Calcitonin Stimulates Multiple Stages of Angiogenesis by Directly Acting on Endothelial Cells

Srinivasulu Chigurupati, Trupti Kulkarni, Shibu Thomas, and Girish Shah

Department of Pharmacology, University of Louisiana School of Pharmacy, College of Health Sciences, Monroe, Louisiana

Abstract

Although a strong correlation between neuroendocrine differentiation and angiogenesis of prostate cancer has been reported, no mechanistic link between the two events has been established. Because neuropeptide calcitonin is secreted by prostate tumors and endothelial cells are known to express calcitonin receptor–like receptor, we examined the potential action of calcitonin on endothelial cells. The presence of calcitonin receptor, calcitonin receptor–like receptor, and receptor activity–modifying proteins in human microvessel endothelial-1 cells was tested by reverse transcriptase-PCR (RT-PCR). The proangiogenic action of calcitonin was examined in several in vitro models of angiogenesis using HMEC-1 cells and also in vivo using dorsal skinfold assays. Calcitonin expression of PC-3M cells was modulated, and its effect on angiogenesis was examined in in vitro as well as in vivo models. The results of RT-PCR and radioligand receptor assays showed the presence of functional calcitonin receptor in HMEC-1 cells. Calcitonin stimulated all phases of angiogenesis through the calcitonin receptor, but its effect on tube morphogenesis by endothelial cells occurred at the concentration of the Kd of calcitonin receptor. Silencing of calcitonin receptor expression in HMEC-1 cells abolished calcitonin-induced tube formation. Vascular endothelial growth factor antibodies attenuated but did not abolish calcitonin-induced tube morphogenesis. PC-3M prostate cancer cells induced angiogenesis in in vivo and in vitro models. Overexpression of calcitonin in PC-3M cells increased their angiogenic activity, whereas the silencing of calcitonin expression abolished it. These results show that prostate tumor–derived calcitonin may play an important role in prostate tumor growth by regulating intratumoral vascularization. (Cancer Res 2005; 65(18): 8519-29)

Introduction

Angiogenesis, the de novo sprouting and remodeling of capillaries, plays a central role in a variety of physiologic and pathologic processes (1, 2). Tumor angiogenesis, which can be broken down into several overlapping phases (3), typically correlates with increased tumor growth and metastasis (4). The first phase begins when angiogenic cytokines activate vascular cytokines. The second phase occurs when activated endothelial cells begin to proliferate and migrate towards the mitogenic stimulus where they form a vascular sprout. This phase also requires the release of proteolytic enzymes, which degrade the basement membrane to facilitate the invasion of endothelial cells. In the third phase, the vascular sprout forms a lumen. Finally, periendothelial cells are recruited to the area to provide further support for the new vessel.

Prostate cancer cells secrete a variety of proangiogenic substances, including vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and extracellular matrix proteinases (5–8). It has been proposed that neovascularization begins in benign prostatic hyperplasia and keeps progressing in a stepwise fashion in proliferative premalignant and malignant stages (9, 10). Although a strong correlation exists between the degree of neuroendocrine differentiation and the metastatic potential of prostate cancer, the results of this study fail to establish a mechanistic link between increased expression of bombesin and neurotensin with increased angiogenesis of the metastatic prostate cancer (10). Our previous studies have shown that calcitonin, a neuropeptide, is secreted by primary prostate epithelial cells, and its secretion from cancer-derived primary prostate cells is several-fold higher than it is from cells of benign prostatic hyperplasia origin (11). High affinity calcitonin receptors have been identified in membrane preparations of primary tumors and of LNCaP and PC-3M cell lines (12, 13). Exogenously added calcitonin stimulates the proliferation of LNCaP cells by stimulating cyclic AMP accumulation and by increasing cytoplasmic Ca2+ transients, and it stimulates their invasion by activating protein kinase A–mediated mechanisms (14). In addition, PC-3M cells stably overexpressing calcitonin form highly vascularized tumor xenografts in nude mice, raising the possibility that calcitonin might induce the angiogenic process in malignant prostate endothelium either by directly acting on endothelial cells or by inducing the secretion of proangiogenic cytokines or growth factors.

Considering that the calcitonin receptor–like receptor (CRLR) is expressed by human endothelial cells (3), we examined the possibility that prostate tumor–derived calcitonin might directly activate angiogenesis by activating either the calcitonin receptor or the CRLR in endothelial cells. We studied the effect of calcitonin on human microvessel endothelial-1 (HMEC-1) cells in in vitro models of different phases of neovascularization, such as migration, proliferation, invasion, and lumen morphogenesis. Finally, we examined the effect of PC-3M-derived calcitonin on angiogenesis in nude mice using a dorsal skinfold assay. The results provide evidence for the direct, receptor-mediated effect of calcitonin on HMEC-1 cells and identify an important role for tumor-derived calcitonin in neovascularization.

