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BLOOD REVIEWS

State of the Art

The Significance of Aminopeptidases and Haematopoietic Cell Differentiation

K. Razak, A. C. Newland

SUMMARY. **Aminopeptidases are a group of enzymes found on the cell surface and in the cytoplasmic compartments of many peripheral blood cell types and their progenitors. Their functional roles include the hydrolysis of several biologically active peptides and growth factors and some have proved to be of diagnostic and prognostic value in leukaemia. These enzymes may also be found in serum as a consequence of non-haematopoietic related diseases and so have been used as indicators of liver damage.**

Haematopoietic cells in the bone marrow go through a process of growth and differentiation before being released into the peripheral circulation where they fulfill many functional roles. The enzyme activities of some aminopeptidases have been shown to modulate the growth of these cells. In addition, the activities of these enzymes themselves can be regulated by haematopoietic growth factors. However, the mechanisms that regulate their expression and activity are not fully understood. In this report the current literature has been reviewed for evidence of expression, regulation and clinical significance.

Aminopeptidases are expressed by diverse cell types on the cell surface membrane or in the cytoplasmic compartment. In the cytoplasm they may be present in granules or in microsomes.[1] Haematopoietic cells in the bone marrow or in the peripheral blood express a number of different types of aminopeptidases that differ in their compartmentation, pH range of activity and their preferred substrate.[2] In the peripheral blood polymorphonuclear granulocytes, mononuclear lymphocytes, monocytes and platelets have all been shown to possess aminopeptidase activity.[3–5] Cells that are in close proximity to these cells such as endothelial cells and fibroblasts may also express the same aminopeptidases.[6,7]

The functional roles of many of these enzymes are not clear but some have been implicated in the regulation of immune function by hydrolysing a number of growth and activation factors or hormones. In this report the current literature is reviewed for evidence of function, regulation of expression and possible clinical value. Many aminopeptidases are poorly characterized and so this report concentrates on those enzymes that have been sufficiently characterized and are related to blood or blood related cells.

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Cell Distribution of Aminopeptidases

Aminopeptidases are defined as enzymes that cleave amino groups near or at the N- or C-terminus of peptides. They include enzymes which hydrolyse the first peptide bond (aminoacyl-peptide hydrolases and iminoacyl-peptide hydrolases), and those which remove dipeptides from polypeptides (dipeptidyl-peptide hydrolases). Some peptidases act only on dipeptides or tripeptides which may also be considered as aminopeptidases (Table 1).

According to the method and conditions of analysis, several forms of aminopeptidase activity can be

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Table 1 Expression and location of some haematopoietic cell aminopeptidases

Enzyme name	Cell compartment	Cell type	Ref
Aminopeptidase-N (CD13)	Surface membrane	Myeloid	46
Leucine aminopeptidase	Cytoplasmic	Lymphoid	34,35
Aminopeptidase A	Surface membrane	B-cells	85,86
Dipeptidylpeptidase IV (CD26)	Surface membrane	T-cells	29
Aminopeptidase P	Surface membrane	Platelets	4
Dipeptidylpeptidase III	Cytoplasmic	Leucocytes	1
Serine di/tri-peptidase	Surface membrane	Myeloid	72
Neutral endopeptidase	Surface membrane	Lymphoid	87
		Granulocytes	88

detected in human plasma and other tissues. This heterogeneity in their structure is a result of several factors, including location to different gene loci, broad substrate specificity and post translational modification. The most characterised aminopeptidase is alanine aminopeptidase (EC 3.4.11.2) and this report will centre on this enzyme.

Human alanine aminopeptidase is expressed by a large number of tissues.[8–10] In particular, high specific activities are present on the brush border membranes of kidney proximal tubules and intestine and in bile canicular membranes.[11,12] This enzyme has also been purified from human liver,[13] kidney,[14,15] intestine,[16] placenta,[10] and blood plasma.[17] Haematopoietic cells in the bone marrow and peripheral blood mononuclear and polymorphonuclear cells also express alanine aminopeptidase. These include mature granulocytes, monocytes, and platelets. Other aminopeptidases have been detected on the surface and in the cytoplasm of lymphoid cells and include cytosolic leucine aminopeptidase in addition to surface membrane bound dipeptyl dipeptidase IV (CD26).[1,18,48]

Characteristics of Aminopeptidase-N (EC 3.4.11.2)

Alanine aminopeptidase is generally found in membrane fractions obtained by ultracentrifugation. It can be solubilised from tissues by autolysis or by proteolytic enzymes such as papain, bromelain and trypsin, or by extraction by detergents.[11]

