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The main neutral aminopeptidase activity of human lymphoid tumour cell lines does not originate from the aminopeptidase N-(APN; CD13) gene

T. Wex *, U. Lendeckel, T. Kähne, A. Ittenson, K. Frank, S. Ansorge

Institute of Experimental Internal Medicine, Department of Internal Medicine, University of Magdeburg, Leipziger Strasse 44, D-39120 Magdeburg, Germany

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Abstract

Lymphocytes and related cell lines are predominantly CD13-negative, however, there are reports describing neutral aminopeptidase activity in or on these cells. The aim of this study was to answer the question, whether this activity originates from APN-gene expression.

The total cellular activities (Ala-pNA hydrolysis) of lymphoid cell lines are up to 15 times higher than that of normal lymphocytes. Despite weak or lacking CD13 surface expression all lymphoid cell lines tested contain APNmRNA as quantified by competitive RT-PCR as well as low enzymatic activity in their particulate fractions. By isoelectric focusing two enzyme species with isoelectric points of 5.4 or between 3.5 to 4.8, respectively, were detected. To investigate whether these activities result from APN-gene we established transfectants lacking cellular APN expression of the CD13-positive histiocytic cell line U937 and the CD13-negative T cell line H9. Studies on these transfectants proved (I) that the main neutral aminopeptidase activity expressed in lymphoid cells is definitively not related to APN and (II) that APN is also expressed in lymphoid cells, although on a low level only.

Keywords: Neutral aminopeptidase; Antisense; CD13; Lymphoid cell; (EC 3.4.11.2)

1. Introduction

APN is expressed in many tissues including intestine, kidney, liver as well as the central nervous system [1,2]. The enzyme is involved in the final hydrolysis of nutrients and the degradation of bioactive molecules such as tuftsin and cytokines [3–5]. Furthermore, APN serves as a receptor for corona viruses [6,7], and it is possibly involved in processes of antigen presentation [8]. Recently, Saiko and coworkers have shown that the CD13 antigen is involved in the regulation of tumour-cell invasion and the degradation of extracellular matrix [9]. In the diagnostic of hematopoietic malignant disorders APN is used as a routine marker for myelomonocytic cells [3,10]. Other cells such as lymphocytes and related cell lines are predominantly CD13-negative [1,10,11]. However, there are several reports describing the

Abbreviations: Ala-βNA, alanine-β-methoxy-naphthylamide; Ala-pNA, alanine-*p*-nitroanilide; APN, aminopeptidase N; HUVE-cells, human umbilical vein endothelial cells; IEF, isoelectric focusing; IP, isoelectric point; PBS, phosphate buffered saline; RT, reverse transcription

* Corresponding author. Fax: +49 391 6713291; E-mail thomas.wex@medizin.uni-magdeburg.de

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occurrence of neutral aminopeptidase activities in or on these cells. Amoscato and co-workers reported on Ala-pNA-hydrolysing activity located on the cell surface of lymphoid cell lines [12]. Other reports extended these findings showing that APN is expressed on peripheral blood lymphocytes and on lymphoid T cell lines as well [13,14]. It was also shown that the CD13 antigen is frequently expressed on the surface of malignant B cells [15], on T lymphocytes derived from inflamed tissues [16] as well as on mitogen activated T cells [17].

Other reports describe, however, that peripheral blood lymphocytes, Jurkat T cells and tumour cells separated from patients suffering from leukeamia express neutral aminopeptidase activity which is distinct from APN with respect to its biochemical properties such as substrate specificity, sensitivity to inhibitors, or CD13-immunoreactivity [18–20].

In the studies presented here, we investigated neutral aminopeptidase activity of cell lines to answer the question, whether this activity originates from the APN-gene and then would represent a modified APN, or whether it has to be attributed to another aminopeptidase.

2. Materials and methods

2.1. Cell cultures

The CD13-positive histiocytic cell line U937-H and the lymphoid cell lines H9, HuT78, Daudi and Jurkat were obtained from American Type Culture Collection (ATTC, Rockville, MD, USA), the CD13-negative cell clone U937-L, as well as the lymphoid cell lines Karpas and PC12 Ichikawa cells were supplied by the German Collection of Microorganisms and Cell Cultures (DSM, Braunschweig, Germany). Cells were grown in IMDM (GibcoBRL, Eggenstein, Germany) supplemented with 10% foetal calf serum (Gibco) and 60 U/ml penicillin (Gibco). Peripheral T cells were purified by nylon adherence as described by others [32].

