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Review Article

ENDOGENOUS ANGIOGENESIS INHIBITOR ENDOSTATIN: AN OVERVIEW

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ABSTRACT

Angiogenesis, the process of new blood vessel formation, plays a central role in both local tumor growth and distant metastasis. Healthy adults require angiogenesis only for wound healing, endometrial proliferation and pregnancy. Thus the inhibition of angiogenesis offers an attractive therapeutic target with little expected toxicity. However, mutant tumor cells may over time produce angiogenic factors. Therefore, for long-term use in cancer, combinations of angiogenesis inhibitors or broad spectrum angiogenesis inhibitors will be needed. The first angiogenesis inhibitor endostatin for cancer was approved by China. Endostatin is a fragment of collagen XVIII A1 that acts as an endogenous inhibitor of angiogenesis and tumor growth, generated by tumor-derived proteases. It shows broad spectrum anti-angiogenesis action and downregulates pathological angiogenesis without side-effects. Endostatin displays a high antitumor activity *in vivo*: it inhibits the progression of more than 60 types of tumors. This review highlights generation, structure, biological role, mechanisms of endostatin's action and its antiangiogenic signaling network.

Keywords: Angiogenesis, Collagen XVIII A1, Endostatin

INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing ones, takes place in various physiological and pathological conditions, such as embryonic development, wound healing, menstrual cycle, chronic inflammation and tumors. Tumor neoangiogenesis is stimulated by protein growth factors released by cancerous cells. These factors include vascular endothelium growth factor (VEGF), basic fibroblast growth factors (FGF-1, FGF-2), angiogenins, epidermal growth factor (EGF), etc. The tumor cell producing angiogenic growth factors also produce a number of angiogenic inhibitors[1]. Therefore, the growth rate of tumor may be the result of balance between the positive and negative effects of angiogenic stimulators and inhibitors[2]. However, at least 16 endogenous angiogenesis inhibitors (Table 1) have been discovered in the circulation and in the extracellular matrix. These may become the safest and least toxic anti-cancer therapies[3, 4]. Endostatin[5, 6] is the first potent endogenous angiogenesis inhibitor discovered as a fragment of the carboxyterminus of collagen XVIIIA1 (*COL18A1*)[7]. All information about this review was collected from various standard journals.

COL XVIII A1

Collagen XVIII A1 a proteoglycan containing heparan sulfate, is widely distributed in vertebrata and is also found in *Caenorhabditis elegans* and *Drosophila.* Along with perlecan and agrin, collagen XVIII is one of three major proteoglycans of basal membranes and the adjacent connective tissue[8]. The sequencing of collagen XVIII performed in the first half of the 1990s[9] revealed that it belongs to a small subfamily of collagens called multiplexins[7], which includes type XV and XVIII collagens have a domain organization that differs from that of other collagens. In molecules of multiplexins, the triple helix is interrupted by regions with non-helical α-chains. These regions are called non-collagenous (NC) domains. Collagen XVIII A1 contains ten collagen domains, with NC-domains between them (Fig 1). Molecules of the majority of collagen types are represented by one large domain organized as a triple helix and are rigid and inextensible; but the discontinuous structure of collagens XV and XVIII seems to provide a significant flexibility of the molecule[10, 11]. The gene of human collagen XVIII A1 includes 43 exons and two promoters, which have been mapped in the region *21q22.3*[12].Variants of gene collagen XVIII *A1* transcription resulted in three different isoforms of collagen XVIII (Fig 2). The variant NC11-303, a product of the transcript containing exons 1, 2, and 4-43 and is a short form of the protein. This transcript is synthesized on activation of the promoter on exon 1 located against the transcription direction. Another promoter located against the transcription direction on exon 3 resulted in production of two other longer isoproteins: NC11-493 and NC11-728[13]. Isoforms NC11-303 and NC11-728 of collagen XVIII were found in the retina of the human eye. However, collagen XVIII A1 isoforms were also found in many other tissues. Thus, the short isoform NC11-303 was detected in the majority of vascular and epithelial basal membranes and the long isoforms NC11-493 and NC11-728 were expressed in the liver [13, 14].