Materials and Methods

Endothelial Cell Lines

Human microvessel endothelial cells (HMEC-1), provided by Dr. Fransisco Candal (Center for Disease Control, Atlanta, GA), were maintained in MCDB 131 formula (Life Technologies Invitrogen, San Diego, CA) supplemented with 10% fetal bovine serum (FBS), epidermal growth
factor (EGF, 10 ng/mL), hydrocortisone (1 μg/mL), and L-glutamine (10 mmol/L). Aliquots of cells were preserved frozen between passages 1 and 5. For all experiments, HMEC-1 cells were used at passages 3 or below and harvested from a confluent dish.

Reverse Transcriptase-PCR for Calcitonin Receptor, Calcitonin Receptor–Like Receptor, and Receptor Activity–Modifying Proteins
Calcitonin is known to interact with calcitonin receptor as well as CRLR (15). In addition, accessory proteins such as receptor activity–modifying proteins (RAMP) regulate the turnover and specificity of these receptors (16, 17). Although evidence for the presence of CRLR in human endothelial cells exists (3), the presence of calcitonin receptor in these cells has not been reported. We examined the presence of transcripts for these receptors as well as RAMPs in HMEC-1 cells.

Total RNA (2 μg) prepared from HMEC-1 cells was reverse transcribed into cDNA with 1 μg oligo dT primers in a 20-μL reaction volume using Invitrogen Superscript cDNA synthesis kit (Life Technologies Invitrogen). The primers were designed to be specific for calcitonin receptor, CRLR, RAMP1, RAMP2, and RAMP3, and not to cross-hybridize with other known sequences as previously described (3, 18). The forward and reverse primer sequences were: calcitonin receptor, 5′-AAGCTTTTGGTCTCTATGGAGCTG-3′ and 5′-GAAATTCCCTCAGTGATCAGAATA-TC; CRLR, 5′-GTAATGTTAACCCACCGAGAAG-3′ and 5′-ATCCCCAGCAAGAAATAATAC-3′; RAMP1, 5′-GCAGACTCTTCAACCCTTACC-3′ and 5′-CTGGCTACTCTG-GACTCTGTG-3′; RAMP2, 5′-CATCCTCTCTTCTACTACCTTG-3′ and 5′-GGCTTTCACTCCGAGACATC-3′; and RAMP3, 5′-GCCAGTGAGGAGAAATGGTATAAG-3′ and 5′-AGGAACACAGAGGTGTCAG-3′.

PCR was done in 50-μL volume with 20 mmol/L Tris-HCl (pH 7.4, 25°C), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.1% Triton X-100, 200 μmol/L each of four deoxynucleotide triphosphates, 1 μmol/L of each primer, cDNA derived from the equivalent of 200 ng total RNA, and 2.5 units of Taq Polymerse (Life Technologies Invitrogen). Samples were subjected to 35 cycles in the iCycler (Bio-Rad, Hercules, CA). Cycle variables were as follows: the initial denaturation step at 95°C for 5 minutes; the repeat cycles consisted of annealing at 55°C, 50°C, and 55°C for calcitonin receptor, CRLR, for 40 seconds followed by extension at 72°C for 1 minute and denaturation at 94°C for 30 seconds; the last extension time was lengthened to 10 minutes. Amplified cDNAs were fractionated on 1.2% agarose gels in 89 mmol/L Tris, 89 mmol/L NaCl, 5 mmol/L HCl, and 5.5 mmol/L each of four deoxynucleotide triphosphates. 1 μmol/L of each primer, cDNA derived from the equivalent of 200 ng total RNA, and 2.5 units of Taq Polymerse (Life Technologies Invitrogen). Samples were subjected to 35 cycles in the iCycler (Bio-Rad, Hercules, CA). Cycle variables were as follows: the initial denaturation step at 95°C for 5 minutes; the repeat cycles consisted of annealing at 55°C, 50°C, and 55°C for calcitonin receptor, CRLR, for 40 seconds followed by extension at 72°C for 1 minute and denaturation at 94°C for 30 seconds; the last extension time was lengthened to 10 minutes. Amplified cDNAs were fractionated on 1.2% agarose gels in 89 mmol/L Tris, 89 mmol/L NaCl, 1 mmol/L CaCl2, 0.5 mmol/L MgCl2, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 100 units/ml aprotinin, 5 mmol/L EDTA, and homogenized by 50 strokes in a glass homogenizer. Membrane fraction was obtained by centrifuging homogenates at 15,000 × g for 7 minutes. Protein concentration was determined by Bio-Rad assay, and the membranes were aliquoted and stored frozen at −70°C.

Radioligand Binding Studies for Calcitonin Receptor
Membrane preparation. HMEC-1 cells in 100-mm dishes were detached with 0.05% EDTA in PBS, harvested by centrifugation at 200 × g for 10 minutes, suspended in cytoplasmic lysis buffer (10 mmol/L HEPES, 10 mmol/L NaCl, 1 mmol/L KCl, 5 mmol/L NaHCO3 1 mmol/L CaCl2, 0.5 mmol/L MgCl2, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 100 units/ml aprotinin, 5 mmol/L EDTA), and homogenized by 50 strokes in a glass homogenizer. Membrane fraction was obtained by centrifuging homogenates at 15,000 × g for 7 minutes. Protein concentration was determined by Bio-Rad assay, and the membranes were aliquoted and stored frozen at −70°C.