2.2. Isoelectric focusing

Cells were solubilized in 1% *n*-octyl-β-D-glucopyranoside in PBS (pH 7.4), and particulate materials were sedimented by ultracentrifugation (30 min, $100\,000 \times g$). IEF and capillary blotting were performed as previously described [14]. Blots were used for activity staining of neutral aminopeptidase by means of the chromogenic substrate Ala-β-methoxy-naphthylamide (5 mg/10 ml) and Fast Blue B (10 mg/10 ml) in PBS (pH 7.4).

2.3. Enzymatic assay

Neutral aminopeptidase activity was determined in triplicate by measuring the hydrolysis of the chromogenic substrate Ala-pNA in a photometric assay as described previously [14]. Briefly, cells or subcellular fractions, respectively, were incubated in 100 μl PBS (pH 7.4) containing 2.5 mM Ala-pNA. Reactions were stopped by adding 0.4 ml 1 M sodium acetate (pH 4.4), either immediately (control) or after 60 min of incubation at 37°C. After centrifugation for 5 min at 4°C, absorption of the cell-free supernatants at 390 nm was determined using a Spekol 21 (Carl Zeiss Jena).

2.4. Cell fractionation

Cells were resuspended in PBS (pH 7.4) and sonicated using a Vibra cell (Sonics and Materials, Danbury, CT, USA) at 40 W for 2 min (3×40 s). Cytosolic and particulate fractions were separated by ultracentrifugation (4°C, $100\,000 \times g$, 2 h) of the lysate.

2.5. Plasmid construction and transfection

The 2785 bp *Hin* dIII-fragment of the APNcDNA (kindly provided by A.T. Look, [29]) was ligated into the plasmid pREP4 (ITC Biotechnology, Germany). The resulting plasmids contained the APNcDNA fragment in either antisense (pREP4AA) or sense (pREP4AS) orientation. Cells were transfected by electroporation (15 μg plasmid DNA per 4×10^6 cells; H9: 960 μF, 300 V; U937: 250 μF, 300 V; cuvette 0.4 cm, Gene Pulser, Bio-Rad) and selected in medium containing 250 μg/ml hygromycin (Boehringer Mannheim, Germany). Typically, transfected cell populations were established 4–6 weeks after electroporation.

2.6. Immunofluorescence staining of cells

Indirect immunofluorescence staining of cells was performed as described previously [21]. APN was detected using monoclonal antibodies (My-7, Coulter; Leu-M7, Becton Dickenson). For control of non-specific or Fc-receptor mediated labelling, binding of irrelevant immunoglobulin was determined. This control value was set to 2%. Cytofluorimetric analyses were performed using the EPICS PROFILE II (Coulter Electronics).

2.7. Competitive PCR

Competitive PCR was performed as described previously [24]. Briefly, RNA was extracted using 'RNeasy' kit (Qiagen). Five hundred ng total RNA from cells was mixed with an aliquot of a given dilution of the standard APN-RNA-fragment, and transcribed into cDNA by 50 U MML-V reverse transcriptase using 1 ng APN-specific primer (5′-gcggtagaagcccgccaggtca-3′). An aliquot of the cDNA mixture was used directly for enzymatic amplifications which were performed in 30 μl reaction volume containing 0.5 U 'Gold-Star' polymerase (Eurogentec), 0.25 mM dNTP, 2.5 mM $MgCl_2$, 0.2 pmol of primers (forward: 5′-gccgtgtgcacaatcatcgcact-3′; reverse: 5′-caccagggagcccttgaggtg-3′) and 1 × reaction buffer (Eurogentec) in an Autogene II (CLF, Emmersacker, Germany). Initial denaturation at 94°C for 3 min was followed by thirty cycles with denaturation at 95°C for 0.7 min, annealing at 54°C for 0.6 min and elongation at 72°C for 1.3 min. The final extension step was at 72°C for 10 min. Electrophoresis of reaction products as well as calculation of the APN-mRNA contents was performed as described previously [24].

