Current Topics

Aminopeptidases in Health and Disease

Role of Aminopeptidase in Angiogenesis

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Microvessels are composed of endothelial cells and surrounding pericytes. Angiogenesis, a neo-vessel formation from pre-existing microvessels, is a complex phenomenon, which requires following sequential steps: detachment of pre-existing pericytes for vascular destabilization, extracellular matrix turnover, migration, proliferation, tube formation by endothelial cells (ECs), and reattachment of pericytes for vascular stabilization. Aminopeptidases regulate the N-terminal modification of proteins and peptides for maturation, activation or degradation, and thereby relate to a variety of biological processes. Recently, three aminopeptidases have been reported to be involved in angiogenesis. They include type 2 methionine aminopeptidase, aminopeptidase N, and adipocyte-derived leucine aminopeptidase/puromycin insensitive leucyl-specific aminopeptidase. This review will focus on the possible role of these aminopeptidases in angiogenesis.

Key words aminopeptidase; endothelial cell; angiogenesis

GENERAL CONCEPT OF ANGIOGENESIS

The vascular system is the first functional organ that starts to develop in the embryo. The extraembryonic mesodermal cells, so-called hemangioblasts, aggregate in the yolk sac to form blood islands, where they differentiate into an external endothelial cell (EC) layer and an inner core of blood cells. The external ECs subsequently constitute the primary capillary plexus. Shortly thereafter, the intraembryonic mesodermal hemangioblasts and angioblasts located at the proximal lateral mesoderm differentiate and organize into the dorsal aorta. These processes are known as “vasculogenesis”. In the following process of “angiogenesis”, neo-vessels are generated from the primary capillary plexus by sprouting or intussusception and become distributed throughout the body. In the final process of vascular development, mesenchymal cells differentiate to mural cells (smooth muscle cells and pericytes), surround blood vessels, and make them mature and stabilized. In the adult, ECs on the inner surface of blood vessels are normally quiescent and play an important role in maintaining the integrity of the vessels. However, these cells in microvessels do have the ability to form neo-vessels.¹⁾

Angiogenesis is regulated by the balance between angiogenic factors and angiogenesis inhibitors. Many factors are reported to regulate angiogenesis. Among them, endothelium-specific factors and their receptors expressed on ECs have been extensively studied. They include vascular endothelial growth factor (VEGF) family members and their receptors, and angiopoietins and TIE receptors. VEGF receptors include VEGF receptor-1 (VEGFR-1, also known as Flt-1), VEGF receptor-2 (VEGFR-2, also known as KDR/Flk-1), VEGF receptor-3 (VEGFR-3, also known as Flt-4), neuropilin-1 and neuropilin-2. VEGFR-1 and VEGFR-2 are expressed on vascular endothelium, whereas VEGFR-3 is preferentially expressed on lymphatic endothelium in the adult. Neuropilins, originally identified as regulators of axon guidance, are also expressed on ECs and act as co-factors of

VEGF receptors. VEGF family members are VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF). VEGF-A binds to VEGFR-1 and VEGFR-2, VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3, and PlGF and VEGF-B bind only to VEGFR-1. Among these VEGF family members, VEGF-A seems to be the most important factor for angiogenesis, stimulating protease synthesis in and migration and proliferation of ECs; and most of the VEGF-A-mediated signals are transduced *via* VEGFR-2.⁸⁾ Angiopoietins are ligands of the TIE-2 receptor. There are 4 members of angiopoietins, and are classified into 2 subgroups. Angiopoietin-1 (Ang-1) and angiopoietin-4 (Ang-4), comprising one subgroup, are agonistic ligands of the TIE-2 receptor; whereas angiopoietin-2 (Ang-2) and angiopoietin-3 (Ang-3), in the other subgroup, exhibit very weak activity and are thought as antagonistic ligands of the TIE-2 receptor.²⁾

The most common sprouting-type angiogenesis includes following sequential steps: detachment of pre-existing pericytes for vascular destabilization, extracellular matrix (ECM) degradation by endothelial proteases, migration of ECs, proliferation of ECs, tube formation by ECs, and reattachment of pericytes for vascular stabilization.³⁾ Hypoxia is one of the most important triggers of angiogenesis, which induces the expression of VEGF-A in various cell types.⁴⁾ The resultant VEGF-A stimulates ECs and induces the expression of various genes including Ang-2.⁵⁾ Ang-2 is an antagonistic ligand of TIE-2, and that makes the detachment of pericytes from the vascular wall for vascular destabilization. Consecutively, VEGF-A stimulates ECs to form neo-vessels by promoting protease synthesis for ECM degradation, migration, and proliferation. Ang-2 itself does not stimulate angiogenesis, but enhances the angiogenic activities of VEGF-A when combined with it. ECs of neo-vessels produce platelet-derived growth factor (PDGF), which attracts pericytes to the neo-vessels. The attracted pericytes produce Ang-1.⁶⁾ When Ang-1 synthesized by pericytes dominates EC-derived Ang-

