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Review*

Following Angiogenin during Angiogenesis: a Journey from the Cell Surface to the Nucleolus

ANTONI WIĘDŁOCHA

Department of Biochemistry, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway

Abstract. Angiogenin is a potent inducer of new blood vessel formation. It binds to high-affinity endothelial cell-surface receptors and, with lower affinity, to extracellular matrix. Angiogenin is the only angiogenic factor known to exhibit ribonucleolytic activity. It belongs to the pancreatic RNase superfamily of proteins. Angiogenin is the only member of the superfamily able to stimulate angiogenesis. Although the catalytic activity of the protein is rather weak, it is critical for its angiogenic properties. Angiogenin is specifically endocytosed by endothelial cells and transported to the nucleus, where it accumulates in the nucleolus. Also, the nuclear location of the angiogenic factor appears to be necessary for its angiogenic activity. The mechanism of action of the protein seems to be unusual, since it does not fit into the current paradigm of how exogenous regulatory polypeptides, including other angiogenic factors, work. Here, the role of transport of angiogenin from the cell-surface into the nucleolus and of its intracellular/nuclear mode of action in stimulation of angiogenesis is discussed.

Key words: angiogenesis; angiogenin; signaling; nuclear transport; nucleolus.

Complexity of Angiogenesis

Angiogenesis is the growth of new blood vessels, namely the formation of new capillaries from existing ones[12, 37]. This process is fundamental for differentiation during embryogenesis and organ development, and in the healthy adult organism for processes involved in wound healing and formation of the endometrium, corpus luetum and placenta. Normal angiogenesis is self-limiting and strictly regulated. Persistent unregulated angiogenesis occurs under pathological conditions during the progression of inflammatory arthritis, diabetic retinopathy, psoriasis and cancer. It is very well documented that the invasive phase of solid tumor development (rapid growth and ability to form metastases in different organs) begins when new blood vessels appear in the primary tumor, i. e. the transition from the pre-vascular to the vascular phase of tumor growth[10, 12]. Angiogenesis starts by local degradation of the vascular basement membrane and interstitial matrix by endothelial cells. Then endothelial cells migrate in the direction of the angiogenic stimulus. Subsequently, they proliferate and form new capillary loops.

Angiogenesis depends upon a balance of angiogenic stimulators (e. g. VEGF, bFGF, aFGF, angiogenin, TNF-α and TGF-(α/β) and inhibitors (e.g. trombospondin-1, interferon α/β, platelet factor-4, 16 kDa N-terminal fragment of prolactin, angiostatin and endostatin)[12]. The new blood vessel formation is actually a complex of many processes involving a number of growth factors and cytokines responsible for differentiation, proliferation, migration, adhesion and other events that are necessary for angiogenesis to occur[37]. Moreover, all of these processes often have to act in a changing micro-

* This review is dedicated to the memory of Prof. Anna D. Inglot.

enviroment of surrounding tissues. Their significant role in the regulation of angiogenic processes has been established in different *in vitro* and *in vivo* experimental models, including athymic, transgenic and knockout mice[12].

Angiogenin and Angiogenesis

Angiogenin has been recognized as a tumor-associated angiogenic factor. Originally it was found in conditioned culture medium from human colon adenocarcinoma cells as a potent inducer of new blood vessel formation in the chicken embryo chorioallantoic membrane (chick CAM assay) and rabbit cornea[45]. In addition, results of anti-angiogenin experimental therapy have shown that it is an *in vivo* angiogenic factor[8, 9, 32]. It was found that the protein could easily stimulate neoangiogenesis in tumors secreting it. It should be noticed that the concentration of angiogenin and the level of its mRNA are increased in cells of certain histological types of human tumors[34]. Under physiological conditions, angiogenin is present in milk and circulates in human plasma at a concentration of about 300 ng/ml[15, 18]. Its turnover rate is very fast, with a half-life <5 min. The high concentration of the protein in human plasma is somewhat unexpected. Angiogenin can induce most of the events necessary for the formation of new blood vessels. It binds specifically to endothelial cells and stimulates cell migration and invasion. The protein is able to promote cell proliferation and differentiation, and it also mediates cell adhesion and activates cell-associated proteases[13, 15, 17]. The angiogenic factor induces plasminogen activator and, thereby, the plasmin system promoting migration and tubular morphogenesis of endothelial cells.

