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REVIEW

Endostatin: Current Concepts about Its Biological Role and Mechanisms of Action

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Abstract—Endogenous inhibitors of angiogenesis are proved to be a major factor preventing the emergence of clinically manifested stages of human cancer. The protein endostatin, a 20-kD proteolytic fragment of type XVIII collagen, is one of the most active natural inhibitors of angiogenesis. Endostatin specifically inhibits the *in vitro* and *in vivo* proliferation of endothelial cells, inducing their apoptosis through inhibition of cyclin D1. On the surface of endothelial cells, endostatin binds with the integrin $\alpha_5\beta_1$ that activates the Src-kinase pathway. The binding of endostatin with integrins also down-regulates the activity of RhoA GTPase and inhibits signaling pathways mediated by small kinases of the Ras and Raf families. All these events promote disassembly of the actin cytoskeleton, disorders in cell–matrix interactions, and decrease in endotheliocyte mobility, i.e., promote the suppression of angiogenesis. Endostatin displays a high antitumor activity *in vivo*: it inhibits the progression of more than 60 types of tumors. This review summarizes results of numerous studies concerning the biological activity and action mechanism of endostatin.

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ANGIOGENESIS AND THERAPEUTIC POTENTIAL OF ENDOSTATIN

Angiogenesis is the process of formation of a new microhemodynamic link as a result of an induced realignment of mature blood vessels produced earlier. Prevention of angiogenesis is one of the most important natural mechanisms suppressing the growth of malignant tumors. Really, an emerged pool of cancerous cell can remain for years in a latent microscopic state (dormancy) if angiogenesis is not induced and the malignant region of tissue realizes its metabolism through diffusion. By contrast, progression of malignancies is associated with a high level of angiogenesis, and these pathologic processes are in direct correlation [1-5].

Tumor neoangiogenesis is stimulated by protein growth factors released by cancerous cells. These factors include vascular endothelium growth factor (VEGF), basic fibroblast growth factor (bFGF), angiogenins, epidermal growth factor (EGF), etc. [1]. According to a current hypothesis, the extremely low intensity of angiogenesis in the normal body is maintained mainly due to the quantitative predominance of anti-angiogenic compounds over pro-angiogenic ones. Paradoxically, cancerous cells also produce inhibitors of angiogenesis, but the significance of this phenomenon is still unclear. Because of different half-life, in the region of the primary tumor stimulants of angiogenesis (possessing a short half-life) dominate, whereas angiogenesis inhibitors (which are relatively stable in the circulation, with half-life in blood plasma to several hours) are prevalent in the systemic blood flow, and this prevents progression of metastases [2, 3]. Extirpation of the primary tumor in some cases decreases the fraction of circulating anti-angiogenic molecules, and this triggers angiogenesis in inactive latent

Abbreviations: bFGF) basic fibroblast growth factor; eESC) endotheliocytes originating from embryonal stem cells; FAK) focal adhesion kinase; HUVEC) human umbilical vein endothelial cells; ECM) extracellular matrix; JNK) c-Jun N-terminal kinase; MAP kinases) mitogen-activated protein kinases; MMP) matrix metalloprotease; TNF-α) tumor necrosis factor α; NC) non-collagenous; t-PA) tissue-type plasminogen activator; VEGF) vascular endothelium growth factor.

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micrometastases and their growth [4]. In normal tissues of an adult human angiogenesis occurs only during growth or tissue regeneration, and in women it also occurs during formation of the corpus luteum and placenta [4, 5]. Therefore, specific inhibition of angiogenesis seems promising as a selective therapeutic influence on solid malignant tumors.

In this context, endogenous inhibitors of angiogenesis are especially interesting. At present, there are described about 25 such compounds, which are present in the body with the normal phenotype and during malignant transformation of tissues [5]. Mechanisms of generation of these compounds by normal cells remain unclear. There are only sporadic observations that facilitate comprehension of the regulation of angiogenesis on the organism's level. The initial studies on angiogenesis (in the 1980s) revealed a significant role of the blood coagulation system. Platelets were shown to secret both angiogenesis inducers (VEGF, bFGF, EGF, angiopoetin-1) and inhibitors (fragments of the hepatocyte growth factor, thrombocytic factor-4, thrombospondin-1, etc.) [6-8]. Angiogenesis inhibitors, such as kininogen and prothrombin fragments and antithrombin III activated by partial proteolysis (the so-called anti-angiogenic antithrombin III), are also produced during different stages of the blood coagulation cascades [9]. The human plasminogen system is also involved in the up- and down-regulation of angiogenesis [10]. More than a half of endogenous angiogenesis inhibitors are really "hidden" fragments of physiological proteins, which can be activated by a partial proteolysis of the precursor molecule resulting in the release of an active anti-angiogenic peptide fragment [4].

The discovery of endostatin (a fragment of collagen XVIII) in 1997 was the first demonstration of the role of extracellular matrix (ECM) proteins in the regulation of angiogenesis [2]. After the discovery of tumstatin (a fragment of collagen IV that is the major component of the blood vessel basal membrane [11]), it became clear that proteolytic fragments of ECM components and basal membrane of vessels are crucial regulators of angiogenesis *in vivo*. Such polypeptides are proposed to be isolated as a separate group of angiogenesis inhibitors originating from the matrix, as differentiated from inhibitors of another origin [5].

