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Regulation of aminopeptidase N (EC 3.4.11.2; APN; CD13) by interferon- γ on the HL-60 cell line

Jelka Gabrilovac^{a,*}, Davorika Breljak^a, Barbara Čupić^a, Andreja Ambriović-Ristov^b

^a*Division of Molecular Medicine, Ruđer Bošković Institute, Laboratory of Experimental Haematology, Immunology and Oncology Bijenička c. 54 HR-10002 Zagreb, POB 180, Croatia*

^b*Division of Molecular Biology, Laboratory for Genotoxic Agents, Ruđer Bošković Institute, Zagreb, Croatia*

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Abstract

Membrane-bound peptidases play important roles in the regulation of local concentrations of various signalling peptides such as the growth factors, hormones, chemokines and cytokines. That is accomplished by means of their enzyme activity. Recently, membrane-bound peptidases have also been shown to act as receptors, receiving signals from as yet undefined ligands and transducing them into the cell interior. By using either or both of these mechanisms, peptidases interact with fundamental cellular functions: growth, differentiation, activation and death. This study addressed the effects of a T-cell derived cytokine, interferon-gamma (IFN- γ) on the activity of aminopeptidase N (APN), an ectoenzyme processing several signal peptides. Cells of a myelo-monocytic cell line HL-60 were used as a model system, and APN was assayed at the levels of mRNA, its membrane marker CD13, and the enzyme activity. Regulation of CD13/APN by IFN- γ was found at all three levels. The direction of regulation was time-dependent: an initial down-regulation seen 24 and 48 hrs after the onset of treatment with IFN- γ was replaced by an up-regulation after 72 and/or 96 hrs. Up-regulation of CD13/APN observed after 96 hrs was preceded by an up-regulation of APN mRNA reaching its maximum after 72 hrs. The IFN- γ -induced regulation of APN was due to membrane aminopeptidase N, since it could be completely abrogated by an APN blocking antibody WM-15. The delayed up-regulation of CD13/APN (observed after 72 and/or 96 hrs), required de novo protein synthesis as it could be abrogated by cycloheximide, an inhibitor of protein synthesis. Possible role of endogenous (IFN- γ -induced) TGF- β in mediating CD13/APN up-regulation could be excluded, since no TGF- β was found in supernatants of IFN- γ treated HL-60 cells. Thus, our data show regulation of CD13/APN on cells of

* Corresponding author. Tel.: +385 1 45 61 082; fax: +385 1 45 6101.
E-mail address: gabril@irb.hr (J. Gabrilovac).

myelo-monocytic origin by a T-cell derived cytokine, IFN- γ . A similar mechanism might play a role in inflammation.

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Introduction

Aminopeptidase N (APN; CD13; EC 3.4.11.2), also called alanyl aminopeptidase, is a zinc-dependent metallopeptidase (clan MA, family M1, ID: M1.001, gluzincins) (Hooper, 1994; Rawlings and Barrett, 1999) that cleaves neutral amino acids from the N-terminus of oligopeptides (Shipp and Look, 1993; Riemann et al., 1999; Lendeckel et al., 2000). Human APN cDNA has been cloned by two independent groups who showed its sequence to be identical to the myeloid marker CD13 (Olsen et al., 1988; Look et al., 1989). Human APN gene, located on chromosome 15 (Look et al., 1986; Watt and Willard, 1990), spans approximately 35 kilobases (kb), contains 20 exons (Lerche et al., 1996) and encodes two RNA transcripts (3.4 and 3.7 kb) that are controlled by two different promoters: myeloid and epithelial (Olsen et al., 1991; Shapiro et al., 1991; Olsen et al., 1994; Shapiro, 1995). APN is membrane-bound 140–160 kDa glycoprotein with type II membrane topology. It is expressed as a non-covalently linked homodimer on the surface of hematopoietic as well as non-hematopoietic cells (Riemann et al., 1999; Van der Velden and Hulsmann, 1999; Riemann, 2002). Outside the hematopoietic system, CD13/APN is expressed on epithelial cells of the intestine and kidney, hepatocytes, osteoclasts, endometrial cells, fibroblasts, endothelial cells, bone marrow stromal cells and on neuronal synaptic membranes (Shipp and Look, 1993; Lendeckel et al., 2000). Although conventionally accepted as a myeloid marker for normal and malignant cells of the myeloid lineage, CD13/APN has recently been detected as well on the lymphocytes, following malignant transformation, inflammation and cell activation (Wex et al., 1997; Lendeckel et al., 1999; Riemann et al., 1999; Lendeckel et al., 2000).

Since APN has an extracellularly oriented catalytic domain, it is classified as an ectopeptidase capable of cleaving various extracellular peptides. Its spectrum encompasses opioid peptides such as enkephalins and endorphins, neuropeptides such as neurokinin A and somatostatin, inflammatory peptides such as tuftsin, N-formyl-Met-Leu-Phe (fMLP) and monocyte chemoattractant protein (MCP-1), vasoactive peptides such as angiotensin III and kallidin, and peptides of the extracellular matrix (Shipp and Look, 1993; Riemann et al., 1999; Lendeckel et al., 2000). Contradictory data on degradation of IL-8 by APN have been reported (Kanayama et al., 1995; Kehlen et al., 2001). Recently, a more complex interaction between the IL-8 and APN in regulating the local concentration of the chemokine has been suggested, showing that inhibitor of APN bestatin, not only decreased degradation, but also increased production of IL-8 (Mishima et al., 2002). Hence, APN is evidently involved in regulation of peptide-mediated cellular responses. By activating/inactivating extracellular peptides endowed with signal functions, APN changes their local concentrations and thereby modifies transduction of their signals (Shipp and Look, 1993; Lendeckel et al., 2000; Riemann, 2002).

Several other membrane-bound ectopeptidases, such as CD10/NEP and CD26/DPPIV are co-expressed with CD13/APN in many cells. Those ectopeptidases may collaborate with CD13/APN in peptide-mediated cellular responses, thus regulating cell proliferation, adhesion, signal transduction,

activation, differentiation and transformation (Kenny et al., 1989; Shipp and Look, 1993; Van der Velden and Hulsmann, 1999; Lendeckel et al., 2000; Riemann, 2002).

CD13/APN has many other functions. It is a receptor for human coronavirus 229 E and cytomegalovirus (Soderberg et al., 1993; Yeager et al., 1992), may serve as a target receptor for drug delivery into tumors, and may contribute to angiogenesis (Pasqualini et al., 2000; Bhagwat et al., 2001; Curnis et al., 2002). CD13/APN is involved in processing and presentation of the peptides bound to major histocompatibility class molecules I or II on antigen-presenting cells such as mononuclear phagocytes and dendritic cells (Larsen et al., 1996; Amoscato et al., 1998), plays a role in cell adhesion (the cell-cell and cell-matrix interactions) and in tumor cell migration (invasion) (Menrad et al., 1993; Saiki et al., 1993; Fujii et al., 1995), controls the cell-cycle and proliferation of myeloid and lymphoid cells (Wex et al., 1997; Lendeckel et al., 1996; Lendeckel et al., 1999; Löhn et al., 2002), and participates in the signal transduction cascade in myeloid and lymphoid cells (Lendeckel et al., 2000; Santos et al., 2000a). As recently described, APN is localized in lipid rafts of the cell membranes (Santos et al., 2000b, Riemann et al., 2001).

In spite of numerous data about the biochemical and functional properties of CD13/APN, little is known about its exact physiological role (or function) on blood cells. It has been suggested that leukocyte APN could degrade (inactivate) pro-inflammatory peptides and thus terminate the inflammatory responses. Indeed, APN reduces chemotactic responses of human neutrophil granulocytes to fMLP, tuftsin and IL-8 (Kenny et al., 1989; Shipp and Look, 1993).