Human angiogenin has been characterized as a heparin-binding protein as well. Studies performed on HT-29 human colon adenocarcinoma cells and angiogenin suggest that the protein could also be a substrate for tumor cell adhesion[42]. It can bind to the tumor cell proteoglycans and to endothelial cells as well as to their extracellular matrix, forming a connection between tumor cells and the endothelium.

Angiogenin is a 14.1 kDa, basic protein, which is a member of the pancreatic ribonuclease superfamily[44]. Human angiogenin resembles human pancreatic RNase: their amino acid sequences are about 35% identical, including the active site residues[1, 44]. Angiogenin is the only member of the superfamily exhibiting angiogenic activity. The enzymatic, ribonucleolytic activity of angiogenin is relatively weak, but it is essential for its ability to induce angiogenesis[11]. Exogenous angiogenin is transported into the nucleus of endothelial cells[28]. The nuclear translocation results in accumulation of the protein in the nucleolus. The ability of angiogenin to be transported from the cell surface into the nucleus and then to the nucleolus appears to be necessary for its angiogenic activity. These observations concerning the nuclear localization and ribonucleolytic activity make the mechanism of action of the protein very unusual in comparison to many other exogenous regulatory polypeptides able to induce signal transduction[13, 14, 31].

Specific Receptors and Signaling

Angiogenin binds specifically to the cell-surface of endothelial cells. Beside proteoglycans, the protein interacts with two specific binding sites/receptors: 1) endothelial cell surface smooth muscle-type α/actin (or an α-actin-like protein) (42 kDa, $K_d = 5\times10^{-10}$ M)[16, 19, 27] and 2) an endothelial cell-surface 170 kDa polypeptide (K_d not measured)[18, 44]. The 170 kDa membrane-associated receptor appears on human endothelial cells when they grow in very sparse cultures. It should be noticed that, although angiogenin as such is a rather weak stimulator of cell proliferation *in vitro*, it is able to increase the level of both DNA synthesis and proliferation of endothelial cells, but mainly in sparse cell cultures (20–25% over control). Only under such conditions is the 170 kDa protein detectable and does angiogenin exhibit specific binding to it[18]. Actin, anti-actin antibody or short peptides resembling angiogenin binding sites on actin inhibit angiogenesis induced by angiogenin in the chick CAM assay[9, 19, 45]. They also suppress the establishment of human distinct tumor cells implanted into athymic mice as efficiently as monoclonal anti-angiogenin antibody[9, 10, 34]. Actin and anti-actin antibody also inhibit the nuclear localization of angiogenin[28]. This suggests that the actin or actin-like protein found at the cell surface of endothelial cells is a functional angiogenin signal-mediating receptor. Although none of these receptors are similar to receptors of other known growth factors and cytokines able to induce proliferation or differentiation of endothelial or other cells, the data available demonstrate that the observed biological activity of angiogenin can be mediated by these two proteins. The role of 170 kDa receptor in mediating angiogenin activity *in vivo* is not clear and is under investigation. Angiogenin contains two sites for specific binding to actin/actin-like protein, namely residues 58–70 and 108–111[19, 36]. It should be noticed that these residues are essential for its angiogenic activity, since mutated molecules with

changes in these short sequences do not bind to actin or actin-like protein at the endothelial cell-surface and they are not active *in vitro* or *in vivo.* It must also be mentioned that it is not clear at all how actin can be localized at the cell surface and that this is still controversial.

