

Extra Cellular Matrix Derived Metabolite Regulates Angiogenesis by FasL Mediated Apoptosis

Raj K. Verma¹, Venugopal Gunda², Smita C. Pawar³, Yakkanti Akul Sudhakar^{4,5*}

1 Irma Lerma Rangel College of Pharmacy, Texas A&M Health Science Center, Kingsville, Texas, United States of America, **2** The Eppley Institute for Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska, United States of America, **3** Department of Genetics, Osmania University, Hyderabad, Andhra Pradesh, India, **4** Cell Signaling Laboratory, Center for Cancer and Metabolism, Bioscience Division, SRI International, Menlo Park, California, United States of America, **5** Cell Signaling and Tumor Angiogenesis Laboratory, Department of Genetics, Boys Town National Research Hospital, Omaha, Nebraska, United States of America

Abstract

Object: Antiangiogenic treatments are beginning to give promising outcomes in many vascular diseases including tumor angiogenesis. In this current study the antiangiogenic and pro-apoptotic actions of $\alpha 1(\text{IV})\text{NC1}$ and its N- and C- peptides $\alpha 1\text{S1}(\text{IV})\text{NC1}$, $\alpha 1\text{S2}(\text{IV})\text{NC1}$ were investigated *in-vitro* and *in-vivo*.

Study Method: Endothelial cells (ECs) were treated with $\alpha 1(\text{IV})\text{NC1}$, $\alpha 1\text{S1}(\text{IV})\text{NC1}$, $\alpha 1\text{S2}(\text{IV})\text{NC1}$ and *in-vitro* proliferation, migration, tube formation and apoptotic assays were executed. FasL, Fas, Caspase-8, -3 and PARP activations were studied using immunoblotting analysis using specific antibodies. Also the *in-vivo* antiangiogenic and pro-apoptotic effects were tested using $\alpha 1(\text{IV})\text{NC1}$ in a mice model.

Results: Like $\alpha 1(\text{IV})\text{NC1}$, its N- and C- terminal $\alpha 1\text{S2}(\text{IV})\text{NC1}$ and $\alpha 1\text{S1}(\text{IV})\text{NC1}$ domains possess anti-proliferative, pro-apoptotic activity and inhibit ECs migration and tube formation *in-vitro*. Both $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains promote apoptosis by activating FasL and downstream apoptotic events including activation of caspase-8, -3 and PARP cleavage in a dose dependent manner *in-vitro* in ECs. Tumors in mice showed apoptotic TUNEL positive microvasculature upon $\alpha 1(\text{IV})\text{NC1}$ treatment, indicating inhibition of tumor angiogenesis and tumor growth. Further, the antitumor activity of $\alpha 1(\text{IV})\text{NC1}$ was abrogated when caspase-3 inhibitor was used. These results conform additional properties of $\alpha 1(\text{IV})\text{NC1}$ as an endogenous angioinhibitor that induces apoptosis *in-vitro* and *in-vivo* by activating FasL mediated caspase-3.

Significance: $\alpha 1(\text{IV})\text{NC1}$ and its N- and C- terminal $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains also possess pro-apoptotic and angioinhibitory activity *in-vitro* and *in-vivo*. $\alpha 1(\text{IV})\text{NC1}$ regulates tumor angiogenesis by activating FasL mediated apoptosis *in-vitro* and *in-vivo*. These results demonstrate that $\alpha 1(\text{IV})\text{NC1}$ and its peptides inhibit neo-vascular diseases.

Citation: Verma RK, Gunda V, Pawar SC, Sudhakar YA (2013) Extra Cellular Matrix Derived Metabolite Regulates Angiogenesis by FasL Mediated Apoptosis. PLOS ONE 8(12): e80555. doi:10.1371/journal.pone.0080555

Editor: Sujit Basu, Ohio State University, United States of America

Received: September 11, 2013; **Accepted:** October 14, 2013; **Published:** December 4, 2013

Copyright: © 2013 Verma et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: sudhakar.yakkanti@sri.com

Introduction

Angiogenesis, the formation of new blood vessels from preexisting blood vessels, is a very stringently controlled program and normally does not occur, except during development and wound repair processes [1,2]. This stringent regulation of angiogenesis is manifested by a balance between pro- and anti-angiogenic factors, which keep angiogenesis in check [2]. However, the dynamic equilibrium between pro-angiogenic and anti-angiogenic factors are controlled under many pathological settings, including tumor angiogenesis in cancer progression and other incidents like as age-related macular degeneration, retinopathy of prematurity and diabetic retinopathy resulting in the growth of abnormal new blood vessels [3–5].

Vascular basement membranes (VBM) constitute an important component of blood vessels [6]. Makeover of VBM can provide vital pro- and anti-angiogenic molecules to control formation of new blood vessels [7–9]. Type IV collagen is a major component of VBM and plays a critical role in new blood vessel development

[6]. Proteolytic degradation of type IV collagen in the VBM generates numerous antiangiogenic molecules [7,10–12]. One such antiangiogenic molecule derived from type IV collagen non-collagenous (NC1) domain $\alpha 1$ chain, $\alpha 1(\text{IV})\text{NC1}$, has been tested in variety of tumor angiogenesis studies in mice [13–15]. However, the molecular and cellular mechanism(s) responsible for inhibition of angiogenesis is not yet clearly understood. The *in-vitro* and *in-vivo* studies have demonstrated that $\alpha 1(\text{IV})\text{NC1}$ can directly affect endothelial cell migration and impact their proliferation and sprouting [14]. Earlier we have demonstrated that $\alpha 1(\text{IV})\text{NC1}$ promotes apoptosis via activation of caspase-3 and PARP cleavage by inhibiting FAK/p38-MAPK/Bcl-2 and Bcl-x_L signaling cascade [15]. These results provide a clear understanding about the apoptotic signaling and therapeutic potential of $\alpha 1(\text{IV})\text{NC1}$ molecule in neovascular diseases. However, the effects of $\alpha 1(\text{IV})\text{NC1}$ and its N- and C-terminal domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ on endothelial cell apoptosis and neo-vascularization have not been previously studied.

In the present study, we demonstrate that $\alpha 1(\text{IV})\text{NC1}$ and its N- and C-terminal domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ are potent inhibitors of endothelial cell proliferation, migration and tube formation *in-vitro* and tumor angiogenesis *in-vivo*. $\alpha 1(\text{IV})\text{NC1}$ promotes apoptosis via activation of caspase-3 and PARP cleavage, presumably by inhibiting FAK/p38-MAPK/Bcl-2 and Bcl-x_L signaling cascade [15]. Here in this study, we show that N- and C-terminal domains of $\alpha 1(\text{IV})\text{NC1}$ cross talk with FasL and activate FasL and its downstream apoptotic machinery including caspase-8, caspase-3 and PARP cleavage *in-vitro*. Furthermore, we identified that $\alpha 1(\text{IV})\text{NC1}$ promotes apoptosis in tumor vasculature and inhibits angiogenesis and this effect was reversed by a caspase-3 specific inhibitor DEVD *in-vivo*. These findings contribute significantly towards understanding the apoptotic activation in proliferating ECs and therapeutic potential of endogenous angioinhibitor $\alpha 1(\text{IV})\text{NC1}$ and its N- and C-terminal $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains in tumor growth and tumor angiogenesis.