Since APN represents a cellular potential for activation or inactivation of inflammatory peptides, modulation of its expression by different agents and stimuli may affect the inflammatory responses. Regulation of APN expression has been studied on epithelial and endothelial cells (Stefanović et al., 1992; Van Hal et al., 1994; Kehlen et al., 1998), and on fibroblasts (Stefanović et al., 1998; Sorrell et al., 2003). The most potent APN regulators were cytokines IL-4 and IL-13. In addition, a strong up-regulation of APN was also obtained with a synthetic glucocorticoid, dexamethasone (Stefanović et al., 1998; Sorrell et al., 2003). Among the cells of hematopoietic origin, regulation of APN was studied on human monocytes/macrophages. Effects of IL-4 (Riemann et al., 1995), IFN- γ (Van Hal et al., 1994) and transforming growth factor-beta (TGF- β) (Kehlen et al., 2000) have been reported.

Although myeloid progenitor cells and mature granulocytes express CD13/APN, regulation of the expression has not been examined in detail. We have addressed, therefore, the effect of a T cell-derived cytokine, IFN- γ , on the expression of CD13/APN on cells of a human myelo-monocytic cell line HL-60 as a model system. These cells have high levels of enzymatically active APN and express strongly the CD13 membrane marker (Laouar and Bauvois, 1992; Xu et al., 1995). APN was determined at the level of mRNA, the CD13 membrane marker, and the enzyme activity.

Materials and methods

Chemicals

Recombinant human interferon-gamma (IFN- γ), FITC-labeled anti-CD13 (clone WM-47) and FITC-labeled isotype control (mouse-IgG1), bestatin, actinonin and cycloheximide were all purchased from Sigma. L-Ala-pNA was purchased from Bachem. Bestatin, actinonin, and L-Ala-pNA were dissolved in phosphate-buffered saline solution (PBS) and stored as stock solutions at $-20\text{ }^{\circ}\text{C}$ until used.

Cycloheximide was dissolved in ethanol and stored at $-20\text{ }^{\circ}\text{C}$ until used. APN-blocking (WM-15) and non-blocking (WM-47) anti-CD13 antibodies, were a generous gift from Dr. E. J. Favaloro (Institute of Clinical Pathology and Medical Research, New South Wales, Australia).

HL-60 cell line

HL-60 cell line (ATCC) was maintained in RPMI-1640 medium containing L-glutamine (3 mM), HEPES (20 mM), penicillin (0.1 g/L), streptomycin (0.1 g/L), and 10% fetal calf serum (FCS). Cells were seeded at $1\text{--}2 \times 10^5$ per mL and the medium was replaced every 48 hours.

Experimental design

HL-60 cells were seeded in 24-well culture-plates in the presence of various concentrations of IFN- γ . Control samples were cultured in medium without IFN- γ . Initial concentration of HL-60 cells varied depending on the length of the treatment (5×10^3 cells/mL to 4×10^5 cells/mL) so as to achieve approximately the same cell density at the end of treatment. This calculation was based on the growth-curves showing a $t_{1/2}$ of about 1 day. At the end of incubation time, the cells were collected, washed in PBS, counted and adjusted to the desired concentration. Subsequently, the cells were analysed for CD13 membrane expression and APN enzyme activity, or RNA was isolated.

Determination of membrane CD13 by FACS

HL-60 cells (10^6 per assay) were incubated with FITC-labelled anti-CD13 (clone WM-47; Sigma, cat. no. F-5671), or the isotype control (mouse IgG1, kappa; Sigma cat. no. F-6397; $0.5\text{ }\mu\text{g}$ per 10^6 cells) for 30 min at $4\text{ }^{\circ}\text{C}$. After 2 washings, the cells were resuspended in PBS with 0.1% BSA and analysed on FACSscan (Becton-Dickinson). All HL-60 cells were CD13 $^+$. The density of CD13 expression was expressed as a mean fluorescence intensity (MFI) obtained with anti-CD13 antibody, corrected for MFI obtained with the isotype control.

Determination of APN enzyme activity

2×10^5 of HL-60 cells in PBS were mixed with the APN substrate L-Ala-pNA in a final volume of $100\text{ }\mu\text{l}$ in 96-well flat-bottomed plates (Falcon). The incubation (60 min) was carried out in water bath at $37\text{ }^{\circ}\text{C}$ with constant shaking. The reaction was terminated by putting the samples into ice-bath and by subsequent addition of ice cold PBS ($150\text{ }\mu\text{L}$ per well). The plates were spun down (5 min at $400 \times g$) and the supernatants ($200\text{ }\mu\text{L}$ per well) were transferred to another 96-well flat-bottomed plate. Optical density (OD) of the cell-free supernatants was measured by using an ELISA reader with selective filter for 405 nm. The data are expressed as the amount of hydrolysed para-nitroanilin (nmol/60 min per 2×10^5 cells) according to para-nitroanilin standard curve.

Semiquantitative RT-PCR analysis

Total cellular RNA was extracted from HL-60 cells using 'High Pure RNA Isolation' kit (Roche) following the manufacturer's instruction. RNA concentration was determined by absorption at 260 nm

using a spectrophotometer. Total cellular RNA (1 µg) was heated at 65 °C for 5 minutes and subsequently used as a template for the first strand cDNA synthesis. The reaction mixture contained 3 µg of random hexadeoxynucleotide primers (Roche), 1 mM dNTP mix (Roche), 40 units RNase-Inhibitor (Roche), 10 mM dithiothreitol (Sigma), 1 × reverse transcription buffer (Roche) and 40 units Moloney murine leukemia virus (M-MuLV) reverse transcriptase (RT) (Roche) in a final volume of 20 µL. The reaction mixture was incubated at 37 °C for 1 hour and heated to 65 °C for 10 minutes. cDNA synthesised as described above was diluted 5 times in DNase and RNase free water (Gibco) and stored at –20 °C. Each RT included two negative controls: RNA sample without M-MuLV RT, and M-MuLV RT without the RNA template. Housekeeping gene ABL was used as an internal standard and as a control for the presence of contaminating DNA (Watzinger and Lion, 1998). The predicted size of PCR amplified ABL cDNA is 193 bp while the same reaction give a 793 bp fragment from genomic DNA. Only RTs that proved to be free of contaminated DNA were subjected to further examination. The predicted size of amplified cDNA of APN was 450 bp.

The sequences of the specific primers and reaction conditions used for PCR are defined in Table 1. PCR was performed using 2 µL of the cDNA diluted as described above on a Perkin Elmer 9600 thermocycler. Initial denaturation at 95 °C for 3 min and the last extension step at 72 °C for 10 min were used for both PCR. Reaction was performed in total volume of 20 µL with 1 unit of *Taq* polymerase (Roche), 0.2 mM dNTP (Roche), 1 × PCR buffer (Roche), 0.20 µM of each primer. Six microliters of each PCR reaction were resolved by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and visualised under ultraviolet light. Densitometric analysis was performed using Image Master VDS software 1.0 (Pharmacia).

In order to obtain semiquantitative results, the PCR products of both the APN and of the housekeeping ABL gene, were measured during the log phase of the reaction, i.e. before saturation was reached. Preliminary experiments were performed in order to determine the number of cycles which would result in linear relationship between the number of cycles and amount of PCR products (data not shown).

Determination of TGF-β by ELISA

Presence of TGF-β in supernatants of HL-60 cultures after treatment with IFN-γ was examined by ELISA following manufacturer's instruction (Promega cat. no. G7590). All samples were run in duplicates.

Statistical analysis

Significance of the observed data was statistically evaluated by Student's t-test. The level of significance was set at $p < 0.05$.

