Induction of the membrane alanyl aminopeptidase gene and surface expression in human T-cells by mitogenic activation

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The metal-dependent membrane alanyl aminopeptidase (aminopeptidase N, APN, CD13; EC 3.4.11.2) is a well-established marker of normal and malignant cells of the myelo-monocytic lineage. It is also expressed by leukaemic blasts of a small group of patients suffering from acute or chronic lymphoid leukaemia. CD13-specific monoclonal antibodies do not bind to the surface of normal B lymphocytes, and APN mRNA was not detectable by Northern analysis in normal lymphocytes or in T-cell lines. Recently the expression of the APN gene in T-cell lines as well as the ability of these cells to cleave chromogenic substrates preferred by APN have been demonstrated [Lendeckel, Wex, Kähne, Frank, Reinhold and Ansgorge (1994) *Cell. Immunol.* **153**, 214–226]. Here, by means of dot-blot hybridization and RNase protection assay, evidence is provided that human peripheral T-cells as well as derived cell lines contain significant amounts of APN mRNA, comparable to that in the promyelocytic cell line U937, and that mitogenic activation of peripheral human T-cells leads to a more than 4-fold increase in their APN mRNA content. In the course of activation, T-cells increase their total

alanine *p*-nitroanilide-hydrolysing activity to approx. 7-fold that of resting cells. Furthermore these cells become immunoreactive towards CD13 to a significant extent (up to 51 %) as shown by surface staining and confirmed by activity staining and immunostaining after isoelectric focusing (pI of T-cell APN = 4.6). In addition it is demonstrated by fluorescence microscopy that viable, activated T-cells effectively cleave the fluorogenic aminopeptidase substrate bis-glycyl-rhodamine 110 and that the corresponding aminopeptidase activity is associated with the cell surface. We show that specific inhibitors of APN, probestin and actinonin, strongly decrease DNA synthesis in phytohaemagglutinin (PHA)-stimulated T-cells. In summary, evidence is presented that in the course of mitogenic activation human peripheral T-cells increase the expression of APN both at the transcriptional level and at the cell surface. This has been demonstrated both at the APN mRNA level and at the protein level with respect to aminopeptidase enzymic activity and CD13 immunoreactivity.

INTRODUCTION

Membrane alanyl aminopeptidase (aminopeptidase N, APN, CD13; EC 3.4.11.2) is a 150 kDa metalloprotease preferentially cleaving neutral amino acids from the N-terminus of oligopeptides. In humans the APN gene is located on the long arm of chromosome 15 (q11-qter) [1]. It is highly expressed in the intestine, where it participates in the final hydrolysis of ingested nutrients, as well as in the kidney and to a smaller extent in other tissues [2,3]. APN is believed to be involved in the degradation of neuropeptides and angiotensin [4,5], but the physiological role of the lymphocyte-derived enzyme remains unclear. Two excellent studies have provided evidence that the CD13 antigen might function as a corona virus receptor in human lymphocytes [6,7]. APN seems to play a role in tumour invasion and was shown to contribute to the degradation of collagen type IV [8]. Furthermore APN has been reported to be implicated in antigen processing [9].

With respect to the haematopoietic system, APN has been accepted as being exclusively expressed on cells of the myelo-monocytic lineage [10]. Subsequently it was shown, however, to be expressed on the surface of malignant B-cells as well [11,12]. Another group reported the expression of the CD13 antigen on

the surface of concanavalin A (ConA)-stimulated T-cells [13,14]. The induction of CD13 in T-cells [15] or natural killer cells [16] derived from the synovial fluid of patients with rheumatoid arthritis might also be a response to some stimulating signal. Moreover the induction of CD13 has been observed on immature thymocytes [17], but this rather reflects the fact that these cells still retain characteristics of multipotent progenitor cells.

It has been demonstrated that the human T-cell lines HuT78 and H9 contain both alanine *p*-nitroanilide (Ala-pNA)-hydrolysing activity and APN mRNA [17a]. Subsequently the copy number of APN gene transcripts in both T-cell lines was determined by competitive PCR [18]. Consistent with these findings is the existence of CD13-positive human T-cell lines commercially available [German Collection of Microorganisms and Tissue Cultures (DSMZ, Braunschweig, Germany); KARPAS (ACC31) and P12/ICHIKAWA (ACC34)]. The human T-cell line Jurkat contains an aminopeptidase activity distinct from APN [19].