3. Results

3.1. Neutral aminopeptidase activity

The neutral aminopeptidase activities of cell lines were investigated by assaying their capability to hydrolyse the chromogenic substrate Ala-pNA. Total cellular activities measured in the lysate of lymphoid cell lines were in the range of 23 to 172 pkat/10^6 cells. In comparison with the corresponding activity of peripheral T cells (12 pkat/10^6 cells), related cell lines showed substantially higher aminopeptidase activities (Table 1). The CD13-positive histiocytic cell line U937-H contained 63 pkat/10^6 cells Ala-pNA hydrolysing activity. Surprisingly, some of the CD13-negative lymphoid cell lines such as the B cell line Daudi, the T cell lines H9, PC12-Ichikawa, and the weakly CD13-expressing T cell line Karpas contained higher neutral aminopeptidase activities than U937-H cells (Table 1). The majority of this activity expressed in lymphoid cells as well as in the CD13-

Table 1
Neutral aminopeptidase activity, APN surface expression and APNmRNA content of different cell lines

Cells	Ala-pNA hydrolysing activity (pkat/10^6 cells)		Cell surface expression (range of CD13-positive cells, %)	APNmRNA content (pg/μg total RNA)
	Lysate	Particulate fraction		
U937-H	63 ± 10	40 ± 2	85–98	47 ± 9
U937-L	42 ± 13	7 ± 2	1–3	0.5 ± 0.4
Daudi	172 ± 90	18 ± 6	1–2	2.0 ± 0.8
Jurkat	23 ± 10	7 ± 2	1–2	1.9 ± 0.6
H9	85 ± 41	6 ± 4	1–2	6.2 ± 5.6
HuT78	41 ± 19	4 ± 1	1–3	6.5 ± 5.0
PC12	74 ± 19	13 ± 5	1	1.9 ± 1.0
Karpas	105 ± 31	13 ± 4	9–27	12.6 ± 3.9
T cells	12 ± 3	3 ± 1	1–3	2.0 ± 0.9

Enzymatic data represent the means of at least 5 experiments. Cell surface expression was determined by immunofluorescence analysis in three separate experiments using My-7 (Coulter) as described in Section 2. APNmRNA contents of cell lines and peripheral T cells are the means of three experiments.

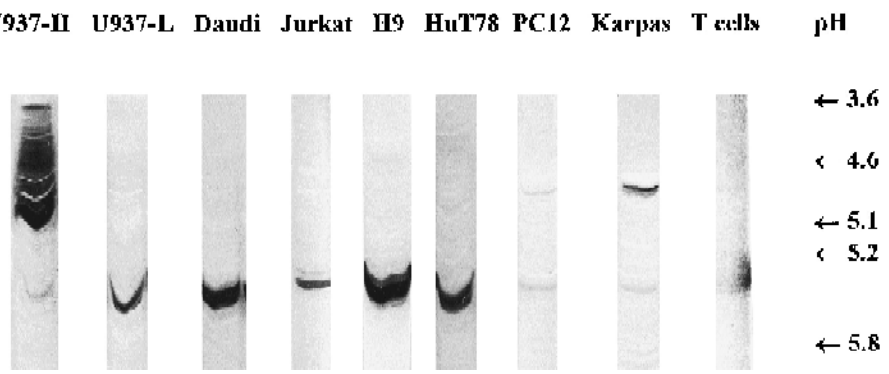

Fig. 1. Detection of neutral aminopeptidase activities (Ala-βNA-hydrolysis) of cell lines and peripheral T cells. Total cell lysate of 10^7 cells from cell lines U937-H, -L, H9, HuT78, Daudi, Jurkat and peripheral T cells as well as 10^6 cells from Karpas and PC12-Ichikawa were isoelectrically focused and blotted. Blots were stained enzymatically as described in Section 2.

negative U937-L cells is located in the soluble fraction. In the case of U937-H cells, 63% of the enzymatic activity were detected in the particulate fraction, whereas most other cells contained only small (Daudi, PC12-Ichikawa, Karpas) or neglectable amounts (Jurkat, H9, HuT78, U937-L, peripheral T

