

Inhibition of Tumor Angiogenesis by Tumstatin: Insights into Signaling Mechanisms and Implications in Cancer Regression

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Abstract. Growing tumors develop additional new blood vessels to meet the demand for adequate nutrients and oxygen, a process called angiogenesis. Cancer is a highly complex disease promoted by excess angiogenesis; interfering with this process poses for an attractive approach for controlling tumor growth. This hypothesis led to the identification of endogenous angiogenesis inhibitors generated from type IV collagen, a major component of vascular basement membrane (VBM). Type IV collagen and the angiogenesis inhibitors derived from it are involved in complex roles, than just the molecular construction of basement membranes. Protease degradation of collagens in VBM occurs in various physiological and pathological conditions and produces several peptides. Some of these peptides are occupied in the regulation of functions conflicting from those of their original integral molecules. Tumstatin ($\alpha 3(\text{IV})\text{NC1}$), a proteolytic C-terminal non-collagenous (NC1) domain from type IV collagen $\alpha 3$ chain has been highlighted recently because of its potential role in anti-angiogenesis, however its biological actions are not limited to these processes. $\alpha 3(\text{IV})\text{NC1}$ inhibits proliferation by promoting endothelial cell apoptosis and suppresses diverse tumor angiogenesis, thus making it a potential candidate for future cancer therapy. The present review surveys the physiological functions of type IV collagen and discovery of $\alpha 3(\text{IV})\text{NC1}$ as an antiangiogenic protein with a comprehensive overview of the knowledge gained by us towards understanding its signaling mechanisms.

KEY WORDS: $\alpha 3(\text{IV})\text{NC1}$; integrin receptors; non-collagenous domains from alpha 3 chain of type IV collagen; signaling mechanisms; tumor angiogenesis; vascular basement membrane (VBM).

INTRODUCTION

The development of extracellular matrix (ECM), a complex structure comprising different proteins, is a crucial event in evolution of multicellular organisms. In addition to providing mechanical support for cells, ECM also influences cell behavior (1). ECM remodeling during physiological or pathological processes generates new signals, particularly between endothelial cells and basement membranes (BMs) (2). BMs are thin layers of specialized ECM associated closely with different cells (3). Different collagens together with laminins, nidogens, heparan sulfate proteoglycans (HSPG), fibulins, dystroglycan and other glycoproteins, are major constituents of BM (4). Some of the non-collagenous (NC1) C-terminal domains from different collagens were

identified to be antiangiogenic and anti-tumorigenic in addition to their biological role in molecular architecture of the BM (5,6).

During angiogenesis, new capillaries sprout from pre-existing blood vessels by an invasive process "neovascularization" that occurs in physiological (embryonic development, reproduction and wound healing) and pathological (tumor growth, metastasis, arthritis, age related macular degeneration etc.) conditions (7). Early reports hypothesized that tumor growth is strictly dependent on neovascularization, and inhibition of vascular supply to growing tumors could suppress tumor growth (7,8). Solid tumors cannot grow beyond 2 to 3 mm in diameter without recruitment of their own blood supply and nutrients, thus tumor growth depends on balance between circulating endogenous pro-angiogenic factors (VEGF, FGF, PDGF etc.) and endogenous angiogenesis inhibitors (antiangiogenic peptides generated from ECM by proteases) (7,9). The ECM derived endogenous angiogenesis inhibitors control three fundamental processes (tumor cell proliferation, apoptosis, and tumor angiogenesis) that are critical for growth of primary tumors and metastases (10). Since these endogenously released circulating peptides play a crucial role in anti-angiogenesis, much effort has been focused on development and identification of endogenous anti-angiogenic molecules from BM in the past decade aiming to produce potential medical treatments. A search for endogenous angiogenesis inhibitors led to the discovery of

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endostatin, a C-terminal NC1 domain from type XVIII collagen $\alpha 1$ chain (5). To date several endogenous angiogenesis inhibitors were identified, some of them were cryptic fragments released by proteolysis of ECM and the others are of non-ECM origin (10). The angiogenesis inhibitors from ECM includes a large multifunctional glycoproteins such as thrombospondin, anastellin, a fibronectin fragment, fibulins (COOH terminal fragments corresponding to fibulin 1D and the domain 111 of fibulin 5), endorepellin, a COOH terminal end of perlecan, (or perlecan domain V) (11–14). Some of the type IV collagen derived NC1 domains possess characteristic antiangiogenic activities that were first reported by Dr. Brook's group (6).

This review will illustrate a comprehensive overview of type IV collagen, its physiological functions with specific emphasis on the generation of $\alpha 3$ chain type IV collagen NC1 domain $\alpha 3(\text{IV})\text{NC1}$ (tumstatin) an endogenous inhibitor of angiogenesis. This review also highlights important features of $\alpha 3(\text{IV})\text{NC1}$ addressing signaling mechanisms in regulation of tumor angiogenesis which would explain how this endogenous angiogenesis inhibitor regulates angiogenic balance in the tumor bed.