Endostatin is the most intensively studied human endogenous angiogenesis inhibitor; it stimulates apoptosis in proliferating endothelial cells of pathologically growing vessels and also possessing other mechanisms of anti-angiogenic action. This protein is interesting first of all as a promising antitumor drug. Just from the discovery of endostatin, there were attempts of its using in therapy of cancer. Intensive preclinical studies demonstrated high efficiency of endostatin in mice with transplanted solid tumors, including those of humans (in some cases therapy with endostatin resulted in a complete regression of tumor foci) [5, 12]. In USA, China, and other countries, endostatin is now under stage I or II of clinical investigations on patients with different oncological diseases [12, 13].

Endostatin can be effective not only in antitumor therapy but also in the experimental treatment of other diseases with neoangiogenesis involved in pathogenesis. In particular, therapy of such an inflammatory disease as rheumatoid arthritis characterized by invasion of inflammatory cells into the joint synovial membrane and associated with a progressing angiogenesis can be based on inhibition of angiogenesis. Endostatin suppressed the development of rheumatoid arthritis in mice with hyperproduction of tumor necrosis factor-α (TNF-α) [14]. Gene therapy with endostatin in mice decreased the volume of the human synovial tissue transplants affected with rheumatoid arthritis [15, 16]. Pathological neoangiogenesis is involved in the progression of Crohn's disease, atherosclerosis, diabetic retinopathy, psoriasis, endometriosis, and obesity. However, intensive laboratory and clinical investigations of the therapeutic potential of endostatin are now performed only with respect to oncological diseases.

Therefore, pronounced interest of clinicians in possibilities of endostatin as a promising drug is quite reasonable. Concurrently, biochemists and other researchers are studying the role of endostatin and other anti-angiogenic fragments of ECM proteins and proteoglycans in the modulation of behavior of endothelial cells and, consequently, their involvement in normal tissue development. No doubt, results of studies on endostatin are necessary for deep understanding the cell–matrix interactions on the molecular level in the embryo and adult human organism, including those in some diseases. Moreover, elucidation of these problems is important for enhancing our knowledge of mechanisms of apoptosis induction.

COLLAGEN XVIII

Collagen XVIII, a proteoglycan containing heparan sulfate, is widely distributed in vertebrata and is found also 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 [17]. The sequencing of collagen XVIII performed in the first half of the 1990s [18] revealed its belonging to a small subfamily of collagens, the so-called multiplexins [19]. Multiplexins, which include 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 contains ten collagen domains, with NC-domains between them.

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 [20, 21].

The gene of human collagen XVIII (*COL18A1*) including 43 exons and two promoters has been mapped in the region *21q22.3* [22]. Variants of gene *COL18A1* transcription result in three different isoforms of collagen XVIII. The variant NC11-303 is a product of the transcript containing exons 1, 2, and 4-43 and presents the so-called 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 results in production of two other longer isoproteins: NC11-493 and NC11-728 [23]. By approaches of molecular genetics, collagen XVIII and its fragment endostatin were shown to be important for the development and homeostasis of eyes. These data were obtained on patients with Knobloch syndrome, an autosomal recessive genetic disease, which is mainly manifested by severe damage of the visual organ (vitreo-retinal degeneration, retinal detachment, myopia, etc.) and also an occipital encephalocele [24]. The Knobloch syndrome is a very rare disease, and by now only 24 patients with this pathology have been described [22]. These patients carry a homozygous mutation (IVS1-2A→T) on the consensus sequence AG of the 3′-end of intron 1 of the gene *COL18A1*. This mutation results in exon 2 deletion and emergence of an untimely stop codon in exon 4 of the *COL18A1* transcript [25]. This is associated with a deficiency only of the short isoform of NC11-303, whereas long isoforms do not change [23].

Isoforms NC11-303 and NC11-728 of collagen XVIII are found in the retina of the human eye. Knobloch syndrome is associated with imbalance and deficiency of collagen XVIII isoforms and, possibly, endostatin shortage in the eyes. However, collagen XVIII isoforms are also found in many other tissues. Thus, the short isoform NC11-303 is detected in the majority of vascular and epithelial basal membranes. The long isoforms NC11-493 and NC11-728 are expressed in the liver [23, 26]. Nevertheless, patients with Knobloch syndrome who carry mutations leading to deficient transcripts of all isoforms of collagen XVIII display no significant phenotypic disorders in other organs, except the eyes and the occipital part of the scull. Isoforms of collagen XVIII, which play a specific role in the visual organ, are under careful studies. Because Knobloch syndrome is caused by inactivating mutations in the gene *COL18A1*, this syndrome is usually studied on *Col18a1*$^{-/-}$-mice [23]. In the *Col18a1*$^{-/-}$-mice morphological defects are observed in nearly all parts of the eye where collagen XVIII is expressed. However, these mice have no occipital encephalocele, which is a specific clinical sign in patients with Knobloch syndrome [27]. A shortage of collagen XVIII is also a factor of predisposition to hydrocephalus, as it has been shown on a line of mice with switched off *Col18a1* gene. However, these mice also had changes in the basal membrane ultrastructure not only in the eyes but also in other organs [28].

