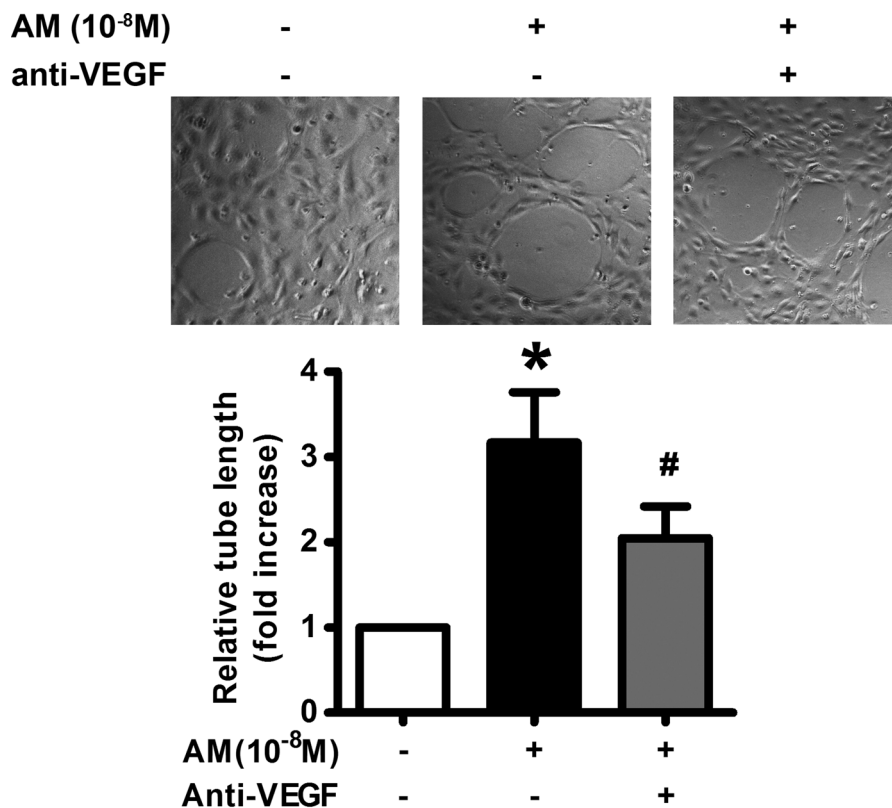
### VEGF Mediated AM-Induced Angiogenesis in Epithelial Ovarian Carcinoma Cells In Vitro

To investigate whether AM could affect the angiogenic property of epithelial ovarian carcinoma, we performed a capillarylike tube formation assay on Matrigel, the most commonly used method for in vitro angiogenesis. EA.hy926 cells were cocultured with HO-8910 cells pretreated with or without AM ( $10^{-8}$  mol/L) for 24 hours. Quantitative analyses revealed that AM treatment could increase the total length of capillarylike tubes in EA.hy926 cells significantly but abolished by VEGF neutralizing antibody (Fig. 5), indicating that AM might promote epithelial ovarian carcinoma angiogenesis through autocrine or paracrine effects of tumor-derived factors, such as VEGF.

### DISCUSSION

The present study demonstrated that AM can induce the expression of VEGF through the JNK/AP-1 pathway in human epithelial ovarian carcinoma cell line HO-8910 cells and subsequently contribute to cancer development by promoting tumor angiogenesis. This conclusion is supported by the following observations<sup>1</sup>: AM up-regulates VEGF mRNA expression in HO-8910 cells in a time- and concentration-dependent manner<sup>2</sup>; AM enhances VEGF promoter activity<sup>3</sup>; the effect of AM on VEGF expression seems to be primarily transcriptional<sup>4</sup>; transcription factor AP-1 mediates the effects of AM<sup>5</sup>; inhibition of JNK signaling pathway blocks the effects of AM on AP-1, therefore attenuating the up-regulation of VEGF expression<sup>6</sup>; blocking of HO-8910 cell-derived VEGF inhibits AM-stimulated capillarylike tube formation of EA.hy926 cells. To the best of our knowledge, this is the first report demonstrating the AM-mediated modulation of VEGF expression in epithelial ovarian carcinoma.