Table 1
Primer sequences and conditions used for PCR amplification

Gene	Sequence (5'-3')	Denaturation	Annealing	Extension	Primer reference
ABL	F. agcatctgactttgagcc	30 s at 95 °C	30 s at 55 °C	45 s at 72 °C	Watzinger and Lion, 1998
	R. cccattgtggattatagcctaagac				
APN	F. gccgtgtgcacaatcatcgact	30 s at 95 °C	30 s at 57 °C	45 s at 72 °C	Wex et al., 1995
	R. caccaggagccccttgaggtg				

Results

CD13 membrane expression

Time- and concentration-dependence of the effect of IFN- γ on membrane expression of CD13 on HL-60 is shown in Figs. 1 and 2. The effect depended on duration of the treatment: short exposure of the cells to IFN- γ (for 24 and 48 hrs) decreased, whereas prolonged treatment (96 hrs) increased CD13 expression on HL-60 cells. In the applied range of concentrations (6 to 100 ng/mL), the effects of IFN- γ were of similar extent in both directions, i.e. in down- as well as in up-regulation (Fig. 3A and B, respectively).

APN enzyme activity

In order to test whether the IFN- γ -regulated CD13 protein was a functionally active, the APN enzyme activity of HL-60 cells was measured. Time- and concentration-dependence were

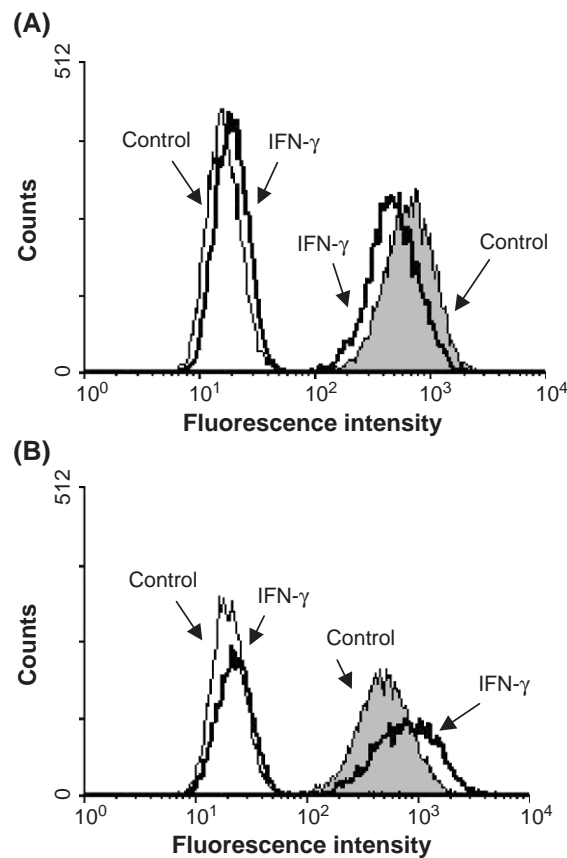


Fig. 1. Time-dependent regulation of membrane CD13 on HL-60 cells induced with IFN- γ . HL-60 cells were incubated with IFN- γ (6 ng/mL) for 24 (A) or 72 h (B). Control cells were incubated in medium. At the end of incubation, the cells were washed and labeled with anti-CD13-FITC or isotype control (mouse-IgG1-FITC). The results are presented as histograms (i.e. distribution of cells according to their fluorescence intensity).

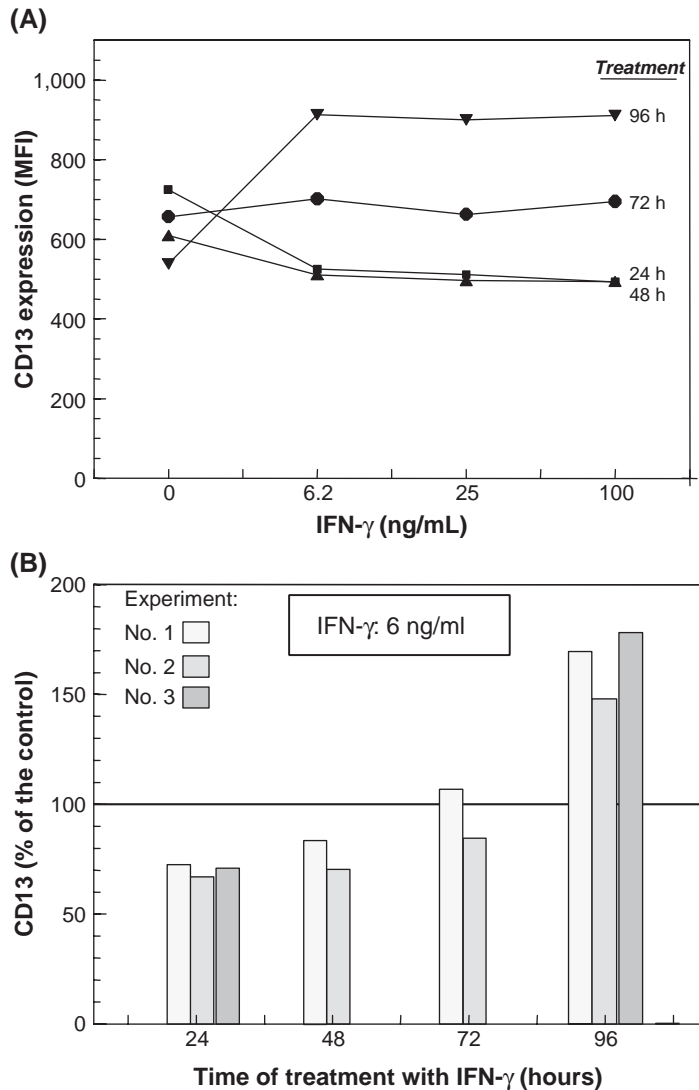


Fig. 2. Kinetics of CD13 modulation on HL-60 cells by IFN- γ . HL-60 cells were incubated with IFN- γ of indicated concentrations for 24, 48, 72 or 96 h. Control cells were incubated in medium. At the end of incubation, the cells were washed and incubated with anti-CD13-FITC or isotype control (mouse-IgG1-FITC). The results are presented as MFI of CD13-positive cells *minus* MFI of the respective isotype control (A), or as the percentages of the control values obtained with cells incubated in medium (B).

examined. A short exposure of the cells to IFN- γ (for 24 hrs) decreased, whereas a prolonged exposure (for 96 hrs) increased the APN enzyme activity (Fig. 4A and B). All tested concentrations (3, 6, 12, 25 and 50 ng/ml) produced similar effects (Fig. 4A and B). Thus, IFN- γ -induced changes in CD13 membrane expression paralleled similar changes in APN activity.

