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ScienceDirect
Biochemical and Biophysical Research Communications 350 (2006) 74–81
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VCC-1, a novel chemokine, promotes tumor growth

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Received 30 August 2006
Available online 12 September 2006

Abstract

We have identified a novel human gene by transcriptional microarray analysis, which is co-regulated in tumors and angiogenesis model systems with VEGF expression. Isolation of cDNA clones containing the full-length VCC-1 transcript from both human and mouse shows a 119 amino acid protein with a 22 amino acid cleavable signal sequence in both species. Comparison of the protein product of this gene with hidden Markov models of all known proteins shows weak but significant homology with two known chemokines, SCYA17 and SCYA16. Northern analysis of human tissues detects a 1 kb band in lung and skeletal muscle. Murine VCC-1 expression can also be detected in lung as well as thyroid, submaxillary gland, epididymis, and uterus tissues by slot blot analysis. By quantitative real time RT-PCR 71% of breast tumors showed 3- to 24-fold up-regulation of VCC-1. *In situ* hybridization of breast carcinomas showed strong expression of the gene in both normal and transformed mammary gland ductal epithelial cells. *In vitro*, human microvascular endothelial cells grown on fibronectin increase VCC-1 expression by almost 100-fold. In addition, in the mouse angioma endothelial cell line PY4.1 the gene was over-expressed by 28-fold 6 h after induction of tube formation while quiescent and proliferating cells showed no change. VCC-1 expression is also increased by VEGF and FGF treatment, about 6- and 5-fold, respectively. Finally, 100% of mice injected with NIH3T3 cells over-expressing VCC-1 develop rapidly progressing tumors within 21 days while no growth is seen in any control mice injected with NIH3T3 cells containing the vector alone. These results strongly suggest that VCC-1 plays a role in angiogenesis and possibly in the development of tumors in some tissue types.

Keywords: Cancer; Angiogenesis; Tumorigenesis; Chemokine; Breast carcinoma; Vascular endothelial growth factor; VEGF; IL-8; GRO1; SCYA16; SCYA17

One definition for a genetic network is regulation of genes by other genes through their protein products [1]. Microarray technology is a compelling tool for the examination of genetic co-regulation since it allows for simultaneous evaluation of all cellular processes. The underlying hypothesis of network analysis is that the more often a pair of genes is observed to have correlated expression under differing conditions or states (e.g., cell type, drug treatment, growth conditions, and disease state) the more likely that these genes are co-regulated.

Cancer is a complicated disease, due to its polygenic nature, and presents itself as an excellent candidate for the assessment of microarray technology. Previous work provided us with a strong indication that this approach would yield new molecular markers and targets that could be used in the prognosis and treatment of cancer [2]. To achieve these goals we examined more than 50 tumors isolated from patients suffering from early to late stage breast, colon, kidney or lung cancer. This approach has allowed us to elucidate several key genes and pathways involved in these cancer types.

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.08.194

Due to the challenges involved in directly inhibiting solid tumor growth (i.e., tumor heterogeneity, incomplete penetrance of a therapeutic agent) the tumor vasculature has arisen as an attractive target mechanism. Angiogenesis appears essential for sustained tumor growth and eventual metastasis of most solid tumor types. Through the disruption of the local blood supply, molecular inhibitors may destroy all tumor cells reliant upon readily accessible vasculature. Among the many proteins involved in angiogenesis, vascular endothelial growth factor (VEGF) has been the most extensively examined and the most critical player in the growth and differentiation of the vasculature. In breast cancer patients, high VEGF levels have been correlated with shorter overall patient survival, shorter relapse-free survival, and increased occurrence of metastasis [3,4]. Therefore, due to its central role in angiogenesis, particular attention was paid to the expression of VEGF. We found VEGF expression to be significantly correlated to the expression of three genes, two known and one novel. The novel gene has similarities to known chemokines, and is therefore named VEGF correlated chemokine 1, or VCC-1.

Cytokines are a family of approximately 45–50 proteins in humans, containing a structural homology of conserved cysteine residues. The CXC chemokines are a sub-family of the cytokines, named due to their conserved Cys-Xaa-Cys sequence near the N-terminus of the protein, which also contain two additionally conserved cysteine residues. The proteins are roughly 70–130 amino acids in size and are secreted with a leader sequence of approximately 20–25 amino acids that is cleaved before release. A characteristic three-dimensional folding of the chemokines is stabilized by the disulfide bonds that form between the conserved cysteine 1 and cysteine 2, and between cysteine 3 and cysteine 4 [5].

All known biological effects of chemokines are exerted through their interaction with a cell surface receptor. There are six CXC chemokine receptors (CXCRs) identified to date [6]. The CXCRs are members of the superfamily of serpentine proteins that signal through heterotrimeric G-proteins. These proteins have been shown to possess the ability to bind multiple chemokines with high affinity, and chemokines have conversely been shown to bind multiple receptors. This creates the possibility of multiple combinations of receptor–ligand combinations and multiple biological outcomes.