Ovarian carcinoma is the fourth most common cause of cancer death among women in the United States, although it is the 12th most common cause of cancer death among women in China, the mortality is more than 70% within 5 years. Adrenomedullin is a multifunctional peptide that is widely expressed in many kinds of tumors.<sup>17</sup> Treatment with antibody against AM caused a dose-dependent suppression of the



**FIGURE 5.** HO-8910 cells–derived VEGF-mediated AM-induced angiogenesis of EA.hy926 cells. HO-8910 cells were pretreated with or without AM ( $10^{-8}$  mol/L) for 24 hours, then EA.hy926 cells were cocultured with or without VEGF neutralizing antibody for 12 hours. The total length of capillarylike tubes was measured and normalized with EA.hy926 cells cocultured with AM-untreated HO-8910 cells. Data are mean  $\pm$  SEM from 3 independent experiments. \* $P < 0.05$  versus EA.hy926 cells cocultured with AM-untreated HO-8910 cells, # $P < 0.05$  versus AM treatment alone.

growth in human tumor cell lines, including the human breast adenocarcinoma MCF-7 cells, the ovarian adenocarcinoma OVCAR-3 cells and the glioblastoma T98G cells.<sup>8</sup> Therefore, endogenously produced AM by tumor cells is proposed to act as a growth stimulator for the tumors. Our study also gave a new clue that AM promotes tumor angiogenesis of ovarian epithelial carcinoma through the autocrine/paracrine effect of up-regulated VEGF, since VEGF-neutralizing antibody could abolish the capillarylike tube formation of EA.hy926 cells cocultured with HO-8910 cells. However, although AM works as a growth factor in epithelial ovarian carcinoma in vitro, as revealed by proliferation assays and cell cycle analysis, unexpectedly, the clinical study revealed that high expression of AM was linked to positive outcome in ovarian carcinoma.<sup>18</sup> Therefore, although in vitro AM was a potential factor in biological aggressiveness, this possibility was not confirmed in vivo; use of an AM antagonist would be inappropriate in managing patients with ovarian cancer.

Vascular endothelial growth factor, one of the most important angiogenic switch molecule, exerts great function on the behaviors of endothelial cells, including migration, proliferation, and differentiation.<sup>19,20</sup> Vascular endothelial growth factor plays a central role in tumor angiogenesis; drugs have been developed to target VEGF, such as bevacizumab, the monoclonal antibody directed at VEGF. Many factors participated in the transcriptional regulation of VEGF. The proximal GC-rich region in the VEGF promoter contains binding sites for AP-2, Egr-1, WT1, nuclear factor kappa B, and SP1/SP3 transcription factors, whereas distal enhancer sites bind AP-1 and HIF-1 $\alpha$ .<sup>16,21–23</sup> c-Jun N-terminal kinase/AP-1 signaling pathway is a classical transduction pathway participating in inflammatory stimulation. In various cell types, JNK is preferentially activated by cytokines (IL-1 $\beta$  and tumor necrosis factor  $\alpha$ ) or stress stimuli such as osmotic shock, UV light and heat. c-Jun N-terminal kinase is an important signaling molecule involved in the regulation of cell proliferation as well as apoptosis.<sup>24,25</sup> In this study, we showed that JNK was activated in response to AM stimulation, and the JNK inhibitor SP600125 obviously attenuated AM-induced VEGF expression in HO-8910 cells. c-Jun N-terminal kinase activates c-Jun by phosphorylation of 2 serine residues at the 63 and 73 positions of the NH2 terminus of c-Jun. Activator protein 1 is a transcriptional activator composed of homodimers and heterodimers of c-Jun and c-Fos proteins.<sup>26</sup> Activator protein 1 enhanced by cytokines has been shown to be a critical regulator of several genes involved in tumors, which bind to cis-acting elements in the promoters of target genes and result in AP-1-dependent transcription.<sup>27</sup> Consistent with these observations, our results showed that AM activates AP-1 via the phosphorylation of JNK.

In summary, the JNK/AP-1 pathway is involved in AM-induced VEGF expression in human epithelial ovarian carcinoma cell line HO-8910 cells. Additional studies are needed to better define how these interactions are initiated and regulated and to demonstrate whether similar effects play a role in vivo.

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