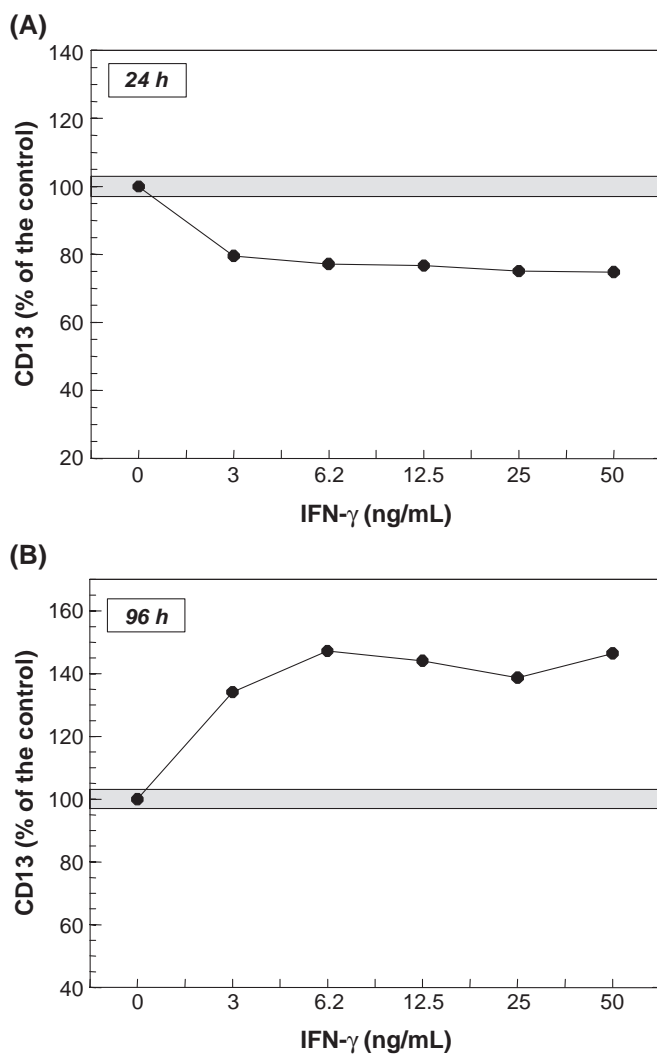


Fig. 3. Concentration-dependence of CD13 modulation on HL-60 cells by IFN- γ . HL-60 cells were incubated with IFN- γ of indicated concentrations for 24 h (A) or for 96 h (B). Control cells were incubated in medium. At the end of incubation, the cells were washed, adjusted to the same concentration, and labeled with anti-CD13-FITC or isotype control (mouse-IgG1-FITC). The results are expressed as MFI (MFI of CD13-positive cells *minus* MFI of the respective isotype control) and presented as percentages of the control (cells incubated in medium). Results of one out of three experiments with similar results are presented.

Specificity of the effect of IFN- γ

Specificity of the effect of IFN- γ on membrane aminopeptidase activity was analysed by using a monoclonal antibody against the active site of APN (clone WM-15) which blocks the APN enzyme activity (Ashmun et al., 1992). This treatment enabled elimination of a possible interference by cytoplasmic aminopeptidases which cross-react with the substrate Ala-pNA. WM-15 in a final concentration 10 μ g/mL, blocked 53% to 58% of the APN enzyme activity in a control sample

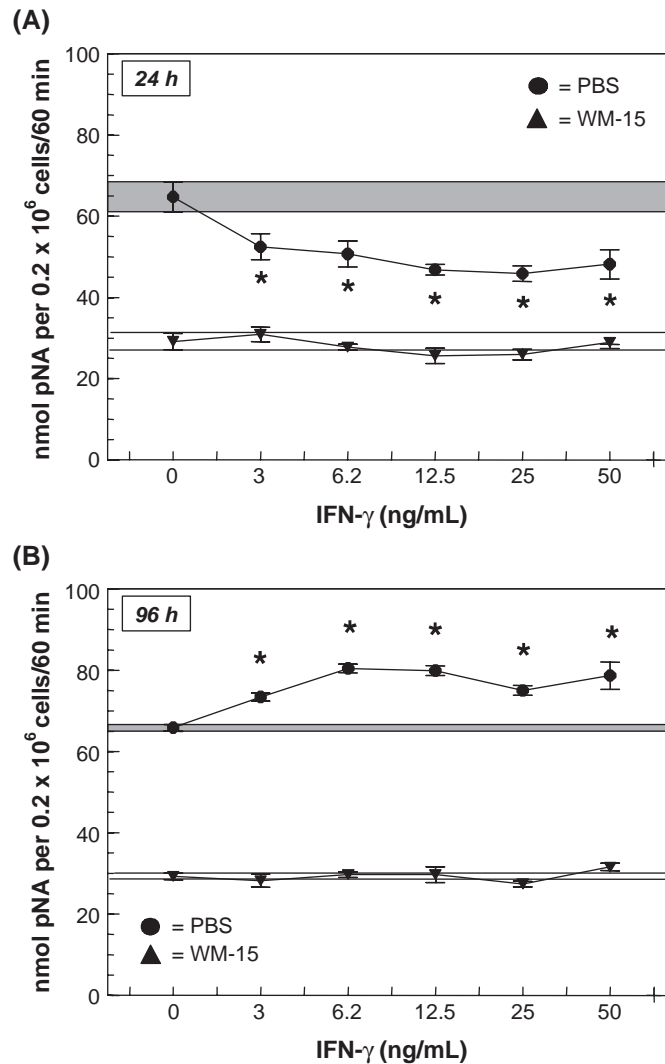


Fig. 4. Down- and up-regulation of APN on HL-60 cells induced by short (24 h) and prolonged (96 h) exposure to IFN- γ : abrogation by a blocking antibody WM-15. HL-60 cells were incubated with IFN- γ of indicated concentrations for 24 h (A) or for 96 h (B). Control cells were incubated in medium. At the end of incubation, the cells were washed, adjusted to the same concentration, preincubated for 30 min at room temperature with the WM-15 blocking antibody (10 μ g/mL) or in PBS, and tested for the APN enzyme activity. The data are expressed as means \pm s.d. of five parallel samples. Results of one out of two experiments with similar results are presented. *Significantly different from the control.

(Fig. 4A and B) and abrogated changes in APN activity induced by IFN- γ treatment. Non-blocking anti-CD13 antibody, clone WM-47, was used as a control. Treatment of cells with this antibody did not have influence on IFN- γ -induced changes in APN activity (Fig. 5). Hence, IFN- γ specifically regulated the enzyme activity of membrane-bound APN.

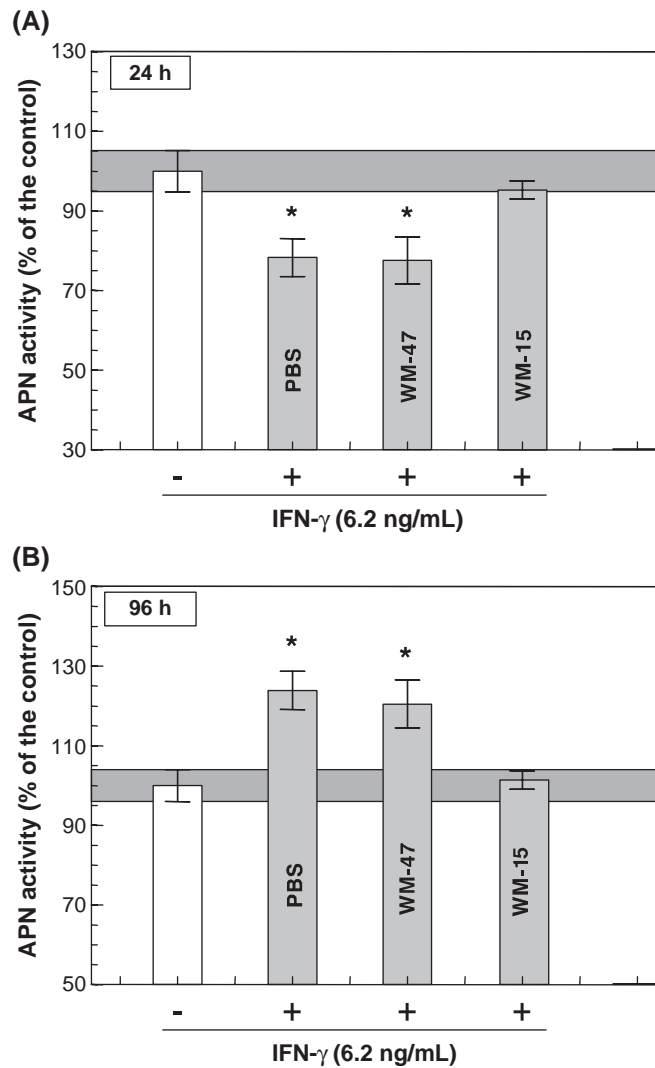


Fig. 5. The specificity of APN blocking by WM-15. HL-60 cells were incubated with IFN- γ (6.2 ng/mL) for 24 h (A) or 96 h (B). Control cells were incubated in medium. At the end of incubation, the cells were washed, adjusted to the same concentration, preincubated with blocking antibody WM-15 or non-blocking antibody WM-47 (10 μ g/mL) and tested for APN enzyme activity. The data are expressed as mean \pm s.d. of five parallel samples. Results of one out of two experiments with similar results are presented. * = significantly different from the control.