Radioligand binding assay. The 125I-mono-iodinated sCT of high specific activity was prepared as previously described (12). Membranes (1 μg protein per tube) were incubated with increasing concentrations of 125I-sCT in the assay buffer (25 mmol/L Tris, 10 mmol/L MgCl2, 1 mmol/L EGTA, 0.25 mmol/L PMSE, 0.85 mg/100 mL aprotinin, 0.85 mg/100 mL leupeptin) for 1 hour at 4°C. The total assay volume was kept at 50 μL. The radioactivity of the membrane fraction was determined after extensive washings. The binding data was analyzed by nonlinear regression and scatchard analysis (Prism, GraphPad, San Diego, CA).

Silencing of Calcitonin Receptor Expression with Small Interfering RNA
Exponentially growing HMEC-1 cells were plated at a density of 2 × 105 cells per well in a 6-well plate for 24 hours. The growth culture medium (MCDB 131 supplemented with 10% FBS, EGF, 10 ng/mL, hydrocortisone, 1 mmol/L, and L-glutamine, 10 mmol/L) was then replaced with serum-free MCDB 131 medium, and the cells were transfected with one of the following small interfering RNA (siRNA) duplexes against calcitonin receptor: 5′-GACACGCCCCUGACCUUCUUCU-3′ and 5′-P-GAAGAUAGUCAGGGCA-GUGCUCU-3′; 5′-GGAAGGGUCUGUACGAAAUUUU-3′ and 5′-P-at-GCAAGACU-AAGCCUCUUCUUU-3′ and 5′-P-AUCCCGGUAUAGGUUUU-3′. After overnight incubation in serum-free medium, the transfectants received the growth medium containing 10% FBS for additional 24 hours. The cells were then harvested to analyze calcitonin receptor mRNA abundance by real-time PCR.
Calcitonin and Angiogenesis

The PCR was done in a 50-µL reaction using 5 µL of the diluted first-strand cDNA template, optimized amounts of primers, and 25 µL of the 2× IQ SYBR Green supermix (Bio-Rad). The reactions were cycled at 50°C for 2 minutes, 94°C for 5 minutes, and 40 cycles of 94°C for 30 seconds, 57°C for 1 minute, and 68°C for 2 minutes. Template concentrations were adjusted to bring PCR products of all samples in the linear range of amplification. The level of transcripts for each gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample. All PCR reactions were done in duplicates and at three doses of the cDNA template. Ct values were determined, and the fold change in mRNA abundance was calculated as described previously (19).

The sequences of specific primer pairs are as follows: calcitonin receptor, 5'-ATCGAGGTCCAAACCACCGTGAAGC-3' and 5'-CAGTATTGACAAAGAGACCCCTG-3'; and GAPDH, 5'-ACGCCGCTTCTTGTGTCG-3' and 5'-ACAGCGGATCTTCTTTGTGC-3'.

In vitro Angiogenesis: Endothelial Cell Network Formation Assays
The assay examined the formation of vascular networks by HMEC-1 cells (20). The cells were plated in 8-well chambers at a density of 4 × 10^4 cells per well and cultured in endothelial growth medium for 4 hours. The cells were then treated with conditioned media (CM) of PC-3M cells or various concentrations of calcitonin (0-100 nmol/L) in serum-free medium for 12 hours. The cells were then washed and stained for H&E with Diff-Quick staining kit (Dade Behring Diagnostics, Aguada, Puerto Rico). The network formation was observed under an inverted phase contrast microscope (Nikon, Tokyo, Japan), and the images were captured with a video graphic system (Retiga 1300 Digital Output Camera). The number of network projections in ten 100× fields were counted for four independent experiments in each group, and the data were obtained using the following formula: angiogenesis = a × b where a = the number of branch points per 100× field and b = the total number branches per point.

Transwell migration assay. Migration assays were done in transwell tissue culture plates (6.5 mm and 8-µm pore size, Becton Dickinson and Co., Franklin Lakes, NJ). The bottom of the transwell chamber was coated with 10 mg/mL of collagen I (Sigma, St. Louis, MO), and uncoated sites were blocked with 10% bovine serum albumin (BSA). Approximately 1 × 10^5 cells per 100 µL were added into each well and allowed to migrate for 6 hours. The cells were then fixed with methanol and stained with crystal violet. The migrated cells in five random 100× fields were counted. Each experiment was done in triplicates, and the experiment was repeated twice. The results are expressed as mean number of migrated cells per field ± SE.

Endothelial cell proliferation assay. The growth/proliferation of HMEC-1 cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (American Type Culture Collection, Manassas, VA). Exponentially growing HMEC-1 cells were plated at the density of 8 × 10^3 cells per well of 96-well plates and grown in complete MCDB131 medium for 24 hours. The complete medium was then replaced with 100 µL of basal MCDB131 medium (containing 0.1% BSA, 4 mmol/L L-glutamine, 100 IU/mL penicillin G, and 100 µg/mL streptomycin). MTT solution was added after 24 hours and incubated at 37°C for 4 hours, the reaction was terminated with the addition of 100-µL solubilization/stop solution, and incubation was continued at 37°C to completely dissolve the formazan product. The plates were then analyzed for absorbance on an ELISA plate reader (Bio-Rad) at 595 nm.