GENERATION OF ENDOSTATIN

Angiostatin supports the theory of a negative factor released by a primary tumor by suppressing the growth of metastases in the urine of mice carrying low-metastatic Lewis lung carcinomas [15]. The molecule angiostatin represents an internal fragment of plasminogen that may be produced by metalloelastase from tumor-infiltrating macrophages[16]. Endostatin was identified as an endogenous angiogenesis inhibitor isolated from the conditioned media of a non-metastatic murine hemangioendothelioma cell line, EOMA. When applied to bovine capillary endothelial cells that have been stimulated with FGF-2, the conditioned media of EOMA cells inhibited the proliferation of the growth factor stimulated cells. Purification and partial amino acid sequencing of endostatin from EOMA culture media showed that endostatin is a fragment of collagen XVIII[5], which was later detected in humans[17]. At present, structures of mouse and human endostatins are characterized in detail[18, 19], where endostatin is a fragment of the C-terminal NC1-domain of collagen XVIII and consists of 183-184 amino acid residues (its molecular weight is 20 kD). Structural analysis of the recombinant C-terminal NC1 domain of mouse collagen XVIII (38 kD) has shown that, it consists of three fragments: the N-terminal trimerization domain (5 kD), the hinge region containing protease recognition sites, and the C-terminal endostatin domain (22 kD). As collagen XVIII A1 contains three polypeptide chains, it generates three NC1-domains which in turn produce three molecules of endostatin. The proteolytic mechanisms by which endostatin is cleaved from collagen XVIII A1 are currently being elucidated [20].

Felbor et al. reported that endostatin was originally isolated from murine hemangioendothelioma cell (EOMA) culture, which was produced from Collagen XVIII due to the proteolytic activity. Using a panel of class-specific protease inhibitors, it was shown that endostatin generation by EOMA cells could be completely inhibited by cysteine protease inhibitors. EOMA cells secrete large amounts of procathepsin L that gets activated to cathepsin L in a slightly acidic medium. Cathepsin L in turn generates endostatin from

collagen XVIII. EOMA cells also secrete matrix metalloproteases (MMPs) which produce a larger, 30 kDa endostatin containing fragment in a parallel processing pathway [21].

Further study, Wen et al. found that endostatin can also be generated following the proteolytic cleavage of collagen XVIII in a two-step mechanism that involves a metal dependent first step followed by elastase cleavage[22]. The fragments (endostatin) released by cleavage of protease-sensitive hinge region of the NC1 domain of collagen XVIII resulted in inhibition of angiogenesis. One argument, the cleavage sites for endostatin in human and mouse collagen XVIII are not identical[23]. Consistent with this observation, the fragment generated from human recombinant NC1 protein by human cathepsin L is precisely 11 amino acid residues longer than mouse endostatin (Fig 3)[21]. Some experiments on animals revealed that; recombinant endostatin effectively inhibits angiogenesis, growth of various primary tumors and their metastases[5]. Boehm *et al*. reported that it has less side effects, less toxicity, no sign of drug induced resistance and the dormancy state, even though therapy was discontinued[6].

STRUCTURE AND BIOLOGICAL ACTIVITIES OF ENDOSTATIN

Hohenester, E. et al.[18] expressed recombinant mouse endostatin in human embryonic kidney cells and determined its crystal structure by x-ray crystallography at 1.5 Å resolution. It was found that endostatin folded into a single globular domain with approximate dimensions of 35×30×30 Å. The structure was composed predominantly of a β-sheet and loops, but also two α-helices, one of them short. A total of 40% of the amino acid residues adopted an extended main chain conformation, but due to many irregularities such as kinks and bulges, only 25% were actually contained in uninterrupted β-strands. The disulfide bond Cys33-Cys173 connected the longer α-helix to the central β-sheet and the shorter circularized a twisted loop containing three strands. The human and mouse protein sequences showed 86% identity and >90% similarity[17] demonstrating a high structural (and possibly functional) relationship. The alignment with mouse endostatin, isolated from EOMA medium, indicated that the N-terminus of circulating human endostatin was 12 amino acids shorter than endostatin from mouse tumors. In comparison to the full cDNA sequence, the C-terminus of human endostatin lacks a single lysine residue as deduced by mass spectrometry, indicating possible processing by carboxypeptidase(s). The difference between mouse and human endostatins might be explained by releasing from different sources. Thus, they may have different properties regarding selectivity or specificity. Moreover, mouse and human peptides may undergo different posttranslational processing events, which are crucial for their respective biological activities.