Regulation of mRNA for APN

Semiquantitative RT-PCR was performed to verify whether the observed regulation of membrane CD13, associated with parallel changes in APN enzyme activity, was due to alteration in the level of mRNA for APN. Time-dependence of the effects was examined. Short exposure to IFN- γ (for 24 hrs) moderately decreased (Fig. 6A and B), whereas a longer exposure (72 and 96 hrs) increased the expression of mRNA for CD13/APN (Fig. 6C and D; Fig. 6E and F,

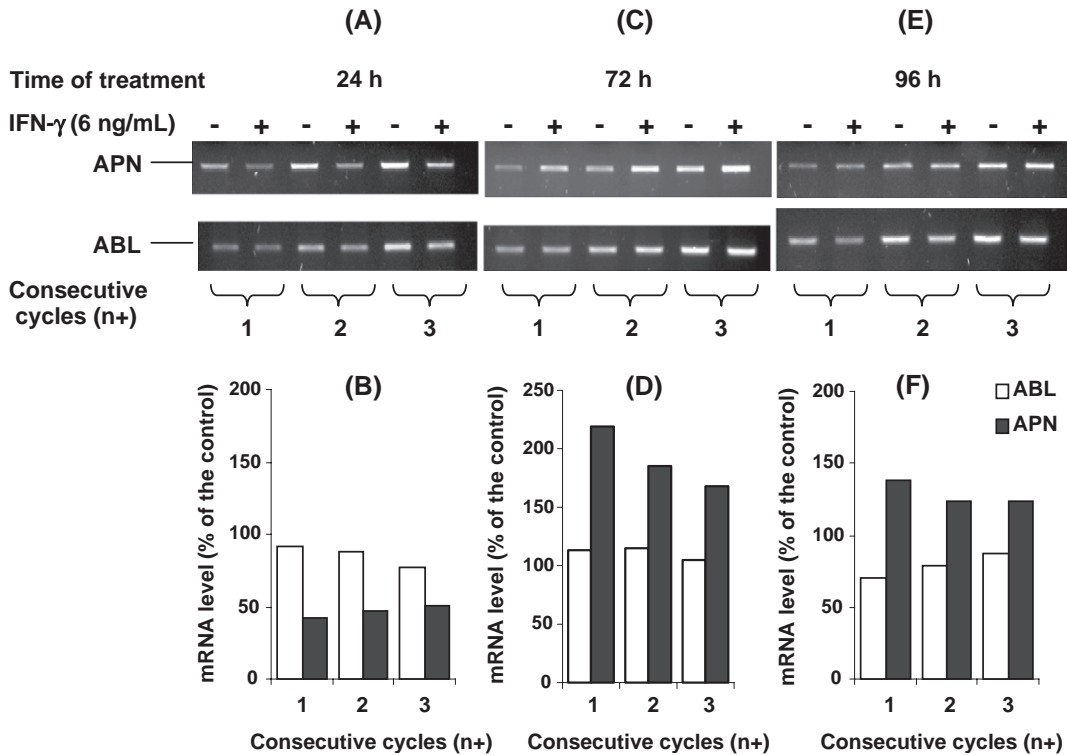


Fig. 6. Time-dependent regulation of APN mRNA in HL-60 cells induced with IFN- γ . HL-60 cells were cultured for 24 h (A and B), 72 h (C and D) or for 96 h (E and F) with IFN- γ (6 ng/mL). At the end of treatment, total cellular RNA was isolated and semiquantitative RT-PCR was performed. Ethidium bromide staining of RT-PCR products; (A), (C) and (E): graphic presentation of the data shown in (B), (D) and (F) obtained by the Image Master VDS. The data are expressed as percentages of the control (cells cultured in medium, designated as 100%). For ABL n = 24; for APN n = 26.

respectively). The up-regulation of mRNA for APN was most pronounced after 72 hrs of treatment (Fig. 6 C and D).

Assessment of mediators responsible for the delayed up-regulation of CD13/APN

Since up-regulation of APN after treatment with IFN- γ was postponed, both at the mRNA (72 and 96 hrs) and at the membrane protein (CD13/APN) level (72 and/or 96 hrs), we proposed that it might be due to an indirect effect caused by de novo synthesised factor(s) induced by IFN- γ . In order to examine this hypothesis, an inhibitor of protein synthesis, cycloheximide, was applied 24 hrs before the CD13 up-regulation becomes detectable. Cycloheximide abrogated the CD13 increase in a dose-dependent manner (Fig. 7). Assuming that the de novo synthesised protein might be TGF- β , a potent CD13/APN stimulator of another cell line of monocytic origin, U-937, we determined its presence in supernatants of IFN- γ -treated HL-60 cells. However, no TGF- β was found at either IFN- γ concentration (25 and 6.2 ng/mL) applied after 48, 72 or 96 hrs (data not shown). The data suggest that IFN- γ does not induce TGF- β secretion from HL-60 cells that would account for the observed CD13/APN up-regulation.

Discussion

In this study, we have shown that T-lymphocyte derived cytokine IFN- γ , modulates expression of CD13 on human myelo-monocytic cell line HL-60 in a time-dependent manner: down-regulation occurs after a short exposure (24 and/or 48 hrs) and up-regulation after a longer exposure (72 and/or 96 hrs). The changes of enzymatically active (APN) membrane CD13 were preceded by similar changes in mRNA for APN, i.e. maximal up-regulation of mRNA was recorded 72 hrs after the exposure to IFN- γ , and maximal up-regulation of CD13/APN occurred after 96 hrs. IFN- γ -induced changes in APN expression can be completely abrogated by an APN blocking antibody WM-15, hence, these changes can be attributed to a membrane aminopeptidase N. To our knowledge this is the first report of regulation of CD13/APN expression by IFN- γ on immature cells of myelo-monocytic origin.

Initial down-regulation of CD13 (for 28% to 38%) was associated with decreased APN activity (for 29% to 32%), as well as with decreased mRNA for APN (for up to 50%). Since the HL-60 cell line, used as a model in this study, is of myelo-monocytic origin, we can speculate that a similar decrease of the APN expression on mature granulocytes and monocytes at the sites of inflammation might help ensure high and sustained concentrations of pro-inflammatory peptides in early stages of the inflammatory process. Indeed, a decreased APN expression was recorded on peripheral blood monocytes after a 3-day treatment with IFN- γ (Van Hal et al., 1994). Several inflammatory peptides have been reported to be substrates for APN, such as chemokine MCP-1 (Weber et al., 1996), chemotactic peptides fMLP (Shipp and Look, 1993) and tuftsin (Xu et al., 1995); and vasoactive peptide angiotensin III (Riemann et al.,