While originally defined as soluble factors able to control the migration of leukocytes, chemokines are now known to play a complex role in a variety of cell types, including tumor cells [7]. The clearest example of chemokines directly modulating tumorigenesis and growth was shown by over-expression of GRO α, β, and γ in human melanocytes, which lead to an anchorage-independent growth phenotype *in vitro* and the ability to form tumors *in vivo* in nude mice [8,9]. Furthermore, both IL-8 and ENA-78 expression in non-small cell lung carcinoma (NSCLC) has been correlated with tumor angiogenesis [10,11].

In this work, we describe the identification and cDNA cloning of a novel chemokine which we named VCC-1. We have examined its expression in a wide variety of tissues as well as in breast and colon carcinomas. We demonstrate that expression of this chemokine is regulated *in vitro* by VEGF and that levels of VCC-1 are increased significantly in endothelial cells undergoing tube formation. Finally, over-expression of this gene is sufficient to initiate tumorigenesis *in vivo*, as demonstrated in a xenograft model system.

Materials and methods

Microarray analysis. Fluorescently labeled (Cy3, Cy5) cDNA probes were generated from the RNA samples (Incyte Genomics). Incyte HG microarray chips were analyzed by competitive hybridization to each of four sets of 10 individuals afflicted with cancerous tumors (breast, colon, lung or kidney) and were compared to a normal tissue pool constructed from six sudden death individuals. After error correction and normalization, the fold increase or decrease of tumor tissue expression/normal tissue was calculated.

Genetic network analysis. A differential expression vector is constructed for all genes represented on the microarrays for a given experiment. An experiment is generally defined as a set of related conditions with a common comparator. A given gene of known function is selected and a Pearson correlation coefficient is calculated between the given gene and all other genes within the experiment. The Pearson correlation coefficient is given by:

$$\bar{r}_{\text{pearson}} = \frac{\left(\sum_{x=1}^{N_{\text{cluster1}}} \sum_{y=1}^{N_{\text{cluster2}}} r_{x,y}\right)}{N_{\text{cluster1}} * N_{\text{cluster2}}}$$

where,

$$\bar{r}_{\text{pearson}} = \frac{\left(\sum_{x=1}^{N_{\text{cluster1}}} \sum_{y=1}^{N_{\text{cluster2}}} r_{x,y}\right)}{N_{\text{cluster1}} * N_{\text{cluster2}}}$$

This analysis is repeated for all experiments. Genes which have a significant correlation coefficient, defined by a p-value less than 0.01 in multiple experiments, are considered to be members of the local network. This process can then be repeated for the members of the local network to expand the network.

Sequence analysis. EST gene assembly was performed utilizing the Crossmatch and Phrap programs [12]. The human genomic structure of VCC-1 was determined utilizing sequence data from Celera Human Genome Assembly Release R26. Markov models of the protein database were constructed utilizing SAM 3.2 [13].

Isolation of cDNA. The 360 bp full-length human VCC-1 was cloned from cDNA generated with SuperScript II reagents (Invitrogen, catalog #11904-018) according to manufacturer's protocol. HT29 human colon cancer cells served as the source of RNA (AATC, catalog #HTB-38), which was isolated using a Qiagen RNeasy Miniprep column (Qiagen, catalog #74104) as per manufacturer's instructions, from cells growing in culture. The forward primer used, hvccf1, was ATGAAAGTTC TAATCTCTTCCCTC. Two reverse primers were used, one containing an in-frame stop codon and one without a stop codon. Primer hvccr1, CTACAAAGGCAGAGCAAAGCTTC, contained a stop codon, while primer hvccr2, CAAAGGCAGAGCAAAGCTTCTTAGC, did not and was designed for in-frame fusion to a myc/HIS tag. Polymerase chain reactions were performed using 0.8 μl cDNA with the following conditions: 1 μl of forward primer at a concentration of 25 μM, 1 μl of either reverse primer hvccr1 at 25 μM or reverse primer hvccr2 at 25 μM, 0.5 μl *Taq*, 1.5 μl of 50 mM $MgCl_2$, 5 μl of 10× PCR buffer (Invitrogen, catalog #10342-053), 1 μl of 10 mM dNTP mix (Invitrogen, catalog #18427-013),

and water to 50 μl. Cycling parameters were: 4 min at 94 °C for hot start, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 3 min at 72 °C, with a final extension of 10 min at 72 °C. Amplification was assessed by running 10 μl of the PCR on a 1.5% agarose gel.

The 360 bp mouse VCC-1 was isolated by PCR from mouse embryo cDNA (Ambion, catalog #7800). The sequence of the murine forward primer, mvccf1, was ATGAAGCTTCTAGCCTCTCCC. Two reverse primers were used, one with a stop codon and one without a stop codon. The sequence for the reverse primer mvccr1, CTATAAGGGCAGCG CAAAGCTTGC, contained a stop codon. Reverse primer mvccr2, TAAGGGCAGCGCAAAGCTTGC, does not contain a stop codon. The PCR conditions for amplification of mouse VCC-1 were the same as described above, except 1 μl of template was used. Cycling parameters were: 4 min at 94 °C for hot start, followed by 30 cycles of 1 min at 94 °C, 1 min at 57 °C, and 3 min at 72 °C, with a final extension of 10 min at 72 °C. Both mouse and human VCC-1 were ligated into pCRII-Topo vector using 2 μl PCR product and 1 μl of vector, according to manufacturer's instructions (Invitrogen, catalog #K4600-40). Potential clones were digested with restriction enzyme *Eco*RI to determine the presence of insert and candidates were sequenced.