Cell invasion assay. These experiments were conducted in 24-well, two-compartment, Matrigel invasion chambers (Becton Dickinson, Bedford, MA) as described before (14). Exponentially growing HMEC-1 cells were serum starved for 24 hours with basal MCDB131 medium, harvested, and seeded at a density of 25 × 10^3 cells per well in the insert of Matrigel invasion chamber. The lower chamber received the chemoattractant medium, which consisted of 90% serum-free MCDB131 medium and 10% conditioned medium from the cultures of PC-3M cells expressing constitutively active Gαs protein (21). The cells also received calcitonin or other agents, which were added to the insert. The incubations were carried out for 24 hours, after which the Matrigel (along with noninvasive cells) was scraped off the inside bottom of the insert using cotton swabs, and outer side of the insert was fixed and stained using Diff-Quick staining kit. The number of cells migrated on the outer bottom side of the insert were counted under the microscope in at least six randomly selected fields

Figure 2. Effect of calcitonin (CT) on network formation by HMEC-1 cells. HMEC-1 cells were cultured and treated with increasing concentrations of CT (0-100 nmol/L) for 24 hours. Representative micrographs show that calcitonin stimulated the network formation (A-D). Columns, mean number of branches per network × number of networks (n = 12); bars, ±SE. Pooled data of four separate experiments is presented in (E). *, P < 0.01, significantly increased over the control value (one-way ANOVA and Newman-Keuls test).
Growth correction. To rule out the possibility that cells migrated during early part of the 24-hour incubation period could proliferate during the remaining treatment period, we determined the growth rate of HMEC-1 early part of the 24-hour incubation period could proliferate during the remaining treatment period.

In vitro tube morphogenesis on Matrigel. The vessel morphogenesis assay was done as previously described (3). HMEC-1 cells were adjusted to a density of $3 \times 10^4$ cells in 200 µL in MCDB 131 (Life Technologies Invitrogen) medium supplemented with 0.5% FBS and either calcitonin or VEGF and added to the wells of eight-well chamber slides precoated with growth factor–depleted Matrigel (7 µg/mL; Becton Dickinson, Bedford, MA). The slides were incubated at 37°C for 18 to 20 hours. The medium was then removed gently without disturbing newly formed tubules. The images were captured at a 40× magnification on Nikon Optiphot II microscope. Total tube length of each well was measured using IP Lab Image Analysis Program (Scanimalsics, Inc., Arlington, VA).

In vivo Angiogenesis
The effect of PC-3M-secreted calcitonin on in vivo angiogenesis was examined using dorsal skinfold assay (22–25). Calcitonin-secreting activity of PC-3M cells was modulated by stably transfecting them with either constitutively active vector expressing calcitonin or hammerhead anti-calcitonin ribozymes. Each modified cell line had vector controls.

Overexpression of calcitonin. The full-length human calcitonin cDNA cloned from primary prostate tumor specimens was inserted into pcDNA3.1 plasmid (Research Genetics, Carlsbad, CA) in the sense direction (26). The orientation of the insert was confirmed by sequencing at the institutional DNA sequencing facility. CT-pcDNA3.1 construct was then transfected into PC-3M cells and G418-resistant colonies were selected as described before (21).

Silencing of calcitonin expression. Two strands of oligonucleotide templates encoding each ribozyme against calcitonin cDNA having a BamHI and HindIII site at the 3′ and 5′ end, respectively, were synthesized at the Genemed Biosynthesis, Inc. (San Francisco, CA). Sense R4, 5′-GAAGATCTTC/CAAAAGCCAG/TTTGTCCCTACGCACTCATCAG/-ATCTGGCT/CCGCTGAGCGG-3′; Antisense R4, 5′-CTTCTAGAAG/GTTCCCGTCA/AAGCAGGATGCCGTGAGAGTC/TAGACCGA/GGCGAG/ 

The oligonucleotides were purified by PAGE, annealed and cloned into BamHI and HindIII sites of pcDNA3.1 construct was then transfected into PC-3M cells and G418-resistant colonies were selected as described before (21).

In vivo Angiogenesis: Dorsal Skinfold Assay
To validate the results of in vitro studies under in vivo conditions, we exposed skinfolds of nude mice to steady concentrations of calcitonin by placing PC-3M variant cells in transparent diffusion chambers as described before (28, 29). The diffusion chambers were prepared by cementing 0.45-µm membranes on both sides of the rim of the “O” ring (Millipore, Bedford, MA). The chambers were then sterilized by UV radiation for 20 minutes and wetted with sterile PBS. PC-3M-R4, PC-3M-CT−, PC-3M-CT+, PC-3M-CT−, PC-3M-CT−, cells (2 × 10^6 cells per 150 µL of sterile PBS) were injected individually into each chamber. A dorsal air sac in an anesthetized mouse was prepared by injecting 10 µL of air in the skinfold. The air sac was then opened to implant the chamber underneath the skin and then incision was sutured. After 10 days, the animals were sacrificed, and carefully skinned around the implanted chambers. The skinfold covering the chambers was carefully removed, stretched on a glass slide and photographed under visible light microscope (20×). New blood vessels on the skinfold were counted and their lengths were determined. Sterile small-animal surgical techniques were followed during the whole procedure, which was approved by the institutional Animal Care and Use Committee.