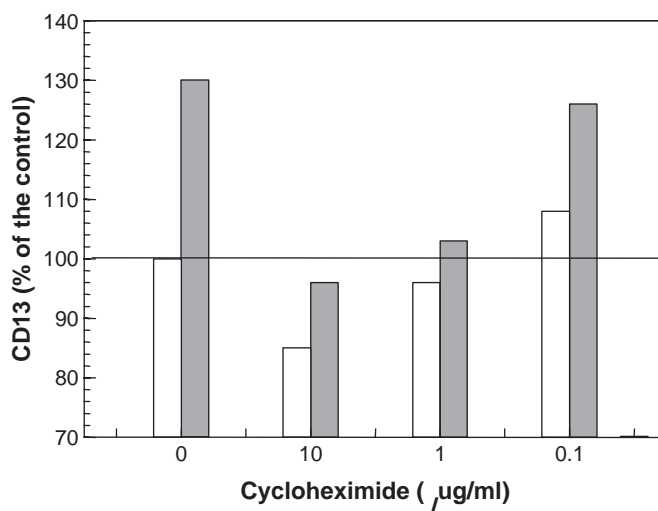


Fig. 7. Abrogation of IFN- γ -induced CD13 up-regulation by cycloheximide. HL-60 cells were incubated with IFN- γ (6 ng/mL) for 96 hrs. Control cells were incubated in medium. Twenty-four hours before the end of incubation, cycloheximide of indicated concentrations was added, and the incubation continued for 24 hrs. At the end of treatment (96 hrs with IFN- γ , 24 hrs with cycloheximide) the cells were washed, adjusted to the same concentration, and labeled with anti-CD13-FITC or isotype control (mouse-IgG1-FITC). The results are expressed as MFI (MFI of CD13-positive cells *minus* MFI of the respective isotype control) and presented as percentages of the control (cells incubated in medium). Results of one out of two experiments with similar results are presented. Open bars: cells treated with cycloheximide only; hatched bars = cells treated with IFN- γ and cycloheximide.

1999). In addition, susceptibility of chemokine IL-8 to degradation by APN has been also reported (Kanayama et al., 1995). On the other hand, data of Mishima et al. (2002) suggest that inhibition of APN is also associated with an increased production of IL-8.

After a prolonged treatment of HL-60 cells with IFN- γ (for 72 and 96 hrs), the initial down-regulation of CD13/APN was replaced by up-regulation. The increased APN enzyme activity (for up to 35%) was associated with increased membrane CD13 (for up to 89%) as well as with increased level of mRNA for APN (for up to 100%). Similar data have been reported by Riemann et al. (1999) and Van der Velden et al. (1998) who found that anti-inflammatory cytokine IL-4 up-regulated CD13 and/or APN on renal and bronchial epithelial cells, respectively, after a 3 to 5 days treatment. Increased CD13/APN, associated with increased APN mRNA, was found by Van Hal et al. (1994) on endothelial cells after a 2 to 4 days treatment with IL-4. Recently, TGF- β has also been reported to up-regulate CD13 on human monocytic cells (Kehlen et al., 2000). The increase could be observed at the mRNA level, as well as at the level of membrane protein and APN enzyme activity. The intensity of changes induced by TGF- β was mRNA > CD13 > APN. The data presented in this study are in line with those reported by Kehlen et al. (2000), i.e. the maximal increase was observed with mRNA (for up to 100%), followed by membrane expression of CD13 (for up to 89%) and APN enzyme activity (for up to 35%).

The regulatory ability of IFN- γ may not be restricted only to HL-60 cell line. In contrast to low-differentiated (myelo-monocytic) HL-60 cells that express high levels of CD13/APN, cells of the U937 line are partly differentiated toward monocytes and express low levels of CD13/APN (data not shown). We show here that IFN- γ moderately increased (for 30% to 89%) the high basal level of CD13 in HL-60 cells. However, IFN- γ strongly increased (up to 5 times) the low basal level of CD13 expressed on U937 cells, but no initial decrease of CD13 was observed on U937 treated with IFN- γ (data not shown; manuscript in preparation). Thus, IFN- γ increased CD13/APN on both cell lines of myelo-monocytic or monocytic origin (HL-60 and U937, respectively), but the extent and kinetics of the increase differed.

Kehlen et al. (2000) studied regulation of CD13/APN by TGF- β on the partly differentiated monocytic cell line U937 and on the more differentiated monocytic line Mono-Mac6 and found that the up-regulation was more pronounced in the immature than in the mature cells. In the view of those, as well as our data, the APN regulation by IFN- γ might be relevant for cells of myelo-monocytic origin.

The increased CD13/APN expression induced by IFN- γ on HL-60 cells found in this study was delayed, suggesting the requirement for de novo synthesis of secondary factor(s) regulating CD13/APN expression directly. Indeed, the IFN- γ -induced CD13 up-regulation could be abrogated by cycloheximide in a dose-dependent manner, demonstrating requirement for de novo synthesis of protein(s). TGF- β , a powerful stimulator of APN on monocytic cell line U937 (Kehlen et al., 2000), was checked as a possible candidate, but was not found in supernatants of IFN- γ -treated HL-60 cells. Thus, the up-regulating effect of IFN- γ on CD13/APN could not be attributed to TGF- β . This conclusion is further strengthened by our observation that HL-60 cells could increase CD13 expression upon stimulation with exogenously added TGF- β (data not shown). IL-4, also reported as potent modulator of CD13/APN on bronchial epithelial cells (Van der Velden et al., 1998) and fibroblasts (Sorrell et al., 2003), could be excluded as a mediator of IFN- γ -induced CD13 up-regulation as well, as it only marginally (up to 10%) and transiently increased CD13 on HL-60 cells after 24 hrs treatment (data not shown). Similarly to the results of this study, Stefanović et al. (1992) observed postponed up-regulation of CD13 on glomerular epithelial cells treated with IFN- γ for 5 to 11 days.

Up-regulation of APN was also observed with some growth factors that initiate differentiation. For example, Laouar et al. (1993) reported up-regulation of APN enzyme activity on HL-60 cells after

induction of their differentiation toward monocytes by the granulocyte-macrophage colony-stimulating factor (GM-CSF). In contrast, a decrease of the APN expression was observed when the cells were driven toward differentiation into granulocytes by retinoic acid.

The most potent regulator of APN expression seems to be the synthetic glucocorticoid dexamethasone. It increased APN on human dermal fibroblasts (Stefanović et al., 1998) and in synergy with IFN- γ increased APN activity in bronchial epithelial cells (Van der Velden et al., 1998). Up-regulation of APN and NEP by dexamethasone has been proposed as an additional mechanism of its anti-inflammatory and immunosuppressive action (Van der Velden et al., 1998).

Modulation of CD13 membrane expression found in this study was paralleled by a modulation of APN mRNA suggesting a causal relationship. However, the mechanism of altered mRNA expression induced by IFN- γ is not clear and may encompass altered transcription and/or altered mRNA stability. The results accrued so far do not allow a distinction between these possibilities. Wex et al. (1997) have shown that activated T lymphocytes up-regulate APN mRNA due to an increased stability of the mRNA.

The specificity of the IFN- γ induced modulation of the APN enzyme activity on HL-60 cells was checked by using an antibody, WM-15, which is specific for the active site of APN and thus blocks its enzyme activity (Favaloro, 1993). Indeed, 53% to 58% of the APN activity could be blocked by WM-15 in HL-60 cells. In addition, the blocking WM-15, but not the non-blocking WM-47 antibody, completely abrogated both the down- and up-regulated APN induced by IFN- γ treatment. These data strongly suggest that the enzyme activity regulated by IFN- γ , found in this study, is due to membrane APN and not to other peptidases with similar substrate selectivity. Namely, intracellular leucine-aminopeptidase which shares substrate selectivity with APN, could also be activated by IFN- γ (Harris et al., 1992). Due to its intracellular localisation, leucine-aminopeptidase could not be blocked by WM-15 antibody.