Generation of expression constructs. Human and mouse VCC-1, both with and without stop codons, were transferred into pcDNA3.1 (Invitrogen, catalog #V800-20). The VCC-1 constructs cloned into pCRII-Topo were digested with *Eco*RI and the insert was gel purified using Qiagen gel extraction columns (Qiagen, catalog #28704) according to manufacturer's instructions. The vector pcDNA3.1 was also digested with *Eco*RI and dephosphorylated with calf intestinal alkaline phosphatase (CIAP) (Promega, catalog #M182A). Vector and insert were ligated at 20 °C and transformed into competent DH5α cells (Invitrogen, catalog #44-0098). Bacteria were grown overnight at 37 °C on LB plates supplemented with ampicillin (Sigma, catalog #A9393). Clones were then picked and grown overnight at 37 °C in LB with ampicillin. DNA from the clones was isolated using Qiagen DNA MiniPrep kit (Qiagen, catalog #27106) and digested with restriction enzyme *Eco*RI to determine the presence of inserts and *Hind*III to determine orientation.

Northern analysis. The full-length mouse or human VCC-1 clone was used as a template for generating radioactive probe. Labeling was performed with [α-^{32}P]dATP utilizing the PRIME-IT reagents (Stratagene, catalog #300385) and probes purified with NucTrap columns (Stratagene, catalog #400701). Either multiple human RNA samples were then analyzed using a Multiple Tissue Northern Blot (Clontech Laboratories, Inc., catalog #7780-1) or mouse RNA samples analyzed using a Mouse RNA Master Blot filter (Clontech Laboratories, Inc., catalog #7771-1). Hybridization was performed overnight at 68 °C. Filter was washed at 68 °C for 1 h in 2× SSC/0.05% SDS before exposing to film.

Transfection and selection of NIH3T3 cells. NIH3T3 cells were transfected using a CalPhos transfection kit (Clontech Laboratories, Inc., catalog #K2051) following manufacturer's instructions and utilizing 4 μg of either VCC-1 cloned into the expression vector pcDNA3.1 with the myc/HIS tag, or expression vector alone. Cells were maintained in DMEM (Gibco-BRL, catalog #11995-040) supplemented with 10% heat-inactivated FBS (Gibco-BRL, catalog #26140-079) and 1% penicillin/streptomycin (Gibco-BRL, catalog #15140-122) at 37 °C and 5% CO_2. Cells were selected in the same media, supplemented with 800 μg/ml G418 (Gibco-BRL, catalog #11811-031) for 17 days. Clonal cells were then pooled and transfected cells are considered stable.

Tube formation assay. Tissue culture dishes (Falcon, 100 mm × 20 mm) were coated with 4 ml of matrigel basement membrane matrix and allowed to gel by incubation in a 5% CO_2 incubator at 37 °C for 45 min to 1 h for the thick coating method. For the thin coating method, matrigel was diluted to 5 μg/ml with serum-free media and added to the dishes. Dishes were then incubated at room temperature for 1 h and the media aspirated before use. Plates were then overlaid with PY4-1 cells (from Victoria Bautch, University of North Carolina at Chapel Hill) at a concentration of 1.6×10^6 cells per dish in DMEM supplemented with 10% fetal bovine serum (Gibco-BRL). After 6 h, when tube-like structures have formed in the thick-coated plates but not in the thin-coated plates, cells were harvested and RNA generated using RNeasy Miniprep Kit (Qiagen) according to manufacturer's instruction.

In vivo tumor formation assay. Cells were harvested with 0.05% trypsin/EDTA, washed in PBS, and resuspended to 1×10^7 cells/ml in 60% cold matrigel in PBS. Cells were then injected s.c. into the flanks of female CD-1 nu/nu mice at concentration of 1×10^6 cells per injection with 27 gauge needle. The diameter of subcutaneous tumors was measured three times a week using vernier calipers.

Endothelial cell infection. Endothelial cells (HUVECs) were seeded in six-well plates at 2×10^3 cells/well and incubated at 37 °C overnight. A recombinant adenovirus-5 expression construct (prepared under custom contract by Galapagos) containing the VCC1 gene, or a control empty control adenovirus, was added in fresh medium (multiplicity of infection = 30). After 4 h, the adenoviruses were removed and fresh media were added. After 24 h, cells were processed to isolate total RNA using a Qiagen RNeasy kit. One microgram of purified RNA was used to produce cDNA (Reverse Transcription Kit; Applied Biosystems Inc), and the cDNA was analyzed by Taqman technology using custom primer/probe (Applied Biosystems Inc.) sets designed for specificity for each of 23 genes. Gene-specific signals were normalized to that of a reference control cDNA, the housekeeping gene cyclophilin, contained in each sample. Differences in the relative quantity of mRNA between control- and VCC1-infected sample preparations were calculated by the $2^{-\mathrm{ddCt}}$ method [14].