PHYSIOLOGICAL FUNCTIONS OF TYPE IV COLLAGEN

Type IV collagen is ubiquitously present in vascular basement membrane (VBM) and is highly conserved among vertebrates and invertebrates, regulating cell adhesion and migration (15–17). Type IV collagen is one of the most abundant constituents of basement membrane (BM) architecture that forms a network like structure in ECM (18,19). Type IV collagen consists of six distinct gene products ($\alpha 1$ to $\alpha 6$ chains) and their genomic localization shows a pair-wise head-to-head arrangement with a bi-directional promoter, that were mapped onto three different chromosomes (20–22). Each α -chain in type IV collagen is composed of three distinct domains, a cysteine-rich N-terminal 7S domain, a central long triple helical domain and a globular C-terminal NC1 domain (Fig. 1). The NC1 domains of type IV collagen are involved in the assembly of α -chains to form heterotrimers and the 7S domain is involved in the covalent assembly of heterotrimers in a complex mesh like network that serves as a scaffold for BM (18,19,23,24). Type IV collagen is found normally in the BM, during certain pathological conditions associated with tumor fibrosis, it accumulates in tumor interstitium (4). $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ collagen chains are most abundant forms of type IV collagen and are widely expressed and co-localized

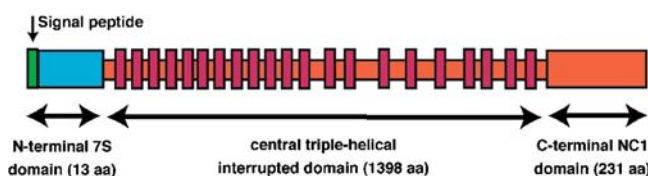


Fig. 1. A hypothetical structure of $\alpha 3$ chain type IV collagen. Linear structure of human $\alpha 3$ chain type IV collagen composed of three distinct domains: a cysteine rich N-terminal 7S domain, a central triple helical domain with multiple small interruptions and a globular C-terminal non-collagenous (NC1) domain.

in numerous tissue types, whereas $\alpha 3$ – $\alpha 6$ chains show temporal and spatial distribution, and expression in physiological as well as pathological processes (25–29).

Several human genetic diseases provided insights into the physiological role of type IV collagen. Mutations or deletions in *Col4a5*, *Col4a3* and *Col4a4* chains are involved in Alport syndrome (defective glomerular BM) and diffused leiomyomatosis (a benign smooth muscle tumor) (30,31). The C-terminal region of $\alpha 3$ chain of type IV collagen has been identified as an autoantigen involved in Goodpasture syndrome (an immune disease characterized by glomerulonephritis and pulmonary hemorrhage) (29). Mouse models for autosomal Alport syndrome have been developed and a phenocopy of human disease was also reported (32).

GENERATION OF $\alpha 3(\text{IV})\text{NC1}$ (TUMSTATIN) FROM TYPE IV COLLAGEN

The proteolytic pathways involved in generation of NC1 fragments from type IV collagen are not well understood. Several NC1 fragments were detected in the serum suggesting that these fragments exist due to physiological cleavage of ECM by proteases. Presumably, several distinct proteolytic pathways are involved in generation of these NC1 domains; however their generation is not extensively tested like as endostatin, which is generated from type XVIII collagen $\alpha 1$ chain NC1 domain (33). To isolate NC1 domain from type IV collagen, researchers isolated vascular basement membrane (VBM) and extracted type IV collagen that was subjected to different proteases. The different protease degradation fragments in the supernatant were separated by gel filtration, anion exchange chromatograph, HPLC etc. Several small peptides were identified by amino acid sequence analysis and immunoblotting using antibodies specific to type IV collagen NC1 domains (34–37) (Fig. 2). Earlier we reported $\alpha 3(\text{IV})\text{NC1}$ as a 28 kDa proteolytic NC1 peptide generated from $\alpha 3$ chain of type IV collagen by MMP-9 and MMP-2 (38,39). Although we gained preliminary clues for *in vitro* processing of type IV collagen NC1 domains, the exact mechanism of generation of these NC1 domains still needs to be extensively investigated.

BIOLOGICAL FUNCTIONS OF $\alpha 3(\text{IV})\text{NC1}$ IN ANTI-ANGIOGENESIS

The biological functions of type IV collagen NC1 domains seem to be conserved throughout evolution. In primitive invertebrate *Hydra vulgaris*, addition of NC1 type (IV) collagen alters morphogenesis, blocking cell aggregation and development (40). *In vitro*, NC1 type (IV) collagen promotes axonal but not dendritic growth in rat embryos sympathetic neurons, hexameric NC1 supports attachment and migration of chicken neural crest cells, but not intact dimers (41,42). These results confirm that the biological functions of NC1 domains are conformation dependent. Proteolytic degradation of type IV collagen may induce exposure of cryptic sites which involved in binding of integrins, and sends new signals between cells and basement membrane (43). Cells bind to type IV collagen, and this binding was inhibited by type IV collagen derived peptides was demonstrated in several cell types that was first reported