The initial down-regulation of APN induced by IFN- γ might reflect a mechanism by which the inflammatory cells ensure sufficient local concentrations of inflammatory peptides at the onset of inflammation. In turn, up-regulation of APN after a prolonged exposure to IFN- γ may reflect a quenching mechanism preventing an excessive response. Thus, the same cytokine appears to be capable of down- and up-regulating cellular armament, by which cells optimise local concentrations of signalling peptides. In addition, the observed APN regulation by IFN- γ might also involve changes in cellular proliferative ability, since APN seems to play a role in the regulation of cell growth (Löhn et al., 2002).

In summary, the data of this study obtained at the level of mRNA, membrane protein and its enzyme activity, have shown that IFN- γ modulates expression of CD13/APN on human myelo-monocytic cell line HL-60 in a time-dependent manner: initial down-regulation (24 and/or 48 hrs) switched to up-regulation after a longer exposure (72 and/or 96 hrs). The postponed increase of CD13/APN seems to be due to de novo synthesised regulatory protein(s) other than TGF- β . Knowledge about the regulatory mechanism(s) of CD13/APN expression on mature monocytic or myeloid cells which participate in the immune and inflammatory responses might help targeted manipulation of CD13/APN in pathological states, such as chronic inflammation, auto-immune and allergic diseases.

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References

- Amoscato, A.A., Prenovitz, D.A., Lotze, M.T., 1998. Rapid extracellular degradation of synthetic class I peptides by human dendritic cells. *The Journal of Immunology* 161 (8), 4023–4032.
- Ashmun, R.A., Shapiro, L.H., Look, A.T., 1992. Deletion of zinc-binding motif of CD13/aminopeptidase N molecules results in loss of epitopes that mediate binding of inhibitory antibodies. *Blood* 79 (12), 3344–3349.
- Bhagwat, S.V., Lahdenranta, J., Giordano, R., Arap, W., Pasqualini, R., Shapiro, L.H., 2001. CD13/APN is activated by angiogenic signals and is essential for capillary tube formation. *Blood* 97 (3), 652–659.
- Curnis, F., Arrigoni, G., Sacchi, A., Fischetti, L., Arap, W., Pasqualini, R., Corti, A., 2002. Differential binding of drugs containing the NGR motif to CD13 isoforms in tumor vessels, epithelia, and myeloid cells. *Cancer Research* 62 (3), 867–874.
- Favaloro, E.J., 1993. CD-13 ('gp150'; aminopeptidase-N): co-expression on endothelial and haemopoietic cells with conservation of functional activity. *Immunology and Cell Biology* 69 (4), 253–260.
- Fujii, H., Nakajima, M., Saiki, I., Yoneda, J., Azuma, I., Tsuruo, T., 1995. Human melanoma invasion and metastasis enhancement by high expression of aminopeptidase N/CD13. *Clinical and Experimental Metastasis* 13 (5), 337–344.
- Harris, C.A., Hunte, B., Krauss, M.R., Taylor, A., Epstein, L.B., 1992. Induction of leucine aminopeptidase by interferon-gamma. Identification by protein microsequencing after purification by preparative two-dimensional gel electrophoresis. *The Journal of Biological Chemistry* 267 (10), 6865–6869.
- Hooper, N.M., 1994. Families of zinc metalloproteases. *FEBS Letters* 354 (1), 1–6.
- Kanayama, N., Kajiwara, Y., Goto, J., Maradny, E., Maehara, K., Andou, K., Terao, T., 1995. Inactivation of interleukin-8 by aminopeptidase N (CD13). *Journal of Leukocyte Biology* 57 (1), 129–134.
- Kehlen, A., Göhring, B., Langner, J., Riemann, D., 1998. Regulation of the expression of aminopeptidase A, aminopeptidase N/CD13 and dipeptidylpeptidase IV/CD26 in renal carcinoma cells and renal tubular epithelial cells by cytokines and cAMP-increasing mediators. *Clinical and Experimental Immunology* 111 (2), 435–441.
- Kehlen, A., Langner, J., Riemann, D., 2000. Transforming growth factor- β increases the expression of aminopeptidase N/CD13 mRNA and protein in monocytes and monocytic cell lines. *Advances in Experimental Medicine and Biology* 477, 49–56.
- Kehlen, A., Gebert, I., Thiele, K., Fischer, K., Riemann, D., Langner, J., 2001. Increased expression of interleukin-8 and aminopeptidase N by cell-cell contact: interleukin-8 is resistant to degradation by aminopeptidase N/CD13. *European Cytokine Network* 12 (2), 316–324.
- Kenny, A.J., O'Hare, M.J., Gusterson, B.A., 1989. Cell-surface peptidases as modulators of growth and differentiation. *The Lancet* 2 (8666), 785–787.
- Laouar, A., Bauvois, B., 1992. Characterization and modulation of cell surface proteases on human myeloblastic (HL-60) cells and comparison to normal myeloid cells. *Immunology Letters* 34 (3), 257–265.
- Laouar, A., Wietzerbin, J., Bauvois, B., 1993. Divergent regulation of cell surface protease expression in HL-60 cells differentiated into macrophages with granulocyte macrophage colony stimulating factor or neutrophils with retinoic acid. *International Immunology* 5 (8), 965–973.
- Larsen, S.L., Pedersen, L.O., Buus, S., Stryhn, A., 1996. T cell responses affected by aminopeptidase N (CD13)-mediated trimming of major histocompatibility complex class II-bound peptides. *The Journal of Experimental Medicine* 184 (1), 183–189.
- Lendeckel, U., Wex, T., Reinhold, D., Kahne, T., Frank, K., Faust, J., Neubert, K., Ansorge, S., 1996. Induction of the membrane alanyl aminopeptidase gene and surface expression in human T-cells by mitogenic activation. *The Biochemical Journal* 319 (3), 817–821.
- Lendeckel, U., Arndt, M., Frank, K., Wex, T., Ansorge, S., 1999. Role of alanyl aminopeptidase in growth and function of human T cells (review). *International Journal of Molecular Medicine* 4 (1), 17–27.
- Lendeckel, U., Kähne, T., Riemann, D., Neubert, K., Arndt, M., Reinhold, D., 2000. Review: the role of membrane peptidases in immune functions. *Advances in Experimental Medicine and Biology* 477, 1–24.