Real time quantitative reverse transcription polymerase chain reaction. Primers were selected using Primer Express software (Perkin-Elmer). RNA samples were isolated and processed with RNeasy Miniprep Kit (Qiagen) and individually transcribed into cDNA using the Taqman Reverse Transcription reagents (Applied Biosystems) as per manufacturer's protocols. The reactions were performed in 100 μl final volume in a PTC-200 Peltier Thermal Cycler (MJ Research) under thermal cycling conditions of 5 min at 25 °C, 30 min at 48 °C, and 5 min at 95 °C. The RT-PCR was then run with 1 μl of the cDNA in an ABI PRISM 7700 Sequence Detection System with primers at 100 nM and enzymes from SYBR-Green Mix (ABI) as per manufacturer's protocol. The cycling conditions were: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Fluorescent signal for the reaction was collected and characterized as cycle thresholds (C_T).

In situ hybridization. The probe for VCC1 consists of the full-length gene cloned into pCRII/Topo, as described above. The plasmid was digested with *Spe*I and labeled with T7 for the sense control probe, and digested with *Eco*RV and labeled with SP6 to generate the antisense probe. Tissue samples were harvested and immediately flash-frozen. Flash-frozen samples were further processed by removing them from a minus 80 °C freezer and adding each sample directly to ice-cold 10% formalin with moderate rocking overnight at 4 °C followed by an additional 4 h at 20 °C. Paraffin tissue blocks were made for each sample by standard paraffin embedding procedures using a Tissue Tek VIP processor. Briefly, samples were dehydrated using a series of ethanol washes 70%, 95%, and 100% at 35 °C followed by two 35 °C xylene washes. Samples were embedded in paraffin using four separate paraffin exchanges at 58 °C followed by paraffin block. Tissue slides were prepared by cutting 5–10 μm thick serial sections and allowed to air-dry overnight at 20 °C. Slides were rehydrated using a standard xylene, ethanol, and water hydration protocol followed by a 4% paraformaldehyde fixation. Fixed tissue sections were washed in PBS Tween 20 (PBT) and digested with proteinase K (20 μg/ml) for 15 min at 37 °C followed by two additional PBT washes and a second fixation step in 4% paraformaldehyde. Acetylation of proteinase K digested and paraformaldehyde fixed tissue sections was done at 20 °C for 15 min followed by two PBT washes and an RNAse free water rinse. Slides were incubated in prehybridization buffer for 1 h at 42 °C. VCC sense and antisense probes were heat denatured at 85 °C for 5 min in hybridization buffer and rapidly cooled on wet ice. After prehybridization all slides were incubated overnight at 42 °C with the appropriate denatured digoxigenin RNA labeled probe. Sense and antisense labeled slides were rinsed separately in 5× SSC (saline sodium citrate buffer) followed by a 30 min incubation in 1× SSC/50% formamide at 20 °C. Formamide was removed by washing all slides in TNE buffer (10 mM Tris, pH 7.6, 500 mM NaCl, and 1 mM EDTA) for 10 min followed by a ×20 min wash in 2× SSC and

2 × 20 min washes in 0.2× SSC. All washes were done at 20 °C. Slides were equilibrated in 1× MABT antibody dilution buffer 2 × 5 min and pre-blocked in 20% sheep serum diluted in 1× MABT for 1.5 h at 20 °C. All slides were rinsed in 1× MABT before the addition of donkey anti-digoxigenin alkaline phosphatase coupled detection antibody diluted 1:2000 in 2% sheep serum/1× MABT overnight at 4 °C. Slides were rinsed in 1× MABT to eliminate excess antibody with 4 × 5 min washes at 20 °C on a plateform shaker. Two additional 10 min washes were done in NTMT (100 mM NaCl, 100 mM Tris–HCl, pH 9.5, 50 mM $MgCl_2$, and 0.1% Tween 20 containing 2 mM levamisole). NBT and BCIP substrate was diluted 1:250 in NTMT and incubated at 20 °C. Slides are developed during the course of the day as substrate conversion is considered optimal. Once developed, all slides were rinsed in NTMT followed by 1 × 5 min wash in PBS. They are then fixed in 4% paraformaldehyde for 30 min at 20 °C and washed once more in PBS for 5 min. Samples were then mounted, air dried, and sealed.

Results

In order to investigate which genes appear to be co-regulated in the human genome, Pearson correlation coefficients were determined utilizing a large number of transcriptional microarray experiments from various normal tissues, cancer tumors, time-course drug treatments, diseased tissues, and cell lines cultured under a wide variety of conditions (Table 1). Due to its central role in angiogenesis, particular attention was paid to the expression of VEGF. A Pearson correlation coefficient was calculated between VEGF and all other genes within each experiment. Genes which had a significant correlation coefficient, defined by a *p*-value less than 0.001 in multiple experiments, were considered to be members of the local network. VEGF was seen co-regulated with three genes, two genes known to be involved in the angiogenesis pathway (IL-8 and GRO1) and one novel gene (VCC-1). The expression of these genes (IL-8, GRO1, and VEGF) was in turn linked to a number of other genes that tie the angiogenesis pathway with the inflammation, apoptosis, and cell cycle pathways.