STRUCTURE AND GENERATION OF ENDOSTATIN

Endostatin was first discovered and isolated from conditioned cultural medium of nonmetastasizing cells of the EOMA line of mouse hemangioendothelioma [2]. Addition of this conditioned medium to endothelial cells of bovine capillaries (cell growth was stimulated by bFGF) inhibited the cell proliferation. Later endostatin was detected in humans [29]. At present, structures of mouse and human endostatins are characterized in detail [30, 31]. 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). Human monomeric endostatin is a globular protein containing two disulfide bonds: Cys162–302 and Cys264–294 [32].

The mechanism of proteolytic generation of endostatin from collagen XVIII is now known. 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) [33]. Because collagen XVIII consists of three polypeptide chains, it contains three NC1-domains and can generate three molecules of endostatin. The EOMA line cells were shown to produce collagen XVIII and a proteolytically active compound, which could generate endostatin from the precursor molecule. Different protease inhibitors were added into the medium, and endostatin generation was completely suppressed by inhibitors of cysteine endopeptidases. It then became obvious that the EOMA line cells produced large amounts of procathepsin L, which in a weakly acidic medium converted to active cathepsin L detaching, in turn, endostatin from collagen XVIII. The ability of generating endostatin from the NC1-domain of collagen XVIII is also inherent in cathepsins B and K [34].

The endostatin production by the EOMA line cells was suppressed in the presence of an elastase inhibitor, elastatinal. It is unlikely that elastase is the main enzyme detaching endostatin from the precursor molecule, because elastase cannot be detected in the EOMA line cells or in the conditioned medium used for cultivation of these cells. However, the *in vitro* addition of porcine elastase to the recombinant mouse NC1 protein resulted in an active production of endostatin [35].

BIOLOGICAL ACTIVITY OF ENDOSTATIN

In experiments on animals, recombinant endostatin effectively inhibits angiogenesis and growth of various primary tumors and their metastases [2], without considerable side effects or toxicity of the drug and without the development of drug resistance [36]. In individuals with Down's syndrome, the circulating endostatin concentration is enhanced, and this seems to be due to the presence of an anomalous third copy of the *COL18A1* gene. Possibly just this is the cause of a very low incidence of solid tumors in such patients [37]. Endostatin inhibits the *in vitro* proliferation and migration of endothelial cells and also prevents the formation of blood vessels [38].

Notwithstanding numerous studies on the molecular mechanism of endostatin action, many questions remain unclear. Endostatin affected bFGF-induced signal transmission, and this inhibited the migration of endotheliocytes [39] and stimulated apoptosis [40] through inhibition of cyclin D1 and arresting the cell cycle of endotheliocytes in the G_1 phase [41]. Endostatin also blocked the VEGF-mediated signaling pathways through direct interaction with the VEGF-R2/KDR/Flk-1 receptor of the endothelial cells of human umbilical vein cell line HUVEC [42]. In the presence of endostatin, the TNF-α-induced activation of c-Jun N-terminal kinase (JNK) is inhibited. This results in suppression of the pro-angiogenic gene expression controlled by JNK [14]. Endostatin rapidly and negatively influences expression of many genes in proliferating endotheliocytes, including the "early response" genes, genes responsible for the cell cycle control, and also the genes of apoptosis inhibitors, mitogen-activated protein kinases (MAP kinases), and the focal adhesion kinase (FAK) [43].

Because various integrins (cell adhesion molecules connecting the cytoskeleton with the ECM and mediating cell translocation, invasion, proliferation, etc.) are involved in pathogenesis and progression of many diseases, including malignant tumors, studies on them is an attractive problem in the field of antitumor therapy [44]. Endostatin has been shown to bind with integrin $\alpha_5\beta_1$ on the surface of proliferating endotheliocytes, and this lowers cell migration. This effect is, in particular, caused by inhibition of signaling pathways mediated by small kinases Ras and Raf. This leads to decrease in the activities of MAP kinases ERK-1 (environment-responsive kinase-1) and p38 [45]. The interaction of endostatin with integrin $\alpha_5\beta_1$ on the endotheliocyte surface triggers integrin clustering, which is accompanied by $\alpha_5\beta_1$ colocalization with the membrane protein caveolin-1. The latter is involved in the transmission of regulatory signals from integrins and their ligands into the cytoplasm. Endostatin binds concurrently with integrin $\alpha_5\beta_1$ and caveolin-1, and this activates the caveolin-1-coupled kinases of the Src family [46, 47]. These nonreceptor tyrosine kinases are involved in the regulation of cell proliferation and differentiation and also of cell mobility [48, 49]. Integrins and Src kinases are closely coupled and provide for "outside-in" signals, into the cells contacting with ECM or to one another [50]. The activation of v-Src/s-Src kinases induces disruption of the actin cytoskeleton, destabilization of the cell focal contacts, and deceleration of the deposition by endotheliocytes of fibronectin matrix, which facilitates cell migration and adhesion during angiogenesis [51]. Fibronectin can be deposited only in the presence of stable focal adhesions of the cell. Such adhesions are also necessary for formation of actin fibers and for cell movement [47].