Here, for the first time, evidence is provided that in the course of mitogenic activation of human peripheral T-cells a strong increase in both APN mRNA content and neutral aminopeptidase activity is accompanied by the presence of CD13 immunoreactivity on the surface of these cells.

Abbreviations used: Ala- β MeNA, alanine β -methoxynaphthylamide; Ala-pNA, alanine *p*-nitroanilide; APN, aminopeptidase N (= CD13); ConA, concanavalin A; Gly-Rho₁₁₀-Gly, bis-glycyl-rhodamine 110; IEF, isoelectric focusing; IL, interleukin; mAb, monoclonal antibody; PHA, phytohaemagglutinin; PMA, phorbol 12-acetate 13-myristate; RPA, RNase protection assay; T-CLL, chronic T-cell leukaemia.

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MATERIALS AND METHODS

Cell culture and stimulation experiments

Peripheral blood T-cells were enriched by the nylon wool adherence technique as described [20]. T-cells were grown in complete Iscove's modified medium supplemented with 10% (v/v) fetal calf serum (Gibco BRL, Eggenstein, Germany), 60 i.u./ml penicillin and 50 µg/ml Ciprobay. Cells were grown in 50 ml culture flasks to densities of approx. 10^6 cells/ml. T-cells were stimulated by the addition of ConA or PHA-L (1 µg/ml each) (Boehringer Mannheim, Mannheim, Germany), PHA-H (5 µg/ml) (Boehringer Mannheim), pokeweed mitogen (2 µg/ml) (Serva, Heidelberg, Germany), interleukin 2 (IL-2; 50 units/ml) (Boehringer Mannheim), anti-CD3 monoclonal antibody (mAb; 100 ng/ml) (Dianova, Hamburg, Germany) or anti-CD28 mAb (100 ng/ml) (Dianova). In all cases phorbol 12-acetate 13-myristate (PMA; Sigma, Heidelberg, Germany) at 10 ng/ml was used as co-stimulant.

To exclude possible cytotoxic effects of the aminopeptidase inhibitors bestatin and actinonin (Sigma) and probestin (kindly provided by T. Aoyagi), we measured the viability of all cell cultures by Trypan Blue staining and tetrazolium salt reaction using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma). In this study the viability of T-cells was not significantly affected by the inhibitors.

Proliferation assay

Purified T lymphocytes (10^5 cells/100 µl) were suspended in serum-free CG-medium (Virotec, Vilshofen, Germany) and stimulated with PHA-L in the presence of different concentrations of the aminopeptidase inhibitors actinonin, probestin and bestatin.

After 90 h the cultures were pulsed for a further 6 h with [3 H]methylthymidine (0.2 µCi per well) (Amersham, Braunschweig, Germany). Cells were harvested on glass fibre filters and the incorporated radioactivity was measured by scintillation counting.

Immunofluorescence studies

Staining of T-cells with the PE-labelled anti-CD13 antibody, clone WM15 (Dianova), was performed at 4 °C for 30 min. After two washes with PBS (0–4 °C), the cells were fixed with 1% (w/v) paraformaldehyde in PBS. Cytofluorimetric analysis was performed on an Epics Profile II (Coulter). 20000 cells per sample were counted. The threshold was defined such that positive staining included no more than 1% of the relevant control.

Isoelectric focusing (IEF) and Western blotting

Cells (2×10^7) were solubilized in 100 µl of 1% (w/v) n-octyl- β -D-glucopyranoside in PBS on ice for 30 min. Insoluble particles were sedimented by centrifugation for 30 min at 4 °C and 100000 g. Portions (20 µl) of the resulting supernatant were applied on a 0.8% agarose gel (IEF-agarose, Pharmacia/LKB) containing 7% (v/v) Servalyte 3-10 and 2% (v/v) Servalyte 4-6 (Serva). Running conditions were: 0 °C, 2 kV, 20 mA, 35 W, 2000 V h.