- Lerche, C., Vogel, L.K., Shapiro, L.H., Norén, O., Sjöström, H., 1996. Human aminopeptidase N is encoded by 20 exons. *Mammalian Genome* 7 (9), 712–713.
- Löhn, M., Mueller, C., Langner, J., 2002. Cell cycle retardation in monocytoid cells induced by aminopeptidase N (CD13). *Leukemia and Lymphoma* 43 (2), 407–413.
- Look, A.T., Peiper, S.C., Rebentisch, M.B., Ashmun, R.A., Roussel, M.F., Lemons, R.S., Le Beau, M.M., Rubin, C.M., Sherr, C.J., 1986. Molecular cloning, expression, and chromosomal localization of the gene encoding a human myeloid membrane antigen (gp 150). *The Journal of Clinical Investigation* 78 (4), 914–921.
- Look, A.T., Ashmun, R.A., Shapiro, L.H., Peiper, S.C., 1989. Human myeloid plasma membrane glycoprotein CD13 (gp 150) is identical to aminopeptidase N. *The Journal of Clinical Investigation* 83 (4), 1299–1307.
- Menrad, A., Speicher, D., Wacker, J., Herlyn, M., 1993. Biochemical and functional characterization of aminopeptidase N expressed by human melanoma cells. *Cancer Research* 53 (6), 1450–1455.
- Mishima, Y., Matsumoto-Mishima, Y., Terui, Y., Katsuyama, M., Yamada, M., Mori, M., Ishizaka, Y., Ikeda, K., Watanabe, J.-I., Mizunuma, N., Hayasava, H., Hatake, K., 2002. Leukemic cell-surface CD13/aminopeptidase N and resistance to apoptosis mediated by endothelial cells. *Journal of the National Cancer Institute* 94 (13), 1020–1028.
- Olsen, J., Cowell, G.M., Königshøfer, E., Danielsen, E.M., Møller, J., Laustsen, L., Hansen, O.C., Welinder, K.G., Engberg, J., Hunziker, W., 1988. Complete amino acid sequence of human intestinal aminopeptidase N as deduced from cloned cDNA. *FEBS Letters* 238 (2), 307–314.
- Olsen, J., Laustsen, L., Kärnström, U., Sjöström, H., Noren, O., 1991. Tissue-specific interactions between nuclear proteins and the aminopeptidase N promoter. *The Journal of Biological Chemistry* 266 (27), 18089–18096.
- Olsen, J., Laustsen, L., Troelsen, J., 1994. HNF1 alpha activates the aminopeptidase N promoter in intestinal (Caco-2) cells. *FEBS Letters* 342 (3), 325–328.
- Pasqualini, R., Koivunen, E., Kain, R., Lahdenranta, J., Sakamoto, M., Stryhn, A., Ashmun, R.A., Shapiro, L.H., Arap, W., Ruoslahti, E., 2000. Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. *Cancer Research* 60 (3), 722–727.
- Rawlings, N.D., Barrett, A.J., 1999. MEROPS: the peptidase database. *Nucleic Acids Research* 27 (1), 325–331.
- Riemann, D., Kehlen, A., Langner, J., 1995. Stimulation of the expression and the enzyme activity of aminopeptidase N/CD13 and dipeptidylpeptidase IV/CD26 on human renal cell carcinoma cells and renal tubular epithelial cells by T cell-derived cytokines, such as IL-4 and IL-13. *Clinical and Experimental Immunology* 100 (2), 277–283.
- Riemann, D., Kehlen, A., Langner, J., 1999. CD13 — not just a marker in leukemia typing. *Immunology Today* 20 (2), 83–88.
- Riemann, D., Hansen, G.H., Niels-Christiansen, L., Thorsen, E., Immerdal, L., Santos, A.N., Kehlen, A., Langner, J., Danielsen, E.M., 2001. Caveolae/lipid rafts in fibroblast-like synoviocytes: ectopeptidase-rich membrane microdomains. *The Biochemical Journal* 354 (1), 47–55.
- Riemann, D., 2002. CD13. *Wiley Encyclopedia of Molecular Medicine*, vol. 5. John Wiley & Sons, Inc., pp. 549–552.
- Saiki, I., Fujii, H., Yoneda, J., Abe, F., Nakajima, M., Tsuruo, T., Azuma, I., 1993. Role of aminopeptidase N (CD13) in tumor-cell invasion and extracellular matrix degradation. *International Journal of Cancer* 54 (1), 137–143.
- Santos, A.N., Langner, J., Herrmann, M., Riemann, D., 2000a. Aminopeptidase N/CD13 is directly linked to signal transduction pathways in monocytes. *Cellular Immunology* 201 (1), 22–32.
- Santos, A.N., Roentsch, J., Danielsen, E.M., Langner, J., Riemann, D., 2000b. Aminopeptidase N/CD13 is associated with raft membrane microdomains in monocytes. *Biochemical and Biophysical Research Communications* 269 (1), 143–148.
- Shapiro, L.H., Ashmun, R.A., Roberts, W.M., Look, A.T., 1991. Separate promoters control transcription of the human aminopeptidase N gene in myeloid and intestinal epithelial cells. *The Journal of Biological Chemistry* 266 (18), 11999–12007.
- Shapiro, L.H., 1995. Myb and Ets proteins cooperate to transactivate an early myeloid gene. *The Journal of Biological Chemistry* 270 (15), 8763–8771.
- Shipp, M.A., Look, A.T., 1993. Hematopoietic differentiation antigens that are membrane associated enzymes: cutting is the key! *Blood* 82 (4), 1052–1070.
- Soderberg, C., Giugni, T.D., Zaia, J.A., Larsson, S., Wahlberg, J.M., Møller, E., 1993. CD13 (human aminopeptidase N) mediates human cytomegalovirus infection. *Journal of Virology* 67 (11), 6576–6585.
- Sorrell, J.M., Brinon, L., Baber, M.A., Caplan, A.I., 2003. Cytokines and glucocorticoids differentially regulate CD13/APN and DPPIV/CD26 enzyme activities in cultured human dermal fibroblasts. *Archives of Dermatological Research* 295 (4), 160–168.

- Stefanović, V., Vlahović, P., Ardaillou, N., Ronco, P., Ardaillou, R., 1992. Cell surface aminopeptidase A and N activities in human glomerular epithelial cells. *Kidney International* 41 (6), 1571–1580.
- Stefanović, V., Vlahović, P., Mitić-Zlatković, M., 1998. Receptor-mediated induction of human dermal fibroblast ectoaminopeptidase N by glucocorticoids. *Cellular Molecular Life Sciences* 54 (6), 614–617.
- Van der Velden, V.H., Naber, B.A., Spoel, P., Hoogsteden, H.C., Versnel, M.A., 1998. Cytokines and glucocorticoids modulate human bronchial epithelial cell peptidases. *Cytokine* 10 (1), 55–65.
- Van der Velden, V.H., Hulsmann, A.R., 1999. Peptidases: structure, function and modulation of peptide-mediated effects in the human lung. *Clinical and Experimental Allergy* 29 (4), 445–456.
- Van Hal, P.T., Hopstaken-Broos, J.P., Prins, A., Favarolo, E.J., Huijbens, R.J., Hilvering, C., Figdor, C.G., Hoogsteden, H.C., 1994. Potential indirect anti-inflammatory effects of IL-4. Stimulation of human monocytes, macrophages, and endothelial cells by IL-4 increases aminopeptidase-N activity (CD13; EC 3.4.11.2). *The Journal of Immunology* 153 (6), 2718–2728.
- Watt, V.M., Willard, H.F., 1990. The human aminopeptidase N gene: isolation, chromosome localization, and DNA polymorphism analysis. *Human Genetics* 85 (6), 651–654.
- Watzinger, F., Lion, T., 1998. Multiplex PCR for quality control of template RNA/cDNA in RT-PCR assays. *Leukemia* 12 (12), 1984–1986.
- Weber, M., Ugucioni, M., Baggolini, M., Clark-Lewis, I., Dahinden, C.A., 1996. Deletion of NH₂-terminal residue converts monocytes chemotactic protein 1 from an activator of basophil mediator release to an eosinophil chemoattractant. *The Journal of Experimental Medicine* 183 (2), 681–685.
- Wex, T., Lendeckel, U., Wex, H., Frank, K., Ansorge, S., 1995. Quantification of aminopeptidase N mRNA in T cells by competitive PCR. *FEBS Letters* 374 (3), 341–344.
- Wex, T., Bühling, F., Arndt, M., Ansorge, S., Lendeckel, U., 1997. The activation-dependent induction of APN-(CD13) in T-cells is controlled at different levels of gene expression. *FEBS Letters* 412 (1), 53–56.
- Xu, Y., Wellner, D., Scheinberg, D.A., 1995. Substance P and bradykinin are natural inhibitors of CD13/aminopeptidase N. *Biochemical and Biophysical Research Communications* 208 (2), 664–674.
- Yeager, C.L., Ashmun, R.A., Williams, R.K., Cardellicchio, C.B., Shapiro, L.H., Look, A.T., Holmes, K.V., 1992. Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature* 357 (6377), 420–422.