Thus, specific kinases and phosphatases with activities mainly depending on integrins regulate the organization of contacts between the cell membrane and ECM, i.e. regions of focal adhesion. Reorganization of actin filaments and cell migration are directly connected with the regulation of focal adhesion. In turn, the cell migration, in addition to proliferation and extracellular proteolysis, also determines the intensity of angiogenesis [52]. On binding with integrin $\alpha_5\beta_1$, endostatin more than twofold increases the kinase activity of a 60-kD Src-kinase coupled with caveolin-1. All this results in suppression of the cell–matrix interactions and mobility of endotheliocytes [47].

But some authors believe [53] that endostatin inhibits the chemotaxis of endotheliocytes not affecting the intracellular signaling pathways of regulation of migration and proliferation, because no endostatin influence has been detected on activities of phospholipase C-γ, Akt-protein kinase B, and MAP kinases p44/42 and p38. Endostatin action depended on the level of E-selectin exposition on the surface of endotheliocytes, although no direct binding of endostatin with E-selectin was observed [54].

Endostatin has been shown to bind with heparin [55] and also (although with low affinity) with all types of heparan sulfate proteoglycans [56, 57], which are involved in the regulation of the cell activity by growth factors. It seems that the anti-angiogenic activity of endostatin is caused by its interaction with heparan sulfate proteoglycans. In fact, the same specific arginine clusters located on the protein molecule surface are crucial in the interaction of human endostatin with integrin $\alpha_5\beta_1$ and heparin [58]. This sequence is thought to determine the inhibitory effect of endostatin on the migration of endotheliocytes [58, 59].

Some works concern identification of sites in the endostatin molecule that are responsible for anti-angiogenic activity. The endostatin molecule is a compact globule containing two disulfide bonds. Endostatin binds one zinc atom through three N-terminal histidine residues (His1, His3, and His11) and Asp76 [31, 57]. Oligomeric endostatin (trimer or dimer) binds mainly with laminin of the basal membrane [60]. This finding is important for understanding the biological activity of

endostatin. Endostatin is likely to realize a number of biological functions through different regions of its molecule.

The antitumor activity of endostatin is associated with the N-terminal sequence of 27 amino acid residues. A synthetic peptide corresponding to this sequence of endostatin has an anti-angiogenic activity that is inherent in the whole-size molecule. However, substitution of the histidine residues by alanines resulting in the loss of the Zn^{2+}-binding ability of this peptide, and consequently, also of endostatin, and completely eliminated this activity [61]. However, the question about the importance of zinc for the biological activity of endostatin remains under discussion. Thus, the substitution of His1 and His3 by alanines is considered [62] to abolish the inhibitory effect of endostatin on the progression of Lewis lung carcinoma (LLC) in mice. However, a mutant endostatin with deletions of five amino acid residues on the N- and C-ends manifested in mice with renal carcinoma Rc-9 antitumor activity comparable with the activity of the full-size protein [63]. In these works, both the models and schemes of endostatin application were different, and this could cause the different conclusions about the role of the Zn^{2+}-binding activity of endostatin.

Contradictory opinions on the role of Zn^{2+} in the biological activity of endostatin are published also in other works. The removal of four N-terminal amino acid residues (His-Ser-His-Arg) from the human endostatin sequence had no effect on the antitumor effect of endostatin [64]; moreover, this artificial endostatin bound two Zn^{2+} per molecule, whereas the native endostatin molecule bound ten zinc cations. According to work [31], one endostatin molecule binds with one zinc atom and the removal of four N-terminal amino acid residues results in the loss of the Zn-binding ability of the protein. The biological activity of endostatin was postulated [58] to depend on the sequence of 11 amino acid residues including several Arg residues and capable of binding heparin. Thus, further studies are likely to elucidate the interrelationship between individual sites of the polypeptide and its anti-angiogenic activity.

Endostatin inhibits activation and activity of some matrix metalloproteases (MMPs), which represent a family of calcium-dependent zinc-containing endopeptidases involved in tissue remodeling, in particular, during tumor progression [65]. The involvement of endostatin was reported in the inhibition of MMP-2, -9, -13, and MT1-MMP, and at least with two of these enzymes (MMP-2 and -9) endostatin interacted directly [66]. However, endostatin had no influence on the activation of MMP-8 [66]. Although endostatin is an inhibitor of MMPs, these endopeptidases seemed to be involved in the *in vivo* production of endostatin, because some MMPs were shown to generate different endostatin-containing polypeptides (20-30 kD) produced by proteolysis of human collagen XVIII [67]. A negative effect of endostatin on the plasminogen-activating proteases has also been reported [68], and this observation can partially explain the anti-angiogenic action of endostatin.

Endostatin suppressed the invasive activity not only of endothelial but also of cancerous cells in experiments on an artificially reconstructed mouse basal membrane, Matrigel. This effect was associated with inhibition of MMPs [69]. 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 metastasizing [5, 66]. During the intravasation, cancer cells penetrate across the blood vessel walls and enter the blood flow.