The crystal structure of mouse endostatin predicted that 11 out of the total 15 arginines are clustered together; this may serve as a binding site for heparin. In order to map the binding epitope, nine solvent-exposed arginines were mutated to alanine[23]. A surprising observation was the existence of a primary binding site centered around Arg158 and Arg270 and a secondary site involving Arg193 and/or 194. The spacial relationship of both binding sites clearly indicated that they could be occupied simultaneously by a single heparin molecule. Olsson et al.[24] showed that inhibition of FGF-2 induced chick chorioallantoic membrane (CAM) angiogenesis by endostatin was dependent on both the major and the minor heparin-binding site, whereas inhibition of VEGF-A induced CAM angiogenesis was dependent only on the minor heparin-binding site. The fact that the major heparin-binding site was critical for the mechanism of action of endostatin only in FGF-2–treated cells implies a high degree of specificity in the interaction between endostatin and heparin/heparan sulfates on the endothelial cell surface. Clamp et al.[25] demonstrate that the effect of oligomeric endostatin can also be inhibited by exogenous glycosaminoglycans in a size-dependent manner, with heparin oligosaccharides containing more than 20 monosaccharide residues having optimal inhibitory activity. The action of oligomeric endostatin on Chinese hamster ovary cells, an epithelial cell line was shown to be dependent on cell surface glycosaminoglycans, principally heparan sulfate with N- and 6-O-sulfation of glucosamine residues rather than iduronate 2-O-sulfation being important for bioactivity.

In another study, endostatin inhibited the chemotactic migration of human umbilical vein endothelial cells (HUVECs) in response to VEGF in a dose-dependent manner and prevented the subcutaneous growth of human renal cell carcinomas in nude mice at concentrations and in doses that are from 1000- to100 000-fold lower than those previously reported[26]. This activity did not depend on heparin binding but was critically dependent on the order in which the cells were exposed to endostatin and VEGF. It is likely, therefore, that endostatin may inhibit angiogenesis by both heparin-dependent and heparin-independent mechanisms. At picomolar concentrations, heparin-binding played no role, but at concentrations several orders of magnitude higher, heparin-binding may well be critical. Another study explains that endostatin has been shown to bind with heparin[27] and also (although with low affinity) with all types of heparan sulfate proteoglycans [28, 23,] they are involved in the regulation of the cell activity by growth factors.

Endostatin has a number of biological functions through different regions of its molecule. Analysis of the structure of human endostatin reveals the presence of the zinc ion near the N-terminal histidine residues (His1, His3, and His11) and Asp76 [19, 23]. It was initially reported that zinc-binding was essential for inhibition of endothelial migration or antiangiogenic action of endostatin[29] but later studies have failed to confirm the relationship between zinc binding and inhibition of endothelial cell migration or angiogenesis[26, 23].

Hohenester, et al.[30] found a structural heterogeneity in the zinc binding site between two different crystal forms of mouse endostatin and concluded that zinc was likely to play a structural rather than a functional role in endostatin. Recently, the effects of Zn(II)-binding on the folding and stability of recombinant human endostatin were studied using thermal- and guanidine hydrochloride (GdmC1)-induced unfolding monitored by differential scanning calorimetry (DSC) and tryptophan emission fluorescence, respectively. The results showed that upon Zn (II)-binding, ES was more stable against heat, denaturant, and certain proteolytic conditions[31]. In order to further explain these inconsistent results, more studies on the effects of Zn (II)-binding on endostatin are needed.

Although several endogenous molecular forms of human endostatin differing in their N-terminal length and their post-translational modifications (18.5-22 kDa) have been discovered, only one recombinant form of 20 kDa is used in clinical trials. This protein, recombinantly expressed in *Pichia pastoris*, contains four cysteines linked as Cys1- Cys4 (Cys33-Cys173) and Cys2- Cys3 (Cys135-Cys165), in a nested pattern[32]. Disulfide bonds are common post-translational modification which play an important role in establishing and maintaining the tertiary structure, and thus essential for the physiological function. Zhou, H. *et al*.[33] constructed three mutants, C33A/C173A, C135A/C165A and all-Ala, to evaluate the contributions of individual disulfide bonds to the structure, stability, and biological functions of endostatin. C135A/C165A and all-Ala mutants, lacking disulfide bond of Cys135-Cys165, lost nearly entire endostatin tertiary structure and most of inhibitory activities both on the migration and proliferation of human microvascular endothelial cells.C33A/C173A mutant, which retained some native-like structures, was less stable and partially active. Javaherian et al. reported that the oligomeric endostatin (trimer or dimer) binds mainly with laminin of the basal membrane [34]. This finding is important for understanding the biological activity of endostatin.