In vitro Angiogenesis: Dorsal Skinfold Assay
To validate the results of in vitro studies under in vivo conditions, we exposed skinfolds of nude mice to steady concentrations of calcitonin by placing PC-3M variant cells in transparent diffusion chambers as described before (28, 29). The diffusion chambers were prepared by cementing 0.45-µm membranes on both sides of the rim of the “O” ring (Millipore, Bedford, MA). The chambers were then sterilized by UV radiation for 20 minutes and wetted with sterile PBS. PC-3M-R4, PC-3M-CT+, PC-3M-CT−, PC-3M-CT−, PC-3M-CT−, cells (2 × 10^6 cells per 150 µL of sterile PBS) were injected individually into each chamber. A dorsal air sac in an anesthetized mouse was prepared by injecting 10 µL of air in the skinfold. The air sac was then opened to implant the chamber underneath the skin and then incision was sutured. After 10 days, the animals were sacrificed, and carefully skinned around the implanted chambers. The skinfold covering the chambers was carefully removed, stretched on a glass slide and photographed under visible light microscope (20×). New blood vessels on the skinfold were counted and their lengths were determined. Sterile small-animal surgical techniques were followed during the whole procedure, which was approved by the institutional Animal Care and Use Committee.

Figure 3. Effect of calcitonin (CT) on in vitro angiogenesis. A, calcitonin and migration of HMEC-1 cells. HMEC-1 cells were plated in the transwell insert and treated with increasing concentrations of calcitonin (0–100 nmol/L). The number of cells passing through the filters after 6 hours was counted. Columns, number of migrated cells per 100× field, pooled results of four separate experiments (n = 12); bars, ±SE. *P < 0.01; **P < 0.001, significantly increased over the control value (one-way ANOVA and Newman-Keuls test). B, effect of calcitonin on proliferation of HMEC-1 cells. HMEC-1 cells were cultured in the presence of increasing concentrations of calcitonin (0–100 nmol/L) for 24 hours. Their cell proliferation was assessed by the MTT assay. Absorbance at 595 nm was measured. Columns, mean of four independent experiments (n = 8); bars, ±SE. *P < 0.01, significantly increased from the control values (one-way ANOVA and Newman-Keuls test). C, effect of calcitonin on invasion of HMEC-1 cells. HMEC-1 cells were seeded on a Matrigel layer of invasion chambers and were treated with increasing concentrations of calcitonin (0–100 nmol/L). Cells migrating through the Matrigel layer were counted after 24 hours. Columns, mean number of invading cells per 100× field, pooled results of four separate experiments (n = 12); bars, ±SE. *P < 0.01, significantly increased from the control values (one-way ANOVA and Newman-Keuls test).
Statistical Analysis
Data were expressed as mean ± SE and were statistically evaluated by one-way ANOVA followed by Newman-Keuls test. The difference was considered significant if \( P < 0.05 \).

Results

Expression of Calcitonin Receptor, Calcitonin Receptor–Like Receptor, and Receptor Activity–Modifying Proteins in HMEC-1 Cells

Total RNA from HMEC-1 cells was tested for the presence of calcitonin receptor, CRLR, and RAMP-1 to RAMP-3 transcripts. We obtained predicted amplified products of the transcripts of following sizes: calcitonin receptor (666 bp), CRLR amplimers (392 bp), and RAMP-3 (192 bp; Fig. 1A). Transcripts of RAMP-1 or RAMP-2 were not amplified.

High-Affinity Binding Sites for \(^{125}\)I-sCT in Membranes of HMEC-1 Cells

Because calcitonin receptor transcript was detected in HMEC-1 cells, we tested its functional presence by the radioligand receptor assay. The HMEC-1 membranes displayed saturable \(^{125}\)I-sCT binding sites (Fig. 1B). Scatchard analysis of the binding data determined the \( K_d \) value of 3.2 nmol/L, confirming the presence of high-affinity calcitonin binding sites (Fig. 1C).

Calcitonin Stimulates the Network Formation by HMEC-1 Cells

As revealed by the results of Fig. 2, increasing concentrations of calcitonin significantly stimulated network formation of HMEC-1 cells in a dose-dependent fashion. The effect of calcitonin on capillary-like formations was evident in the number of network formation per field as well as on the thickness of these tube-like structures.

Because angiogenesis involves multiple processes associated with neovascularization including migration of endothelial cells, their proliferation, invasion and lumen morphogenesis on Matrigel, we examined the effects of the CM of calcitonin-treated PC-3M cells in each of these phases.