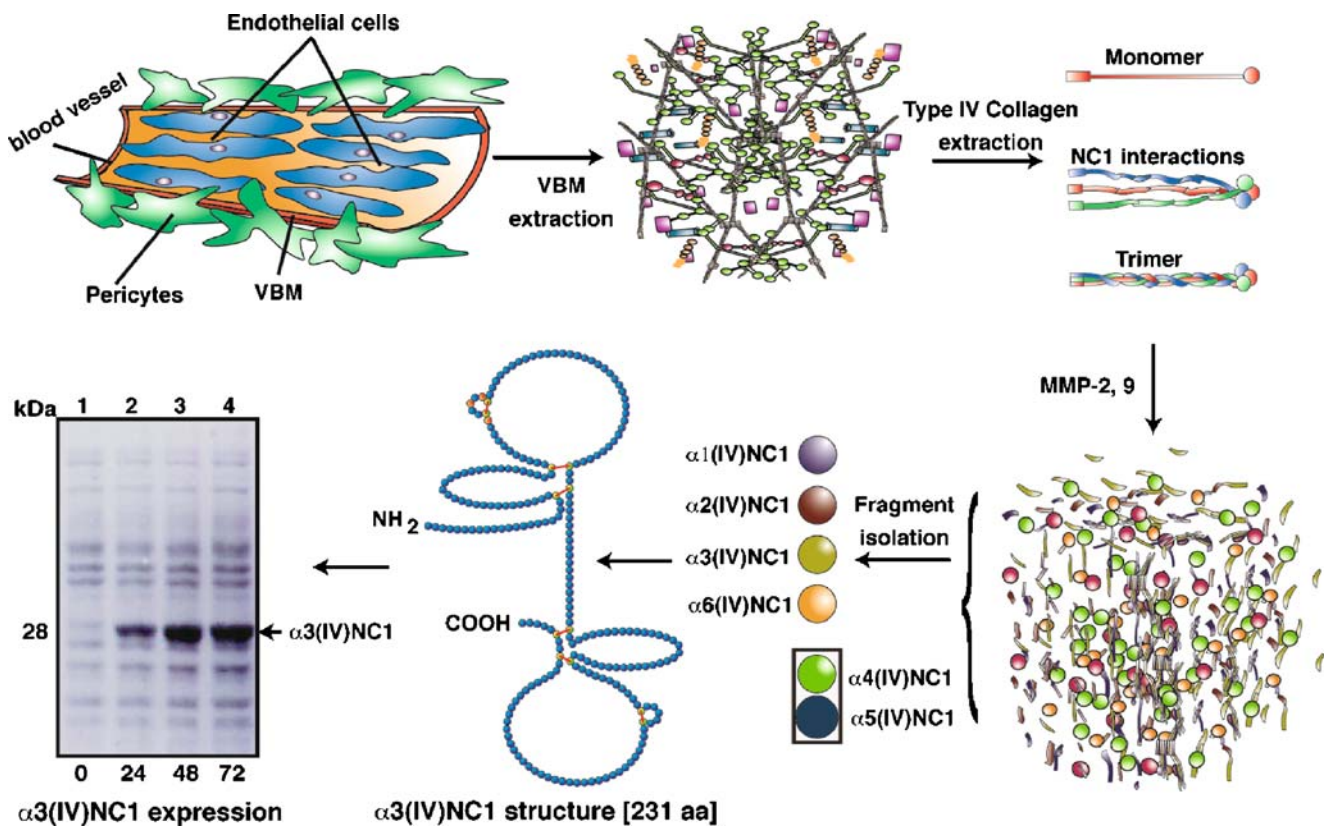


Fig. 2. Generation of antiangiogenic cryptic domains from VBM. Vascular basement membrane was extracted to isolate type IV collagen. The assembled types IV collagen (monomer or trimer) has no antiangiogenic activity. Interestingly when type IV collagen was digested with matrix metalloproteinases (MMP-2/-9), antiangiogenic fragments were identified. Four out of six non-collagenous (NC1) domains of type IV collagen were discovered as antiangiogenic ($\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 6(\text{IV})\text{NC1}$) whereas $\alpha 4$ and $\alpha 5(\text{IV})\text{NC1}$ were not reported to be showing similar functions. The lengths of NC1 domain are approximately 231 amino acids shown in the structure. $\alpha 3(\text{IV})\text{NC1}$ (tumstatin) a 28 kDa protein was cloned in baculovirus expression system and identified that it binds to cell surface integrins to mediate its antiangiogenic activity (66,100,101).

in 1986 (44,45). A synthetic peptide encompassing residues 183–205 of $\alpha 3$ chain type IV collagen NC1 domain was shown to specifically inhibit activation of polymorphonuclear leukocytes (46). This peptide binds to an integrin complex, promotes adhesion, chemotaxis and inhibits proliferation of various human cancer cell lines (47,48). In addition a peptide derived from $\alpha 3(\text{IV})\text{NC1}$ was shown to prevent glomerular hypertrophy in the early stages of diabetic nephropathy (49).