The molecular sequence of aminopeptidase-N (EC 3.4.11.2) has homology with the CD13 molecule which characterises haematopoietic cells of the myeloid lineage.[16,18] The cDNA sequence predicts a 967 amino acid integral membrane protein. The gene has a first coding exon of 613bp and additional exons span >30 KB on the long arm of chromosome 15.[20,21] A highly significant homology with *E.Coli* aminopeptidase-N, a cytoplasmic enzyme involved in intracellular protein turnover, suggests evolutionary conservation. Human aminopeptidase-N (EC 3.4.11.2) is however ninety seven amino acid residues longer than that of *E.Coli*. This extra segment includes the cytoplasmic and transmembrane sequence. The molecular sequence has a characteristic zinc binding motif and a number of sites are available for glycosylation. Different forms of aminopeptidases occur as a result of post translational modifications by variable O-glycosylation of the molecule and this has been used to explain the different molecular weights of cytoplasmic and surface membrane bound forms of aminopeptidase-N.[22] Aminopeptidases also form dimers prior to entering the golgi apparatus prior to surface expression, where they may remain as dimers or separate to monomeric form.[23]

Aminopeptidase-N/CD13 contains a plasma membrane anchoring domain of 23 hydrophobic amino acids. The catalytic domain was shown to be positioned extracelluarly which can be released from the membrane by proteolytic cleavage. The relative molecular mass estimations of this enzyme range from 150 000[24] obtained from nonpurified serum enzyme to 240 000[13,25] for purified enzyme by gel filtration. Recent work using transfection technology demonstrated the requirement for a pentapeptide zinc binding region for enzyme activity.[26]

Antibody Detection of Aminopeptidases

Historically, CD13 was identified by monoclonal antibodies binding to haematopoietic cells of the myeloid lineage. Antibodies were raised to leukaemic cell lines by several investigators independently and were found to react with the same cell types. In international workshops these were clustered as binding to the same molecular group.[27–29] As undifferentiated and differentiated cells were used in these analysis, these antibodies were assigned a cluster of differentiation number (CD) hence CD13. Subsequently, this molecule was sequenced and the homology to aminopeptidase-N revealed. CD13 is expressed by normal human granulocyte-monocyte progenitors (CFU-GM) and their more differentiated progeny. Lymphoid cells do not express cell surface CD13/aminopeptidase-N.[30–32]

More recently, studies have been undertaken to investigate the functional activity of different epitopes on this enzyme on different cell types. Five epitopes have been detected for aminopeptidase-N. Studies on cell lines have found that antibodies can be subdivided on the basis of whether they block enzyme activity

or not.[33] Deletion of the zinc binding region of the molecule resulted in loss of both the enzyme activity and the binding of a subgroup of monoclonal antibodies to the cell membrane bound molecule.[26] In addition, in our laboratory we have found that by using flow cytometric techniques that these antibodies can be sub-classified on the basis of their ability to inhibit the binding of other competing CD13 antibodies (paper in preparation). Further studies are required to establish the possible functional role of these different epitopes. Some are obviously related to enzyme activity but others may be framework epitopes, necessary for binding to other cell surface or substrate molecules for full functional activity to occur.

Peptidyl dipeptidase IV (CD26) found in lymphoid cells, has also been shown to have more than one epitope for antibody binding.[29] Many aminopeptidases however have only been sufficiently characterized to establish a molecular weight and substrate specificity.[34,35]

Clinical Significance

In the early 1960s alanine aminopeptidase activity was used as a specific marker for carcinoma of the head of the pancreas. Hepatic and biliary tumours and drug induced hepatitis were found to also have elevated alanine aminopeptidase activities.[36] In the serum aminopeptidase activities increase in childhood and decrease after puberty. Their levels are also higher in men than in women and may be increased by smoking, alcohol consumption or drug intake.[24] Leucine aminopeptidase is a liver cytosolic enzyme and can therefore be used as a marker of hepatic cell lysis. High activities of leucine aminopeptidase in serum is seen in patients with acute hepatitis from a variety of causes or following embolised hepatoma. Also in cases of active cyclomegalovirus infection leucine and alanine aminopeptidase activities are higher in peripheral blood lymphocyte cells than normal.[37]

In serum, immune complexes containing aminopeptidases have been described in cases of patients with rheumatoid arthritis and aminopeptidases have also been implicated in the pathogenesis of autoimmune thyroid disease, where autoantibodies arise that are directed to peptide sequences common to several metalloproteases.[38,39] In support of the involvement of CD13, activated macrophages with elevated levels of this molecule have been detected in inflamed synovial joints of rheumatic patients.[40] We have previously shown that levels of this molecule on granulocytes determined in whole blood are low compared with granulocytes activated by dextran sedimentation before determination of expression.[3] CD13/aminopeptidase-N may therefore also be an activation marker for peripheral blood granulocytes.