It was also reported that copper and zinc increased binding of the angiogenic factor to endothelial cells[41]. Metal-ion affinity chromatography and atomic absorption spectrometry showed the direct interaction of the protein with copper and zinc ions. Since copper is a known modulator of angiogenesis *in vivo*, it may be engaged in the regulation of angiogenin activity.

Binding of angiogenin to the cell surface of endothelial cells leads to activation of phospholipase C (PLC)[15, 26] and phospholipase A2 (PLA2)[3]. Activation of PLA2 by angiogenin also results in increased secretion of prostacyclin by endothelial cells.

Nuclear Transport

It has been demonstrated in immunofluorescence studies with anti-human angiogenin monoclonal antibody that angiogenin is transported to the nucleus when it is added to *in vitro* growing endothelial cells[28–30]. After binding to the cell-surface, human angiogenin is internalized by receptor-mediated endocytosis and is then localized in the nucleus (finally accumulating in the nucleolus) of the proliferating (subconfluent) endothelial cells. No nuclear angiogenin is detectable in the nuclei of nonproliferative (confluent) cells. This indicates that the transport of angiogenin to the nucleus/nucleolus is specific to proliferating endothelial cells and does not occur in confluent, nonproliferating cells. Binding of exogenous angiogenin to specific binding sites at the cell surface is crucial for its endocytosis and nuclear localization. Thus, angiogenin mutants unable to bind specifically to endothelial cell-surface receptors are not internalized and are not detectable in the nuclei of treated cells[23, 28, 29].

Angiogenin contains a signal in its amino acid sequence mediating import of the molecules into the nucleus. Such a signal, called the nuclear localization sequence (NLS), was found at the N-terminal end of human angiogenin – residues 31–35: RRRGL[29]. The angiogenin NLS peptide (aNLS) is similar to other NLS peptides found in many nuclear proteins[25]. When linked to non-nuclear proteins, it is able to direct these proteins towards the nucleus[29]. The import of angiogenin from the cytosol to the nucleus is a saturable process, because the synthetic aNLS peptide as such inhibits the nuclear transport of angiogenin and albumin-aNLS conjugate when added in excess to digitonin-permeabilized CPAE and HUAE cells[28, 29]. It might be concluded that this inhibitory effect is due to titering out a cytosolic nuclear import-mediated receptor by synthetic aNLS peptides. Thus, the nuclear import receptor could not bind to the cargo (angiogenin) via its NLS in order to bring it into the nucleus. Such a mechanism based on a NLS-receptor has already been described for different nuclear proteins containing different types of NLS[25]. Moreover, when the R33A mutant of the aNLS peptide was used instead, the nuclear import of angiogenin and reported non-nuclear aNLS-containing protein was not affected[29]. As expected, the R33A mutant of angiogenin was not imported to the nucleus, either. However, when the wild type of aNLS was crosslinked to the angiogenin R33A mutant, the nuclear translocation and nucleolar accumulation were restored[29]. Therefore, it appears that angiogenin NLS belongs to the strong and classical type of NLS[20, 25]. Deletion mutagenesis and reporter localization studies with aNLS have defined the residue R33 as essential for nuclear import. This residue is conserved in all species analyzed. Since the aNLS is able to transport non-nuclear proteins also to the nucleolus, it shows that the angiogenin nuclear localization signal is a nucleolus targeting sequence as well[23, 28, 29]. An important observation was the finding that translocation of angiogenin to the nucleus in permeabilized endothelial cells does not occur at 4°C[29]. This demonstrates that the import of the angiogenic factor to the nucleus is most likely an energy-dependent active transport mechanism, and that it does not enter the nucleus by free diffusion, which in principle could be the case, because of the low molecular weight of the protein (ions, metabolites and macromolecules smaller than 60 kDa can cross the nuclear envelope by diffusion through channels provided by nuclear pore complexes, the only known gates for nucleocytoplasmic transport, located in the nuclear envelope[20]). All these findings imply that the import of angiogenin from the cytosol to the nucleus is NLS-dependent, carrier-mediated and energy-dependent, like a large number of karyophilic macromolecules (proteins and ribonucleoparticles) actively transported into the nucleus[25]. Interestingly, it was recently reported that activation of PLC is required for the nuclear translocation of angiogenin, but not for its internalization[15]. Evidence obtained in these studies also suggests that inhibition of PLC might lead to accumulation of the protein in the cytosol with simultaneous decrease in nuclear angiogenin. This indicates that import of the protein from the cytosol to the nucleus is regulated by angiogenin itself, since it activates PLC by binding to