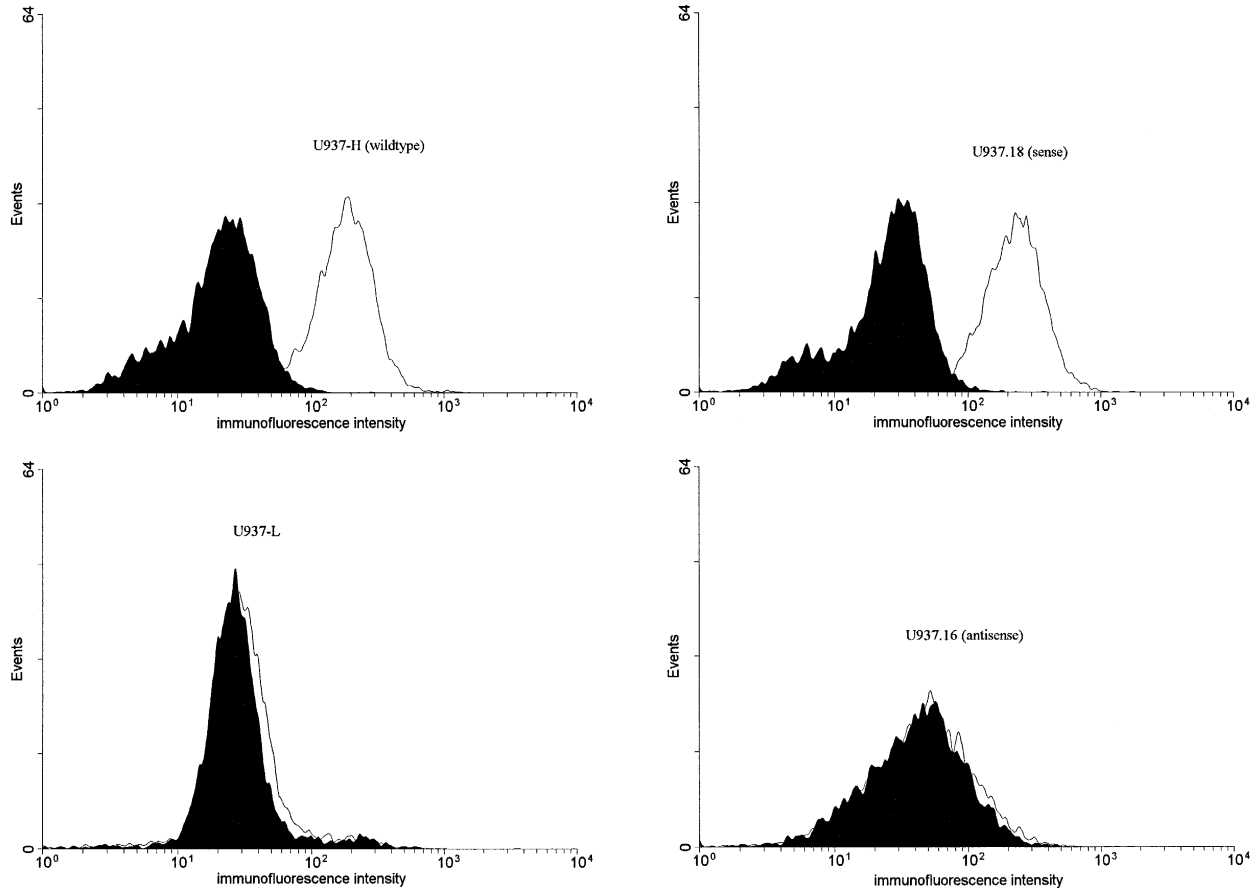

Fig. 2. Immunofluorescence analysis of the U937-H cells, the derived transfectants and U937-L cells. CD13 expression of cells was determined using the anti CD13 monoclonal antibody LeuM7 (Becton Dickinson) as described in Section 2. Control values are black filled.

cells) of Ala-pNA-hydrolysing activity in this fraction (Table 1).