2, pericytes attach firmly to neo-vessels and stabilize them. In addition, Ang-1 can directly stimulate the migration and survival of ECs. When pericytes and ECs contact directly in this manner, latent transforming growth factor- β (TGF- β) is efficiently activated, which may participate in the maturation of neo-vessels.⁷⁾

Physiological angiogenesis in the adult is observed only in restricted places such as the endometrium and ovarian follicle, and this process is normally transient. However, persistent angiogenesis plays a crucial role in various pathological conditions including tumors, diabetic retinopathy, and rheumatoid arthritis. In particular, the induction of angiogenesis, or so-called "angiogenic switch", is recognized as a critical step for tumor progression,⁸⁾ and the anti-angiogenesis therapy has become a promising strategy for cancer treatment.⁹⁾ In contrast, the pro-angiogenesis therapy is anticipated for ischemic diseases including arteriosclerotic occlusion of lower limb or angina pectoris/myocardial infarction in order to rescue the ischemic tissue by developing collateral circulation.¹⁰⁾

PROTEASES AND ANGIOGENESIS

Two classes of protease system have been vigorously investigated in relation to angiogenesis. They include plasminogen activators (PAs)/plasmin and matrix metalloproteinases (MMPs). These 2 classes of protease systems involve in extracellular proteolysis.

PAs belong to the large serine proteinase family, which convert plasminogen to plasmin. In particular, urokinase-type PA (uPA) and its specific cell surface uPA receptor (uPAR) are inducibly expressed in ECs during angiogenesis. uPA is capable of initiating a cascade of proteolysis that involves the generation of plasmin and activation of pro-MMPs. Moreover, uPA is able to modulate cell adhesion and migration through uPAR. Recent data suggest that this non-proteolytic activities of the uPA/uPAR are coupled to intracellular signaling through integrins.¹¹⁾

MMPs, zinc dependent proteolytic enzymes, cleave ECM (collagen, laminin, fibronectin, *etc.*) as well as non-matrix substrates (growth factors, cell surface receptors, *etc.*). The activities of MMPs are tightly regulated at the gene transcription levels, zymogen activation by proteolysis, and inhibition of active forms by endogenous inhibitors, tissue inhibitor of metalloproteinase (TIMP), and RECK. There are more than 20 of MMPs have been identified to date. Among them, MMP-2 (gelatinase A), MMP-9 (gelatinase B) and MMP-14 (membrane type-1 MMP) play important role in angiogenesis.¹²⁾

Recent reports suggest that aminopeptidases also take part in angiogenesis. At least 3 aminopeptidases are reported to be involved in angiogenesis. They include type 2 methionine aminopeptidase, aminopeptidase N, and adipocyte-derived leucine aminopeptidase/puromycin insensitive leucyl-specific aminopeptidase.

AMINOPEPTIDASE

Aminopeptidases are metalloproteinases that remove amino acids from unblocked N-termini of peptides or proteins. This N-terminal modification of peptides or proteins

occurs either co-translationally or post-translationally. Aminopeptidases utilize conserved amino acid residues to generate a scaffold capable of binding one or two divalent metal ions such as Zn^{++} , Co^{++} and Mn^{++} . Spectroscopic and kinetic analyses indicate that some aminopeptidases require two metal ions for full activity. In other instances, a single metal ion is essential for enzymatic activity while the second metal ion modulates enzymatic activity either positively or negatively.

Aminopeptidases distribute widely in animal cells as well as in plant cells, bacteria and fungi. With respect to the localization, some are secreted, but most are cell-associated (either cytosolic, microsomal or membrane-bound). Aminopeptidases can be categorized based on their substrate specificity. Cleavage of the scissile peptide bond is dependent upon the chemical structure and composition of the amino acid residues flanking the cleavage site. The "pita-bread" enzymes such as methionine aminopeptidase (MetAP) process substrates restricted to particular N-terminal and penultimate residues. In contrast, the broad-range enzymes such as leucine aminopeptidase are capable of cleaving hydrophobic amino acids from the N-terminus, without strict requirement for the penultimate residue.