Calcitonin directly increases the migration of endothelial cells. The results of Fig. 3A show that calcitonin stimulated migration of HMEC-1 cells in a dose-dependent fashion. Calcitonin-induced increases in the migration were 60% and 100% over the controls at 50 and 100 nmol/L calcitonin, respectively, and were statistically significant.

Effect of calcitonin on proliferation of HMEC-1 cells. At 10 nmol/L, calcitonin did not have any effect on proliferation of HMEC-1 cells. At 50 nmol/L, calcitonin induced a marginal increase in cell proliferation. Only at the highest tested concentration of 100 nmol/L, calcitonin induced a moderate but significant 50% increase.

---

Figure 4. Morphogenic Activity of VEGF and calcitonin (CT). HMEC-1 cells were seeded on Matrigel precoated wells and cultured in the presence of low-serum conditions with either VEGF (0-6 ng) or calcitonin (0-100 nmol/L). Representative photomicrographs of HMEC-1 cultures were taken after 18 hours after the treatment (40×). Diameter of the vessels was also recorded. Columns, mean branch points \( \times \) number of branches per 100× field (n = 12); bars, ±SE (A and C). Columns, mean vessel density (μm, n = 12); bars, ±SE (B and D). The effect of VEGF on tube morphogenesis presented as branchpoints \( \times \) number of networks formed (A) or microvessel density (B). The effect of calcitonin on tube morphogenesis presented as branchpoints \( \times \) number of networks formed (C) or microvessel density (D). *, \( P < 0.01 \), significantly increased from the control values (one-way ANOVA and Newman-Keuls test).
Calcitonin increases the invasion of HMEC-1 cells through Matrigel. The results of Fig. 3C show that at 50 and 100 nmol/L, calcitonin caused significant, 75% and 128% increase in invasion of HMEC-1 cells, respectively. The results are corrected for possible cell proliferation during the experimental period.

Calcitonin Mimics the Action of Vascular Endothelial Growth Factor by Stimulating Tube Morphogenesis on Matrigel

When challenged with VEGF, HMEC-1 cells migrate throughout the Matrigel surface and align to form vascular cord-like structures (3). The micrographs presented in Fig. 4 show that HMEC-1 cells do not form cord-like structures in the absence of angiogenic stimulus. However, they formed these structures when treated with various concentrations of VEGF (Fig. 4A-B) and calcitonin (Fig. 4C-D). VEGF stimulated tube formation at 3 and 6 ng/ml concentrations (Fig. 4A-B). Similarly, calcitonin also increased these formations at all tested doses, and 100 nmol/L calcitonin caused over 10-fold increase in tube formations compared with vehicle controls (Fig. 4C). VEGF as well as calcitonin also increased the thickness (microvessel density) of these structures. (Fig. 4B-D).

Silencing of Calcitonin Receptor Expression in HMEC-1 Cells Abolishes Calcitonin-Induced Tube Morphogenesis on Matrigel

To confirm that the effect of calcitonin on tube formation is specific and receptor-mediated, we silenced calcitonin receptor expression in HMEC-1 cells using siRNA against calcitonin receptor. Our earlier studies with other cell lines including prostate cell lines have shown that siRNA3 was most potent and reduced calcitonin receptor mRNA expression by >95%. siRNA2 was also effective but less potent than siRNA3. siRNA1 was completely ineffective and was used as a negative control. The results of Fig. 5A have shown that siRNA1 was ineffective in silencing calcitonin receptor gene expression. siRNA2 attenuated calcitonin receptor mRNA abundance by 75% whereas siRNA3 decreased it by 94% (Fig. 5A).

Sham-transfected HMEC-1 cells and those receiving siRNA1 formed almost equal number of tubes with a similar microvessel density (Fig. 5B). Treatment with 50 nmol/L calcitonin increased the number of tube structures by almost 5-fold, which was similar to the responses in previous experiments (see Fig. 4). However, HMEC-1 cells receiving either siRNA2 or siRNA3 displayed no tube

Figure 5. Silencing of calcitonin (CT) receptor (CTR) expression and morphogenic activity of calcitonin. HMEC-1 cells were transfected siRNA duplexes against calcitonin receptor mRNA sequence. After 48 hours, the transfectants were plated on Matrigel-precoated wells and treated with/without 50 nmol/L calcitonin for additional 18 hours. Representative photomicrographs of tube-like formations by HMEC-1 cells (40×). A, calcitonin receptor mRNA abundance in HMEC-1 cells after transfection with siRNAs. B, columns, mean branchpoints × number of branches per field for each treatment (n = 6); bars, ±SE. *, P < 0.01; **, P < 0.001, significantly increased from the control values (one-way ANOVA and Newman-Keuls test).
formation at all either with/without calcitonin stimulation, suggesting that calcitonin-mediated tube formation by HMEC-1 cells cannot occur in the absence of calcitonin receptor. The pooled data from all experiments is presented in Fig. 5B.