CD13 expression on leukaemic blast cells has been used to provide diagnostic information for leukaemia classification. The presence of this molecule on a leukaemic blast cell usually indicates that the cell belongs to the myeloid cell lineage.[41] High levels of CD13 in conjunction with low levels of CD33 expression have been shown to indicate a poor prognosis for patient survival.[42] In addition, lymphoid leukaemias expressing CD13 have also been shown to have poorer prognosis than lymphoid leukaemias not expressing this molecule.[43]

Recent work has shown that CD13 is a major receptor for coronaviruses. These viruses bind to the enzyme active site on the molecule causing either lung infection or gastroenteritis, suggesting a pivotal role of CD13 in determining the tissue tropism of transmissible gastroenteritis virus and related infections.[44,45]

Regulation of Expression

Very little is known of the mechanisms that regulate the lineage restricted patterns of expression of cell surface peptidases in haematopoietic cells or how these differ from the regulatory elements controlling their expression in non-haematopoietic tissues. At the molecular level of CD13 control, Shapiro et al[46] described the existence of two separate promoter regions, resulting in independent transcripts differing only in their 5′-untranslated sequences. They found that in small intestinal epithelial cells, transcription is controlled by a classical promoter containing a TATA box immediately upstream from the translation initiation codon. This promoter directs the expression of very high levels of aminopeptidase-N on the intestinal brush border membrane. By contrast, aminopeptidase-N transcripts in myeloid cells and fibroblasts are derived from multiple sites in a region 8Kb upstream from the ATG codon, resulting in a longer mRNA that includes sequences from a unique untranslated exon. However, the transcription of DNA to RNA does not always result in translation to protein molecules. Danielson et al[47] found that mRNA for aminopeptidase-N was present in both fetal and adult gut tissues of pigs but the protein product was only expressed in the adult.

Studies in our laboratories and by others have shown that CD13 surface expression may be modulated by the tumour promoting phorbol ester 12-O-tetradecanoyl-phorbol-13-ascitate on both myeloid leukaemic blast cells or on normal peripheral blood granulocytes.[3,48,49] Growth factors such as GM-CSF or G-CSF (and anaphyatoxins[50]) can also stimulate expression on normal granulocytes. More recently, others have reported the release of aminopeptidases from granulocytes following stimulation with either TPA or the chemotactic peptide fMet-Leu-Phe. The enzymes appear to originate from granules together with vitamin B12-binding protein.[51] The ability of these growth factors to stimulate CD13/aminopeptidase N on leukaemic cells is variable and dependant on the nature of the blast cell (unpublished obser-

vations). In addition, time course studies following the activity of aminopeptidase-P and peptidyl dipeptidase IV during stimulation of human T lymphocytes by phytohaemagglutinin indicated the involvement of these two enzymes in the proliferation process of these cells.[52]

In an attempt to further understand the movement of aminopeptidase-N during haematopoietic differentiation we performed experiments with the myeloid cell line HL60, often used as a model of cell differentiation,[53] and observed the CD13 expression during differentiation events when stimulated by the agent retinoic acid. We found that CD13 expression was lost by a large proportion of these cells (unpublished observations). Our results collectively, support a model whereby immature myeloid haematopoietic cells express high levels of surface aminopeptidase but as these cells mature the expression is lost from the surface, but may be retained cytoplasmically, mature peripheral blood granulocytes and monocytes may then express this enzyme when the cell has been activated by an appropriate stimuli.