Direct interaction between $\alpha 3(\text{IV})\text{NC1}$ domain and $\beta 3$ integrin signaling inhibited focal adhesion kinase (FAK) and phosphorylation of phosphatidylinositol 3-kinase (PI-3K) (50). Furthermore, inhibition of cell migration was reported in melanoma and fibrosarcoma cells using native type IV collagen or 185–205 peptide, with a decrease in expression of membrane-bound metalloproteinase (MT1-MMP) and $\beta 3$ integrin subunit with a decrease in the levels of activated membrane-bound matrix metalloproteinase-2 (MMP-2) (51). MMP-2 is involved in tumor progression and metastasis and its activation is dependent on MT1-MMP/TIMP-2 (tissue inhibitor of metalloproteinase-2) complexes (52,53). $\alpha 3(\text{IV})\text{NC1}$ inhibited expression of MT1-MMP in bronchial tumor cell line and 185–205 peptide inhibited their invasion on [$\alpha 1(\text{IV})$]₂ $\alpha 2(\text{IV})$ collagen (54). Altogether, this data indicates the ability of this peptide to inhibit proliferation and regulates cellular adhesion and motility. The $\alpha 3$ type IV collagen chain has specific interaction with invasive cancer

cells. In the context of tumor progression and metastasis, the presence of $\alpha 3(\text{IV})\text{NC1}$ may negatively regulate the invasion process. Interestingly, in lungs, where $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$ and $\alpha 5(\text{IV})$ collagen chains are expressed in normal alveolar BM, development of bronchoalveolar carcinoma correlates with loss of $\alpha 3$, $\alpha 4$ and $\alpha 5$ chain expression and an increase in $\alpha 1$ and 2 chain expression (55). The heterotrimer [$\alpha 1(\text{IV})$]₂ $\alpha 2$ is permissive for the invasion of different cancer cell lines and mediates pro-MMP-2 activation (56).

Synthesis of type IV collagen by VBM is a prerequisite for angiogenesis (57,58). Several groups have focused their attention on potential anti-angiogenic properties of NC1 domains. Dr. Brook's group first generated all six type IV collagen NC1 domains and described the antiangiogenic effects of $\alpha 2$, $\alpha 3$ and $\alpha 6(\text{IV})\text{NC1}$ by chorioallantoic membrane (CAM) assays (6). Later several researchers including myself re-conformed anti-angiogenic activities of these domains and nomenclatured as $\alpha 1(\text{IV})\text{NC1}$ (arresten), $\alpha 2(\text{IV})\text{NC1}$ (canstatin) and $\alpha 3(\text{IV})\text{NC1}$ (tumstatin) (50,59–65). These type IV collagen derived NC1 domains inhibit endothelial cell proliferation and migration (38,50,59–62). $\alpha 3(\text{IV})\text{NC1}$ seems to be studied more extensively compared to other domains of type IV collagen. Interestingly, none of the whole NC1 domains inhibited proliferation of cancer cell lines, as observed with the 185–205 ($\alpha 3(\text{IV})\text{NC1}$) peptide, indicating that this effect is dependent on partial degradation of the NC1 domain.

INHIBITION OF ANGIOGENESIS BY $\alpha 3(\text{IV})\text{NC1}$

$\alpha 3(\text{IV})\text{NC1}$ inhibits formation of tubular structures in mouse aortic endothelial cells embedded in Matrigel and block the recruitment of capillaries in Matrigel plugs and inhibits growth of different tumors in mouse models (6, 38, 62, 63, 66). But what are the cell surface integrin receptor(s) involved in these antiangiogenic actions of $\alpha 3(\text{IV})\text{NC1}$? It is clear that different integrins are key targets of $\alpha 3(\text{IV})\text{NC1}$. $\alpha 3(\text{IV})\text{NC1}$ binds to $\alpha \text{V}\beta 3$ integrin in an RGD-dependent and independent manner (67). Integrin $\alpha \text{V}\beta 3$ interacts with $\alpha 3(\text{IV})\text{NC1}$ through two distinct regions, comprising residues 54–132 and 185–203 amino acids. The first site is involved in the anti-angiogenic activity, whereas the second site is involved in anti-proliferative activity on cancer cell lines (68). Adhesion of endothelial cells to $\alpha 3(\text{IV})\text{NC1}$ also seem to occur through $\alpha 6\beta 1$ and $\alpha \text{V}\beta 5$ integrins, but the significance of these integrins interaction is not yet clear (63, 67). It is also known that type IV collagen NC1 domain, interacts with cells *via* $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins (69). During angiogenesis cryptic integrin-binding sites in type IV collagen are exposed that induce a switch in integrin recognition, with a loss of $\alpha 1\beta 1$ binding site and a gain of $\alpha \text{V}\beta 3$ binding site that might be due to denaturation and concomitant degradation of type IV collagen by MMPs (70,71). These results indicate that $\alpha 3(\text{IV})\text{NC1}$ -integrin interactions are involved in the regulation of angiogenesis.