Analogous to many other angiogenesis inhibitors, endostatin has high affinity for heparin. Experimental studies showed that recombinant endostatin made from baculovirus-infected insect cells, specifically inhibits the proliferation of endothelial cells in a dose-dependent fashion similar to that of endostatin purified from EOMA cell culture media. Recombinant endostatin obtained from bacteria is largely insoluble, but still efficient in arresting tumor growth *in vivo* after injection into mice[6, 5]. Endostatin inhibits the *in vitro* proliferation, migration of endothelial cells and also prevents the formation of blood vessels[35]. Intermittent therapy with recombinant-bacterially produced endostatin reduces several experimental tumors, including Lewis lung carcinoma to a dormant state. Endostatin (20kD) was isolated from large volumes of human plasma hemofiltrate[17] and in immunological studies[20]. Physiological levels of circulating endostatin in the plasma of healthy individuals ranges from 10-50 ng/ml (0.5-2.5 nM), with a wide range of variation within the population[36, 37]. Endostatin levels are elevated in certain cancer, chronic inflammatory diseases, eg. rheumatoid arthritis[38] and diabetic retinopathy[39]. Platelets are found to sequester endostatin[40] for later release, eg: to modulate wound healing and also to suppress vascular permeability[41]. A number of studies reported the elevated levels of circulating endostatin in various types of human cancer. The elevated levels of endostatin correlate to tumor aggressiveness and poor prognosis[42, 43, 44, 45, 46, 12]. Another study reported that, Down syndrome is a complex developmental disorder, most commonly caused by a duplication of one of the copies of chromosome 21, where the COL18A1 gene is located. Down syndrome patients have decreased incidence for solid tumors and concomitantly increased serum levels of endostatin. No recognized tumor suppressor genes localize to this chromosome, implying that circulating endostatin might act as a protective agent for the development of solid tumors[37]. Endostatin may also regulate tissue morphogenesis. The ureteric bud produces the endostatin fragment, which inhibits hepatocyte growth factor induced migration and branching morphogenesis of renal epithelial cells and the ureteric bud. These effects are dependent on the presence of the heparan sulfate proteoglycan syndecan-3[47].

MECHANISMS ACTION OF ENDOSTATIN

Numerous studies have indicated that recombinant endostatin is a very potent inhibitor of tumor angiogenesis. The lack of dramatic vascular effects of endogenous endostatin is a result of its sequestration in the basement membranes, where it is not accessible for interaction with cell surface receptors[48]. In addition, the concentrations of endostatin used to achieve anti-tumor effects are 10-fold higher than the levels of endostatin in the circulation, suggesting that the pharmacological effects of high concentrations of endostatin might be distinct from its physiological effects. The ability of endostatin to inhibit tumor growth and angiogenesis has been extensively demonstrated in animal models and human tumors in mice by different investigators [49, 50, 51]. All the reports emphasize the lack of toxicity.

However, endostatin also inhibited tumor cells directly by suppressing tumor cell migration and invasion, as well as by down-regulating gene expression of several pro-migratory molecules and upregulating AP-1 in the tumor cells. Wilson, et al., demonstrated that in some tumors, endostatin's clinical efficacy may extend beyond its antiangiogenic activity and antitumorigenic activity, as well as its non-toxicity to other tissues [52]. The possible cell biological mechanisms underlying the anti-angiogenic effects of endostatin include inhibition of endothelial cell migration, induction of cell cycle arrest, and promotion of apoptosis [53, 27, 54, 5].