Activity-staining of isoelectrically focused cell lysates using Ala-βNA revealed the presence of different enzyme species. The main enzymatic activity of U937-H cells represents proteins with isoelectric points between 4.8 and 3.5 (Fig. 1). This activity is also expressed in the lymphoid cell lines PC12 and Karpas and at a very low level in the T cell lines HuT78 and H9 as well as in peripheral T cells (Figs. 1 and 3). The main neutral aminopeptidase activity of most lymphoid cells, however, represents an enzyme form with an IP of 5.4 (Fig. 1). Using enriched particulate and cytosolic fractions of the cell lines U937-H and H9 for isoelectric focusing it was proven that the cytosolically located Ala-pNA-hydrolysing activity is identical to the corresponding activity with an IP of 5.4 and that the enzyme form with an IP of 4.8 represents membrane-bound APN (data not shown).

3.2. Detection of APN surface expression and APN-mRNA

The cell surface expression of cell lines was measured using anti-CD13 monoclonal antibodies. The level of cell surface expression mainly corresponded to the presence of Ala-pNA-hydrolysing activity detected in particulate fractions. Strong CD13-surface expression was found on the histiocytic cell line U937-H only, and to some lower extent on the T cell line Karpas, expressing Ala-pNA hydrolysing activity of 40 pkat/10^6 cells or 13 pkat/10^6 cells in their particulate fractions, respectively (Table 1). Although Daudi and PC12 express activities comparable to that Karpas cells, both cell lines were CD13-negative (Table 1). All other lymphoid cell lines tested as well as peripheral T cells were negative in immunofluorescence analysis and contain only neglectable amounts of enzymatic activity in the particulate fraction (Table 1).

To investigate whether the neutral aminopeptidase activity detected in the soluble fraction of lymphoid cells results from APN-gene expression, we quantified APNmRNA contents by competitive PCR (Table 1). The CD13-positive U937-H cells contained 47 pg APN-transcript per μg total RNA, whereas 13 pg APN-transcript per μg total RNA were determined in the T cell line Karpas, which expresses CD13 at a lower level than U937-H cells. The APN-transcript levels of the T cell lines H9 and HuT78 were 6 or 7 pg per μg total RNA, respectively, whereas corre-

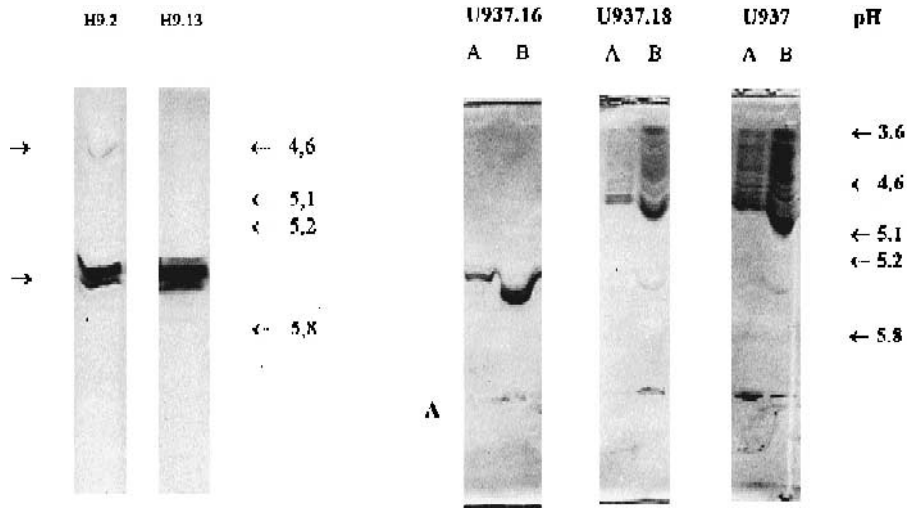

Fig. 3. Isoelectric focusing and detection of Ala-βNA hydrolysing activity from the cell lines H9 and U937 as well as from the derived transfectants. The pattern of the wildtype H9 cells was identical to that of the H9.2 cells (not shown). Used cells: H9.13 (antisense), H9.2 (sense): 10^7 cells; U937 (lanes B) wildtype: 5×10^6 cells, U937.16 (antisense), U937.18 (sense): 2×10^6 cells (lanes A represent 1/4 of the cells used in lanes B).

sponding values of all other cells are in the range from 0.5 to 2 pg APN-transcript per μg RNA.

