Blockade of Endothelin-1 Release Contributes to the Anti-Angiogenic Effect by Pro-Opiomelanocortin Overexpression in Endothelial Cells

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Pro-opiomelanocortin (POMC) is the precursor of several neuropeptides, such as corticotropin (ACTH), α-melanocyte–stimulating hormone (MSH), and the endogenous opioid, β-endorphin (EP). ACTH-dependent Cushing's syndrome is characterized by ACTH overproduction and is associated with an increased risk of cardiovascular disease. Endothelial dysfunction has been recognized as an early marker of cardiovascular disease. However, the mechanism underlying endothelial dysfunction by ACTH overexpression in Cushing's patients remains elusive. Endothelial cells, the primary cells producing endothelin (ET)-1, are both the source and target of POMC-derived peptides. In the present study, we generated adenovirus vectors (Ad) encoding POMC (Ad-POMC) and green fluorescent protein (GFP; Ad-GFP) to investigate whether POMC gene transfer altered the ET-1 homeostasis and angiogenic functions in human EA.hy926 endothelial cells. *Via* adenovirus gene delivery, the POMC-transduced EA.hy926 cells released significantly elevated ACTH and β-EP levels ($P < 0.001$). In addition, POMC gene delivery significantly decreased the ET-1 release ($P < 0.001$) without affecting the ET-1 messenger RNA (mRNA) level. Despite no effect on the secretion of matrix metalloproteinases (MMPs) and cell proliferation, POMC gene delivery significantly inhibited the migration ($P < 0.01$) and tube-forming capability ($P < 0.01$) of endothelial cells. Moreover, the POMC-induced inhibition of tube formation could be partially reversed by adding exogenous ET-1 ($P < 0.05$). In summary, the attenuated ET-1 release and angiogenic processes by POMC overexpression may contribute to endothelial dysfunction, thereby providing a link between Cushing's syndrome and cardiovascular diseases. Exp Biol Med 231:782–788, 2006

Key words: Cushing's syndrome; POMC; endothelin-1; endothelial cells; gene delivery

Introduction

Endogenous Cushing's syndrome can result from excessive corticotropin (ACTH) production by a pituitary adenoma or by ectopic tumors secreting ACTH or corticotropin-releasing hormone (CRH). ACTH is known to stimulate the adrenal gland to release cortisol. The subsequent hypercortisolism contributes to the pathogenesis of Cushing's syndrome. Recent evidence indicates that Cushing's syndrome is underestimated and is strongly associated with increased incidence of cardiovascular diseases such as diabetes or heart disease (1).

Endothelial injury or dysfunction is an important initiating step in atherogenesis and many cardiovascular diseases, including Cushing's syndrome. Angiogenesis, the process of growing new blood vessels, is highly relevant to endothelial dysfunction and comprises several distinct steps in the endothelium, including secretion of matrix metalloproteinases (MMPs), migration, proliferation, and tube formation (interaction with extracellular matrix; Ref. 2).

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Endothelin (ET)-1, a major vasoconstrictor peptide first identified in endothelial cells, is a potent activator of angiogenesis (3–5). In addition, ET-1 is a potent mitogen for endothelial cells, smooth muscle cells, and many other types of cells via their ETA and ETB receptors, in a paracrine or autocrine mode (3, 6, 7). Because ET-1 contributes to vascular remodeling and cardiovascular disease, blockade of ET receptors has been proposed as a therapeutic alternative to acute and chronic cardiovascular disease (7).

ACTH is derived from a 31-kDa prohormone, pro-opiomelanocortin (POMC), which is also processed into various neuropeptides, including melanotrophins (α-, β-, and γ-melanocyte–stimulating hormone [MSH]), lipotropins, and β-endorphin (EP; Refs. 8, 9). POMC peptides possess pleiotropic functions, including pigmentation, adrenocortical function, and regulation of energy stores, the immune system, and the central and peripheral nervous systems (9). POMC is expressed not only in the pituitary gland but also in a variety of nonpituitary tissues. The specific patterns of posttranslational POMC processing dictate whether an individual cell releases α-MSH, ACTH, or other melanocortin peptides, which then control overlapping arrays of endocrine, metabolic, and neurologic endpoints. In the present study, the antiangiogenic functions of POMC gene delivery were explored in cultured endothelial cells. Our results present evidence for the first time that POMC gene delivery perturbs the ET-1 release and angiogenic processes in endothelial cells, which may contribute to the pathogenesis of endothelial dysfunction. In addition, the reduced ET-1 participated in POMC-induced inhibition of tube formation.