Materials and Methods

Fetal calf serum (FCS), Endothelial basal medium (EBM-2) and Endothelial cell growth medium (EGM-2) were obtained from Fischer Scientific Inc. Penicillin and streptomycin and low melting agarose were purchased from Sigma-Aldrich and cell stains hematoxylin and eosin (H&E) were purchased from Fischer Scientific Inc. Sephadex™-G 100, -G 25 and -G 200 were purchased from GE Healthcare Bio-Sciences AB. BD Matrigel™ Matrix (14.6 mg/ml) was purchased from BD Biosciences Discovery Laboratory. T₄-DNA ligase (bacteriophage ligase), different restriction enzymes and polymerases were purchased from New England Biolabs. SCC-PSA1 tumor cells were purchased from ATCC. Caspase inhibitors z-DEVD-fmk was from Enzyme System Products. H&E staining kit from Fisher Scientific Inc. CD31 antibody was purchased from Upstate. Caspase-3, -8, PARP, FasL and Fas antibodies were purchased from Cell Signaling. MTT assay kit was purchased from Chemicon.

Ethics Statement. Boys Town National Research Hospital IACUC committee approved all mice studies.

Tissue culture procedure

SCC-PSA1 cells were maintained in 10% FCS with penicillin/streptomycin (100units/ml each) and maintained at 37°C incubator with 5% CO₂. Primary mouse choroidal endothelial cells (EC) were maintained as describe previously [16,17]. EC were maintained in 40% HAM's F-12, 40% DME-Low Glucose, 20% FCS supplemented with heparin (50 mg/L), endothelial mitogen (50 mg/L), L-glutamine (2.0 mM), penicillin/streptomycin (100units/ml each), Na Pyruvate (2.5 mM), NEAA (1X), 5.0 µg/L of murine INF-γ and cultured on 0.8% gelatin coated plates at 33°C with 5% CO₂.

Cloning, Expression and Purification of $\alpha 1(\text{IV})\text{NC1}$ and its N- and C- terminal domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$

The coding sequence corresponding to the N- and C-terminal non-collagenous domains (NC1) from human Collagen type IV $\alpha 1$ chain was isolated from the placental cDNA using one-step reverse transcriptase-PCR (RT-PCR) (Invitrogen, CA). The N- terminal subunit 330-bp from full length $\alpha 1(\text{IV})\text{NC1}$ was amplified using the forward primer: ATACATATGGGCTTCCTTGTGAC-CAGGCATA and the reverse primer: CACAAGCT-TAGGCGCCTCACACACAGCAC and cloned between 'NdeI'

and 'XhoI' sites of pET22b. The C- terminal subunit 330-bp from full length $\alpha 1(\text{IV})\text{NC1}$ was amplified using the forward primers: GTGCATATGGTGCACAGCCAGCCACAT and the reverse primer: GTGGCAGCAGCCAACTCA and cloned between 'NdeI' and 'XhoI' sites of pET22b. Amplification and cloning was carried out similarly as reported in our earlier publication [18]. The positive clone was used to transform *E. coli* strain BL21 for protein expression and purification was performed similarly as reported earlier [18].

Purification of $\alpha 1(\text{IV})\text{NC1}$ and $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains

Inclusion bodies of $\alpha 1(\text{IV})\text{NC1}$, $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ were prepared with minor modifications as reported [18]. In addition to the renaturation by stirring method, on-column renaturation was performed for simultaneous renaturation and purification of the $\alpha 1(\text{IV})\text{NC1}$, $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains. Denatured $\alpha 1(\text{IV})\text{NC1}$, $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ protein in 800 µl aliquots was loaded onto the Sephadex G-100, Superdex-200 followed by Sephadex G-25 columns similarly as reported [18]. The fractions containing $\alpha 1(\text{IV})\text{NC1}$, $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ were pooled and further concentrated by lyophilization as reported. Endotoxin levels in the final purified $\alpha 1(\text{IV})\text{NC1}$, $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains samples were estimated using the Limulus Amebocyte Lysate (LAL) QCL-1000 assay kit (Lonza) according to the manufacturer instructions and also similarly reported in earlier publication [18].

Proliferation assay

A suspension of 7000-cells/well mouse choroidal endothelial cells (ECs) in a 96 well plate was used in proliferation assay. Cells were grown in 96 well plate under 0.5% FBS supplemented with heparin, endothelial mitogen, glutamine and penicillin/streptomycin. After 24-hrs, medium was replaced with ECs medium containing 10% FCS and different concentrations of $\alpha 1(\text{IV})\text{NC1}$ or its N- and C-terminal $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains (0.25 and 2.0 µM) and after 48-hrs relative levels of methylene blue incorporation was measured as reported [14,17].

Migration assay

About 1.0×10^4 cells/well of ECs were seeded in serum free medium with and without recombinant $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ (1.0 µM). Medium containing 10 ng/ml of VEGF was placed into the bottom wells of the Boyden chamber and incubated for about 48-hrs at 37°C with 5% CO₂. The numbers of ECs that were migrated and attached to the bottom side of the Boyden chamber membrane were counted as reported earlier [14,19].

Tube formation assay

Briefly, Matrigel matrix about 250 µl was thawed overnight on ice-cold room and added to each well of a 24-well plate and allowed to solidify for 30-min at 37°C culture incubator. A suspension of about 50×10^3 ECs in medium without antibiotic was plated on top of the Matrigel matrix. The ECs were then incubated with and without $\alpha 1(\text{IV})\text{NC1}$ or its N- and C-terminal $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains (1.0 µM) for 48-hrs at 37°C and viewed using a CK2 Olympus microscope [14,20,21].

Cell viability assay

Endothelial cell viability was assessed by MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide] following

the manufacturer's protocol instructions. About 7.0×10^3 EC cells/well were plated on a 96-well plate, over night serum starved and stimulated with 10% FCS containing medium. After 24-hrs different concentration of $\alpha 1(\text{IV})\text{NC1}$ or its N- and C-terminal $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains (0.25–2.0 μM) were added and incubated for 48-hrs. Apoptosis was monitored by trypan blue exclusion using the cell death detection ELISA kit [22].