Recent studies have shown that a cytosolic leucine aminopeptidase with a molecular weight of 53 kDa can be induced in fibroblastic cells by the immunoregulatory cytokine gamma-interferon. The function of this enzyme is not known but it has been suggested that its activity may contribute to the direct antiviral effects of gamma-interferon by degrading viral proteins. This in turn may aid the processing and presentation of antigen via major histocompatibility complex (MHC) proteins to T- lymphocytes.[34] Lymphocytes have also been shown to have various aminopeptidase activities in their cytosolic compartments and several recent publications have reported the identification of additional gamma-interferon inducible cytosolic proteases that like leucine aminopeptidase (53 kDa), may be involved in the processing of antigens for presentation on class I MHC molecules. The genes encoding some of these proteases are located within the MHC region. However, the largest of these proteases is approximately 30 kDa and none show the characteristic zinc binding motif of metalloproteases.[1,19,54,55]

Oncogenes in Regulation of Aminopeptidases

The proto-oncogenes c-myc, c-myb, c-fos, c-fms and c-fes and their viral oncogene counterparts have been implicated in normal myelopoiesis and in myeloid leukaemia.[56,57] The expression of these proto-oncogenes has been extensively analyzed upon chemically induced differentiation of myeloid leukaemic cells.[58,59] However, the expression of only a few of these have been analyzed during in-vitro physiologically induced myelopoiesis of normal myeloid progenitor cells.[60,61] The c-fes oncogene is of particular interest because it encodes a P93 gene product with tyrosine kinase activity. The c-fes gene has also been mapped to chromosome 15, in close proximity to the gene encoding the CD13/Aminopeptidase-N molecule. Liebermann and Hoffman-Leibermann[62] found that myeloid precursor enriched bone marrow cells express c-fes mRNA at low levels but this could be increased as an early event following stimulation of these cells by GM-CSF. Evidence that this oncogene may play a functional role in cell differentiation is inferred from the work of Yu et al.[63] They were able to transfect the c-fes gene into the K562 cell line, a highly undifferentiated cell of the erythroid/granulocytic lineage, and showed that these cells then acquire the ability to differentiate as well as respond to agents such as PMA, a phorbol ester. In our laboratories, inhibitors to tyrosine kinases induced higher than control levels of aminopeptidase-N enzyme activity (unpublished observations). These results imply that tyrosine phosphorylation of the aminopeptidase-N molecule may be a method of regulating enzyme activity and defects in a tyrosine kinase gene may therefore contribute to abnormal enzyme activity which in turn would alter the balance of regulatory factors in the bone marrow or peripheral blood that are substrates for these enzymes.

Substrates of Aminopeptidases

Several synthetic substrates have been used to analyze the activity of aminopeptidases. The most frequently used substrates are 4-nitroanalides and beta-naphthylamides of alanine and leucine. Leucyl-beta-naphthylamide was used by Goldberg et al.[64] to analyze leucine aminopeptidase. Later it became clear that leucine could be hydrolysed by another aminopeptidase[65] which furthermore hydrolysed alanyl-substrates even faster than leucine based ones. This broad substrate specificity has resulted in some confusion in terminology and characterisation. There is now some agreement on the use of alanyl-4-nitroanilide as a substrate for determination of alanine aminopeptidase activity in serum and urine whilst alanyl-beta-naphthylamide is used for histochemical analysis.[2]

The removal of one or two amino acids from the N-terminal of polypeptides can result in the destabilisation of the substrate molecule by conformational change or permitting access of the substrate to further degradation by other enzymes.[66] Outside the haematopoietic system, aminopeptidase N is thought to participate in the degradation of several regulatory peptides. These include met-lys-bradykinin and lys-bradykinin,[67] met-and leu-enkephalin.[68] These opioid compounds can cause peripheral blood granulocytes to change shape, and this phenomenon is interperated as cell activation.[69] Interestingly, enkephalins can also modify granulocyte-endothelial interactions by stimulating endothelial release of the vasodilator prostacyclin.[70] The presence of aminopeptidase on both these cells may, then, modulate the response to these compounds. Aminopeptidase N (EC 3.4.11.2) has also been shown to cause the lowering of blood

pressure in normotensive and hypertensive rats. The mechanisms underlying this effect are not clear but partly dependant upon the brain angiotensin system.[71]

In recent work by Bauvois and co-workers the effects of several surface peptidases on the degradation of tumour necrosis factor-alpha (TNF-alpha), interferon-gamma (IFN-gamma) and interleukin 1-alpha (IL-1-alpha) was investigated.[72] These cytokines are involved in a wide variety of physiological and pathological processes, including, immunoregulation, haematopoiesis and inflammation.[73,74] Using the myeloid cell line U937 they found that aminopeptidase-N did not degrade any of these cytokines. They did, however, find that a serine dipeptidase (similar to Dipeptidase IV) and a tripeptidyl endopeptidase did degrade TNF-alpha. Dipeptidylpeptidase IV/CD26 preferentially cleaves N-terminal Gly-Pro-dipeptides. Hendriks et al have speculated that this molecule might be increased in activated lymphoid cells, because here this enzyme molecule has the task of cleaving active peptides, including interleukin-2 (IL-2), which bear the N-terminal x-Pro sequence.[75] Moreover, others have reported that CD10/neutral endopeptidase (EC 3.4.24.11) has the ability to inactivate IL-1-alpha/beta. This latter enzyme is expressed by normal granulocytes and early lymphoid cells while monocytes and their precursors are negative.[76]