Endostatin inhibits angiogenesis by inhibiting the endothelial cell proliferation and migration. Endostatin binding with the integrin α5β1 activated the Src-kinase pathway, which also down-regulates the activity of RhoA GTPase and inhibits signaling pathways mediated by small kinases of the Ras and Raf families[55]. Wickstrom S. A. et al.[56] found that exogenous recombinant endostatin associated with caveolin-1 and α5β1 integrin in microvascular endothelial cells. Most of the ovarian cancer cells express significant amounts of α5β1 integrin, which is important for ovarian cancer cells to attach to the peritoneal wall. Yokoyama Y et al. showed[57] that ovarian cancer cell, attachment to fibronectin-coated wells can be inhibited by α5β1 integrin specific antibodies as well as endostatin. Additionally, Shichiri, M. et al.[58] demonstrated that endostatin rapidly down-regulated many genes in exponentially growing endothelial cells. Endostatin potently inhibited endothelial cell migration partly via down regulation of c-*myc* expression and inhibition of MAPK (p38 and JNK).Endostatin also inhibits bFGF in VEGF induced migration in several different endothelial cell types *in vitro*[53, 27, 5, 59 26], but it does not seem to affect the key signaling pathways involved in growth factor stimulated cell migration[60].

Endostatin inhibits angiogenesis by inhibiting the actions of angiogenic inducers such as VEGF and FGF-2. Endostatins could antagonize the proangiogenic actions of VEGF or FGF-2 by simply competing with these ligands for binding to either glypican or their high-affinity receptors[61].Another reported showed that endostatin treatment inhibited FGF-and VEGF-mediated migration of primary human microvascular endothelial cells and affected vascular formation in the embryoid body mode[60]. Kim, Y. M. et al.[62] showed that endostatin blocked VEGF-induced tyrosine phosphorylation of KDR/Flk-1 and activation of ERK, p38MAPK and p125FAK in human umbilical vein endothelial cells. The direct interaction of endostatin with KDR/Flk-1 may be involved in the inhibitory function of endostatin toward VEGF actions and responsible for its potent anti-angiogenic and anti-tumor activities *in vivo*. Urbich, C. et al.[63] showed that endostatin reduced VEGF-induced phosphorylation of the endothelial NO synthase (eNOS) at Ser1177, interfered with the activation of eNOS, and significantly reduced VEGF-induced NO-release. So endostatin may inhibit VEGF-induced endothelial cell migration and angiogenesis via NO-synthesis.

Several reportes are showed that endostatin inhibits angiogenesis by inducing the endothelial cell apoptosis. Cyclin D1 plays a key role in the transition of cells from G1 to S, and abrogation of its expression leads to G1 cell cycle arrest. Endostatin was unable to arrest cyclin D1 overexpressing endothelial cells, suggesting that cyclin D1 is a critical target for endostatin action. Using a series of deletion and mutant promoter constructs, Hanai et al. identified the LEF1 site in the cyclin D1 promoter as essential for the inhibitory effect of endostatin[54]. They used this target to analyze events affected by endostatin upstream of cyclin D1, such as the important intracellular mediator β-catenin. They proposed that endostatin targeted β-catenin and subsequently transcription of genes such as c-*myc* and cyclin D1 containing LEF1/TCF binding sites in their promoters. Dixelius, M. et al. showed that endostatin induced tyrosine kinase activity and the formation of multiprotein signaling complexes in endothelial cells. One component identified in these complexes was the Shb adaptor protein, which previously had been implicated in apoptosis. Induction of apoptosis was dependent on the expression of Shb with a functional Src homology2 (SH2) domain and the heparin-binding ability of endostatin. Their data indicated that endostatin bound to the endothelial cell surface via heparan-sulfated proteoglycans, and thereby directly or indirectly induced tyrosine kinase activity, which may lead to apoptosis, dependent on the proliferative state of the cells [27]. In addition Anti-apoptotic members such as Bcl-2 and Bcl-XL prevent programmed cell death in response to numerous stimuli. Conversely, pro-apoptotic proteins such as Bax and Bak can accelerate cell death [64]. Endostatin treatment can lead to a marked reduction of anti-apoptotic members such as Bcl-2 and Bcl-XL and their phosphorylation status[53]. Caspase-3 is an intracellular protease activated early during apoptosis of mammalian cells and initiates cellular breakdown by degrading specific structural regulatory and DNA repair proteins. Endostatin treatment also increased the activity of the intracellular protease caspase-3, resulting in DNA

degradation in the nucleus. Studies are in progress to delineate the importance of other anti-apoptotic and pro-apoptotic proteins and their phosphorylation status in response to endostatin treatment.