Aminopeptidases participate in a wide range of biological processes. Some of them act as ectoenzymes that may participate in the degradation of extracellular matrices, and some act as intracellular enzymes that may regulate the metabolism of bioactive molecules including hormones and neurotransmitters.¹³⁾

METHIONINE AMINOPEPTIDASE TYPE 2

Methionine aminopeptidases (MetAPs) are ubiquitously expressed Co^{++} dependent cytoplasmic enzymes and exhibit narrow substrate specificities when compared to the other aminopeptidases. The removal of the N-terminal methionine by MetAPs, which primarily occurs co-translationally, is a critical step in the maturation of many proteins and polypeptides. The N-terminal sequence analysis revealed that MetAPs remove the N-terminal methionine only when the second residue is small and uncharged (*i.e.*, Gly, Ala, Ser, Thr, Val, Pro, Cys). Eukaryotic organisms express two isoforms of MetAP; type 1 MetAP (MetAP1) and type 2 MetAP (MetAP2). Classification of MetAPs is based on the comparison of their sequences. The type 2 enzyme, in contrast to the type 1 enzyme, has an α -helical domain of approximately 60 residues in length inserted within a surface loop of the C-terminal half of the molecule.¹⁴⁾

The reason why MetAP2 has attracted the attention in relation to angiogenesis is because MetAP2 but not MetAP1 has been shown as a target molecule of anti-angiogenic fumagillin and ovalicin.^{15,16)} A variety of biochemical and structural analyses have shown that these epoxide-containing anti-angiogenic substances inhibit the MetAP2 activity by the direct covalent binding. The inactivation of MetAP2 by fumagillin was initially identified in cultured bovine and human ECs.¹⁷⁾ Experiments on isogenic yeast strains containing either yeast MetAP1 or yeast MetAP2 also showed that only MetAP2 was modified and inhibited by fumagillin.¹⁵⁾ The N-terminal sequence and mass spectroscopic analyses of the proteolytic fragments of the fumagillin-human MetAP-2 complex

showed that fumagillin attached to His231 of MetAP-2.¹⁷⁾ The crystal structure of the fumagillin-human MetAP-2 complex further revealed that fumagillin spanned the active site from His231 to His339, thus preventing the binding of substrates to MetAP-2.¹⁸⁾

Despite of those detailed analyses, the direct evidence that MetAP2 regulates angiogenesis is still missing. Fumagillin and its derivative TNP-470 rather selectively inhibit the proliferation of ECs.^{19,20)} However, the expression of MetAP2 is not restricted to ECs.²¹⁾ Moreover, the study on the null mutation of yeast MetAPs showed that MetAP1 and MetAP2 were redundant and exhibited the similar substrate specificity.²²⁾ Therefore, it is still not clear whether or not fumagillin and TNP-470 exhibit their anti-angiogenic activities *via* the specific inhibition of MetAP2.

M1 ZINC-AMINOPEPTIDASES

M1 zinc-aminopeptidases consist a subfamily of aminopeptidases, which contain a consensus HEXXH(18X)E motif for Zn⁺⁺ binding. Zn⁺⁺ ion is essential for their enzymatic activity. Eight mammalian enzymes of this subfamily have been identified, and are subdivided into membrane-bound ectoenzymes and cytoplasmic enzymes. Membrane-bound M1 zinc-aminopeptidases include aminopeptidase N, aminopeptidase A, TRH-degrading ectoenzyme and insulin-regulated membrane aminopeptidase/human placental leucine aminopeptidase/oxytocinase whereas cytoplasmic M1 zinc-aminopeptidases include aminopeptidase B, leukotriene-A4 hydrolase, and puromycin sensitive aminopeptidase. Adipocyte-derived leucine aminopeptidase (A-LAP)/Puromycin insensitive leucyl-specific aminopeptidase (PILSAP) is the last member of M1 zinc-aminopeptidases.^{23,24)} Although PILSAP is originally described as a secreted protein,²⁴⁾ the following studies including by ours suggest that it is a cytoplasmic enzyme.^{25,26)}

Among M1 zinc-aminopeptidase, APN and A-LAP/PILSAP are reported to be involved in angiogenesis.