Materials and Methods

Cell Cultures and Reagents. For production and propagation of adenovirus (Ad)-5, E1a-transformed human embryonic kidney 293 cells were purchased from Microbix Biosystems Inc. (Toronto, Canada) and maintain at a low passage number. Human endothelial EA.hy926 cells (kindly provided by Dr. C.J.S. Edgell, University of North Carolina, Chapel Hill, NC) were maintained at a low passage number and cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Rockville, MD) containing 10% fetal calf serum (PAA, Austria), 100 μ*M* sodium hypoxanthine, 0.4 μ*M* aminopterin, 16 μ*M* thymidine (HAT; Gibco BRL), 2 m*M* glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL) in 5% CO_2 at 37°C (10, 11). Recombinant basic fibroblast growth factor (bFGF) was purchased from R&D Systems (Minneapolis, MN). Matrigel was obtained from BD PharMingen (La Jolla, CA); and ET-1 from Peninsula Laboratories (Belmont, CA).

Generation of Recombinant Ad-POMC. Generation and characterization of Ad-POMC was performed as recently described (11). After homologous recombination, the virus plaques were verified by checking for cytopathic effect and polymerase chain reaction (PCR). The virus was amplified by two rounds of cesium chloride ultracentrifugation and desalted by G-25 gel-filtration chromatography. The titer of virus solution was determined by measuring the optical density at 260 nm and plaque-forming assay on 293 cells before storage at −80°C.

Immunoassays. β-EP concentrations in cultured media were determined using radioimmunoassay (RIA) kits (Nichols Institute Diagnostics, San Juan Capistrano, CA) with a linear range of measurement between 5 and 1000 pg/ml for β-EP. ACTH was measured by chemiluminescent immunoassay (Immulite 2000; Diagnostic Products Corporation, Los Angeles, CA). ET-1 was extracted and measured as described elsewhere (12) by the quantitative enzyme immunoassay technique (R&D Systems). The intraassay and interassay coefficients of variation were 4.5% and 5.5%, respectively. The minimum detectable dose is typically less than 1.0 pg/ml. A standard curve was constructed for each assay. Experiments were performed at least three times.

Quantitative Reverse-Transcription (qRT)-PCR. The total RNA was isolated from cells using RNAzol (TEL-TEST Inc., Friendswoods, TX). For reverse transcription, 5 μg of total RNA was used for reverse transcription with Superscriptase II (Invitrogen, Carlsbad, CA) using oligo-dT and random primers. One-twentieth of reverse-transcription products were used as a template for RT-PCR in an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA) using a SYBR green assay. PCR reaction was performed in 50 ml SYBR Green PCR Master Mix (Applied Biosystems) containing 10 μ*M* forward and reverse primers and approximately 30 ng complementary DNA (cDNA). Amplification and detection were performed by: 1 cycle of 95°C for 10 mins and 40 cycles of 95°C for 15 secs, 62°C for 20 secs, and 72°C for 15 secs. After completion, a final melting curve was performed by denaturation at 95°C for 15 secs and was recorded by cooling to 60°C and heating slowly until 95°C for 20 mins, according to the dissociation protocol of the ABI Prism 7700 instrument. The primer sequences for ET-1 were: forward primer, 5′-CTTCTGCCACCTGGACATCA-3′; reverse primer, 5′-GGCTTCCAAGTCCATACGGA-3′, which amplified a 100-base pair (bp) ET-1 cDNA fragment. The β-actin mRNA level was determined using: forward primer, 5′-TCACCCACACTGTGCCCATCTACGA-3′; and reverse primer, 5′-CAGCGGAAC CGCTCATTGC-CAATGG-3′, which amplified a 295-bp β-actin cDNA fragment.