In addition, endostatin can also act as a proteinase inhibitor. It inhibits MMP-2 (matrixmetalloproteinases-2) activity by binding to its catalytic domain and also inhibition of MMPs-9 and -13 has been observed and it has no influence on the activation of MMP-8[65]. The affinity of endostatin binding to MMP-2 is significantly weaker than that of the endogenous inhibitor TIMP-2 (tissue inhibitor of metalloproteinases) [66, 67]. Matrixmetalloproteinases (2, 9, and 13) are inhibited by endostatin, but some MMPs generate different endostatin containing polypeptides (20-30 kD) by proteolysis of human collagen XVIII[68]. Endostatin not only suppressed the invasive activity of endothelial cells but also cancerous cells produced on an artificially reconstructed mouse basal membrane, Matrigel. This effect was associated with inhibition of MMPs[66]. Thus, the complex mechanism of the antitumor effect of endostatin includes not only its interaction with endothelial cells, but also the direct suppression of cancer cell migration. Moreover, endostatin inhibits the intravasation of malignant tumor cells, which is one of the main stages of carcinoma progression preceding metastasis[4, 63]. Wickström, et al. revealed that the antiangiogenic activity of endostatin involved down-regulation of the urokinase plasminogen activator system and modulation of focal adhesions and actin stress fibers. Endostatin modulated the distribution of soluble and cell surface-associated urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor, type 1 (PAI-1), resulting in the redistribution of receptor-bound uPA from focal contacts. Accordingly, immunofluorescence staining of the urokinase receptor revealed that endostatin treatment removed urokinase-type plasminogen activator receptor (uPAR) from focal adhesions[56].

ENDOSTATIN'S ANTIANGIOGENIC SIGNALING NETWORK

Abdollahi et al reported[69, 70,] endostatin down-regulates many signaling pathways in human microvascular endothelium associated with proangiogenic activity. Using custom microarrays covering over 90% of the human genome, they reported that ~12% of all genes are significantly regulated in human microvascular endothelial cells. They noted that the upregulated genes as a group include the known angiogenesis inhibitors, while the downregulated genes include the known stimulators. They revealed that, the networked action of endostatin cannot reduce single gene responses. Endostatin, down-regulates hypoxia inducing factor-1(HIF-1α) and reinforces its effects by up-regulating HIF-1α AN (antagonist). Other pathways have found to interact to bring about antiangiogenic response by apoptotic induction, cell cycle arrest and decreasing motility. It decreases the survival factors including HIF-1α, NF-κB, Ets-1 and STATs and upstreams the antiapoptotic genes, eg.Bcl-2, Cox-2, c-myc and iNOS. Further, STATs, NF-κB and AP-1 promotes proliferation, by regulating cyclins like Cyclin D. Endostatin down-regulates these pathways as well as the Id family of transcription factors and is consistent with observed cell cycle arrest. Simultaneously, endostatin up-regulates many antiangiogenic genes, such as kininogen (KNG), At-III, ChromograninA ((CHGA), precursor molecule of vasostatin) and maspin etc. Fig. 4 showed that selected genes are associated with endostatin signaling[69].

ANTITUMOR THERAPY BY ENDOSTATIN GENE THERAPY

Endostatin appears to be an ideal candidate for gene therapy of the full-length protein which has significantly inhibited the growth of primary tumors and their metastases. The animal studies reported that the inhibition was up to 86% reduction in tumor volume and/or complete prevention of pulmonary metastases. Lowest inhibition of tumor growth was ~ 40–45%[71]. In transgenic mice, over-expressing endostatin, a small increase in circulating endostatin of approximately 1.6-fold is sufficient to confer dramatic protection against tumor growth[72]. Several novel approaches for administering endostatin gene therapy have been reported. Intra-arterial delivery of endostatin gene therapy to rat brain tumors resulted in 80% reduction in tumor volume, enhanced survival time up to 47%, and 40% decrease in number of tumor vessels [73]. Another study reported that endostatin directly inhibits the tumor cells and antiangiogenic activity in colon cancer (murine C51, human HT29)[74]. Some reports explained that the endostatin gene therapy failed to inhibit murine primary tumors such as fibrosarcoma T241[75], Murine lung cancer[76], Lew lung carcinoma (weak antitumor activity) [77] and human tumors (Acute lymphocytic leukemia[78], Breast cancer (MDA-MB-231) (minimal effect) [79], Neuroblastoma[80]. Kuo et al.[77] (from the Folkman lab) and Pawliuk et al[75], one explanation is that, the circulating endostatin levels were too high. After these papers were published, it has been found that endostatin's antiangiogenic and antitumor efficacy was biphasic and the circulating levels of endostatin too high or too low are inactive. Another observation explained that, the normal range of endostatin in mouse blood is ~ 5–15 ng/ml and effective therapeutic levels are up to ~ 80–450 ng/ml[81].