After capillary blotting of IEF gels on nitrocellulose membranes (BA 85, Schleicher and Schüll) and blocking in 7.5% (w/v) milk powder in PBS, APN was detected with the anti-CD13 mAb clone My7 (Coulter). The detection was performed by means of goat anti-(mouse alkaline phosphatase) conjugate and NBT/BCIP as the chromogenic substrate.

Duplicates of these blots were used for staining APN enzymically with the chromogenic substrate alanine β -methoxynaphthylamide (Ala- β MeNA) and Fast Blue B.

Ala-pNA-hydrolysing activity

Ala-pNA-hydrolysing activity was measured in triplicate. Cells (100 µl) suspended in PBS, pH 7.4, were mixed with 100 µl of 5 mM Ala-pNA. Reactions were stopped by the addition of 400 µl of 1 M sodium acetate, pH 4.4, either immediately (substrate blank) or after 60–120 min of incubation at 37 °C. After spinning down all particulate material (90 s at 10000 g), the absorbances of the supernatants were read at 390 nm with a Spekol 21 photometer (Carl Zeiss Jena).

Bis-glycyl-rhodamine 110 (Gly-Rho₁₁₀-Gly)-hydrolysing activity

Gly-Rho₁₁₀-Gly was synthesized as described [21], with minor modifications. t-Butoxycarbonyl-glycine was coupled with rhodamine 110 (Synthon AcMaRi Chemie, Wolfen, Germany) by the mixed anhydride method. The t-butoxycarbonyl group was removed by treatment with HCl in acetic acid. Structure and purity were confirmed by HPLC, mass spectroscopy and NMR spectroscopy.

Resting or PHA-L/PMA-activated T-cells were left for 1 day in supplemented Iscove's modified medium at 37 °C and then washed three times in PBS. Cells were resuspended in PBS to a density of 10^6 /ml. Cell suspension (100 µl) was loaded in a well of a 96-well culture plate (Greiner, Germany) and allowed to settle for 10 min. Fluorescence development was followed by video-microscopic observation. Immediately before the addition of Gly-Rho₁₁₀-Gly to a final concentration of 20 µM the starting fluorescence was captured with a standard fluorescein isothiocyanate filter set ($t = 0$ min). Thereafter, fluorescence signals were captured every 300 s up to a total reaction time of 20 min.

RNA isolation and RNase protection assay (RPA)

RNA from 5×10^6 cells in each experiment was prepared by means of guanidinium rhodanide/acidic phenol in accordance with standard protocols [22]. Aliquots of 10 µg of total RNA were used in the RPA, which was performed with the RPA II kit (Ambion, Austin, TX, U.S.A.) exactly following the protocol recommended by the supplier. A 32 S-labelled APN anti-sense RNA fragment (718 bp, 6×10^7 c.p.m.) was used as the probe. Protected fragments were loaded on a 6% (w/v) standard sequencing gel (in 1 × Tris/borate/EDTA buffer at a constant 50 W). The fixed and dried gel was exposed to X-ray film (Kodak) for 3–12 days.

Dot-blot hybridization

Total RNA was transferred to Qiabran® (Qiagen, Hilden, Germany) by standard techniques [22] and hybridized overnight at 68 °C in Quickhyb® (Stratagene, Heidelberg, Germany) to a 32 P-labelled probe, obtained by random primed labelling of full-length APN cDNA cloned into pBluescript [10]. The membrane was exposed to X-ray film (Kodak) for 2 days.

RESULTS

Aminopeptidase activity

Freshly isolated peripheral blood T-cells exhibit only low levels of Ala-pNA-hydrolysing activity, on average 4.8 pkat per 10^6 cells. This activity is markedly increased during T-cell activation. The increase in Ala-pNA-hydrolysing activity was found to be

Table 1 Induction of Ala-pNA hydrolysing activity in peripheral human T cells by various stimulating agents after 3 days of culture

All values are given as means $\pm$ S.E.M. Each value represents at least six experiments. Abbreviations: PWM, pokeweed mitogen; n.d., not determined.