(1) Aminopeptidase N (APN) APN is a type II membrane ectoenzyme.²⁷⁾ CD13, a lineage specific marker of human leukemia is identical to APN.²⁸⁾ It was reported that the treatment of animals bearing carcinoma xenografts with APN functional inhibitors impaired the tumor growth.²⁹⁾ Thereafter, APN was shown to express exclusively in ECs of neo-vessels but not of normal vasculature.³⁰⁾ The following studies by the same group demonstrated that anti-APN blocking monoclonal antibodies or functional inhibitors of APN abrogated network formation by ECs *in vitro*.³¹⁾ Moreover, basic fibroblast growth factor and its downstream Ras-dependent signals mediating EC network formation induced APN expression in ECs.³²⁾ These observations may indicate that APN expressed on ECs participates in the regulation of angiogenesis.

The precise mechanisms how APN regulates angiogenesis is remained to be elucidated. APN has only 9 amino acids in its intracellular domain. This fact suggests that APN itself does not act as a signaling molecule but is more likely as an ectoenzyme. APN trims amino acids from N-terminus of peptides or proteins in the extracellular space and for this reason, its function is determined by the substrates available in the immediate milieu.

(2) Adipocyte-Derived Leucine Aminopeptidase (A-LAP)/Puromycin Insensitive Leucyl-Specific Aminopeptidase (PILSAP) From the search of a novel M1 zinc-aminopeptidase, two groups independently isolated the same aminopeptidase of different species. One group searched from the human expressed sequence tag (EST) database,²³⁾ and the other group performed the PCR amplification from rat pituitary mRNA and the subsequent cDNA-library screening.²⁴⁾ The former group designated the novel enzyme as adipocyte-derived leucine aminopeptidase (A-LAP). The latter group designated the enzyme as puromycin insensitive leucyl-specific aminopeptidase (PILSAP), since the biochemical analyses showed that efficient hydrolysis was observed for Leu and the enzymatic activity was insensitive to puromycin. However, neither of the groups determined the biological function of A-LAP/PILSAP at that moment.

In order to find novel angiogenesis regulators expressed in ECs, we planned to isolate genes that were exclusively expressed in ECs during differentiation. Mouse embryonic stem (ES) cells can be differentiated to Flk-1(+)/VE-cadherin(-) endothelial progenitors and then to Flk-1(+)/VE-cadherin(+) ECs under the two-dimensional culture condition.³³⁾ Using a subtraction strategy, we isolated several novel genes whose expressions were up-regulated in ECs during differentiation from mouse ES cells *in vitro* (Fig. 1). Among those genes, we found one candidate of angiogenesis regulators, which turned out to be a mouse counterpart of A-LAP/PILSAP.²⁵⁾ Thus, our approach is not specified to aminopeptidase but rather cell-type and function oriented. Since the expression of this enzyme is not restricted to adipocyte, the term PILSAP will be used in the following part.

Our functional analyses revealed that PILSAP was induced in ECs by VEGF-A *in vitro*, and was expressed in ECs at the site of angiogenesis *in vivo*. Furthermore, the specific elimination of the expression of PILSAP abrogated VEGF-A-

Fig. 1. Strategy for the Isolation of Genes Expressed during Endothelial Differentiation

Flk-1(+)/VE-cadherin(-) endothelial progenitors and Flk-1(+)/VE-cadherin(+) ECs were purified by using the cell sorter during the differentiation of mouse ES cells. PCR-coupled subtractive hybridization was then used to identify genes expressed abundantly by the Flk-1(+)/VE-cadherin(+) cell population compared to the Flk-1(+)/VE-cadherin(-) cell population. One of the genes that abundantly by the Flk-1(+)/VE-cadherin(+) cell population appeared to be the mouse counterpart of PILSAP. RT-PCR analysis confirmed that the expression of mouse PILSAP was augmented in Flk-1(+)/VE-cadherin(+) cell population.

Fig. 2. PILSAP is Required for VEGF-Stimulated Proliferation and Migration of ECs

(A) Antisense oligodeoxynucleotide (AS-ODN) to PILSAP specifically inhibited VEGF-induced proliferation of mouse ECs, whereas Sense oligodeoxynucleotide (S-ODN), mismatch oligodeoxynucleotide (MIS-ODN) and scramble oligodeoxynucleotide (SCR-ODN) exhibited no effect. Values are the means and S.D.s of triplicated samples. The asterisks indicate significant difference compared with the VEGF+ group ($p < 0.001$). (B) AS-ODN specifically inhibited VEGF-induced migration of mouse ECs, whereas S-ODN, MIS-ODN and SCR-ODN exhibited no effect. Values are the means and S.D.s of 4 fields. The asterisks indicate significant difference compared with the corresponding VEGF+ group ($p < 0.001$).