SUMMARY AND FUTURE DIRECTION

In summary, the endostatin, (broad spectrum endogenous antiangiogenic molecule) is the first endogenous angiogenesis inhibitor discovered as a fragment of the extracellular matrix. Sund et, al showed that elevating the circulating levels of endostatin (by genetic over expression in endothelium) by less than two-fold can suppress tumor growth by 2-3 fold[82]. Recombinant endostatin protein is currently undergoing clinical development. The ongoing phase 1 trial for recombinant human endostatin protein is designed to determine the safety, pharmacokinetics and pharmacodynamic properties of daily administration in patients with refractory cancers. In September 2005, endostatin (Endostar) was approved by the state FDA in china for the treatment of non-small-cell lung cancer[82] and several clinical trials are ongoing throughout the world at different clinical phases (*ClinicaltrialsFeed.org*). Although Endostar is widely sold in China, its clinical efficacy in the absence of co-administration of other traditional chemotherapeutics has yet to be shown. Several reports suggest that certain small molecules taken orally will raise the endogenous expression of specific angiogenesis inhibitors or raise their plasma or serum levels by alternative means, such as mobilization from matrix or platelets. For example, celecoxcib can increase serum endostatin levels[40], Prednisolone and salazosulfapyridine can increase the endostatin levels in joint fluid[83]. Therefore, possible new pharmaceutical field could be developed around the future discovery of drugs that could increase endogenous angiogenesis inhibitors to protect against cancer as well as other angiogenesis-dependent diseases. Hence, biologists, pharmaceutical companies and physicians all over the world are interested in endostatin research.

Table 1: Endogenous angiogenesis inhibitors

Name	Ref.
Alphastatin	84
Angiostatin	15
Arresten	85
Anti-thrombin III (truncated)	85
Canstatin	86
Endostatin	5
Fibulin	87
Interferon-beta	88
2-methoxyestradiol	89
Pigment epithelia derived factor (PEDF)	90
Platelet factor 4 (PF4)	91
Terahydrocortisol	91
Thrombospondin- 1	92
Thrombospondin- 2	93
TIMP-2	94
Tumstatin	95
Fragment of histidine-rich glycoprotein	96

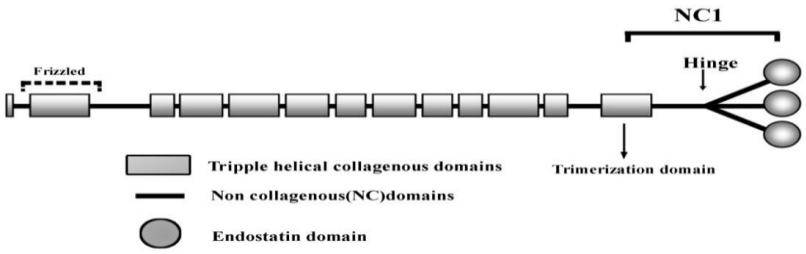

Figure 1: Schematic structure of type XVIII collagen. Ten collagenous domains are interrupted by 11 NC domains. The long isoform contains the alternatively spliced Frizzled domain. The NC1 domain contains the trimerization domain, the protease sensitive hinge domain and the 20 kDa endostatin fragment (Modified from Oh et al., 1994).

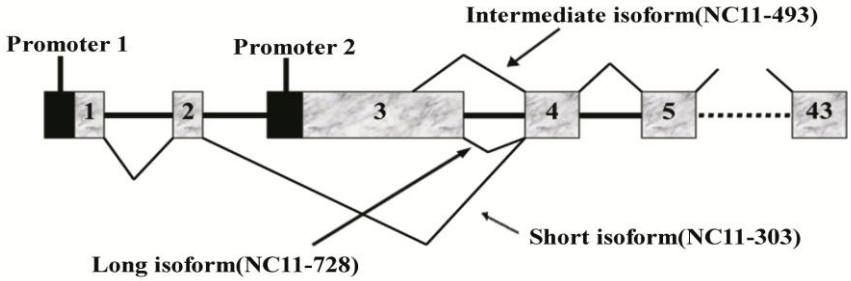

Figure 2: Schematic representation of the two promoters and the splicing events giving rise to 3 different isoforms of COLXVIII A1 transcripts (Modified from Suzuki et al., 2002).