Cell Proliferation MTT Assay. Endothelial cells were cultured in a 96-well plate at a density of 4×10^4 cells/ml. After infection with adenovirus vectors, cells were supplemented with fresh medium containing 0.456 mg/ml 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) and incubated for 1–2 hrs at 37°C. The formazan in viable cells was dissolved with 100 μl of dimethylsulfoxide and determined by reading optical densities in microplate reader (DYNEX Technologies Inc., Chantilly, VA) at an absorption wavelength of 570 nm.

Gelatin Zymography. Secretion of MMPs by endothelial cells was assessed by 0.1% gelatin–sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) zymography, as previously described (13). Briefly, endothelial cells at near 80% confluence were infected with adenovirus vectors for 24 hrs and supplemented with serum-free media for an additional 24 hrs. Aliquots of conditioned media were subjected to separation with 10% SDS-PAGE containing 0.1% type-A gelatin (Sigma Chemical Co., St. Louis, MO). After electrophoresis, the gel was washed twice with 2.5% Triton X-100, incubated in buffer containing 40 m*M* Tris-HCl, pH 8.0; 10 m*M* $CaCl_2$; and 0.01% sodium azide at 37°C for 12–24 hrs, stained with 0.25% Coomassie blue R-250 in 50% methanol and 10% acetic acid for 1 hr, and destained with 10% acetic acid and 20% methanol. The gelatinolytic regions by MMPs were visualized as white bands in a blue background and quantified by densitometer.

Cell Migration Assay. The cell migration assay was performed as previously described (13). Endothelial cells were infected with adenovirus vectors for 24 hrs and seeded in triplicate in the upper compartment of the chamber (1.2×10^5 cells in 400 μl). The lower compartment was filled with 200 μl of DMEM media containing 100 ng/ml bFGF (R&D Systems) as the chemoattractant. A polycarbonate filter (8-μm pore size Nucleopore; Costar, Cambridge, MA), coated with 0.005% gelatin to allow cell adhesion, separated the compartments. After incubation for 2–4 hrs in a humidified 5% CO_2 atmosphere chamber at 37°C, cells on the upper side of the filter were removed, and cells that had migrated to the lower side were fixed in absolute ethanol, stained with 10% Giemsa solution (Merck, Germany), counted, and reported as mean ± SEM per filter under five different high-power fields.

Tube Formation Assay. The tube formation assay was performed as previously described (13). Briefly, Matrigel (Becton Dickinson, Bedford, MA) was diluted with cold serum-free medium to 10 mg/ml. The diluted Matrigel solution was added to 24-well plates (200 μl per well) and allowed to form a gel at 37°C for 30 mins. After infection for 24 hrs, EA.hy926 cells (1.5×10^5 cells/ml) were incubated with Matrigel, then added to each well and incubated for 6–8 hrs at 37°C in 5% CO_2. Under these conditions, endothelial cells form delicate networks of tubes that are detectable within 2–3 hrs and are fully developed after 8–12 hrs. After incubation, the endothelial tubes were fixed with 3% paraformaldehyde and counted in three to four different high-power fields.

Statistical Analysis. Results are expressed as mean ± SEM values. The significance of the differences was assessed by ANOVA. *P* values less than 0.05 were considered significant.

Results

Adenovirus-Mediated POMC Expression Increased the Production of ACTH and β-EP in Endothelial Cells. The replication-defective recombinant adenovirus encoding POMC, Ad-POMC, was generated for gene delivery studies. By infection with adenovirus encoding green fluorescent protein (GFP; Ad-GFP) at various multiplicities of infection (MOI), the optimal condition for adenovirus vectors to infect EA.hy926 cells was determined at a MOI of 100–200 (Fig. 1A). To evaluate the efficacy of POMC processing in endothelial cells, the levels of POMC peptides in the cultured media from infected EA.hy926 cells were determined by RIAs. The POMC-transduced EA.hy926 cells released significantly increased levels of ACTH (274.5 ± 13.6 pg/ml) and β-EP (1992.3 ± 76.7 pg/ml), which were 20- to 100-fold higher than those in cells of control groups ($P < 0.001$; Fig. 1B). These results indicate that the POMC gene was effectively transduced by adenovirus vectors and processed into various neuropeptides in endothelial cells.