**Immunoneutralization of Vascular Endothelial Growth Factor Attenuates but Does Not Abolish Calcitonin-Induced Tube Morphogenesis**

To examine a possibility that calcitonin may stimulate the tube formation by inducing VEGF secretion by PC-3M cells, we tested the effect of calcitonin on tube formation in the presence or absence of anti-VEGF serum (80 ng/mL). The cells were pretreated for 1 hour with anti-VEGF serum before the addition of calcitonin. Anti-VEGF serum partially attenuated but did not abolish the stimulatory effects of calcitonin on *in vitro* angiogenesis (Fig. 6).

**PC-3M-Derived Calcitonin and In Vivo Angiogenesis**

Modulation of calcitonin expression in PC-3M cells. The results depicted in Fig. 7A show the abundance of calcitonin mRNA in PC-3M variant cell lines and their controls. As expected, PC-3M cells expressing CT-pcDNA 3.1 plasmid (CT+) displayed markedly higher abundance of calcitonin mRNA than those expressing empty vector (PC-3M-v). Likewise, the abundance of calcitonin mRNA in cells expressing inactive ribozyme (R4) was similar to PC-3M-v, but CT− cells expressing potent ribozyme (R5) displayed almost complete absence of calcitonin mRNA (Fig. 7A). Consistent with the levels of calcitonin mRNA, PC-3M-CT+ cells secreted 4-fold greater levels of calcitonin compared with PC-3M-v (Fig. 7B). Again, secretion of calcitonin by R4 cells was similar to PC-3M-v, but that of PC-3M-CT− cells was undetectable (Fig. 7B).

Conditioned media of PC-3M cells stimulates the network formation by HMEC-1 cells: its modulation by calcitonin expression. To compare proangiogenic actions, PC-3M cell secretions, we collected the CM from cultures of PC-3M, PC-3M-CT+, PC-3M-R4, and PC-3M-CT− cells after 24 hours and tested first for *in vitro* angiogenic activity. The CM of PC-3M, PC-3M-v, and PC-3M-R4 cells exhibited similar network-forming activity (Fig. 7C). However, The CM of PC-3M-CT+ cells displayed 7-fold higher angiogenic activity. In contrast, the CM of PC-3M-CT− cells could not stimulate angiogenesis, suggesting the absence of factors stimulating the angiogenesis.

**In vivo Angiogenesis: Dorsal Skinfold Assay**

To test the biological significance of calcitonin-induced angiogenesis, we examined the effect of implantation of PC-3M-v, PC-3M-CT+, PC-3M-R4, and PC-3M-CT− cells on neovascularization in

---

*Figure 6.* Immunoneutralization of VEGF and morphogenic activity of calcitonin (CT). HMEC-1 cells were plated on Matrigel-precoated wells as described in Materials and Methods and cultured in low serum conditions and with/without anti-VEGF serum (80 ng/mL). The cells were then treated with increasing concentrations of calcitonin (0-100 nmol/L), and their effects on tube formation were examined. Representative photomicrographs of HMEC-1 cultures after 18 hours of treatment (40×). Columns, mean branchpoints × number of branches per 40× field; mean microvessel density, pooled results of three separate experiments (n = 9); bars, ±SE. *P < 0.01; **P < 0.001, significantly increased from the control values (one-way ANOVA and Newman-Keuls test).
The secretions of PC-3M cell variants induced the formation of new vessels in skinfolds. However, the response seemed to vary with calcitonin expression in PC-3M cells. For example, angiogenesis in mice implanted with PC-3M-CT+ cells was observably greater than those receiving either PC-3M-v or PC-3M-R4 cells (Fig. 8). In contrast, mice receiving PC-3M-CT- cells did not display any angiogenic response. The length of new vasculature in skinfolds of mice treated with the PC-3M cell variants was also determined (Fig. 8).

**Discussion**

Calcitonin is essential for the maintenance of calcium balance (15, 30). It has also been implicated in the regulation of several functions in reproductive and neuroendocrine tissues (31–35). Overexpression of calcitonin and calcitonin receptor has been reported in several malignancies including breast and prostate cancers (26, 36–38). Our earlier studies have shown that the expression of calcitonin in primary prostate tumors is epithelium specific (26). The number of cells expressing calcitonin and/or calcitonin receptor mRNAs correlate positively with the tumor grade, and cells of aggressive tumors coexpress calcitonin and calcitonin receptor mRNAs (26). We examined the potential role of calcitonin in angiogenic process because our recent results suggest that PC-3M-CT+ cells form larger xenografts with markedly higher vascularization than those formed by PC-3M-v cells. The present results for the first time show that HMEC-1 cells express calcitonin receptor and respond to calcitonin with angiogenesis. Moreover, modulation of calcitonin expression in PC-3M cells alters their ability to stimulate angiogenesis. These
results strongly suggest that prostate-derived calcitonin functions as an important angiogenic factor.