endothelial cell-surface receptors. So far, PLC is the only known enzyme able to regulate nuclear translocation of the angiogenic factor. Inhibitors of tyrosine kinases, phosphotyrosine phosphatases and protein kinase C (PKC) did not inhibit nuclear translocation of angiogenin[15], but they can block the nuclear import of a number of proteins involved in gene activation and transcription[20, 23].

In nonpermeabilized endothelial cells, nuclear angiogenin was detectable when the angiogenic factor was added to the cells at concentrations of 50–100 ng/ml. At these concentrations, the protein appeared in the nucleus after 5–10 min and reached saturation level after 30–60 min of incubation at 37°C[23, 28]. This rapid kinetic of the nuclear localization process of angiogenin observed in immunofluorescent studies indicates an efficient transport mechanism of the protein from the cell-surface to the nucleus or nucleolus.

Since angiogenin is localized in the nucleus when it is externally added to cells, the question arises as to where the protein crosses the membrane in order to gain access to the cytosol. Receptor-mediated endocytosis and NLS-mediated nuclear import of angiogenin are believed to be the first and last steps in the transport of the protein from the cell surface into the nucleus. It should be mentioned that transport of exogenous angiogenin to the nucleus of unpermeabilized endothelial cells was not affected by agents (colchicine, nocodasole or taxol) disrupting the microtubular part of the cytoskeleton[23, 28]. This observation seems to be significant, since transport of endocytosed ligands to early endosomes does not require an intact microtubule network, whereas transport to late endosomes and lysosomes does. Therefore, it may be concluded that translocation of angiogenin across the membrane takes place during the early steps of the internalization process, since the transport of the protein from the cell-surface into the nucleus does not depend on the cellular membrane traffic mediated by microtubules. This suggests that the early endosomes may be the place where the protein crosses the membrane in order to enter the cytosol. Treatment of endothelial cells with lysosomotropic agents such as chloroquin, ammonium chloride and bacitracin, which decrease the degradation potential of lysosomes by increasing the internal pH, or by leupeptine, an inhibitor of the main lysosomal protease cathepsin B, did not significantly change the efficiency of nuclear transport of endocytosed angiogenin[23]. Thus, lysosomal functions are most likely not involved in this process. Although these data provide some evidence that translocation of angiogenin to the cytosol takes place in early endosomes, direct proof remains to be obtained. It is not excluded that cytoskeleton-independent vesicular transport between different compartments, including the nucleus can exist. In any event, wherever and however angiogenin crosses the membrane or is delivered by intracellular vesicles directly to the nucleus, it must be a strictly regulated process, because the protein can be cytotoxic when provided in excess into cells[39].