POMC Gene Delivery Inhibited the ET-1 Release in Endothelial Cells. To investigate whether POMC overexpression modulated the ET-1 secretion, the cultured media of infected EA.hy926 endothelial cells were collected for RIA of ET-1. The ET-1 level in the cultured media of Ad-POMC–infected cells (23.7 ± 2.33 pg/ml) was significantly lower than the level in control groups (42.15 ± 0.93 and 43.7 ± 3.7 pg/ml for control and Ad-GFP groups, respectively; $P < 0.01$; Fig. 2A). To further delineate whether the reductions in ET-1 secretion were caused by decreased mRNA transcription, quantitative RT-PCR analysis revealed no significant changes in the ET-1 mRNA level between the Ad-POMC and control groups (Fig. 2B). Thus, POMC gene delivery decreases the ET-1 release without affecting its *de novo* mRNA synthesis.

POMC Gene Delivery Perturbed Migration and Tube Formation in Endothelial Cells, Which Was Partially Restored by Addition of Exogenous ET-1. The effects of POMC gene delivery in various angiogenesis processes were studied, including MMP secretion, proliferation, migration, and tube formation. As assessed by gelatin zymography, no obvious discrepancies in MMP-2 and MMP-9 levels in cultured media between Ad-POMC–infected and control groups of endothelial cells were observed (Fig. 3A), suggesting that POMC gene transfer had no effect on the MMP secretion in endothelial cells. After infection, the proliferation of Ad-POMC–infected cells was similar to that of the endothelial cells in control groups (Fig. 3B), indicating that POMC gene transfer did not affect the proliferation of endothelial cells. In contrast, the endothelial migration was significantly attenuated by POMC gene delivery; the Ad-POMC–infected cells showed substantially retarded migration (102.2 ± 6.8 cells) compared with cells of control groups (245.3 ± 15.5 and 247.2 ± 12.2 cells for control and Ad-GFP groups, respectively; $P < 0.01$; Fig. 3C).

The formation of tube-like structure by endothelial cells in extracellular matrix is considered a pivotal process during angiogenesis. To investigate the influence of POMC