Angiogenesis is multiphasic process involving migration of endothelial cells in tumor, their proliferation and invasion in lumen, and the formation of new blood vessels. We examined the effect of calcitonin on each of these processes individually. We have shown that calcitonin stimulated all these processes and led to the formation of vascular tubules on Matrigel. These actions of calcitonin were mediated by calcitonin receptor as silencing of calcitonin receptor expression completely abolished calcitonin response. However, the magnitude of calcitonin response in each of these processes was different. For example, the effect of calcitonin on proliferation of endothelial cells was marginal and significant only at the highest tested concentration of 100 nmol/L. In contrast, the effect of calcitonin on migration and invasion was stronger, increasing by 2- to 3-fold in response to 100 nmol/L calcitonin. However, the most potent action of calcitonin was on the network formation or tube morphogenesis on Matrigel, and calcitonin also increased the thickness of these microvessels. This action was significant even at the lowest tested concentration of 10 nmol/L. Considering that calcitonin could markedly stimulate this process at concentrations of the $K_d$ of calcitonin receptor, it is likely that this effect has an important pathophysiologic significance (12). These actions of calcitonin were similar to VEGF, which is considered as principal growth factor for blood vessel formation (39). Because prostate cancer cells as well as endothelial cells were shown to express VEGF (39–41), we examined a possibility that VEGF may mediate proangiogenic actions of calcitonin by assessing calcitonin-induced tube formation in the presence of anti-serum to VEGF. This partially attenuated but did not abolish calcitonin action, suggesting that calcitonin may stimulate angiogenesis directly as well as indirectly.

To evaluate the biological significance of calcitonin expression by prostate cancer cells, we modulated calcitonin expression in PC-3M cells and examined its effect in an in vivo model of angiogenesis (22, 24, 42). The implantation of PC-3M cells induced neovascularization in mouse skinfold and that overexpression of calcitonin significantly increased this activity. In contrast, silencing of calcitonin expression abolished angiogenic response, reinforcing a possibility that calcitonin is a potent stimulator for angiogenesis and that tumor cell–derived calcitonin may play a key role in prostate cancer progression by stimulating intratumoral vascularization.

The cells of neuroendocrine phenotype are present in androgen-independent prostate carcinoma and are considered as early indicators for androgen independence (43–45). They are shown to secrete paracrine peptides such as bombesin, neurotensin, calcitonin, and others that may support androgen-independent growth of prostate cancer cells (26, 46–48). Recent evidence has identified very high correlation between neuroendocrine expression, microvessel density and VEGF expression in prostate carcinoma (9, 41), suggesting a potential role of neuroendocrine secretions in angiogenesis of prostate cancers. However, bombesin and neurotensin could not stimulate angiogenesis in a model using human umbilical vascular endothelial cells (10). The present results have shown that calcitonin potently stimulated angiogenesis in an in vivo model as well as an in vitro model using HMEC-1 cells. The

![Figure 8. PC-3M cell variants and in vivo angiogenesis (dorsal skinfold assay). Photomicrographs of dorsal skin-folds were taken 10 days after the implantation of cell chambers. The photographs ($20 \times$) show changes in vessel density of mice skinfolds in response to the secretions of PC-3M cell variants. Length of newly formed vessels was measured and plotted against a PC-3M variant cell lines. * , $P < 0.01$, significantly different from control values (PC-3M-v and R4; one-way ANOVA and Newman-Keuls test).](image-url)
specificity of calcitonin-induced angiogenesis was shown by the results that the silencing of calcitonin receptor in HMEC-1 cells completely abolished calcitonin-induced angiogenic response. In addition to its direct actions on endothelial cells, calcitonin activates other processes that promote angiogenesis. For example, calcitonin stimulates matrix metalloproteinase (MMP) expression of prostate cancer cells (48–53) and the secretion of activated MMP-2 and MMP-9 (14). Increased secretion of activated MMPs leads to the proteolytic degradation of extracellular matrix and the basement membrane, which enables endothelial cells to invade tumor and form new vessels (54). In addition, the present results that immunoneutralization with anti-VEGF serum significantly reduced calcitonin-induced tube formation raise a possibility that calcitonin may also stimulate the secretion of VEGF by PC-3M cells.

Considering the presence of calcitonin receptor, CRLR, and RAMP-3 in HMEC-1 cells, it is conceivable that other ligands for calcitonin receptor, CTR-RAMP3 or CRLR-RAMP-3 heterodimers may also induce angiogenic response. However, there is no evidence for the expression of amylin in prostate cancer and there may also induce angiogenic response. Moreover, PC-3M-derived calcitonin stimulates angiogenesis in vitro as well as in vivo models of angiogenesis. Considering the presence of calcitonin in prostate tumor microenvironment, calcitonin may significantly influence tumor growth by regulating intratumoral vascularization.

Acknowledgments

Received 3/16/2005; revised 6/20/2005; accepted 6/27/2005.


The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

38. Findlay DM, Michalangieli VP, Eisman JA, et al. Calcitonin and 1,25-dihydroxyvitamin D3 receptors in
Calcitonin Stimulates Multiple Stages of Angiogenesis by Directly Acting on Endothelial Cells

Srinivasulu Chigurupati, Trupti Kulkarni, Shibu Thomas, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/18/8519

Cited articles
This article cites 53 articles, 14 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/18/8519.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/18/8519.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.