The linkage of expression of the VCC-1 ESTs with three genes, known to play a role in the process of angiogenesis, led us to determine the complete human cDNA sequence (GenBank Accession No. AY598463). The sequence was determined from an assembly of all private and public EST sequences and an analysis of the human genomic Celera sequence data. This human cDNA sequence was then used as a template to determine the mouse cDNA sequence from mouse genomic and EST data (GenBank Accession No. AY598464). The accuracy of these predictions was then verified by cloning of the VCC-1 cDNA by PCR from the human HT29 colon cancer cell line, human lung tissue, and mouse embryonic RNA. Amplified product was then ligated into the pCRII-Topo vector and bacterial clones were isolated. The clones were then sequenced, confirming the predicted nucleic acid sequences. An examination of the Celera genomic sequence data and a comparison with our determined cDNA sequence information indicated that VCC-1 is comprised of four exons spanning about 15 Kb located in human 19q11.

An analysis of the human and mouse VCC-1 cDNA sequences shows that both code for protein of 119 amino acids. Consequently, the predicted precursor proteins of the human and mouse gene are similar in size, 13,820 and 13,628 Da, respectively. An analysis of the proteins by the SignalP program indicates with high probability (>93%) that the protein in both species contains a cleaved signal peptide sequence of 22 amino acids resulting in a predicted mature human protein of 11,418 Da and a predicted mature mouse protein of 11,164 Da. A comparison of the precursor human and mouse protein sequences shows that they are 72.3% identical and 85.7% similar when conservative amino acid replacements are considered (Fig. 1A). A closer examination of the human sequence reveals that it contains six cysteine residues, with four of them occurring in two CXC motifs, which is characteristic of proteins of the chemokine family. The importance of these cysteine residues is further underscored by the fact that they are absolutely conserved in the mouse sequence.

A linear hidden Markov model (HMM) was constructed for all sequence families present in the Swissprot database using the sequence alignment and modeling (SAM) software system. Families were defined by sets of sequences that demonstrate at least 35% sequence identity. A comparison of VCC-1 with these families identified a homologous two-member group (Fig. 1B). An alignment of VCC-1 with this family shows that it is 19.4% identical and 29.8% similar to the known chemokine SCYA17 (NM_002987) and 10.9% identical and 24.0% similar to the known chemokine SCYA16 (NM_004590). A comparison of these chemokines to one another shows they are 25% identical and

Table 1
Correlation of VEGF expression with VCC-1, GRO1 or IL-8

Gene 1	Gene 2	Source	Correlation coefficient	*p*-Value
VEGF	VCC-1	Lung tumors	0.64	8.30E-05
VEGF	VCC-1	Breast tumors	0.56	1.90E-03
VEGF	VCC-1	Esophageal tumors	0.74	8.80E-07
VEGF	VCC-1	Endothelial cell lines	0.93	5.40E-08
VEGF	GRO1	Lung tumors	0.77	2.80E-06
VEGF	GRO1	Esophageal tumors	0.83	5.20E-08
VEGF	GRO1	Endothelial cell lines	0.9	1.80E-07
VEGF	IL-8	Lung tumors	0.63	9.00E-05
VEGF	IL-8	Esophageal tumors	0.64	1.60E-05

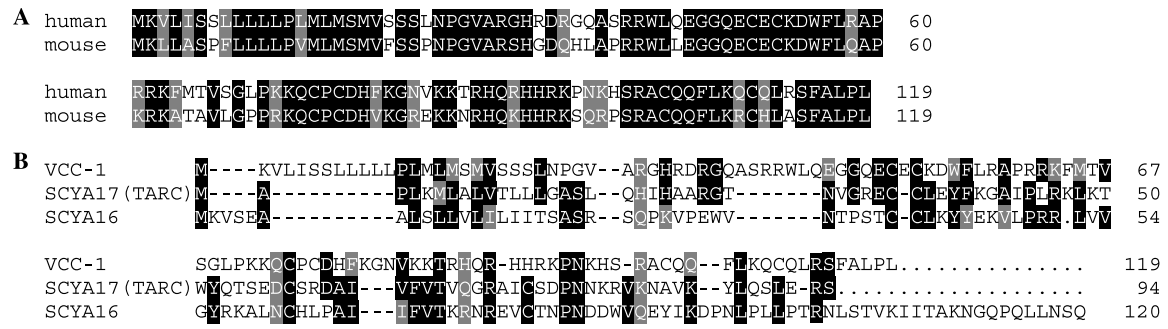

Fig. 1. Conservation and homology of VCC-1. Comparison of human and mouse VCC-1 proteins (A) with identical regions in both proteins represented by black boxes and homologous regions shown as gray boxes. The amino acid number is indicated to the right of each sequence. (B) A multiple alignment based on the HMM using the TZ algorithm of the novel gene VCC-1 and the two known chemokines SCYA17 and SCYA16. Black boxes represent identical regions while gray boxes represent similar regions found in at least two of the sequences. Gaps are introduced into the sequences to maximize aligned regions and are indicated by a dash.

33.1% similar and thus slightly more related to each other than to VCC-1.

In order to determine the normal endogenous expression profile of VCC-1 in mouse and human tissue, Northern blots were hybridized with radiolabeled VCC-1 probes. These probes consisted of the full-length mouse and human open-reading frames. The Northern blot of human tissue RNA shows a single band at approximately 1 kb in samples from lung and skeletal muscle. No signal was seen in RNA from brain, heart, colon, thymus, spleen, kidney, liver, small intestine, placenta, or peripheral blood lymphocytes (data not shown). A blot of mouse tissue RNA yielded signal in samples from the lung, thyroid, submaxillary gland, epididymis, and uterus with weaker signal in the ovary and prostate (data not shown).