FasL and caspase activation assay

About 1.0×10^6 serum starved EC were collected and suspended in serum free medium. These ECs were pretreated with $\alpha 1(\text{IV})\text{NC1}$ N- and C-terminal $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains (1.0 μM) and incubated on 10-cm² dishes for 6 and 18-hrs. After 6 and 18-hrs, the floating ECs were collected, adherent cells washed once with cold PBS. Adhering and floating cells were lysed on ice in RIPA lysis buffer and centrifuged at 4°C for 30-min at 13000-rpm. About 30 μg of cytosolic extract/lane was separated using 10% SDS-PAGE followed by immunoblotting with anti-FasL, Fas, caspase-8, caspase-3 and PARP antibodies. Immunoreactivity of FasL, Fas, cleaved caspase-8, caspase-3 and PARP proteins were visualized using ECL detection kit as reported [15].

In-vivo tumor studies

About 1.0×10^6 SCC-PSA1 teratocarcinoma tumor cells were implanted subcutaneously into each mouse at dorso-lateral sides. Six male 129/Sv mice were used in each treatment and control groups. Once the tumors reached around 100-mm³ sizes, 30 μg of $\alpha 1(\text{IV})\text{NC1}$ or in combination with 10 μg of caspase-3 specific inhibitor DEVD in 100 μl total volume was intravenously injected to each mouse daily for 15-days. All the experimental mice were scarified and tumors and other organs were collected [21,31].

Immunohistochemical staining

Tumors from control and $\alpha 1(\text{IV})\text{NC1}$ or in combination with DEVD treated mice were embedded in OTC compound and snap-frozen in liquid nitrogen. Tumor sections were incubated for 2-hrs with 5% BSA in PBS to block any nonspecific binding and then incubated with anti-CD-31 antibody for 1-hr followed by incubation with peroxidase-labeled goat anti-rabbit secondary antibody at 37°C. The number of CD-31 positive blood vessels per 10-microscopic fields from four tumor sections per condition was counted [14,23].

TUNEL assay for apoptosis

To evaluate the relative levels of CD-31 positive vascular or cellular apoptosis, and to quantify cellular apoptosis in tumors from control and $\alpha 1(\text{IV})\text{NC1}$ or in combination with DEVD treated mice with and without $\alpha 1(\text{IV})\text{NC1}$ treatment, we used ApopTag apoptosis detection kit. Briefly the tumor sections were incubated with 5% BSA in PBS to block any nonspecific binding and stained with ApopTag apoptosis detection reagents following the manufacturer's instructions. To determine whether CD-31 positive blood vessels were undergoing apoptosis, control and $\alpha 1(\text{IV})\text{NC1}$ or in combination with DEVD treated tumor sections were co-incubated with both ApopTag reagents and anti-CD-31 antibody. Tumor sections were washed twice and incubated with rhodamine-conjugated goat anti-rabbit secondary antibody for 1-hr at 37°C. The fluorescence staining was analyzed using a fluorescence microscope [14,23].

Results

Distinct anti-angiogenic activities of $\alpha 1(\text{IV})\text{NC1}$ and its N- and C- terminal domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$

$\alpha 1(\text{IV})\text{NC1}$ is an endogenous metabolite generated from the non-collagenous (NC1) domain of $\alpha 1$ chain of type IV collagen by matrix metalloproteinases-9 (MMP-9) [24,25]. It was discovered as an angioinhibitory protein with substantial anti-tumor activities [13,14]. The present study was aimed at understanding the molecular mechanism(s) underlying angioinhibition by $\alpha 1(\text{IV})\text{NC1}$ and its N- and C- terminal $\alpha 1\text{S1}(\text{IV})\text{NC1}$, $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains and its implications in prevention of tumor angiogenesis. Our studies tried to identify the angioinhibitory and pro-apoptotic activities of $\alpha 1(\text{IV})\text{NC1}$ and its N- and C- terminal $\alpha 1\text{S1}(\text{IV})\text{NC1}$, $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains in mouse choroidal endothelial cells (ECs).

We have performed different angiogenesis experiments to determine the angioinhibitory potential of $\alpha 1(\text{IV})\text{NC1}$ and its N- and C-terminal domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ using ECs. Many serum proteins are major pro-angiopromoting factors, elevated levels or imbalance of serum proteins (example VEGF and bFGF) are responsible for the majority of ocular angiogenesis driven by ischemia and also tumor angiogenesis. We first determined serum stimulated angioinhibitory activity of $\alpha 1(\text{IV})\text{NC1}$ and its N- and C-terminal domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ by measuring ECs proliferation. The anti-proliferative effect of $\alpha 1(\text{IV})\text{NC1}$ and its N- and C-terminal domains were tested in ECs using methylene blue incorporation. Fetal calf serum (10% FCS) stimulated proliferation of ECs were significantly inhibited by $\alpha 1(\text{IV})\text{NC1}$ its N- and C-terminal $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains in a dose dependent manner (**Figure 1A**). Interestingly, both N- and C-terminal $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains were showing similar proliferation inhibitory activity when compared to its parent molecule $\alpha 1(\text{IV})\text{NC1}$.

Migration of endothelial cells is basically essential during neovascularization [14,26]. Migration of ECs across a PVD membrane towards VEGF in a Boyden chamber was inhibited by 1.0 μM N- and C-terminal $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains (**Figure 1B**). We additionally confirmed the angioinhibitory action of $\alpha 1(\text{IV})\text{NC1}$ and its two domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ by a functional assay of ECs tube formation [20]. Tube formation on Matrigel is associated with ECs proliferation, migration and survival [1]. Treatment of ECs with $\alpha 1(\text{IV})\text{NC1}$ and its domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ inhibited tube formation equally on Matrigel matrix (**Figure 1C**). Our previous study reported that the angioinhibitory activity of $\alpha 1(\text{IV})\text{NC1}$ is mediated through $\alpha 1\beta 1$ integrin [14,21]. Surprisingly, in this study we noticed that ECs when treated with N- and C-terminal domains and full length $\alpha 1(\text{IV})\text{NC1}$ (1.0 μM) were appeared rounded (apoptosis-like) and some of the cells detach from the Matrigel matrix (**data not shown**). This might be due to activation caspases and apoptosis in ECs treated with $\alpha 1(\text{IV})\text{NC1}$ or its N- and C- terminal domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ similarly as reported earlier [15].

Further we also tested the angioinhibitory activity at different doses of $\alpha 1(\text{IV})\text{NC1}$ and its N- and C- terminal domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ (0, 0.25, 0.5, 1.0, 1.5, 2.0 μM) by MTT cell viability assay after 10% FCS stimulation in ECs. The results reveal that ECs proliferation was significantly increased by FCS stimulation which was inhibited by $\alpha 1(\text{IV})\text{NC1}$ and its N- and C-terminal domains dose dependently after 24-hrs (**Figure 2**).