Aminopeptidase-P is expressed by platelets and hydrolyses penultimate proline residues present on the terminal ends of many proteins.[4] Several cytokines and growth factors have proline residues that are accessible for this enzyme, these include IL-1 (Ala-Pro), IL-2 (Ala-Pro), TNF-beta (Leu-Pro), G-CSF (Ala-Pro), insulin like growth factor (Gly-Pro), bradykinin (Arg-Pro) and substance P (Arg-Pro).[77] Factors such as G-CSF can activate normal granulocytes as well as myeloid leukaemic blast cells[78,79] and aminopeptidase-P may therefore have an important role in inactivating this and the other factors mentioned.

Aminopeptidase activity can pose problems for thrombotic studies as they can degrade factors for investigations. Recent studies have attempted to produce aminopeptidase resistant arg-gly-asp-peptide analogs that are stable in plasma. These would prove to be useful tools in antithrombotic studies involving the platelet associated molecules gpIIb/IIIa.[80] Collectively, these results suggest that aminopeptidases may be of importance in altering both the magnitude and quality of several cellular responses by mature and immature haematopoietic cells.

Inhibitors of Aminopeptidases

One of the most widly used inhibitors of aminopeptidases is a chemical called Bestatin (Ubenimex).[81–83] The use of this inhibitor has been used to study the effects of aminopeptidases on cell growth. Using bestatin, studies on T cell activation and growth have shown that peptidyl dipeptidase IV plays a key role in these cell processes. Moreover, the action of the cytokines IL-1, IL-2 and IL-6 was shown to be suppressed by this inhibitor.[52] The growth of leukaemic and choriocarcinoma cell lines can also be inhibited.[82] By contrast, bestatin has also been shown to significantly enhance the G-CSF and GM-CSF induced colony formation of human bone marrow cells. To explain these observations it has been suggested that the mechanisms of bestatin action in haematopoietic progenitor cells is by up regulation of the high affinity receptor for GM-CSF whilst in leukaemic cell lines suppression of amino acid incorporation via peptidase regulation occurs.[83]

The chemical modifying agents 2,3-butanedione, diazoacetamide and a soluble carbodiimide have recently been used to further characterize the active site of aminopeptidase-N. These studies have suggested the presence of functional arginyl, histidyl, tyrosyl and aspartyl/glutamyl residues. Steady state kinetics have shown that the arginine, histidine and acidic residues are involved in substrate binding, whilst the tyrosine may play a role in the catalytic process.[84]

Conclusions

Aminopeptidases are found to be expressed on all types of haematopoetic cells. These enzymes have however been difficult to characterize as many have overlapping substrate specificity. As a result there has been some confusion in the literature as to the terminology used to describe the enzyme under investigation. For example, there are many papers that confuse leucine aminopeptidase (EC 3.4.11.1) activity with that of alanine aminopeptidase activity (EC 3.4.11.2). As can be seen from Table 2 a number of aminopeptidases are also able to cleave the same naturally occuring activation and growth factors. The in vivo biological effects of the hydrolysis of these factors at different sites on the molecule is however not yet clear. It would seem likely that several aminopeptidases may be required to complete some physiological cascades.

The majority of work has however concentrated on enzymes that have been shown to have homology with differentiation related antigens such as CD13, CD10 and CD26. More work is required not only

Table 2 Substates of some common aminopeptidases found on haematopoietic cells and discussed in text

Enzyme	Substrates
Leucine aminopeptidase	Enkephalins
Aminopeptidase A	Angiotensin II
peptidyl dipeptidase IV	Substance P
Cysteine protease	IL-1-beta
Serine di-&tri-peptidases	TNF-alpha
Aminopeptidase-P	x-proline (growth factors?)
Aminopeptidase N/CD13	Enkephalins/Bradykinins
Neutral endopeptidase/CD10	Enkephalins IL-1-alpha/beta

on the function of these surface membrane bound enzymes but also on cytoplasmic aminopeptidases, which may well have a role in the processing of internalized factors from the cell surface. The presence of these enzymes on haematopoietic cells does suggest a pivitol role during the process of growth, differentiation and activation.

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