3.3. Antisense-mediated inhibition of APN-gene expression

Two plasmids were constructed containing a 2.8 kb APNcDNA-insert in either antisense (pREP4AA) or sense (pREP4AS) orientation under the control of the RSV-LTR. Both variants were used to transfect the cell lines H9 and U937-H. Both the presence of the plasmids and the transcription of the inserted APN fragment in the transfectants (antisense: H9.13 and U937.16; sense: H9.2, U937.18) were confirmed by in situ-hybridization (data not shown).

The inhibition of the cellular APN expression in the U937-H transfectants was shown by indirect immunofluorescence analyses. The antisense transfectants U937.16 show a marked decrease of their CD13-immunoreactivity compared to wildtype cells or sense transfectants U937.18 (Fig. 2). Furthermore, loss of the APN-derived enzymatic activity could be demonstrated using IEF analysis. The Ala-βNA hydrolysing activities with IPs between 3.6-4.8 were completely absent in antisense transfectants U937.16 (Fig. 3). On the contrary, these activities are still present in the U937 (wildtype) cells as well as in the sense transfectants U937.18 (Fig. 3). Furthermore, IEF analyses revealed that the antisense H9 cells (H9.13) also completely lacked their minor Ala-βNA hydrolysing activity with an IP 4.8, whereas this activity was still present in the wildtype cells H9 as well as in the control cells H9.2 (Fig. 3). This result strongly underlines that the Ala-βNA hydrolysing activity with an IP of 4.8 which is expressed in H9 cells represents APN. Interestingly, the activity with an IP of 5.4 is increased in the H9.13 cells. An induction of this activity was also observed in U937.16 cells (Fig. 3).

4. Discussion

We investigated neutral aminopeptidase activities of different hematopoietic cell lines. Although APN expression within the immune system is largely restricted to cells of the myelomonocytic lineage, there are data providing evidence that lymphoid cells express APN on their surface after mitogenic activation [17,25,28] or malignant transformation [15,22]. This induction of CD13-expression is always accompanied by an increase of corresponding enzymatic activity [17].

Data presented here show that neutral aminopeptidase activities of lymphoid cell lines are 2–15 times higher compared to peripheral T or B cells, respectively [23]. On the contrary, corresponding activities of the histiocytic cell lines U937-H and -L were found similar to those determined on normal monocytes [23]. Interestingly, with the exception of Karpas cells enzymatic activities of all lymphoid cell lines were not accompanied by the appearance of CD13-immunoreactivity. The absence of CD13-surface expression correlates with low enzymatic activity detected in the particulate fractions of most lymphoid cells except Daudi and PC12. At least for Daudi this fact is due to the higher aminopeptidase activity of these cells (172 pkat/10^6 cells).

To investigate whether high enzymatic activities located in the soluble fraction are related to APN expression we studied APN-transcript levels by competitive RT-PCR. The CD13-positive cell line U937-H contains APN-transcript in amounts comparable to renal carcinoma cells, which strongly express CD13 [25]. The APNmRNA-contents of most lymphoid cell lines studied here are below 7 pg/μg total RNA. These values are in accordance to data reported from Riemann and co-workers, who estimated the APN-mRNA content as 4–6 pg/μg total RNA in the T cell line HuT78 (personal information). These low APN-transcript levels suggest that the considerable amounts of neutral aminopeptidase activity detected in the cytosol of lymphoid cell lines could hardly originate from the APN-gene expression.