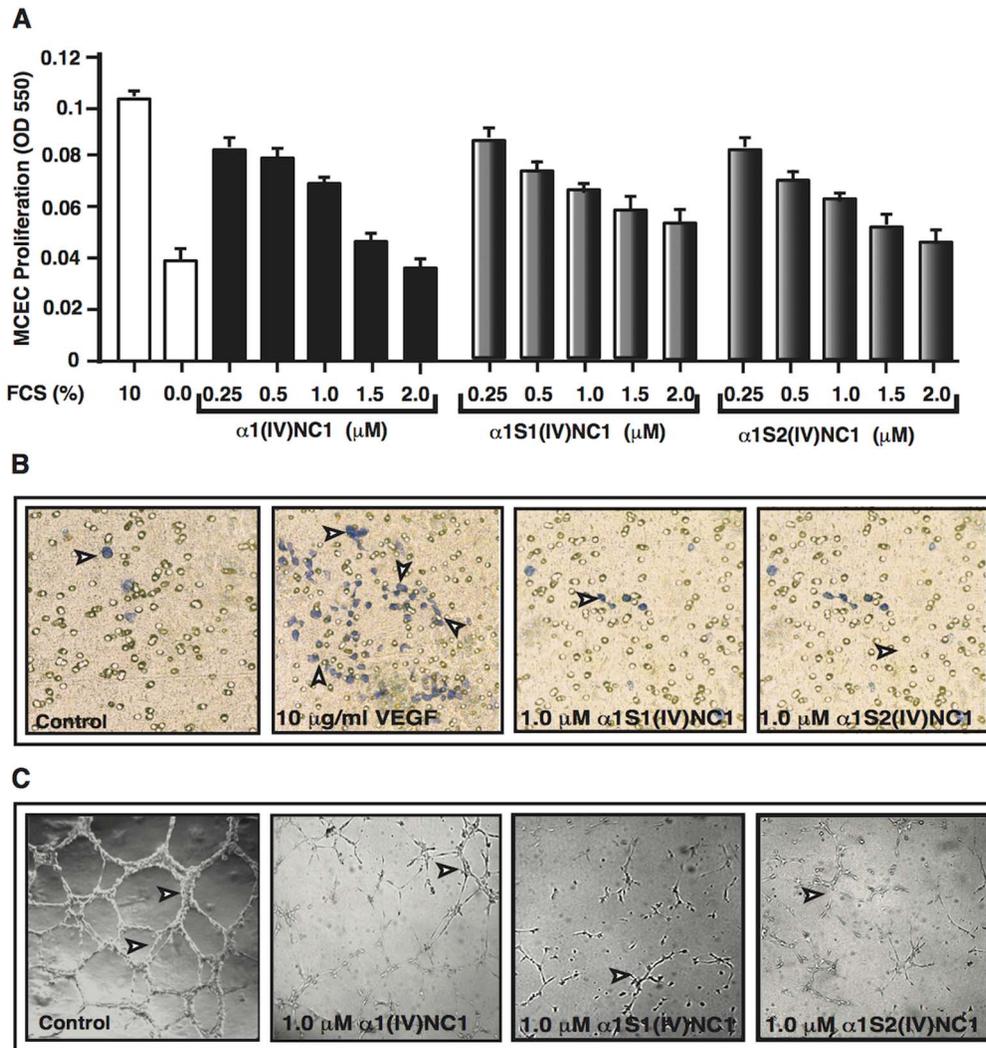


Figure 1. Proliferation (A). Graph summarizes relative levels of methylene blue incorporation in mouse choroidal endothelial cells (ECs) treating with different concentrations of $\alpha 1(\text{IV})\text{NC1}$ and its N- and C terminal $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains compared with and without FCS controls after 48-hrs. All groups represent triplicate samples and data in the graphs are represented as mean \pm SD. **Migration (B).** Number of ECs with and without $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains migrated towards VEGF on the underside of Boyden chamber membrane were shown and cells viewed using a light microscope after 48-hrs representative fields (100 \times magnification) shown. **Tube formation (C).** ECs were plated on Matrigel coated plates in endothelial cell medium as control or with $1.0 \mu\text{M}$ $\alpha 1(\text{IV})\text{NC1}$, $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ proteins. Tube formation was assessed using a light microscope after 48-hrs, and representative fields at 100 \times magnification were shown. doi:10.1371/journal.pone.0080555.g001

FasL mediated *in-vitro* apoptotic activity of $\alpha 1(\text{IV})\text{NC1}$ and its N- and C- terminal $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains

Caspase-3 is a pivotal molecule mediating cellular apoptosis [27]. Earlier we demonstrated that $\alpha 1(\text{IV})\text{NC1}$ induces apoptosis in endothelial cells by activating Caspase-3 [15]. Here in this study, we tested, whether $\alpha 1(\text{IV})\text{NC1}$ and its N- and C-terminal domains possess pro-apoptotic activity or not? Interestingly, ECs incubated with $\alpha 1(\text{IV})\text{NC1}$ and its N- and C-terminal domains showed dose and time dependent activation of FasL without affecting Fas expression compared to control untreated cells (Figure 3A & B). These results suggest both $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains may cross talk with death receptor, activating FasL mediated apoptosis in ECs. These results demonstrate that $\alpha 1(\text{IV})\text{NC1}$ and its two domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$

and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ promoted apoptosis through FasL in addition to MAPK signaling inhibition [15].

We further assessed whether $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ induces activation of FasL downstream different caspases such as caspase-8 and caspase-3. We treated ECs with $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ and observed that activation of caspase-8 (Figure 4A and B). These results demonstrate that full length $\alpha 1(\text{IV})\text{NC1}$, both its N- and C-terminal domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ induced cellular apoptosis by activating FasL and activating downstream caspase-8. Among the known caspases, caspase-3 is an important effector molecule for most cellular apoptosis [27]. To study whether caspase-3 could be activated by $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains, we treated ECs with different doses of $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ and observed activation of caspase-3 in a time dependent manner (Figure 4C and D). Here, we also identified that activation PARP cleavage which is further down stream to FasL, caspase-8 and caspase-3 in

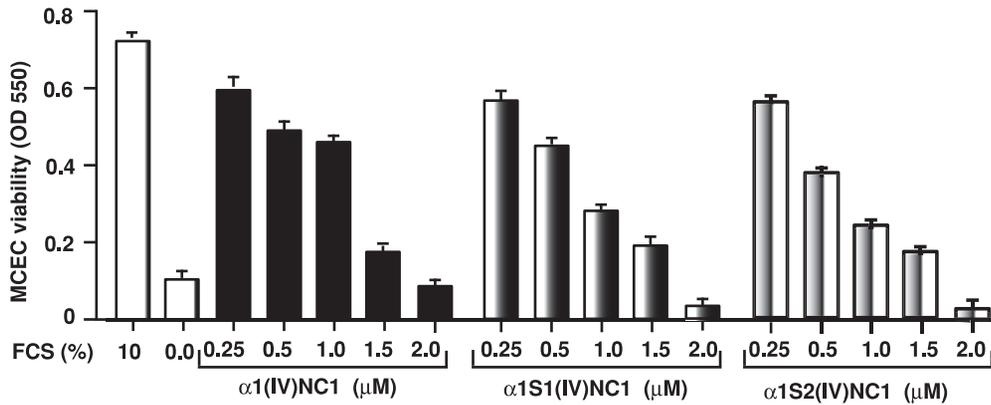


Figure 2. Cell viability. MTT assay was performed to evaluate mouse choroidal endothelial cells (ECs) viability after treatment with different concentrations of $\alpha 1(\text{IV})\text{NC1}$ and its N- and C- terminal domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$. ECs grown with and without FCS as positive and negative controls. Experiments were performed with three replicates and data in the graphs are represented as mean \pm SD. doi:10.1371/journal.pone.0080555.g002

ECs treated with $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ time and dose dependently (**Figure 5A and B**). These results demonstrate that activation of ECs apoptosis by $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains by activating FasL and its downstream caspases and PARP cleavage.