Intranuclear Mode of Action

Although the catalytic site of the angiogenic factor is not required for nuclear localization to occur, it is critical for its angiogenic properties. Angiogenin is a ribonucleolytic enzyme, but its specificity and potency differ markedly from other members of the pancreatic ribonucleases[1, 11, 44]. It is probably due to the lack of one of the four-disulphide loops of RNase A. Instead, the corresponding residues of the angiogenic factor form part of the cell-surface binding region[1, 11, 36]. A number of studies have provided evidence that the catalytic activity of angiogenin is a crucial component of the molecular mechanism of how angiogenin induces neovascularization. A specific substrate for angiogenin has still not been identified. On the other hand, angiogenin, or its proteolytic fragment containing mainly the catalytic site both show ribonucleolytic activities toward tRNA[39], 28S and 18S rRNA and dinucleoside phosphates[11, 44, 45]. Therefore, it seems reasonable to believe that the putative substrate is localized in the nucleolus, where angiogenin is finally located. It should be noticed that the nucleolus not only contains ribosomal RNA, but, as has recently become evident, several nonribosomal RNA processing and assembly functions are also located there. If so, it is not excluded that a different kind or kinds of RNA present in this intranuclear structure might be a potential specific target for the catalytic action of angiogenin. It must be kept in mind that angiogenin mutants with destroyed NLS have full enzymatic activity and the capacity to bind to their receptors at the cell surfaces of endothelial cells. They are endocytosed (and possibly translocated into the cytosol), but they do not localize in the nucleus/nucleolus and they do not induce angiogenesis in the chick CAM assay[28, 45]. Together, this demonstrates that the ribonucleolytic activity of the protein acting in an appropriate intracellular location might be required for its angiogenic activity.

Evolutionary Implications

The transport of regulatory polypeptides from the exterior of the cell into the cytosol/nucleoplasm has

been controversial, since it does not fit well into the current paradigm of how growth factors or growth factor-related proteins work[13, 14, 31]. These proteins have been recognized as regulatory agents operating at the cell surface inducing phosphorylation cascades and other second messengers without the capability of penetrating through the hydrophobic barrier of the cellular membrane to enter the cytosol/nucleoplasm, where they could exert additional activity. Consistent with this, a ligand-receptor complex is internalized by receptor-mediated endocytosis and finally degraded in the lysosomal compartment or recycled back to the cell surface. Apart from bacterial and plant protein toxins[24], there are only a few examples of exogenous proteins (see below), where transport from the cell-surface into cells has been convincingly proved, although in most cases the molecular mechanisms as well as the place of membrane translocation are not well defined. Recently, an increasing amount of evidence has been accumulated demonstrating that some growth factors or growth factor-like proteins can, at least in certain cells, be transported from the cell-surface into the nucleus (aFGF, bFGF, IL-1α, Schwannoma derived growth factor, HIV-Tat protein, *Antennapedia* homeoprotein, lactoferrin and heregulin are some examples)[2, 5, 22, 35, 38, 40, 43, 46–48]. This indicates that some proteins can act in different cellular compartments and likely exhibit distinct action therein.