Changes in the expression levels of VCC-1 during tumorigenesis were assessed by microarray analysis on the Incyte HG chip and indicated that it was significantly up-regulated in various breast and colon tumors (data not shown). The microarray observations were then further validated by quantitative real time reverse transcription polymerase chain reaction (RT-PCR). One of the four colon tumors showed significant over-expression of human VCC-1, compared to the levels seen in a pool of normal colon tissue (Fig. 2A). Five out of seven breast tumors also showed significant up-regulation of human VCC-1 RNA, compared to a pool of normal breast tissue. Levels of over-expression in breast tumors ranged from 3-fold to more than 24-fold (Fig. 2B).

In order to gain a better understanding of the VCC-1 expression in these breast carcinomas, probes were generated for *in situ* hybridization. The antisense VCC-1 probe yielded strong signal in both normal mammary gland ductal epithelia (Fig. 2C–E) and transformed mammary gland epithelial cells (Fig. 2F–H). The probe proved to be highly specific for the epithelial cells in this tissue.

RT-PCR was also performed on an array of endothelial and epithelial cell lines with the objective of determining the cell type that expresses human VCC-1. When compared to a pool of non-transformed epithelial cells, human microvascular endothelial cells (HMVEC) grown on fibronectin displayed almost 100-fold over-expression of VCC-1 (Fig. 3A). Sephranose vascular endothelial cells (SPVEC) and human umbilical vascular endothelial cells (HUVEC) also displayed significant, though more modest, over-expression of the gene. RT-PCR was also utilized to determine if VCC-1 levels differ in endothelial cells undergoing proliferation or tube-formation. RNA was harvested from a mouse angioma endothelial cell line, PY4.1, under various culture conditions. No difference in murine VCC-1 was seen between quiescent versus actively proliferating cells. However, VCC-1 appears over-expressed by 28-fold in RNA from these cells 6 h after they have been induced to form tubes in a two-dimensional assay (Fig. 3B).

In order to determine the effect VCC-1 may have on transcriptional levels of known angiogenesis related genes, adenovirus with the full-length gene was used for infection. HUVEC were infected with either an empty control adenovirus or an adenovirus containing VCC-1, and given 24 h in culture. RT-PCR was then performed on the RNA from these cells in order to examine the transcript levels of 23 genes known to be involved in angiogenesis. Significant changes of gene expression after infection with VCC-1 can be seen in the case of VEGF-A, basic FGF, ang-2, uPA, and uPAR (Fig. 3C).

To assess the potential of VCC-1 to induce tumor formation, the gene was shuttled into a CMV promoter-driven expression construct and transfected into NIH3T3 cells. The level of VCC-1 expression in untransfected and empty vector-transfected NIH3T3 cells was shown to be barely detectable while transgene expression of VCC-1 in stable pools of NIH3T3 cells was increased about 800-fold on average (data not shown). A pool of vector alone-transfected and a representative pool of VCC-1-transfected NIH3T3 cells were then injected subcutaneous into each flank of female CD-1 nu/nu mice which were evaluated over time for tumor growth. The NIH3T3 cells that over-expressed the VCC-1 gene showed significant enhancement of growth and indicate that VCC-1 over-expression can cause tumor formation in fibroblasts "in vivo" (Fig. 4).