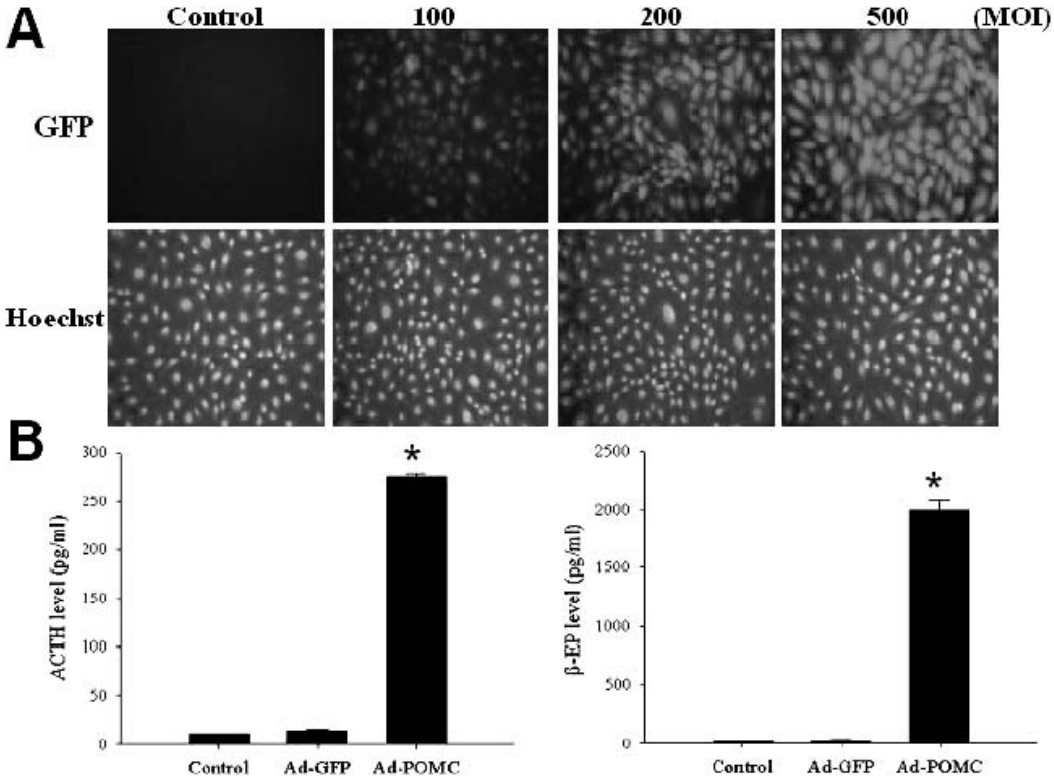

Figure 1. Effect of POMC gene delivery in endothelial cells. (A) Determination of the optimal condition for adenovirus vectors to infect endothelial cells. After infection with Ad-GFP at various MOIs, the green fluorescence of EA.hy926 endothelial cells was recorded under a fluorescence microscope. The nucleus of endothelial cells was monitored using Hoechst 33258 staining (blue). (B) Effect of ACTH and β-EP secretion in endothelial cells after POMC gene delivery. After infection with adenovirus vector for 24 hrs, the conditioned media of EA.hy926 endothelial cells were harvested and analyzed for the levels of ACTH and β-EP by immunoassay ($n = 3$). *$P < 0.001$ vs. control and Ad-GFP groups.

expression on tube formation, the infected endothelial cells were incubated with Matrigel and monitored for the growth of capillaries network. As shown in Figure 4, there was active formation of vessel network in control groups (152.2 ± 10.1 and 247.2 ± 12.3 tubes per field for the control and Ad-GFP groups, respectively), whereas the Ad-POMC–infected endothelial cells were rounded up and exhibited significantly decreased numbers of tubes in Matrigel (41.4 ± 25.1 tubes per field; $P < 0.01$). To study whether the reduced ET-1 release contributed to the perturbed tube formation, exogenous ET-1 was added to Ad-POMC–treated cells and partially restored the tube-forming

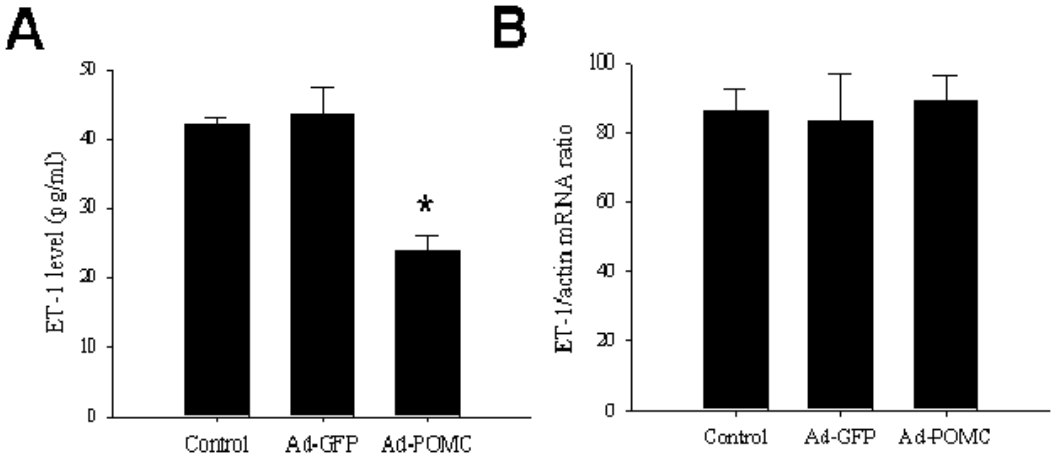

Figure 2. Effect of POMC gene delivery on ET-1 secretion by endothelial cells. After infection with adenovirus vectors for 24 hrs, the conditioned media of EA.hy926 cells were harvested and analyzed for ET-1 levels by immunoassay ($n = 3$). *$P < 0.01$ vs. control and Ad-GFP.