A peptide composed of residues 45–132 of $\alpha 3(\text{IV})\text{NC1}$ fragment is sufficient to inhibit *in vitro* and *in vivo* angiogenesis by increasing apoptosis in endothelial cells (72). These results confirm specific regulatory sub-domains in $\alpha 3(\text{IV})\text{NC1}$ controlling adhesion, proliferation or apoptosis in various cell types. The functional specificity of these sub domains from

$\alpha 3(\text{IV})\text{NC1}$ in endothelial or cancer cells is very interesting. Indeed, the recently published 3D crystal structure of type IV collagen NC1 domain reveals N and C homologous sub domains. The major difference between these sub-domains for each chain is in the region from residues 86–95 in the N sub-domain and 196–209 in the C sub-domain. These regions overlap two sequences that were previously identified to be having anti-angiogenic and anti-proliferative effects in cancer cells (73). $\alpha 3(\text{IV})\text{NC1}$ or its peptides interaction with integrins seems to be involved in the disruption of contacts between endothelial or tumor cells and the BM, leading to apoptosis in these cells.

$\alpha \text{V}\beta 3/\alpha 3\beta 1$ INTEGRIN MEDIATED SIGNALING REGULATED BY $\alpha 3(\text{IV})\text{NC1}$

The signaling mechanism involved in inhibition of endothelial cell-specific protein synthesis by $\alpha 3(\text{IV})\text{NC1}$ by binding to $\alpha \text{V}\beta 3$ integrin was reported by us (65). In addition soluble $\alpha 3(\text{IV})\text{NC1}$ induces endothelial cell apoptosis by interacting with $\alpha \text{V}\beta 3$ integrins and inhibits adhesion to VEGF in the matrix, and this effect was potentiated by anti- $\alpha \text{V}\beta 3$ blocking antibody. Immobilized VEGF almost abolished endothelial cell apoptosis through interactions with these integrins. The inhibition of $\alpha \text{V}\beta 3$ engagement with immobilized VEGF by $\alpha 3(\text{IV})\text{NC1}$, inhibited most of its survival activity (74) (Fig. 3). These mechanisms have since been implicated in inhibition of tumor growth from several tumor cell lines such as renal cell carcinoma (786-O), CT26 (colon adenocarcinoma), prostate carcinoma (PC3), Lewis lung carcinoma (LLC), human lung cancer (H1299), human prostate cancer (DU145), human fibrosarcoma (HT1080) and teratocarcinoma (SCC-PSA1) by inhibiting tumor angiogen-

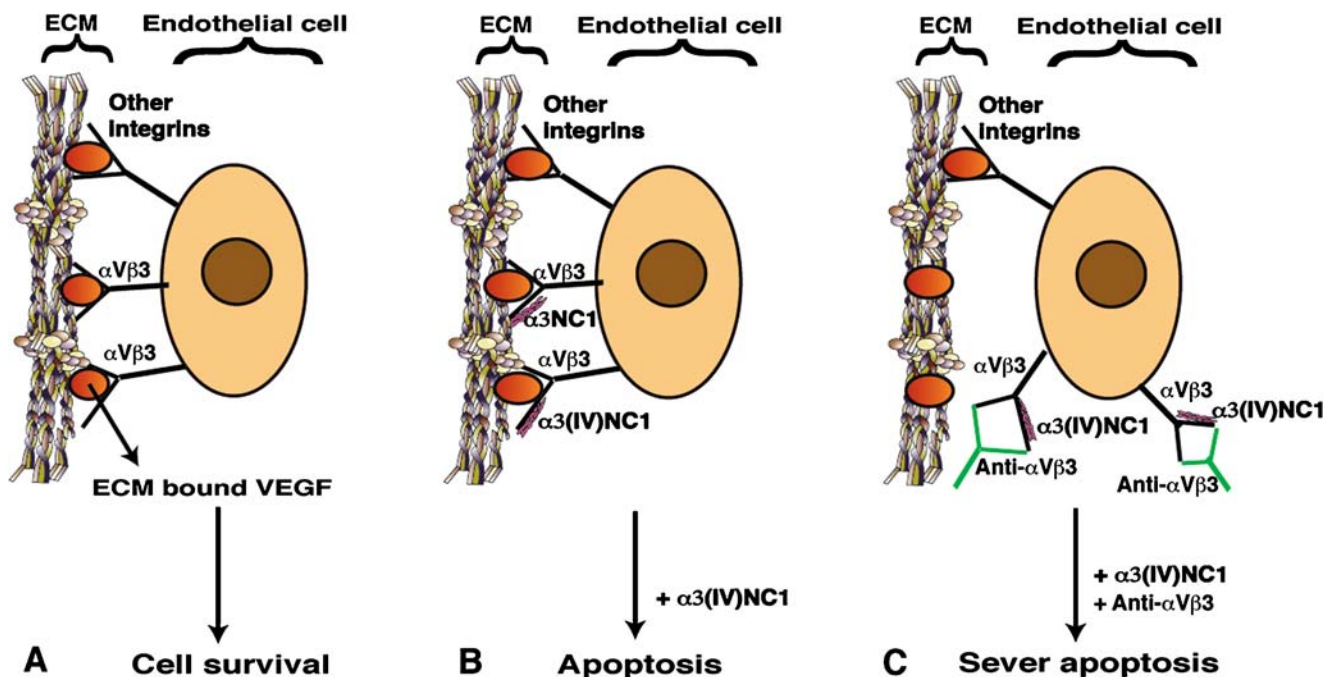


Fig. 3. Schematic illustration of apoptotic effects of $\alpha 3(\text{IV})\text{NC1}$ in endothelial cell. **a** Proliferating endothelial cells binds to extra cellular matrix (ECM), bound VEGF or to immobilized VEGF by integrins leading to cell survival, whereas floating cells will die. **b** When integrin $\alpha \text{V}\beta 3$ is engaged with ECM bound VEGF, $\alpha 3(\text{IV})\text{NC1}$ can induce endothelial cell apoptosis. **c** Whereas the interaction between ECM-bound VEGF and integrin $\alpha \text{V}\beta 3$ when blocked by anti- $\alpha \text{V}\beta 3$ integrin antibody, $\alpha 3(\text{IV})\text{NC1}$ induced apoptosis is significantly enhanced.