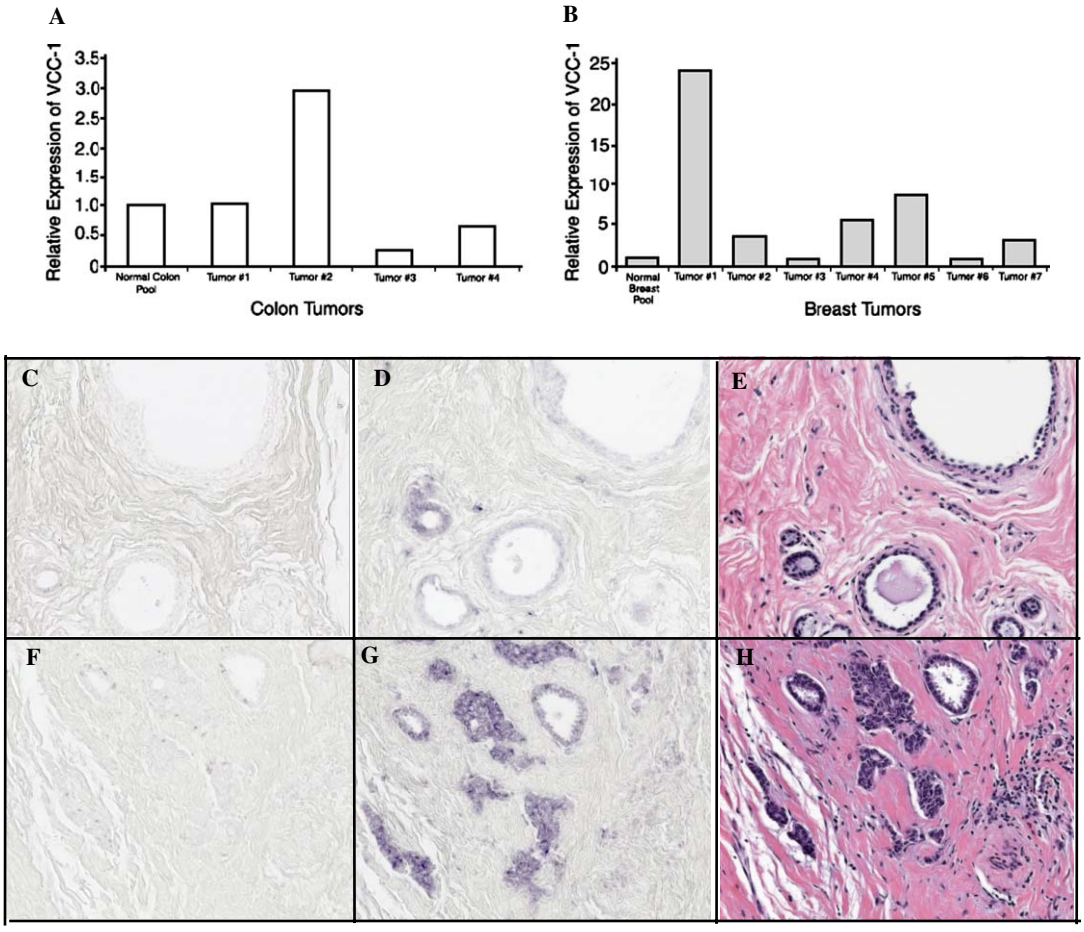

Fig. 2. Expression of VCC-1 by quantitative real time reverse transcription polymerase chain reaction and *in situ* hybridization. Expression of VCC-1 was measured and normalized to expression of the housekeeping gene GUS. The level of VCC-1 expression for the normal tissue pool was then designated as 1. Relative levels of expression of VCC-1 in (A) colon tumor samples and (B) breast tumor samples are shown. *In situ* hybridization using VCC1 sense control probe showed no staining on normal mammary tissue (C), or in the breast carcinoma (F), while the antisense VCC1 probe yield light staining in the ductal epithelial cells of the normal mammary gland (D) and in almost all of the tumor cells from the mammary carcinoma (G). Serial sections of both the normal and carcinoma tissue were stained with hematoxylin and eosin (H and E).

Discussion

Angiogenesis is the process of growth and proliferation of new blood vessels and is vital to a number of processes ranging from wound repair to embryonic development. Angiogenesis is also a fundamental event in the progression from quiescent cells to the uncontrolled neoplastic growth and metastasis of tumor cells. This process is the result of a complicated regulatory network of both positive and negative influences which appear to be controlled by many opposing soluble factors or cytokines. VCC-1 appears to belong to the chemokine subfamily of cytokines due to the presence of the conserved Cys-X-Cys motif. Known cytokines which have been shown to promote the survival and proliferation of endothelial cells include basic fibroblast growth factor (bFGF), growth related oncogene peptide (GRO-1), interleukin 8 (IL-8), platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF).

Our metaanalysis of transcriptional microarray data from lung, breast, and esophageal patient tumor samples and from endothelial cell lines using a genetic regulatory network model has proven useful in the identification of several novel genes which appear to be involved in the mechanism or pathway of angiogenesis (data not shown). One gene which is described here, VCC-1, encodes a novel chemokine which is co-regulated with three well-known genes, IL-8, GRO-1, and VEGF, involved in angiogenesis and which are linked by co-regulation of the inflammation, apoptosis, and cell cycle pathways. Furthermore, these results that suggest that these seemingly disparate pathways are interconnected via these chemokine modulators.

Through the use of HMMs formed from all known proteins, the VCC-1 protein was shown to be strikingly related to two known chemokines, SCYA17 and SCYA16. A multiple sequence alignment generated from this analysis shows alignment of three of the six VCC-1 cysteines with three of the four cysteines in the other two chemokines and overall shows 24–30% similarity between the three proteins. The SCYA17 chemokine shows chemotactic activity towards T cells but not monocytes and binds to the chemo-