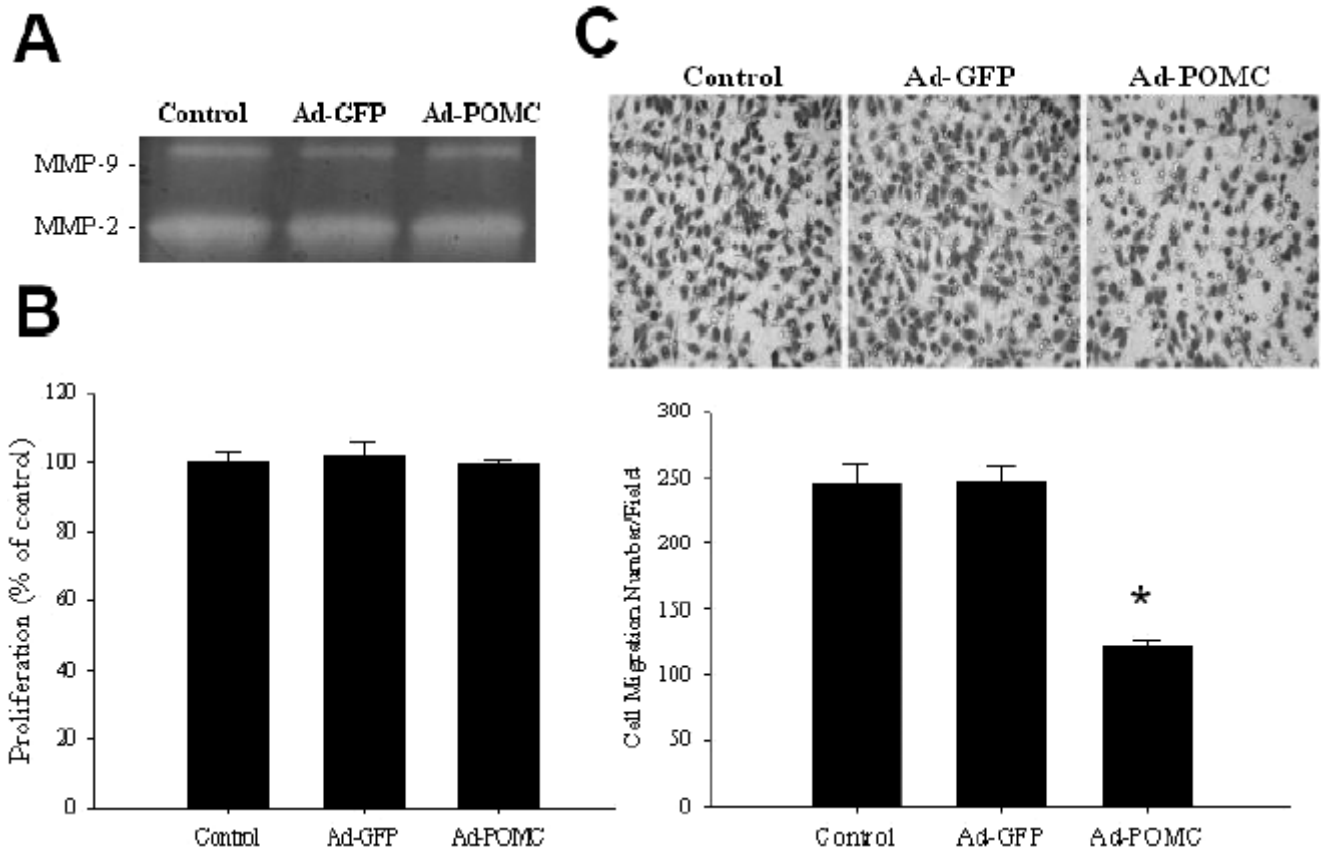

Figure 3. Effect of POMC gene delivery on MMP secretion, proliferation and migration, and tube formation in endothelial cells. (A) Effect of POMC gene delivery on MMP expression in endothelial cells. After infection for 24 hrs, cultured media were collected, normalized for the cell number, and analyzed for MMP-2 and MMP-9 activities in endothelial cultures *via* gelatin zymography. (B) Effect of POMC gene delivery on the proliferation in endothelial cells. After infection for 24 hrs, the proliferation of endothelial cells was determined by MTT assay and expressed as mean ± SEM percentages of control in triplicate. (C) Effect of POMC gene delivery on the migration in endothelial cells. After incubation in a Boyden chamber for 6 hrs, the migrated cells on the filter were stained and counted in three high-power fields. (Upper panel) The representative profile of migrated endothelial cells after gene delivery. (Lower panel) Quantification of migrated cells. Each point represents the mean ± SEM in high-power fields from triplicate experiments. Asterisks indicate statistical significance vs. control groups ($^*P < 0.01$).

capability of the endothelial cells (from 41.4 ± 25.1 to 108.3 ± 17.4 tubes per field; $P < 0.05$). These results indicate that POMC gene transfer potently perturbed the tube formation in endothelial cells, and this perturbation could be partially reversed by addition of exogenous ET-1.