It has already been hypothesised that the transport of different angiogenic factors into the nucleus/nucleolus of endothelial cells is an essential step in the mechanism of angiogenesis[28, 30]. According to this hypothesis, angiogenic factors not only signal to the nucleus by the activation of intracellular messengers[31], but also by the direct transport of themselves into the nucleus[30]. However, among angiogenic polypeptides, only in the cases of bFGF, aFGF and angiogenin has nuclear transport been convincingly shown[38]. So far, there is no convincing evidence that VEGF, TNFs, TGFs and others can be transported from the exterior of the cell into the nucleus. Therefore, the nuclear transport appearing as specific to a few angiogenic factors is not easy to understand in the context of current knowledge concerning signal transduction as well as protein membrane translocation mechanisms. Apparently, certain growth factors and related polypeptides are able to make such a journey from the cell-surface to the nucleus, but the rest of them probably are not. If so, it is tempting to speculate that this may reflect a step in evolutionary development from intracellular factors operating in the nucleus, targeting directly from the cytosol to some regulatory sites on the genome, for instance, to exogenous growth factors and cytokines. Such a fully intracrine pathway of cellular (intracellular) signaling has been recognized in the case of the single-cell eukaryote[33]. Therefore, those polypeptides which are able to enter cells and, in parallel, induce phosphorylation cascades as a consequence of activation of specific cell-surface receptors might represent a sort of intermediate in evolution from fully intracellularly acting proteins to exclusively extracellularly operating growth factors, cytokines and protein hormones, which have lost the capability of being transported to the nucleus. Polypeptide agents with such a dual mode of action might still be well integrated into the regulatory systems present in a highly organized multicellular organism. Although the proposed phylogenesis of polypeptide growth factors is very speculative, it might be interesting to consider it, because it may help to understand why some of the exogenous regulatory proteins can be detected in nuclei of mammalian cells. Perhaps nuclear transport is not a necessary event in the stimulation of angiogenesis for all of the known angiogenic factors (since they might be able to evoke similar effects using different mechanisms). Finally it should be noticed and considered that: 1) HIV-Tat protein and *Antenapedia* homeoprotein are examples of leaderless secretory transcription-like factors, which have been shown to reach the nucleus of target cells when added exogenously[38]; 2) aFGF can enter the nucleus as an endogenous and as exogenous protein. This growth factor contains a NLS, and at least its import from the cytosol to the nucleus is dependent on it. The transport of aFGF from the cell-surface to the nucleus requires binding to FGF tyrosine-kinase receptors and the presence of the aFGF NLS as well. Binding of the growth factor to specific receptors also induces phosphorylation cascades and expression of early genes[46–48]; 3) bFGF is synthesised in four different forms, generated by alternative initiation of translation (18, 20, 22, 24 kDa)[5]. Only the 18 kDa bFGF is released from cells producing it and, as an exogenous protein, it also binds to the FGF tyrosine-kinase receptor, activating second messengers and the transcription of early genes, while high molecular forms of the growth factor are detectable exclusively in the nucleus as endogenous proteins. The 18 kDa form of bFGF is also transported to the nuclear compartment, but only as an exogenous protein, so export from the cell, prior to nuclear transport, seems to be required for its nuclear localization[2, 43]. An interesting feature of aFGF and bFGF as well as of HIV-Tat and *Antenapedia* homeoprotein is that all of them are produced as leaderless proteins, like other cytosolic and nuclear polypeptides. Therefore, the export mechanism for these poly-

peptides appears to be different from the classical ER-Golgi secretory pathway[6, 7, 21].

It seems that angiogenin might also be included in this category of exogenous regulatory polypeptides with an intracellular mode of action. Because of its enzymatic activity[32] and threedimensional relationship to ribonuclease A[1], the protein might be another important example of the origin of extracellularly acting growth factor-like polypeptides from fully intracellular/nuclear forms.

A Possible New Therapeutic Target

It is believed that neoangiogenesis can be a very promising target for anticancer therapy that might protect against the invasive phase of tumor development[4, 10]. Since angiogenin is an important player in the regulation of the growth of new blood vessels, it is therefore, reasonable to utilize the intracellular steps of angiogenin action as new targets for the therapeutic purpose of preventing pathological angiogenesis. The study already mentioned[15] can be an example of such an approach, although additional control experiments and techniques that are more advanced concerning detection of nuclear angiogenin could still improve it. Hence, as was reported, an aminoglycoside antibiotic, i. e. neomycin, interfering with the activity of PLC, was able to inhibit angiogenin-induced angiogenesis in the chick CAM assay through its inhibition of the nuclear translocation of angiogenin in endothelial cells. Neomycin did not inhibit the ribonucleolytic activity of the protein even at high concentration (50 μM), in the presence of which the angiogenic activity was already abolished. In the CAM assay 20 ng of the antibiotic per egg inhibited *de facto* angiogenin-induced angiogenesis. This indicates that the mechanism of the nuclear transport of angiogenin is a promising therapeutic target and it should not encounter the difficulties associated with circulating angiogenin. On the other hand, the quoted studies may indicate that it is possible to develop neomycin and/or its analogs as anti-angiogenin therapeutic agents.

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