Endostatin is present in the body as a monomer and trimer, and the biological activities of these forms are quite different. As discriminated from the monomer, the endostatin trimer does not inhibit the migration of endotheliocytes but even stimulates it due to induction of dissociation and dispersion of these cells from capillary-like structures, as shown *in vitro* [70]. An artificial endostatin dimer has biological features similar to those of the trimer. The trimeric NC1-domain of the human collagen XVIII homolog in *C. elegans* (*cle-1*) induced migration of neurons, whereas the monomer inhibited it [71]. Endostatin can also exist as a soluble globule and also in an insoluble form with a high content of cross β-structures. This promotes aggregation of such endostatin form into intermolecular formations similar to amyloid β (Aβ) aggregations, which play the most important role in progression of Alzheimer's disease [72]. The insoluble form of endostatin can contribute to the activation of plasminogen under the influence of tissue-type plasminogen activator (t-PA). In addition, t-PA was shown to be really a multiligand receptor of the cross β-structures, and upon the t-PA binding with polypeptides of this conformation plasminogen was activated with production of plasmin. Insoluble endostatin was also shown to stimulate plasminogen activation, whereas the soluble globular endostatin had no influence on the activation. It was supposed that t-PA should play the major role in the extracellular degradation of proteins containing cross β-structures (e.g. inadequately folded) via induction of their proteolysis by plasmin. This mechanism is essential for preventing the deposition of protein aggregates on blood vessel walls [72].

A PROBLEM OF PREPARATION OF SOLUBLE ENDOSTATIN

Because of low concentration of endostatin in biological material, initial studies on the native protein prepared from the urine of tumor-carrying mice were limit-

ed by determination of the primary structure [2]. The therapeutic potential was studied on a recombinant endostatin prepared in *Escherichia coli*. For a long time preparation of soluble endostatin was an unsolved problem. It was shown earlier that the subcutaneous injection of mice with insoluble endostatin of toothpaste-like consistency produced an induration 2-3 mm in diameter, which dissolved within two to three days. Every day injections of endostatin resulted in the complete regression of tumor, but on completion of the endostatin injections, the tumor growth recommenced. Restarting the therapy again resulted in disappearance of the tumors in the mice [36]. These observations indicated the absence of drug resistance to endostatin and also of the lack of its toxicity even on injections for 100 days and more [12].

Effective approaches for preparation and purification with high yield of a biologically active soluble recombinant human endostatin in such producers as *E. coli* have been described recently [38, 40, 73]. In work [73], soluble endostatin was prepared in *E. coli* by co-expression of molecular chaperons and fermentation at low temperatures. The recombinant human endostatin was co-expressed using two groups of molecular chaperons (a trigger factor and GroEL-GroES (GroEL/ES) and also DnaK-DnaJ-GrpE and GroEL/ES). The co-expression was performed at 37, 25, and 16°C. Aggregation of the recombinant endostatin was maximally prevented, and the yield of the soluble product was ~36 mg per liter of the cultural medium under conditions of the biomass increasing at 16°C and endostatin co-expression with chaperons DnaK-DnaJ-GrpE and GroEL/ES [73].

However, the earlier attempts to prepare soluble human endostatin in *E. coli* were rather unsuccessful, and virtually all researchers now prefer working with the recombinant endostatin prepared in the yeast *Pichia pastoris* [12]. The aggregation of endostatin during its renaturation from inclusion bodies in *E. coli* is caused by production of occasional intermolecular disulfide bonds [61]. Some contradictions between results of the earlier and modern studies on biological features of endostatin can be due to the different nonspecific aggregation of the endostatin preparations under study, because virtually all works are performed with recombinant endostatin. Such nonspecific aggregation, which is characteristic for endostatin, decreases or perverts the biological activities of human or mouse endostatin preparations.

ROLE OF ENDOSTATIN IN EMBRYOGENESIS: FIRST RESULTS

Endostatin is clearly characterized by versatile biological activity and, possibly, it acts through a number of mechanisms. Recent publications complicated these problems still more. Thus, the effect of endostatin on embryogenesis was shown to be opposite to its influence on the tumor neoangiogenesis. Low concentrations of endostatin (50 ng/ml) stimulated the development of the vascular system of embryos [74]. Phenotypes of endothelial cells are very different depending on their histological macro- and microlocation in blood vessels and on the maturation stage [75]; therefore, effects of human and mouse recombinant endostatins have been studied on the HUVEC line endotheliocytes and on endotheliocytes of the eESC's line originating from differentiated stem cells, i.e., immature endotheliocytes [76]. Endostatin at all concentrations (50-2000 ng/ml) inhibited proliferation of the HUVEC cells, whereas the proliferation of the eESC's cells was inhibited by endostatin concentrations of 1000 and 2000 ng/ml, but the concentration of 50 ng/ml markedly increased the number of endotheliocytes ($142 \pm 12\%$ relative to the control, $p = 0.01$). The effect of endostatin on the endotheliocyte migration determined using a modified Boyden chamber assay, was also ambiguous. High concentrations of endostatin (1000 and 2000 ng/ml) inhibited the migration of the HUVEC cells, whereas 50 ng/ml had no effect. But endostatin at the concentration of 50 ng/ml increased twofold the migration of eESC's cells ($200 \pm 32\%$ compared to the control, $p = 0.02$), whereas its high concentrations inhibited the migration of these cells [76].