Stimulant	Ala-pNA-hydrolysing activity (pkat per 10^6 cells)	CD13 immunoreactivity (% positive cells)
None	4.9 ± 0.8	< 1
PHA-L/PMA	31.4 ± 2.8	51 ± 17
ConA/PMA	18.9 ± 4.2	42 ± 11
PHA-H/PMA	16.2 ± 3.6	32 ± 7
IL-2/PHA	12.4 ± 3.6	n.d.
PWM/PMA	10.8 ± 1.6	24 ± 7
Anti-CD3/PMA	8.6 ± 1.4	14 ± 4
Anti-CD28/PMA	7.2 ± 2.1	n.d.

Figure 1 IEF of solubilized T-cells followed by immunostaining or enzymic staining

Left panel: resting (lane 2) and two independent samples of PHA/PMA-stimulated (lanes 3 and 4) T-cells stained enzymically with Ala- β MeNA and FBB. Included as a reference is HuT78 solubilize (lane 1), which shows aminopeptidase activity at a pI of 5.2. Right panel: blot identical with that shown in the left panel except that HuT78 cells were not included and immunostaining was performed with the anti-CD13 mAb (clone My7), which was detected with goat anti-(mouse alkaline phosphatase) and NBT/BCIP.

dependent on the stimulating agent used. As shown in Table 1, the combined action of PHA-L/PMA seemed to be most effective in this regard and resulted in an activity of 31.4 pkat per 10^6 cells.

The observed increase in aminopeptidase activity could also be demonstrated by IEF of T-cell lysates and subsequent blotting of the proteins on nitrocellulose membranes. By means of activity staining of these blots with the chromogenic substrate Ala- β MeNA, aminopeptidase activity could be detected in peripheral T-cells stimulated by PHA/PMA. In contrast, resting T-cells showed no Ala- β MeNA-hydrolysing activity. As shown in Figure 1 (left panel), the Ala- β MeNA-hydrolysing activity of stimulated T-cells appeared on the blot as a single band, the location of which implied a pI in the region of 4.6. Furthermore this activity was immunoreactive with the anti-CD13 mAb clone My7 (Coulter Laboratories), as revealed by immunostaining of an identical part of the same IEF gel (Figure 1, right panel). Therefore the T-cell aminopeptidase activity detected in the IEF analyses is assumed to be identical with APN. This view is supported by the finding that the previously reported pI of the major aminopeptidase activity of U937 cells is in the same region [17a].

These findings were extended by fluorescence microscopy, showing that viable activated T-cells readily cleaved the neutral

Figure 2 Induction of aminopeptidase activity in activated T-cells

PHA-L/PMA-activated (A, C, E) or resting (B) T-cells were incubated in PBS (pH 6.5) for 20 min after the addition of the fluorogenic substrate Gly-Rho₁₁₀-Gly (final concentration 1 μ M). (D, F) Bright fields corresponding to (C) and (E) respectively. Magnifications: (A) $\times$ 480; (B-F) $\times$ 1200.

Figure 3 Influence of APN inhibitors on DNA synthesis in PHA-stimulated T-cells

Purified T-cells were stimulated with PHA-L (1 μ g/ml) in the presence of different concentrations of actinonin (■), probenstin (◆) and bestatin (●) as outlined in the Materials and Methods section. [3 H]Thymidine incorporation (in c.p.m.) is indicated as means $\pm$ S.E.M. for three different experiments. The values are expressed as a percentage of the [3 H]thymidine incorporated compared with control cultures without inhibitor. [3 H]Thymidine incorporation in control cultures: $32\,400 \pm 4\,700$ c.p.m.

aminopeptidase substrate Gly-Rho₁₁₀-Gly, whereas resting cells did not. Figure 2 clearly shows fluorescence signals due to the removal of Gly-residues by PHA-L/PMA-stimulated T-cells. The aminopeptidase activity is localized near the cell surface.

Surface expression of CD13

T-cell activation leads to a significant increase in the surface expression of CD13. Whereas resting T-cells are practically lacking surface expression of CD13, we found up to 51% CD13-positive T-cells 3 days after activation (Table 1). All stimulants used induced considerable Ala-pNA-hydrolysing activity and surface expression of CD13 on peripheral T-cells, but PHA-L (L₄E₀) and ConA were the most effective.