Discussion

The present study demonstrates that POMC gene delivery inhibits the ET-1 release and perturbs angiogenic processes in endothelial cells, and, thereby, may induce endothelial injury. The increased ACTH and β-EP production in the POMC-transduced endothelial cells suggests that endothelial cells are capable of processing and producing functional POMC neuropeptides. This is consistent with a recent notion that POMC peptides can be processed and produced by dermal microvascular endothelial cells, thereby modulating the biologic functions of endothelial cells (14).

One novel finding in this study is the antiangiogenic function of POMC gene transfer in cultured endothelial cells. We demonstrated recently that intratumor POMC gene transfer led to suppression of the growth and metastasis of melanoma (11). One of the antineoplastic pathways of POMC gene transfer in melanoma is through blockage of blood vessel formation in melanoma (M.H. Tai *et al.*, unpublished data). Therefore, the antiangiogenic function of POMC gene transfer occurs not only in cultured endothelial cells, but also in melanoma grown in mice. Future studies should be performed to delineate the antiangiogenic mechanism underlying POMC gene transfer.

Because ET-1 is a potent angiogenic factor, it seems plausible that a reduction in ET-1 secretion might directly contribute to, rather than casually correlate with, the POMC-mediated angiogenesis inhibition in endothelial cells. Indeed, ET-1 supplementation partially reverted the attenuated tube formation by POMC gene transfer, further supporting the hypothesis that altered ET-1 homeostasis is involved in the antiangiogenesis function of POMC. This finding is also in agreement with the essential role of ET-1 in the formation of capillaries networks (15). However, the mechanism underlying decreased ET-1 release in Ad-POMC–infected endothelial cells remains unclear. Because the level of the ET-1 mRNA transcript was unchanged, it