These results indicated that the effect of endostatin (pro- or angiogenic) depended, first, on the protein concentration and, second, on the type of endothelial target cells. The HUVEC cells are mature endotheliocytes, while the eESC's cells have an embryonal phenotype. The cited work is the first study on the *in vitro* antiangiogenic activity of endostatin on endotheliocytes generated from embryonal stem cells. Commenting on these findings, the authors of work [77] write that to better understand mechanisms of the endostatin action it is necessary to perform further studies on the biological activity of endostatin using the eESC's cell line, including its influence on the gene expression and determination of the type and number of endostatin receptors.

CIRCULATING ENDOSTATIN AND INFLUENCE OF DRUGS ON ITS LEVEL

Endostatin can be released from platelets under the influence of thrombin. Thrombin was shown [7] to induce endostatin release from rat platelets through activation of the PAR-4 receptor by an ADP-independent mechanism. Endostatin circulates in the blood flow of healthy persons, and its concentration in blood serum is 10-50 ng/ml. Note that therapeutically effective and suppressing tumor growth endostatin concentrations are 0.2-20 mg/ml. On the one hand, anti-angiogenic and antitumor effects of endostatin can be a pharmacological effect of its high doses unrelated with physiological functions of the protein [78]. This is supported by data on the absence

of influence of physiological levels of endostatin on the growth of fibrosarcoma and melanoma in mutant mice deficient in collagen XVIII and endostatin [5, 27]. On the other hand, the stimulated hyperexpression (no more than double physiological values) of endostatin in endotheliocytes can cause a two-to-threefold slowing tumor growth [79].

Elevated blood levels of endostatin are observed even during progression of some tumors [80]. A considerably increased level of circulating endostatin was described in patients with renal carcinoma [81]. Moreover, an increase in the serum endostatin level accompanied by thrombocytosis was significantly associated with a poor clinical prognosis in patients with non-small cell lung cancer [82].

Although gene therapy with endostatin of malignant tumors in mice and rats was successful in the majority of works [12, 83, 84], there are also some reports about the inefficiency of this treatment [85-87]. Most likely, this inefficiency was caused by a strong excess of the circulating endostatin over its curative doses. Doses below the therapeutically effective ones (the physiological level) or significantly above them were inefficient. This regularity was postulated by J. Folkman and experimentally confirmed for endostatin [61, 88] and another endogenous inhibitor of angiogenesis, interferon-α [4] (a biphasic (U-shaped) dose–effect relationships). The analysis of publications about endotoxin does not unambiguously answer the question of the molecular mechanisms of this phenomenon. It is only obvious that in every case the optimal therapeutic dose of endostatin can be chosen based on the tumor type and stage of its development and on other factors. In the overwhelming majority of cases, this dose is three-fivefold higher than the physiological level of endostatin. It is concluded that such an increase in the blood plasma endostatin concentration should be favorable for preventing the development of primary tumor foci and metastases.

In this connection, there are interesting pleiotropic effects of some drugs that considerably increase the content of circulating endostatin. Thus, prednisolone combined with salazosulfapyridine increased the level of endostatin in synovial fluid [89] and celecoxib – in serum [90]. The content of another inhibitor of angiogenesis, thrombospondin-1 (see below) was increased under the influence of doxycycline [91] and rosiglitazone [12]. The mechanisms of the influences of these drugs on endostatin level are unknown. Common features of prednisolone, salazosulfapyridine, celecoxib, and rosiglitazone are only their relatively low molecular weight and peroral introduction (in the above-mentioned studies). Because these molecules could not have a proteolytic activity, it was obvious that they influenced the tissue level of endostatin indirectly, stimulating its release from ECM or platelets. If this is so, the development of low-molecular-weight peroral inducers of serum endostatin or another endogenous angiogenesis inhibitor seems very promising for creation of a new group of efficient antitumor drugs. Such drugs would be promising as preventive agents in persons with oncological risk.

OTHER ANGIOGENESIS INHIBITORS PRODUCED FROM ECM PROTEINS

During searches for endostatin homologs, a 22-kD fragment of collagen XV was found, which was called restin, or endostatin-like fragment of collagen XV (EFC XV). EFC XV is a fragment of the NC1-domain of the α1-chain of collagen XV. The polypeptide is 70% homologous with endostatin and inhibits the migration of endotheliocytes without affecting their proliferation. In studies on the chorioallantoic membrane chicken membrane, EFC XV inhibited angiogenesis induced by bFGF or VEGF [92]. Used as a drug, EFC XV suppressed progression of renal carcinoma [93]. In the NC1-domain of collagen XV, similarly to the NC1-domain of collagen XVIII, a trimerization domain and hinge region are described, and the latter is more resistant to proteases in collagen XV than in collagen XVIII. However, as differentiated from endostatin, EFC XV does not bind Zn^{2+} or heparin [92].

A number of anti-angiogenic fragments originate from collagen IV. The α_1- and α_2-chains of collagen IV isolated from some sarcomas inhibited the proliferation of capillary endotheliocytes [94]. Arresten, a 26-kD fragment of the NC1-domain of the α_1-chain of collagen IV, inhibited the proliferation of endotheliocytes and suppressed angiogenesis *in vitro* and *in vivo*. However, even high doses of arresten did not affect the proliferation of malignant tumor cells [95]. Arresten interacted with integrin $\alpha_1\beta_1$ and prevented its binding with collagen I [95]. The biological activity of arresten could also be due to its interaction with heparan sulfate proteoglycans [95], because heparan sulfate proteoglycan could bind with the NC1-domain of the α_1-chain of collagen IV [96].