Neutralizing anti-tumorigenic activity of $\alpha 1(\text{IV})\text{NC1}$ by caspase-3 inhibitor

To further confirm the pro-apoptotic activity of $\alpha 1(\text{IV})\text{NC1}$ also partly regulated by caspase-3 activation and apoptosis, we carried *in-vitro* tumor studies with $\alpha 1(\text{IV})\text{NC1}$, or $\alpha 1(\text{IV})\text{NC1}$ co-administrated with caspase-3 inhibitor DEVD to tumor bearing mice. Consistent to our earlier findings, administration of $\alpha 1(\text{IV})\text{NC1}$ significantly inhibited SCC-PSA1 tumor growth and tumor angiogenesis *in-vivo* [27]. In contrast, treatment with caspase-3 specific inhibitor DEVD alone showed significant effect on inhibition of tumor growth. The antitumor activity of $\alpha 1(\text{IV})\text{NC1}$ was significantly attenuated when $\alpha 1(\text{IV})\text{NC1}$ and DEVD were co-administrated to tumor bearing mice (data not shown).

To assess the antitumor effects of $\alpha 1(\text{IV})\text{NC1}$ and its ability to activate microvasculature apoptosis, control and $\alpha 1(\text{IV})\text{NC1}$ treated mice tumor sections were stained with anti-CD31 antibody (**Fig. 6, middle panel**). In control tumors, less TUNEL positive apoptotic staining was detected whereas elevated levels of apoptosis was observed in tumors that were treated with $\alpha 1(\text{IV})\text{NC1}$ (**Fig. 6**). DEVD treatment alone meagerly affected overall tumor cell apoptosis when compared with $\alpha 1(\text{IV})\text{NC1}$. Further, a significant number of ECs in tumor vasculature underwent apoptosis upon $\alpha 1(\text{IV})\text{NC1}$ treatment when compared to control tumors, as observed through CD31/TUNEL dual staining (**Fig. 6, right panel yellow**). Interestingly, co-administration of $\alpha 1(\text{IV})\text{NC1}$ with DEVD to tumor bearing mice significantly reduced EC apoptosis in microvasculature that was induced by $\alpha 1(\text{IV})\text{NC1}$. Further apoptotic tumor cells were observed in $\alpha 1(\text{IV})\text{NC1}$ treated tumors using TUNEL staining. This could be the result of an indirect effect since the microvasculature is affected by $\alpha 1(\text{IV})\text{NC1}$ treatment, and the tumor cells possibly suffer from oxygen supply and nourishment resulting in apoptosis. This effect was observed at elevated levels in cells undergoing apoptosis, other than CD-31 positive ECs (**Fig. 6, left and right panels**). We also quantified the microvascular density and number of apoptotic vessels through TUNEL staining and by counting the number of CD-31 positive blood vessels (data not shown). Collectively, our results demonstrate that $\alpha 1(\text{IV})\text{NC1}$ and its -N and -C terminal domains induced activation of FasL mediated caspase-3 activation and apoptosis in proliferating endothelial cells.

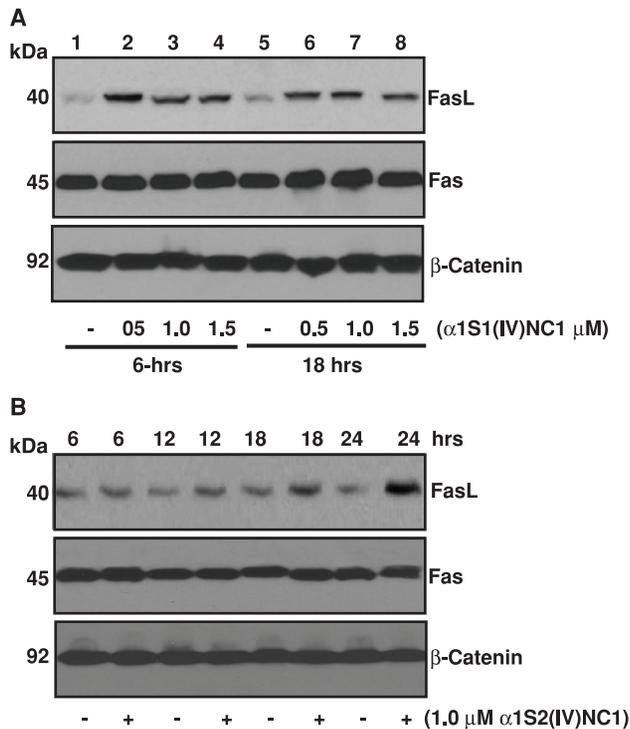


Figure 3. FasL activation (A and B). Mouse choroidal endothelial cells were incubated with and without different doses of $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains for 6 and 18-hrs, and total cells were collected, lysed for 30-min in ice-cold RIPA lysis buffer and about 25 μg of total cytosolic extract per lane was separated and immunoblotted with primary antibodies against FasL and Fas. In panel A and B, β -catenin was shown as loading control. doi:10.1371/journal.pone.0080555.g003