Table 1. Effect of Various Inhibitors on the PILSAP Activity

Inhibitor	Concentration (μ M)	PILSAP activity (%)
None		100
Bestatin	1	84.3 $\pm$ 5.2*
	10	66.9 $\pm$ 4.3**
Amastatin	1	85.0 $\pm$ 6.3
	10	8.1 $\pm$ 3.4**
Leucinethiol	1	60.0 $\pm$ 1.6**
	10	7.4 $\pm$ 2.2**
Fumagillin	1	79.5 $\pm$ 5.4*
	10	60.1 $\pm$ 5.5**
Puromycin	10	97.5 $\pm$ 3.8

* $p < 0.05$ vs. control, ** $p < 0.005$ vs. control.

stimulated proliferation of, migration of and network formation by ECs *in vitro*, and inhibited angiogenesis *in vivo* (Fig. 2).²⁵ These results indicate that PILSAP expressed in ECs plays an important role in the biology of endothelium. We also showed that fumagillin inhibited the enzymatic activity of PILSAP (Table 1).²⁵ As described above, the target molecule of fumagillin was shown to be MetAP2. However, the expression of MetAP2 was not restricted to ECs and the selective inhibition of EC proliferation by fumagillin was not related to differential expression of MetAP2. We speculate that fumagillin exerts its anti-angiogenic activity at least in part by inhibiting PILSAP in ECs.

We are now characterizing the molecular mechanisms how PILSAP regulates the migration and proliferation of ECs. Specific elimination of the expression of PILSAP by antisense oligodeoxynucleotide (AS-ODN) or the enzymatic activity by leucinethiol (LT) did not affect the attachment but did significantly inhibit the spreading of ECs when they were plated on vitronectin-, fibronectin- or type-1 collagen. ECs express type-1 collagen receptor integrin $\alpha 2\beta 1$, fibronectin receptor $\alpha 5\beta 1$, and vitronectin receptors $\alpha v\beta 3$ and $\alpha v\beta 5$. However, AS-ODN and LT did not affect the expression of integrin $\alpha 2$, $\alpha 5$, αv , $\beta 1$, $\beta 3$, and $\beta 5$ subunits on the cell surface. AS-ODN and LT also inhibited the tyrosine phosphorylation of focal adhesion kinase (FAK) when cells were plated

on vitronectin, fibronectin or type-1 collagen.³⁴ These results suggest that PILSAP is involved in the regulation of endothelial integrin function during cell adhesion and migration. Moreover, our recent data further indicate that PILSAP is involved in the G1/S transition during cell proliferation one of the substrates of PILSAP is an intracellular signaling molecule that mediates the regulation of cell cycle (manuscript in submission).

Recently, two molecules with distinct functions have been reported to be identical to PILSAP, *i.e.* aminopeptidase regulator of TNF receptor-1 shedding (ARTS-1)³⁵ and the aminopeptidase associated with antigen processing in the endoplasmic reticulum (ERAAP).²⁶ ARTS-1 is expressed as a type II integral membrane protein in pulmonary epithelial cells as well as in ECs. This protein forms a molecular complex with TNF receptor-1 and promotes TNF receptor-1 sheddase activity.³⁵ ERAAP is expressed in killer T cells and trims intracellular pathogens for MHC class I molecules in the endoplasmic reticulum.²⁶ Thus, PILSAP seems to have multiple functions depending on which cell-type it is expressed.

CONCLUDING REMARKS

Aminopeptidase is recognized as the third protease system following PA and MMP, which are related to angiogenesis. The role of PA and MMP in angiogenesis is rather specified to the degradation of extracellular matrices. In contrast, the role of aminopeptidase in angiogenesis is not clear, because the function of aminopeptidase spreads in numerous divergences. Two classes of aminopeptidases are reported. One is methionine aminopeptidase (MetAP2) and the other is M1-zinc aminopeptidases (APN and PILSAP). In addition, APN is an ectoenzyme enzyme, while, PILSAP is a cytoplasmic enzyme. Thus, the function of each aminopeptidase must be distinct. In order to understand the precise role of aminopeptidase in the regulation of angiogenesis, physiological substrates of each aminopeptidase in ECs need be determined.

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