Synthetic peptides corresponding to the structure of a certain region of the NC1-domain of the α_3-chain of collagen IV (from the 183rd to the 205th amino acid residue) inhibited the *in vitro* proliferation of cells of melanoma and other tumors of epithelial origin [97]. Tumstatin, a 28-kD fragment of the α_3-chain of collagen IV, inhibited angiogenesis and suppressed the growth of human renal carcinoma and prostate carcinoma cells in mice [11]. The angiogenic activity of tumstatin is due to the N-terminal sequence from the 54th to 132nd amino acid residue capable of binding with the integrin $\alpha_v\beta_3$. The C-terminal region of the tumstatin molecule (amino acid residues 185-203), which also binds with the integrin $\alpha_v\beta_3$ is responsible for the cytotoxic effect of the protein on the cells of malignant tumors [98]. Tumstatin and endostatin are only 14% homologous. Nevertheless, tum-

statin suppressed angiogenesis through inhibition of the proliferation and induction of apoptosis of endotheliocytes not affecting their migration, whereas endostatin suppressed the migration of endothelial cells [5].

Anti-angiogenic activity in collagen IV is also inherent in canstatin, a 24-kD fragment of the NC1-domain of the α_2-chain [87], and in a ~25-kD fragment of the NC1-domain of the α_6-chain [99].

The 81-kD endorepellin, a C-terminal fragment of perlecan, inhibited the migration of endotheliocytes and angiogenesis in *in vivo* experiments. Endorepellin also suppressed the adhesion of endotheliocytes to fibronectin and collagen I without an immediate interaction with these matrix proteins [100]. Endorepellin also bound endostatin through the LG2-domain, and this abolished the angiogenic activity of endostatin [100]. Integrin $\alpha_2\beta_1$ acted as a receptor of endorepellin, and upon their binding an increase was observed in the cytoplasmic level of cAMP. Endorepellin and endostatin inhibited the migration of endotheliocytes by destruction of the actin cytoskeleton and destabilization of the adherence clitellums, but the intracellular signaling cascades of these proteins are different: endorepellin activated the enzymes FAK and MAP kinase p38, and the latter also phosphorylated the small heat shock protein HSP27 [101].

The anti-angiogenic activity is also described for fragments of some other proteins of the ECM and basal membrane, in particular, for fragments of fibulin 1D and fibulin 5 [102], fibrin [103], fibrinogen [104], and fibronectin [105]. The homology of these fragments usually is rather low, and mechanisms of suppression of proliferation and migration of endothelial cells are unknown in most cases. The common feature of these angiogenesis regulators is their being proteolysis products of the above-mentioned group of proteins. It should be noted that fragments of some other proteins also display the anti-angiogenic activity not inherent in the initial molecule: such are angiostatin and other fragments of plasminogen [5], vasostatin, which is the N-terminal domain of calreticulin [106], prolactin fragments [107], etc. However, the group of angiogenesis inhibitors of matrix origin is much more numerous.

Moreover, not only fragments but also intact proteins of the ECM display anti-angiogenic activity [108]. A multifunctional glycoprotein of ECM, thrombospondin-1, was among the first detected endogenous angiogenesis inhibitors [109]. Thrombospondin-1 seems to possess both pro- and anti-angiogenic activity depending on its exposure to certain proteases [110], although mice deficient in this protein have an increased vascularization of skin and Langerhans islets [111]. Thrombospondin-2 also inhibited *in vivo* angiogenesis and tumor growth [112]. The proteoglycan decorin of the ECM also inhibited the migration of endothelial cells and their ability for producing tube-like structures, bound with thrombospondin-1, and elevated the anti-angiogenic activity of the latter [113].

However, the ECM proteins fibronectin, various collagens, laminin, and vitronectin not only inhibit angiogenesis, but in some cases stimulate it. Thus, fibronectin from the fraction circulating in the blood plasma can be deposited in many tissues and form a kind of fibronectin matrix. Fibronectin is also synthesized and released into the intercellular space by smooth muscle cells of blood vessels and also by endotheliocytes [114]. The fibronectin matrix provides for adhesion, proliferation, and migration of endotheliocytes. Consequently, it is necessary for angiogenesis; the absence of fibronectin enhances apoptosis of endotheliocytes during angiogenesis [115]. Fibronectin also directly interacts with VEGF and potentiates the VEGF-induced migration of endotheliocytes and activation of MAP kinases [99]. Therefore, preventing the deposition and formation of the fibronectin matrix or its destruction leads to a dramatic decrease in endotheliocyte proliferation. As mentioned, endostatin suppressed the fibronectin matrix formation by endotheliocytes via activation of Src-kinases.