esis (6,63,66,67,75). The antiangiogenic activity of $\alpha 3(IV)NC1$ was conferred by its interaction with integrin $\alpha V\beta 3$ and inhibiting activation of focal adhesion kinase (FAK), PI-3K, Akt/protein kinase B, mammalian target of rapamycin (mTOR) and prevents dissociation of eukaryotic translation initiation factor 4E (eIF4E) from 4E binding protein (4E-BP1) leading to the inhibition of Cap-dependent translation specifically in proliferating endothelial cells (65,76). Furthermore, these findings indicate a specific role for integrins in mediating cell specific inhibition of protein translation suggesting a potential specific mechanism of $\alpha 3(IV)NC1$ on endothelial cells.

The antiangiogenic activity of $\alpha 3(IV)NC1$ is localized on two distinct integrin binding regions on the molecule that is separate from the region responsible for the anti-tumor cell activity (62,63). $\alpha V\beta 3$ integrin binds to the NH₂-terminal end comprising amino acid residues 54–132 region of the $\alpha 3(IV)NC1$ that is presumably associated with Cap-dependent translation inhibition and antiangiogenic activity (65). Whereas $\alpha 3\beta 1$ integrin binds to C-terminal region 185–203 residues associated with antitumor activity (66,77). These data correlate with earlier observations that $\alpha 3(IV)NC1$ binds to $\alpha 3\beta 1$ integrin and transdominantly inhibits expression of $\alpha V\beta 3$ integrin (73). Several previous studies have investigated the role of $\alpha 3(IV)NC1$ and its peptides in tumor growth suppression due to direct pro-apoptotic effects on endothelial cells (72,78,79). Interestingly recent studies clearly show that in addition to tumor suppressive action of $\alpha 3(IV)NC1$ or its peptide (T3; C-terminal end comprising amino acid residues 133–244 region of the $\alpha 3(IV)NC1$) directly inhibits growth of glioma cells (80). In addition a cyclopeptide derived from $\alpha 3(IV)NC1$ (YSNSG) was also shown to inhibit human melanoma cell proliferation about 45% (81). These results indicate that $\alpha 3(IV)NC1$ affects endothelial and non-endothelial cells, and does not appear to be specific for endothelial cells.

Surprisingly, abnormal tumorigenesis is observed in mice lacking $\alpha 3(IV)NC1$, the secret behind this abnormal tumor growth needs to be investigated. No significant tumor angiogenesis effect was observed in mice lacking collagen XVIII indicating that $\alpha 3(IV)NC1$ is playing a role in pathological angiogenesis to decrease tumor progression (38,82–85). However, in a controlled angiogenic process such as wound healing, $\alpha 3(IV)NC1$ does not affect the overall neovascularization (38). Although $\alpha 3(IV)NC1$ is efficient in reducing tumor neovascularization, its exact role needs to be deciphered.

Recently we identified that $\alpha 3(IV)NC1$ inhibits hypoxia induced cyclo-oxygenase-2 (COX-2) expression in endothelial cells via FAK/Akt/NF κ B (nuclear transcription factor-kappa B) pathways, leading to decreased tumor angiogenesis and tumor growth in an $\alpha 3\beta 1$ integrin dependent manner (66). Hypoxic COX-2 expression was inhibited in $\beta 3$ integrin null endothelial cells upon treatment with $\alpha 3(IV)NC1$, indicating that COX-2 mediated signaling is not regulated through $\alpha V\beta 3$ integrin (66). Interestingly COX-2 expression was also not affected when hypoxic $\alpha 3$ integrin null endothelial cells were treated with $\alpha 3(IV)NC1$ protein, confirming that COX-2 expression was regulated by $\alpha 3\beta 1$ integrin (66). In addition to COX-2 inhibition, the down stream VEGF and bFGF protein expression was also inhibited upon $\alpha 3(IV)NC1$ treatment to endothelial cells (66).