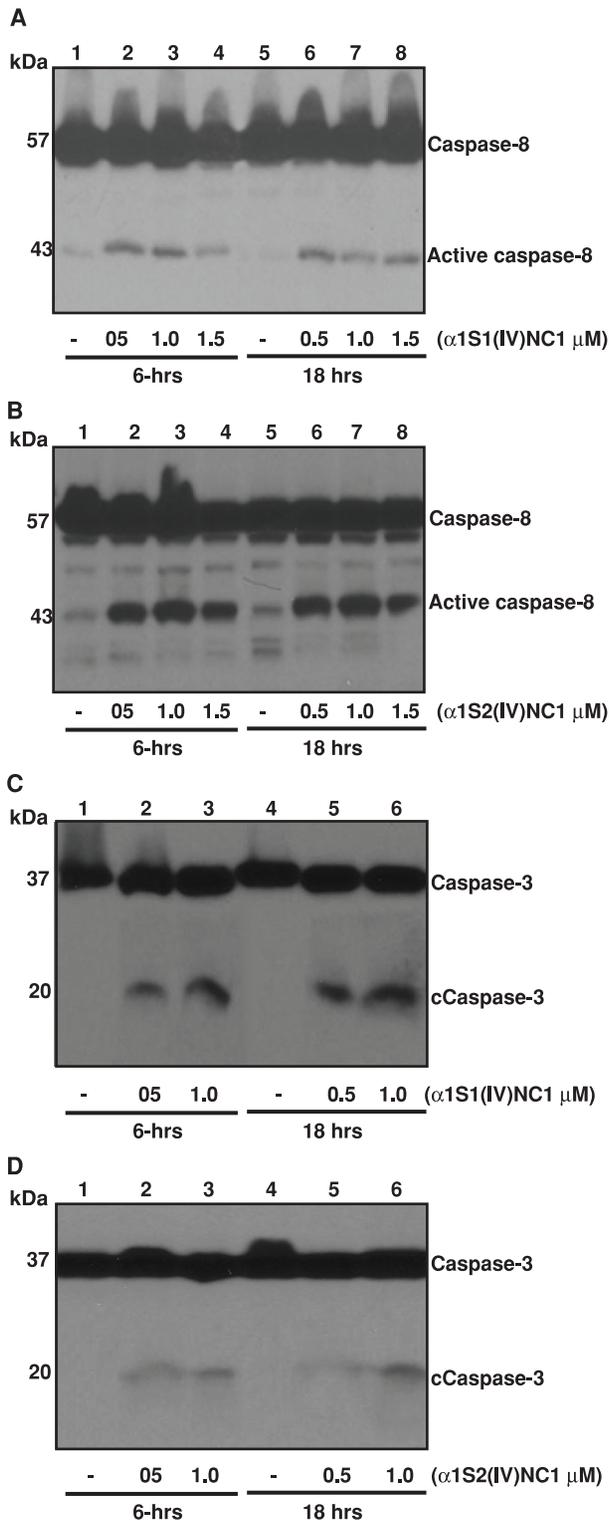


Figure 4. Caspase-8 activation (A and B). Mouse choroidal endothelial cells (ECs) were incubated with and without different doses of $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains for 6 and 18-hrs, and total cells lysed for 30-min in ice-cold RIPA lysis buffer and about 25 μg of cytosolic extract per lane was separated and immunoblotted with primary antibodies against caspase-8. **Caspase-3 activation (C and D).** ECs were incubated with and without different doses of $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains and total cytosolic extract immunoblotted with primary antibodies against caspase-3. doi:10.1371/journal.pone.0080555.g004

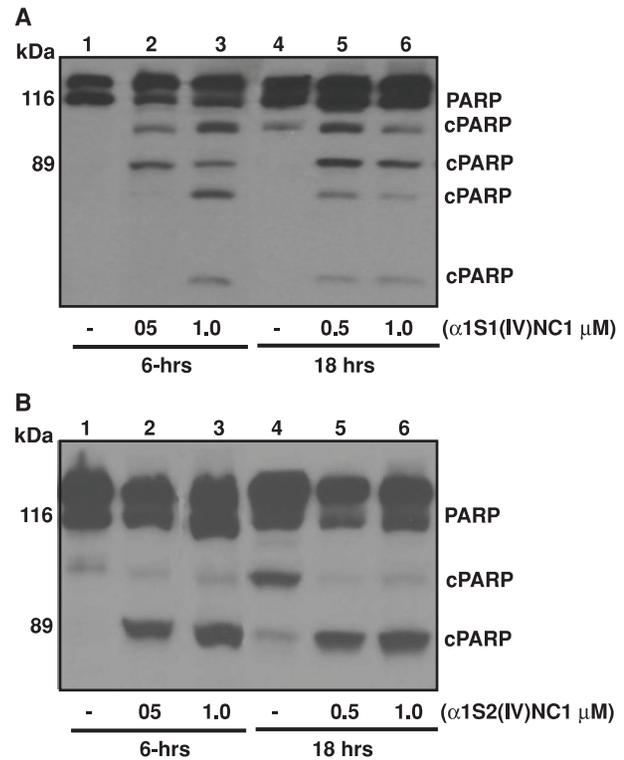


Figure 5. PARP activation (A and B). Mouse choroidal endothelial cells (ECs) were incubated with and without different doses of $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains for 6 and 18-hrs, and total cells lysed for 30-min in ice-cold RIPA lysis buffer and about 25 μg of cytosolic extract per lane was separated and immunoblotted with primary antibodies against PARP. doi:10.1371/journal.pone.0080555.g005

Discussion

Many endogenous metabolites generated from type IV collagen were identified as pro-apoptotic, antiangiogenic and anti-tumorigenic in nature [12–15,21,25,28–32]. The non-collagenous (NC1) domain released by proteolytic activity from type IV collagen $\alpha 1$ chain ($\alpha 1(\text{IV})\text{NC1}$) was identified as an antiangiogenic molecule, where as its N- and C terminal domains apoptotic activity is not yet studied. Antiangiogenic activity of $\alpha 1(\text{IV})\text{NC1}$ is mediating through $\alpha 1\beta 1$ integrin [14,21]. However, it is critical to examine $\alpha 1(\text{IV})\text{NC1}$ and its both its N- and C- terminal domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ effects in several well defined relevant *in-vitro* experiments before confirming that these domains are also antiangiogenic. In this study, we tested the antiangiogenic/pro-apoptotic effects of $\alpha 1(\text{IV})\text{NC1}$ and its N- and C-terminal domains in different *in-vitro* and *in-vivo* experiments.

We demonstrate in this study for the first time, that $\alpha 1(\text{IV})\text{NC1}$ and its N- and C- terminal domains inhibits serum induced EC proliferation, migration and tube formation. In addition, we also identified that both $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains activates caspase-8, caspase-3/PARP cleavage through FasL activation in proliferating endothelial cells. This is coherent with the earlier studies revealing that antiangiogenic activity of $\alpha 1(\text{IV})\text{NC1}$ is mediated through $\alpha 1\beta 1$ integrin signaling and apoptosis [14,33]. $\alpha 1(\text{IV})\text{NC1}$ and its N- and C- terminal $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains activated FasL mediated apoptosis in ECs. Understanding the mechanism(s) of action of $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ domains is crucial for their therapeutic development and use. Thus, $\alpha 1(\text{IV})\text{NC1}$ and its N-