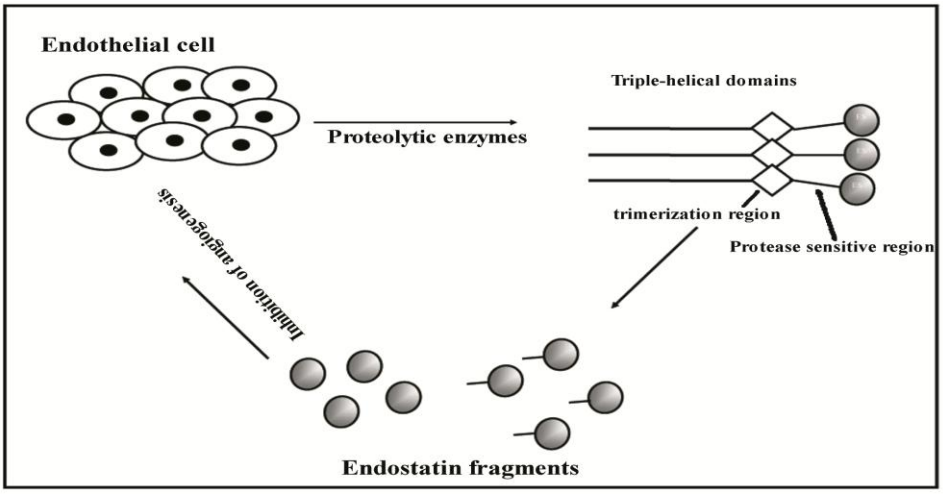

Fig 3: Diagram showing the proposed role of collagen XVIII/endostatin as a local regulator of angiogenesis. Local secretion of cathepsins and matrix metalloproteases (MMPs) by stimulated endothelial cells leads to cleavage within the protease-sensitive C-terminal hinge region of collagen XVIII. This results in release of endostatin and inhibition of angiogenesis which in turn causes a reduction in the release of cathepsins and MMPs (Modified from Felbor et al., 2000).

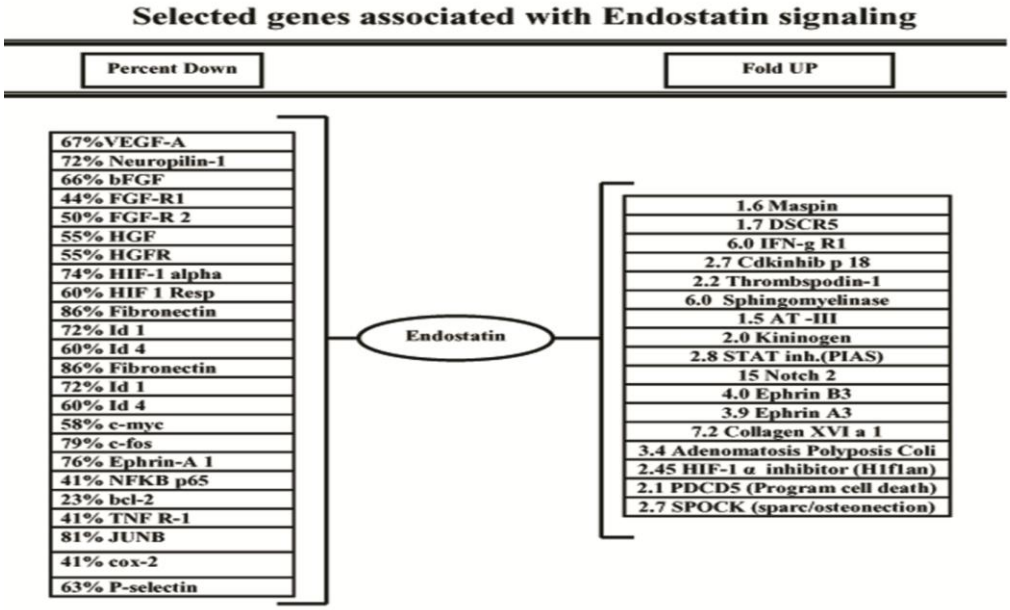

Fig 4: Selected genes associated with endostatin signaling (Modified from Abdollah et al., 2004)

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