INTERCELLULAR MATRIX AND ANGIOGENESIS: TOWARDS UNDERSTANDING THEIR INTERACTIONS

In total, ECM molecules play one of key roles in the proper regulation of angiogenesis. ECM remodeling under the influence of various proteases can display both pro- and anti-angiogenic effect [99]. Proteases promote angiogenesis, first, due to destruction of the ECM and, second, through releasing pro-angiogenic factors sequestered in the ECM, such as bFGF and heparin-binding VEGF isoforms [116, 117]. Moreover, plasmin manifesting a number of pro-angiogenic effects can directly induce the migration of endotheliocytes due to activation of receptors of the PAR family [118]. At the same time, various proteolytic fragments of ECM proteins suppress angiogenesis inhibiting the proliferation or migration of endothelial cells or reducing their capability of producing vessel-like structures.

Elucidation of angiogenesis regulation on the level of cell–matrix interactions will promote not only better understanding tumor growth biology, but also facilitate progress in searching for new drugs, because many angiogenesis inhibitors, including endostatin, are now under clinical investigation in some countries. Studies on physiological angiogenesis resulted in the discovery of a phenomenon known as “vascular pruning”. The essence of this phenomenon is a strict regulation of angiogenesis by the balance of pro- and anti-angiogenic factors depending on the stage of the organism’s development. The initially induced proliferation and migration of endotheliocytes accompanied by other angiogenic processes lead to formation of functioning vessels with the endothelium consisting of mature cells [119]. Endotheliocytes stimu-

lated by pro-angiogenic factors change their phenotype, and this is manifested by increase in proliferation, integrin expression, migration, and other features favorable for angiogenesis. Such endotheliocytes are highly sensitive to inducers of apoptosis, whereas endotheliocytes from preexistent and already formed vessels are rather resistant to apoptotic stimuli, similarly to cells of post-angiogenic vessels with mature endotheliocytes [120]. Until a certain moment, no apoptosis of activated endotheliocytes occurs, and the available mitogens and ECM proteins, including the fibronectin matrix, are favorable for cell survival. Even a slight disorder in the coordinated angiogenesis (e.g. blockage of signalization from adhesion receptors) can induce apoptosis of endotheliocytes [120, 121] and degradation of a whole vessel during its formation. Thus, apoptosis is responsible for the "vascular pruning" through a severe regulation of angiogenesis under natural conditions, such as the development and maturation of follicles in the ovaries [122]. The increase in sensitivity of mitogen-stimulated endotheliocytes to apoptotic stimuli is explained by activation of the homeobox-containing genes, especially *HoxD3* and *HoxB3* [123].

Endotheliocytes are connected with the ECM through different adhesion molecules, among which integrins play the key role. Various integrins are involved in the promotion of apoptosis if the leading edge of the cell fails to meet the corresponding matrix providing for the migration or differentiation of endotheliocytes. Thus, integrin $\alpha_v\beta_3$, unbound with the ligand or bound with an individual molecule of the ligand, i.e. with the ECM-uncoupled ligand, can induce apoptosis of the cell [124]. In other words, such ligands of integrins as collagen IV or collagen XVIII will facilitate angiogenesis and increase the survival of activated endotheliocytes, whereas fragments of these ECM proteins, canstatin or endostatin, induce apoptosis of the same endotheliocytes. There is no common viewpoint on mechanisms of the anti-angiogenic action of endostatin, and the extremely manifold effects of this protein on different cells described in the continuously increasing number of publications do not allow us to adopt the above-mentioned "integrin" pathway of induction of endotheliocyte apoptosis as the major factor of the antitumor potential of endostatin. Nevertheless, the finding that many poorly homologous angiogenesis inhibitors of matrix origin display anti-angiogenic activity through interaction with different integrins has been already called "revolution" [17].

Endostatin binds with integrin $\alpha_5\beta_1$, in particular, through activation of Src-kinases and inactivation of RhoA GTPase, and induces a destruction of bundles of active microfilaments involved in cell movement and maintaining focal adhesions [125]. Arresten interacts with integrin $\alpha_1\beta_1$. Tumstatin binds with integrin $\alpha_v\beta_3$ through a certain N-terminal sequence, and integrin $\alpha_2\beta_1$ acts as a receptor of endorepellin. These data suggest that in the complicated system of angiogenesis regulation integrins and C-terminal NC1-domains of many ECM and basal membrane proteins play the key role. However, it should be elucidated, especially for endostatin, what role is played by other membrane and extracellular receptors in the induction of the intracellular signalization in endotheliocytes. Nevertheless, although it is still far to description of a full pattern of the cell–matrix interactions, there is definite progress in the establishment of the structure and origin of a number of endogenous angiogenesis inhibitors of matrix origin.

Among endogenous angiogenesis inhibitors, endostatin attracts the greatest attention of researchers because of its prospects as an antitumor drug. It is possible that further investigations will show the efficiency of endostatin also in other diseases and reveal a whole picture of endostatin action on endotheliocytes at different stages of development.

Studies on endostatin are also useful for enlarging our concepts about ECM functions. Some of these functions are obviously directly associated with the regulation of angiogenesis. Endostatin and other proteolytic fragments of the ECM proteins and also the intact matrix proteins are involved in this regulation. We may expect impressive discoveries of various new functions of angiogenesis inhibitors during embryogenesis. Studies in this tempting field are only starting. The explanation in detail of the recently found functions of the ECM associated with cellular signalization is also of help in understanding the pathogenesis of such diseases as cancer, rheumatoid arthritis, atherosclerosis, etc. All this is a reason for urgent interest in endostatin of biologists and physicians all over the world.

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