COX-2 is induced by a variety of factors, including cytokines, growth factors, and tumor promoters (86). Hypoxia induced COX-2 expression regulated by NF κ B (87). There is ample evidence that COX-2 over expression contributes to carcinogenesis and its disruption can both prevent and treat a variety of solid tumors (88,89). COX-2 was also reported to play a key role in tumor angiogenesis (90). Moreover, several investigators have demonstrated that blockade of COX-2 mediated pathway serves as a therapeutic benefit in different cancer models and potential target for tumor angiogenesis (91,92). These findings indicate that there may be several targets for the inhibitory effects of $\alpha 3(IV)NC1$ in tumor-angiogenesis, including or in addition to COX-2, VEGF and bFGF (66). The above studies supports the antiangiogenic and anti-tumorigenic activity of $\alpha 3(IV)NC1$ mediated through $\alpha V\beta 3$ and $\alpha 3\beta 1$ integrins (Fig. 4). Integrin $\alpha 3\beta 1$ mediates signaling events that influence downstream effects of COX-2 expression that is central to the mechanism of $\alpha 3(IV)NC1$ regulating tumor-angiogenesis (66).

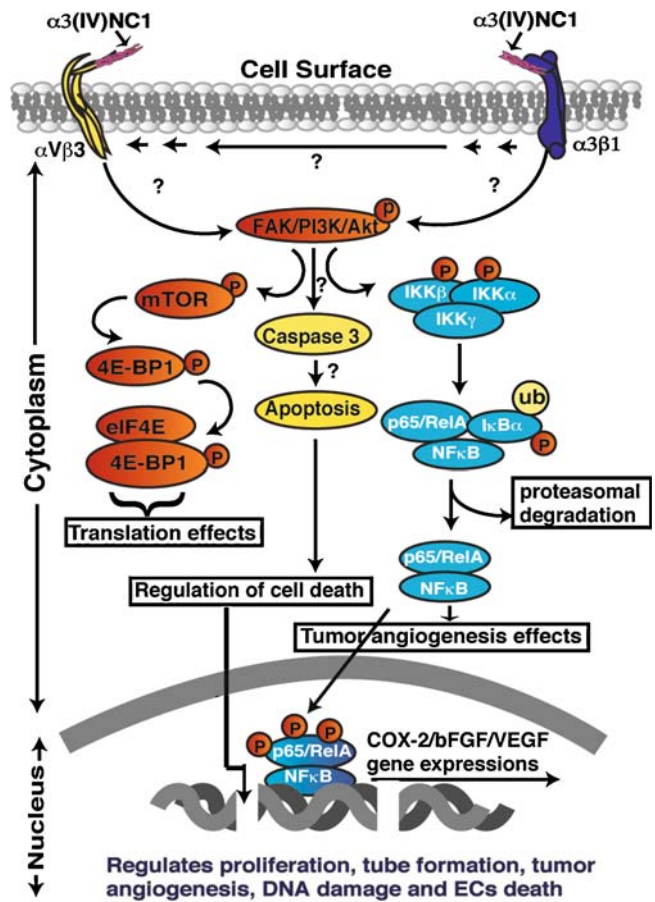


Fig. 4. Schematic illustration of distinct molecular signaling pathways mediated by $\alpha 3(IV)NC1$. $\alpha 3(IV)NC1$ binds to different cell surface integrins, when it binds to $\alpha V\beta 3$ integrin in endothelial cells it inhibits phosphorylation of FAK. Inhibition of FAK activation leads to inhibition of FAK/PI-3K/Akt/mTOR/eIF4E/4E-BP1 cap dependent translation resulting in activation of apoptosis and cell death. When $\alpha 3(IV)NC1$ binds to $\alpha 3\beta 1$ integrin transdominantly inhibits $\alpha V\beta 3$ expression in cells and inhibits NF κ B mediated signaling in hypoxic conditions leading to inhibition of COX-2/VEGF/bFGF expression, resulting in inhibition of hypoxic tumor angiogenesis.

Three possible conclusions can be drawn from the signaling mechanisms of $\alpha 3(\text{IV})\text{NC1}$ in regulating hypoxic tumor-angiogenesis in addition to Cap-dependent translation in endothelial cells. $\alpha 3(\text{IV})\text{NC1}$ binds to cell surface integrins and inhibits hypoxia induced tumor angiogenesis by (1) inhibiting $\text{NF}\kappa\text{B}$ activation, leading to inhibition of COX-2 expression, which in turn results in (2) down regulation of hypoxia induced VEGF/bFGF expression in addition to inhibition of cap dependent translation (Table I). (3) In addition, $\alpha 3(\text{IV})\text{NC1}$ possibly by binding to its several receptors, crosstalk with other cell surface receptors such as VEGF and bFGF, and activate specific caspase mediated signaling and regulate cell functions, as similarly shown by another type IV collagen NC1 domain, $\alpha 2(\text{IV})\text{NC1}$ (93). The decrease in COX-2 expression under hypoxia results in decreased VEGF/bFGF expression representing one of the primary molecular mechanisms by which $\alpha 3(\text{IV})\text{NC1}$ inhibits pathological angiogenesis that is essential for the growth of tumors (66).

Besides $\alpha\text{V}\beta 3$ and $\alpha 3\beta 1$ integrins, another common target for $\alpha 3(\text{IV})\text{NC1}$ seems to be inhibition of MMP-2 activation. A direct interaction with the catalytic domain has been shown in case of endostatin, such an interaction has not been demonstrated for $\alpha 3(\text{IV})\text{NC1}$, and needs further investigation in this direction. An increase in the basal level of pro-MMP-2 activity was also observed in $\alpha 3(\text{IV})\text{NC1}$ null mice (94). Similar possible interaction with other MMPs might exist in the generation of $\alpha 3(\text{IV})\text{NC1}$ and warrants further investigation.