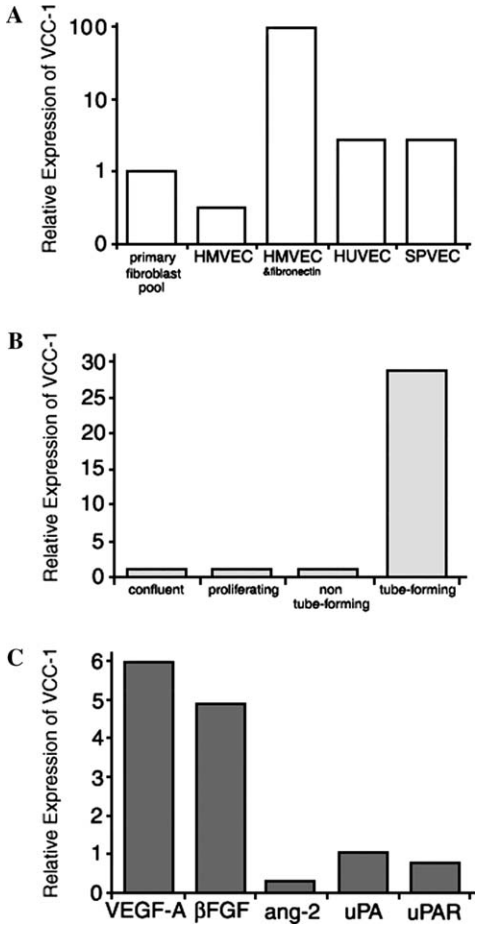

Fig. 3. Quantitative PCR of VCC-1 expression in quiescent and proliferating cells. (A) Quantitative real time reverse transcription polymerase chain reaction performed on human derived endothelial cells in culture. Expression of VCC-1 was measured in all samples and normalized to expression of the housekeeping gene cyclophilin. The value for the level of VCC-1 expression for primary fibroblast pool (consisting of RNA isolated from human synovial fibroblasts, normal human dermal fibroblasts, and human foreskin fibroblasts) was designated as 1. (B) Quantitative real time reverse transcription polymerase chain reaction performed on PY4.1 cells under various conditions using murine VCC-1 primers. Expression of VCC-1 was measured in all samples and normalized to expression of the housekeeping gene cyclophilin. The value for the level of VCC-1 expression in the quiescent cells was designated as 1, and the value for the level of VCC-1 expression in proliferating cells was then compared to that of the quiescent cells. Similarly, the value for the level of VCC-1 expression in the non-tube forming cells was designated as 1 for comparison to the level of VCC-1 in the tube forming cells. (C) Effect of VCC1 over-expression in endothelial cells on mRNA levels of other genes. HUVEC were infected with adenovirus containing the human VCC1 gene or control adenovirus lacking any cDNA insertion. After 24 h of continued culture, RNA was prepared and mRNA levels were compared by quantitative real time reverse transcription polymerase chain reaction. The housekeeping gene cyclophilin was used to normalize differences in tested RNA quantities. Dotted line indicates the relative value obtained for each gene using cells infected with the control. Values shown represent means of two independent assays ± STD. The five genes shown here represent those with the greatest differential expression among 24 tested.

kine receptors CC4 and CC8. Thus, it appears that this chemokine is important in T cell development and perhaps in the activation of mature T cells. The function of the SCYA16, on the other hand, appears to have suppressive

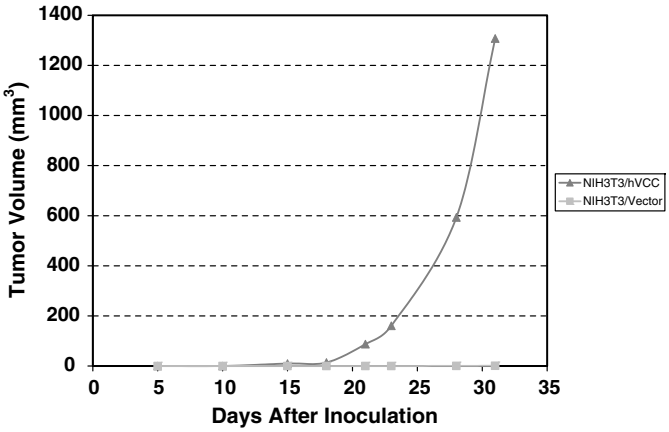

Fig. 4. NIH3T3 cell transformation in nu/nu female mice. NIH3T3 cells, transfected with vector alone or with vector containing the VCC-1 gene under control of CMV promoter, were evaluated for tumor formation over 31 days. Transfected cells were subcutaneously injected into both sides of 5 vector control and 15 vector containing VCC-1 mice. Tumor mass was then measured at each time point by calipers on each flank of the five control mice ($n = 10$) and the 15 VCC-1 transfected ($n = 30$) mice.

activity and inhibit proliferation of myeloid progenitor cells. It is unclear how the angiogenic activity of VCC-1 relates to the activities of the two previously described chemokines, if at all.

Understanding the process by which microvascular endothelial cells are recruited by cancer tissues to support angiogenesis is key to the development of therapeutic agents which target this crucial survival mechanism that is absolutely required by tumor cells. In particular, one advantage of targeting endothelial cells is that they are readily accessible via the bloodstream and they do not develop drug resistance [15,16]. The research community has been keen to recognize the potential advantages of anti-angiogenic therapy and have led to the development of over 200 anticancer agents that are in various stages of clinical investigation [17]. It is clear that if this angiogenic activity could be repressed or abrogated with a therapeutic strategy then the metastatic activity of tumor could be eliminated and might lead to the cessation of tumor growth or even death by an apoptotic mechanism. We assert that VCC-1 is one likely target for an anti-angiogenic agent and future studies should prove the success or failure of such a strategy. This assertion is based on our results, showing VCC-1 over-expression has a profound effect on expression levels of known angiogenic factors such as VEGF, bFGF, and ang-2 among others. VCC-1 expression regulation also appears limited to endothelial tube formation *in vitro*, a common model system for the study of angiogenesis. Last, over-expression of this novel chemokine in NIH3T3 cells implanted in nude mice induces tumor formation.

Finally, since VCC-1 is a secreted molecule it should readily appear in the blood and urine, and may also be useful as a surrogate marker which could easily be monitored to assess the efficacy of various anti-cancer therapies.

Subsequent validation of it as an indirect marker for various cytotoxic and anti-angiogenic therapies should indicate its usefulness as a prognostic indicator of clinical response.

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