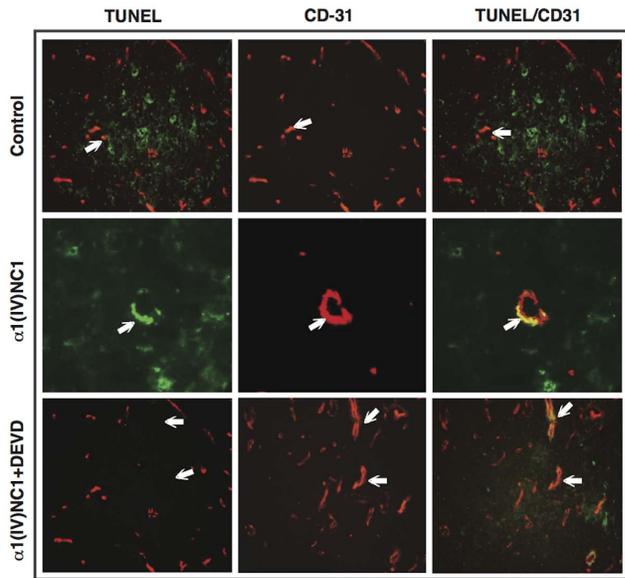


Figure 6. Attenuation of antitumor activity and tumor vasculature apoptosis by caspase-3 inhibitor. $\alpha 1(\text{IV})\text{NC1}$ alone or together with DEVD was injected into SCC-PSA1 tumor bearing mice daily for 15-days. Frozen sections (4.0 μm) of tumors were examined through immunohistochemistry. Apoptotic TUNEL positive cells (green, left), and CD-31 positive tumor blood vessel counts were scored from 10-microscopic fields and four tumors for each experimental condition (red, middle) and co-localization (yellow, right) was shown at 100 \times magnification. Scale bar corresponds to 50 μm .
doi:10.1371/journal.pone.0080555.g006

and C- terminal domains promotes apoptosis in proliferating ECs and inhibits tumor angiogenesis, these endogenous molecules may be an effective therapeutic candidate for treatment of many neovascular diseases. Further evaluation through extensive laboratory studies on these molecules is needed to address the function of these angioinhibitors to be considered for the clinical trials.

References

- Folkman J, Haudenschild C (1980) Angiogenesis by capillary endothelial cells in culture. *Trans Ophthalmol Soc U K* 100: 346–353.
- Folkman J (2006) Angiogenesis. *Annu Rev Med* 57: 1–18.
- Garner A (1986) Ocular angiogenesis. *Int Rev Exp Pathol* 28: 249–306.
- Jampol LM, Ebrown DA, Goldbaum MH (1994) Peripheral proliferative retinopathies: an update on angiogenesis, etiologies and management. *Surv Ophthalmol* 38: 519–540.
- Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1: 27–31.
- Paulsson M (1992) Basement membrane proteins: structure, assembly, and cellular interactions. *Crit Rev Biochem Mol Biol* 27: 93–127.
- Ingber D, Folkman J (1988) Inhibition of angiogenesis through modulation of collagen metabolism. *Lab Invest* 59: 44–51.
- Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. *Nature* 407: 249–257.
- Maragoudakis ME, Missirlis E, Karakiulakis GD, Sarmonica M, Bastakis M, et al. (1993) Basement membrane biosynthesis as a target for developing inhibitors of angiogenesis with anti-tumor properties. *Kidney Int* 43: 147–150.
- Pedchenko V, Zent R, Hudson BG (2004) Alpha(v)beta3 and alpha(v)beta5 integrins bind both the proximal RGD site and non-RGD motifs within noncollagenous (NC1) domain of the alpha3 chain of type IV collagen: implication for the mechanism of endothelial cell adhesion. *J Biol Chem* 279: 2772–2780.
- Sudhakar A (2009) The matrix reloaded: new insights from type IV collagen derived endogenous angiogenesis inhibitors and their mechanism of action. *J Bioequi Availab* 1: 052–062.
- Sudhakar A, Boosani CS (2007) Signaling Mechanisms of Endogenous Angiogenesis Inhibitors Derived from Type IV Collagen Gene Regulation and System Biology 1: 217–226.
- Colorado PC, Torre A, Kamphaus G, Maeshima Y, Hopfer H, et al. (2000) Anti-angiogenic cues from vascular basement membrane collagen. *Cancer Res* 60: 2520–2526.
- Sudhakar A, Nyberg P, Keshamouni VG, Mannam AP, Li J, et al. (2005) Human alpha1 type IV collagen NC1 domain exhibits distinct antiangiogenic activity mediated by alpha1beta21 integrin. *J Clin Invest* 115: 2801–2810.
- Boosani CS, Nalabothula N, Munugalavada V, Cosgrove D, Keshamouni VG, et al. (2009) FAK and p38-MAP kinase-dependent activation of apoptosis and caspase-3 in retinal endothelial cells by alpha1(IV)NC1. *Invest Ophthalmol Vis Sci* 50: 4567–4575.
- Su X, Sorenson CM, Sheibani N (2003) Isolation and characterization of murine retinal endothelial cells. *Mol Vis* 9: 171–178.
- Gunda V, Verma RK, Sudhakar YA (2013) Inhibition of Elastin Peptide-Mediated Angiogenic Signaling Mechanism(s) in Choroidal Endothelial Cells by the alpha6(IV)NC1 Collagen Fragment. *Invest Ophthalmol Vis Sci*. Accepted, unpublished paper “in Press”.
- Gunda V, Boosani CS, Verma RK, Gunda C, Sudhakar YA (2012) L-arginine mediated renaturation enhances yield of human, alpha6 Type IV collagen non-collagenous domain from bacterial inclusion bodies. *Protein Pept Lett* 19: 1112–1121.
- Boosani CS, Sudhakar A (2006) Cloning, purification, and characterization of a non-collagenous anti-angiogenic protein domain from human alpha1 type IV collagen expressed in Sf9 cells. *Protein Expr Purif* 49: 211–218.
- Chow J, Ogunshola O, Fan SY, Li Y, Ment LR, et al. (2001) Astrocyte-derived VEGF mediates survival and tube stabilization of hypoxic brain microvascular endothelial cells in vitro. *Brain Res Dev Brain Res* 130: 123–132.
- Boosani CS, Mannam AP, Cosgrove D, Silva R, Hodivala-Dilke KM, et al. (2007) Regulation of COX-2 mediated signaling by alpha3 type IV noncollagenous domain in tumor angiogenesis. *Blood* 110: 1168–1177.

Earlier lessons from preclinical trials of angiostatin, endostatin, Thrombospondin-1 (ABT-510) and 2-ME suggest that more basic laboratory studies are required to better understand the mechanism of actions associated with these angioinhibitor molecules. Presently, some of the angioinhibitory agents such as Bevacizumab and VEGFR tyrosine kinase inhibitors; Vatalanib (PTK787/ZK 222584), Semaxanib (SU5416), Sunitinib (SU11248), Sorafenib (BAY 43-9006) are in clinical trials [34,35]. Many other angioinhibitory drugs [Macugen (pegaptanib sodium), Lucentis (formerly RhuFab V2), tryptophanyl-tRNA synthetase (TrpRS), VEGF-TRAP, AdPEDF, AG-013958, Avastin (bevacizumab), JSM6427 etc] inhibit ocular neovascularization and prevent leakiness of retinal blood vessels by preventing binding of VEGF to its receptors on endothelial cells [15,36–42]. Endogenous metabolite, $\alpha 2(\text{IV})\text{NC1}/\alpha 3(\text{IV})\text{NC1}$ were also reported to laser induced CNV by promoting apoptosis of endothelial cells *in-vivo* [10].