The enzymatic pattern of the isoelectric focusing revealed the presence of two enzyme species which both represent neutral aminopeptidase activity. As we showed previously, the species with an IP of 4.8 represents the membrane-bound APN in U937-H cells [14]. Although we used different monoclonal antibodies, we did never detect CD13-immunoreactivity of the enzyme form with an IP of 4.8 in lymphoid cells. Perhaps, this effect could be explained by the weak APN expression in lymphoid cells. Recent biochemical studies using inhibitors and the inhibitory mono-

clonal anti-CD13 antibody WM15 underlined the different nature of the two neutral aminopeptidase activities, but they do not definitively answer the question, whether both enzyme species represent APN. The detection of additional aminopeptidase activities in the lymphoid cells is in agreement with data from other groups. Favaloro and co-workers [27] have shown that only 20–40% of the total neutral aminopeptidase activity of acute myeloid or lymphoid leukaemic cells could be attributed to APN. The authors discussed a high complexity of the protease expression pattern of the malignant clones. Using the inhibitory monoclonal antibody WM15 they obtained quite similar results concerning neutral aminopeptidase expression of peripheral T cells [20]. Interestingly, Belhacene and co-workers showed that aminopeptidase B is the main aminopeptidase activity of the T cell line Jurkat [19]. Furthermore, Murray and co-workers described an aminopeptidase activity of Jurkat cells which has to be distinguished from APN [18].

To definitively clarify the possible relation between the cytosolically located activity detected in lymphoid cell lines and the APN we established transfectants lacking cellular APN expression. Functioning of our antisense model was confirmed by transfecting CD13-positive U937-H cells. The strong reduction of CD13-immunoreactivity on the antisense transfectants U937.16 as well as the complete absence of the enzyme species with IP between 3.5 and 4.8 in these cells demonstrate the capability of our antisense system to in inhibit cellular APN expression. Furthermore, the observed variability concerning the isoelectric points (between 3.5 and 4.8) implies that the APN-derived activity represents more than one enzyme species. For the first time, the occurrence of different molecular forms of the APN was shown in an enzymatic assay. The heterogeneity observed could be caused by a different extent of glycosylation as shown by O'Connell and co-workers. They demonstrated that changes in the glycosylation pattern of the APN expressed on HUVE-cells create five APN subpopulations defined by different CD13 monoclonal antibodies [26].

Until recently, it was assumed that APN expression within the hematopoietic system is restricted to cells of the myelomonocytic lineage [1,3]. Results presented here, prove that the Ala-βNA hydrolysing activity with an IP of 4.8 expressed in H9 cells also represents APN. On the basis of the IEF analysis it could be concluded that a small fraction of the cellular enzymatic activity in H9 cells only could be attributed to the APN.

The antisense mediated inhibition of the APN expression clearly demonstrated that the Ala-β-NA hydrolysing activity with an IP of 5.4 is not due to APN expression because this activity was not decreased in the antisense transfectants derived from U937-H and H9 cells. Therefore, the high neutral aminopeptidase activity expressed in H9 cells has to be attributed to another enzyme. The induction of this activity observed in the U937-H transfectants could be considered, perhaps, as a 'by pass phenomenon' or 'compensation' which is occasionally observed in 'knock out' experiments [30,31].

Due to the fact that neutral aminopeptidase activities of all lymphoid cell lines investigated have biochemical features such as cellular location and isoelectric point similar to those of the T cell line H9, we conclude that the main neutral aminopeptidase activity of all lymphoid cells has to be attributed to another enzyme which is not related to the well-characterised APN (EC 3.4.11.2).

Taken together, the data presented here and our former results, support the model of a low-level constitutive APN expression in T cells and related cell lines which is increased after mitogenic stimulation or malignant transformation as it was demonstrated by others [13,17,25,28]. This increase is, however, always associated with the appearance of CD13-immunoreactivity on the cell surface. However, besides the APN at least one other cytosolically located enzyme contributes to the total neutral aminopeptidase activity in lymphocytes and related cell lines. This enzyme represents the main neutral aminopeptidase activity of CD13-negative lymphoid cell lines. The nature of this Ala-βNA hydrolysing activity is still unclear.

Preliminary biochemical studies (gel chromatography, sensitivity towards inhibitors and metal ions) of this aminopeptidase activity enriched from H9 cells have revealed that it could be distinguished from other well-characterised aminopeptidases such as leucine-aminopeptidase (EC 3.4.11.1), cytosolic alanine-aminopeptidase (EC 3.4.11.14) and puromycin-sensitive aminopeptidase. Purification and subsequent

biochemical analyses of this aminopeptidase will be the next challenge.

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