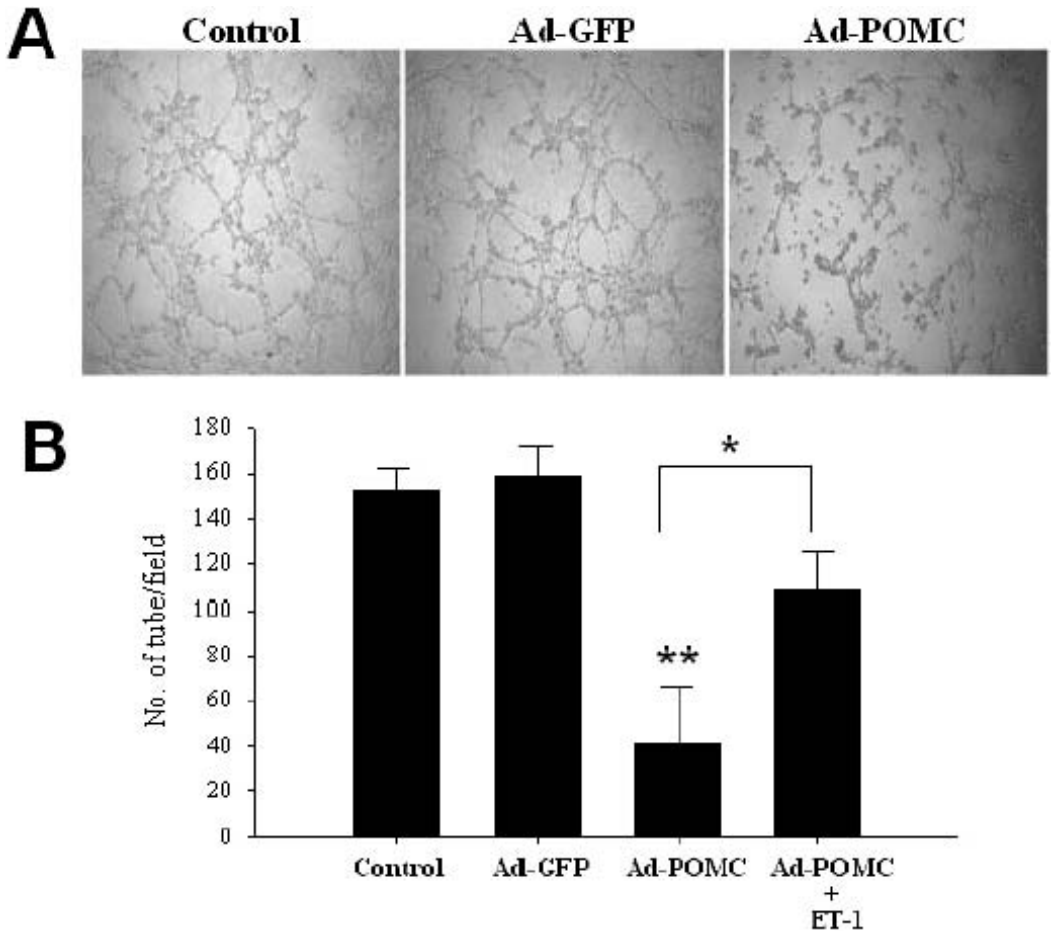

Figure 4. Effect of POMC gene delivery on tube formation in endothelial cells. (A) The representative profile of tube formation in endothelial cells infected with Ad-POMC. (B) Quantification of tube formation of POMC-expressing cells in the absence or presence of ET-1 (50 n*M*). Each data point represents the mean ± SEM of the tube number from quadruplicate experiments. *$P < 0.05$ vs. the Ad-POMC group; **$P < 0.01$ vs. control groups.

seems plausible that POMC gene transfer might affect the ET-1 secretion at posttranscriptional levels, including *de novo* protein synthesis, processing, and vehicle transport during the release to extracellular space. One probable pathway is the inhibition of ET-converting enzyme in endothelial cells by POMC gene delivery. Future studies are necessary to elucidate the influence of POMC expression on the processing enzyme of ET-1 precursors as well as the signaling pathway of ET-1, including expression of ET-1 receptors.

Because POMC encodes several neuropeptides with downstream effectors that have diverse cellular functions, the pathway(s) underlying POMC-mediated inhibition of angiogenic processes in cultured endothelial cells could be extremely complicated. One probable pathway is the cortisol generation by ACTH production in endothelial cells. However, such a possibility seems relatively remote because expression of aldosterone synthase or aldosterone biosynthesis is not detectable in endothelial cells (16). Recent evidence indicates that the ACTH pathway confers protection to vascular endothelium and controls the coordinated development of the vasculature and the endocrine tissue mass (17). In addition, ACTH depletion impairs adrenal endothelium through repression of vascular endothelial-cadherin transcription in mice (18). One likely candidate for an anti-angiogenic factor is β-EP, because application of β-EP inhibited the blood-vessel proliferation in chicken chorioallantoic membrane (19). Further investigations are necessary to elucidate the antiangiogenic function of β-EP. However, the involvement of interactions between multiple neuropeptides could not be ruled out in POMC-induced endothelial dysfunction.

ET-1 is a potent angiogenesis activator and enhances cell proliferation by reducing apoptosis in human umbilical vein endothelial cells *via* the ETB receptor and NADPH oxidase pathways (4, 20). Although the present study demonstrated that local POMC gene transfer attenuates the ET-1 secretion in cultured endothelial cells, a recent report indicates that the circulating ET-1 levels are elevated in patients with Cushing's syndrome (21). The discrepancies in ET-1 studies between cell culture studies and human subjects could be attributed to the chronic and complex

interaction in vascular endothelium of live organisms. Another possibility is that the high circulating ACTH or cortisol levels in patients with Cushing's syndrome may actually downregulate the local formation of endothelial POMC peptides (22), thus, may increase the ET-1 levels instead. Cardiovascular accidents represent the primary cause of death in patients with Cushing's syndrome (1, 23). Thus, the increased plasma ET-1 level may have a role in the pathogenesis of accelerated atherosclerosis development in this disorder, or it may simply be a marker of generalized endothelial dysfunction or damage. The pathophysiologic effects of POMC gene transfer in endothelial culture implicate a role for it in the initiation and progression of atherosclerosis *in vivo*.

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