Our findings suggest that $\alpha 1(\text{IV})\text{NC1}$ and its N- and C- terminal domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ may also be effective in inhibition of tumor angiogenesis by activating caspase-3 in new blood vessels. This is further supported by earlier findings that another angioinhibitor, $\alpha 3(\text{IV})\text{NC1}$ regulate angiogenesis in a number of *in-vitro* and *in-vivo* models [10]. Regression of tumor growth in mice upon $\alpha 1(\text{IV})\text{NC1}$ treatment is associated with reduced tumor vasculature and increased TUNEL positive endothelial and tumor cells when compared to control tumors, indicating *in-vitro* activation of caspase-3. This suggests that $\alpha 1(\text{IV})\text{NC1}$ activates caspase-3 and inhibits tumor angiogenesis and tumor growth. Thus, this work not only supports our efforts in development of $\alpha 1(\text{IV})\text{NC1}$, its N- and C- terminal domains $\alpha 1\text{S1}(\text{IV})\text{NC1}$ and $\alpha 1\text{S2}(\text{IV})\text{NC1}$ as a potential candidate for tumor angiogenesis neovascular diseases.

Author Contributions

Conceived and designed the experiments: YS. Performed the experiments: RKV VG SCP YS. Analyzed the data: YS. Wrote the paper: YS. Prepared the figures: YS.

22. Borza CM, Pozzi A, Borza DB, Pedchenko V, Hellmark T, et al. (2006) Integrin alpha 3beta 1: a novel receptor for alpha3 (IV) noncollagenous domain and a trans-dominant inhibitor for integrin alpha vbeta 3. *J Biol Chem*.
23. Boosani CS, Mannam AP, Cosgrove D, Silva R, Hodivala-Dilke KM, et al. (2007) Regulation of COX-2 mediated signaling by alpha3 type IV noncollagenous domain in tumor angiogenesis. *Blood* 110: 1168–1177.
24. McCawley IJ, Matrisian LM (2001) Matrix metalloproteinases: they're not just for matrix anymore! *Curr Opin Cell Biol* 13: 534–540.
25. Hamano Y, Zeisberg M, Sugimoto H, Lively JC, Maeshima Y, et al. (2003) Physiological levels of tumstatin, a fragment of collagen IV alpha3 chain, are generated by MMP-9 proteolysis and suppress angiogenesis via alphaV beta3 integrin. *Cancer Cell* 3: 589–601.
26. Huang Q, Sheibani N (2008) High glucose promotes retinal endothelial cell migration through activation of Src, PI3K/Akt1/eNOS, and ERKs. *Am J Physiol Cell Physiol* 295: C1647–1657.
27. Nunez G, Benedict MA, Hu Y, Inohara N (1998) Caspases: the proteases of the apoptotic pathway. *Oncogene* 17: 3237–3245.
28. Maeshima Y, Sudhakar A, Lively JC, Ueki K, Kharbanda S, et al. (2002) Tumstatin, an endothelial cell-specific inhibitor of protein synthesis. *Science* 295: 140–143.
29. Maeshima Y, Colorado PC, Kalluri R (2000) Two RGD-independent alpha vbeta 3 integrin binding sites on tumstatin regulate distinct anti-tumor properties. *J Biol Chem* 275: 23745–23750.
30. Sudhakar A, Boosani CS (2008) Inhibition of tumor angiogenesis by tumstatin: insights into signaling mechanisms and implications in cancer regression. *Pharm Res* 25: 2731–2739.
31. Sudhakar A, Sugimoto H, Yang C, Lively J, Zeisberg M, et al. (2003) Human tumstatin and human endostatin exhibit distinct antiangiogenic activities mediated by alphaV beta3 and alpha5 beta1 integrins. *Proc Natl Acad Sci U S A* 100: 4766–4771.
32. Lima ESR, Kachi S, Akiyama H, Shen J, Aslam S, et al. (2006) Recombinant non-collagenous domain of alpha2(IV) collagen causes involution of choroidal neovascularization by inducing apoptosis. *J Cell Physiol* 208: 161–166.
33. Nyberg P, Xie L, Sugimoto H, Colorado P, Sund M, et al. (2008) Characterization of the anti-angiogenic properties of arreten, an alpha1 beta1 integrin-dependent collagen-derived tumor suppressor. *Exp Cell Res* 314:3292–305.
34. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, et al. (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 350: 2335–2342.
35. Morabito A, De Maio E, Di Maio M, Normanno N, Perrone F (2006) Tyrosine kinase inhibitors of vascular endothelial growth factor receptors in clinical trials: current status and future directions. *Oncologist* 11: 753–764.
36. Deissler HL, Lang GE (2008) [Effect of VEGF165 and the VEGF aptamer pegaptanib (Macugen) on the protein composition of tight junctions in microvascular endothelial cells of the retina]. *Klin Monatsbl Augenheilkd* 225: 863–867.
37. Mantel I, Zografos L, Ambresin A (2008) Early clinical experience with ranibizumab for occult and minimally classic neovascular membranes in age-related macular degeneration. *Ophthalmologica* 222: 321–323.
38. Konstantinidis L, Mantel I, Pournaras JA, Zografos L, Ambresin A (2008) Intravitreal ranibizumab (Lucentis(R)) for the treatment of myopic choroidal neovascularization. *Graefes Arch Clin Exp Ophthalmol*.
39. Siemann DW, Shi W (2008) Dual targeting of tumor vasculature: combining Avastin and vascular disrupting agents (CA4P or OX14503). *Anticancer Res* 28: 2027–2031.
40. Nguyen QD, Shah SM, Hafiz G, Quinlan E, Sung J, et al. (2006) A phase I trial of an IV-administered vascular endothelial growth factor trap for treatment in patients with choroidal neovascularization due to age-related macular degeneration. *Ophthalmology* 113: 1522 e1521–1522 e1514.
41. Campochiaro PA, Nguyen QD, Shah SM, Klein ML, Holz E, et al. (2006) Adenoviral vector-delivered pigment epithelium-derived factor for neovascular age-related macular degeneration: results of a phase I clinical trial. *Hum Gene Ther* 17: 167–176.
42. Lu F, Adelman RA (2009) Are intravitreal bevacizumab and ranibizumab effective in a rat model of choroidal neovascularization? *Graefes Arch Clin Exp Ophthalmol* 247: 171–177.