CONCLUSIONS AND PERSPECTIVES

In the last decade several different endogenous angiogenesis inhibitors have been discovered from ECM. Researchers identified that these endogenous angiogenesis inhibitors, through pharmacological studies, showed promising anti-tumor activity, but their mechanism of action and physiological role is not yet understood. Type IV collagen derived endogenous angiogenesis inhibitor, $\alpha 3(\text{IV})\text{NC1}$, binds to different cell surface integrins and exerts its effects through multiple mechanisms including induction of endothelial cell apoptosis, inhibition of cell proliferation, tube formation in endothelial cells, and inhibit or alter the functions of pro-angiogenic growth factors. In this regard $\alpha 3(\text{IV})\text{NC1}$ demon-

strates a genetic evidence for its physiological function in negative regulation of tumor growth and progression in mice (66,73). However, the detailed physiological and biological functions of $\alpha 3(\text{IV})\text{NC1}$ are not yet completely identified. The anti-angiogenic and anti-tumorigenic activities of $\alpha 3(\text{IV})\text{NC1}$ is dependent on $\alpha\text{V}\beta 3$ and $\alpha 3\beta 1$ integrins (66,73). In mice deletion of $\beta 3$ integrin or deletion of $\alpha 3(\text{IV})\text{NC1}$ enhanced tumor angiogenesis, suggesting a role for this integrin and this NC1 domain in limiting angiogenesis *in vivo* (38,95) The anti-angiogenic activities of $\alpha 3(\text{IV})\text{NC1}$ is partly dependent on binding to $\alpha\text{V}\beta 3$ integrin which also supports this hypothesis. Whereas anti-tumorigenic activities of $\alpha 3(\text{IV})\text{NC1}$ are dependent on its binding to $\alpha 3\beta 1$ integrin which transdominantly inhibits $\alpha\text{V}\beta 3$ integrin (73). Another feature of $\alpha 3(\text{IV})\text{NC1}$ is inhibition of various signaling molecules which are involved in cell survival. However anti-angiogenic and anti-tumorigenic activities of $\alpha 3(\text{IV})\text{NC1}$ also seems to be because of the inhibition of different locally released growth factors (66).

In addition several angiogenic inhibitors including αV integrin antagonist EMD 121974, 2-methoxyestradiol (panzam) and MMP-2, -9 inhibitor COL-3 etc. are currently in one of two phases in human clinical trails (96). A recent study suggests that up-regulation of specific pro-angiogenic factors is a common mechanism for colorectal and renal carcinoma cells to evade inhibition by several of extracellular derived endogenous angiogenesis inhibitors (97). Questions regarding resistance to these angiogenesis inhibitors do remain unanswered; however a combination of radiation therapy with other anti-angiogenic therapies may also prove to be clinically useful and effective (97). Earlier lessons from preclinical trials of angiostatin, endostatin, thrombospondin-1 (ABT-510) and 2-ME suggest that more basic research is required for better understanding of the mechanisms of action associated with each of these endogenous angiogenesis inhibitor molecules. Presently, some of the anti-angiogenic agents such as Bevacizumab and several other VEGFR tyrosine kinase inhibitors; vatalanib (PTK787/ZK 222584), semaxanib (SU5416), sunitinib (SU11248), sorafenib (BAY 43-9006) etc., are in clinical trials (98,99). Further extensive evaluation of $\alpha 3(\text{IV})\text{NC1}$ through pharmacokinetic studies is needed to address this molecule as an inhibitor of angiogenesis and to be considered for the clinical trials in the context of tumor angiogenesis and cancer.

Table I. Signaling Mechanisms Mediated by Angiogenesis Inhibitor $\alpha 3(\text{IV})\text{NC1}$

Angiogenesis inhibitor name	Human $\alpha 3(\text{IV})\text{NC1}$ (tumstatin)
$\alpha 3(\text{IV})\text{NC1}$ origin	$\alpha 3$ chain type IV collagen non-collagenous domain
Generation of $\alpha 3(\text{IV})\text{NC1}$	From ECM by MMP-9, -2 and ??
$\alpha 3(\text{IV})\text{NC1}$ binding integrins	CD47/IAP, $\alpha\text{V}\beta 3$, $\alpha 6\beta 1$, $\alpha\text{V}\beta 5$, $\alpha 3\beta 1$
Endothelial proliferation	Effects by $\alpha 3(\text{IV})\text{NC1}$
Endothelial migration	Insignificant effect by $\alpha 3(\text{IV})\text{NC1}$
Endothelial tube formation	Effects by $\alpha 3(\text{IV})\text{NC1}$
Endothelial cells apoptosis	By caspases-3 activation, mechanism not yet known?
Antiangiogenic signaling mediated by human $\alpha 3(\text{IV})\text{NC1}$ domain	FAK, Akt, PI3K/mTOR/eIF-4E/4E-BP1 and $\text{NF}\kappa\text{B}$ /COX-2 mediated signaling

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