Figure 4 Activation-dependent increase in T-cell APN mRNA content

Upper panel: total RNA isolated from resting (control) or ConA/PMA-activated T-cells was immobilized on a nylon membrane and hybridized with a ^{32}P -labelled APN cDNA fragment. A more than 4-fold increase in APN mRNA in the stimulated T-cells can be seen. Lower panel: RPA. Total RNA (10 μg) from resting or activated T-cells (left four lanes) or poly(A) $^{+}$ RNA (2 μg) from different cell lines and a case of T-CLL (right five lanes, as indicated above each lane) were analysed with a ^{32}P -labelled APN cRNA probe [where cRNA is complementary RNA (anti-sense)] and the RPA II kit. Whereas resting T-cells lack detectable amounts of APN mRNA (T cells 1), PHA-L/PMA stimulated T-cells (T cells 2) contain easily detectable amounts of APN transcripts after 3 days of culture. The stimulation of T-cells by ConA/PMA or PHA-L/PMA in the presence of cycloheximide further increased APN mRNA levels (T cells 3 and 4). For details see the Materials and methods section.

Effect of aminopeptidase inhibitors on DNA synthesis in stimulated T-cells

The influence of APN inhibitors on DNA synthesis of PHA-L-stimulated T lymphocytes was studied by incubation of viable cells for 96 h in the presence or absence of various concentrations of actinonin, prostest and bestatin. As shown in Figure 3, DNA synthesis in PHA-L-stimulated T-cells was strongly suppressed by all the inhibitors used (60–70 % inhibition at 10 μM).

APN mRNA contents

The induction of aminopeptidase activity is accompanied by a more than 4-fold increase in APN mRNA content. This was shown by both the RPA and the dot-blot hybridization experiments, which revealed an increase in APN mRNA content of T-cells after stimulation by ConA/PMA in comparison with resting cells (Figure 4). The latter ones contained only trace amounts of APN mRNA. The activation-dependent rise in APN mRNA content could be further increased by the exposure of T-cells to cycloheximide during activation by either ConA/PMA or PHA/PMA (Figure 4, lower panel, lanes 4 and 5 from the left).

The T-cell lines Molt16, Molt17 and Jurkat as well as peripheral T-cells from a patient suffering from chronic T-cell leukaemia (T-CLL) were also found to contain significant amounts of APN mRNA, which were not different from those of the highly CD13-positive myeloid cell line U937 (figure 4, lower panel, lanes 5–9).

DISCUSSION

The results presented here demonstrate clearly an activation-dependent increase in neutral aminopeptidase activity in human peripheral T-cells. The induction was observed with all stimulants used, which is suggestive of a link between T-cell activation and induction of aminopeptidase activity. In support of this view, T-cells have been reported by others to increase their surface expression and enzymic activity of aminopeptidase B and aminopeptidase P in the course of activation [24,25]. In our experiments, PHA and ConA were the most effective in inducing Ala-pNA-hydrolysing activity. With PHA we found PHA-L (L_4E_0) to be much more effective than PHA-H, which consists of both L and E subunits. The maximum effect seen with PHA-L could not be achieved even with higher doses of PHA-H (results not shown).

The fact that the presence of relevant neutral aminopeptidase activity is restricted to activated T-cells was further confirmed by the observed cleavage of the fluorogenic aminopeptidase substrate Gly-Rho $_{110}$ -Gly by viable T-cells, which is in accordance with the observed induction of surface expression of CD13.

Both Ala-pNA/Ala- β -MeNA and Gly-Rho $_{110}$ -Gly are substrates of limited specificity, being readily hydrolysed by a number of aminopeptidases. However, IEF and subsequent activity staining revealed that under the conditions applied only a single aminopeptidase species (pI approx. 4.6) effectively cleaves Ala- β -MeNA. The immunoreactivity with anti-CD13 mAb (clone My7) of this aminopeptidase activity implies that this aminopeptidase is in fact APN. In support of this view, this aminopeptidase activity was hardly affected by the aminopeptidase B-specific inhibitor arphamenine B (at 50 μM) and failed to hydrolyse Arg- β -MeNA or the corresponding Glu derivative, which is strongly preferred by aminopeptidase A.

Additional evidence supports the idea that APN is induced in or on activated T-cells. First, a significant fraction of activated T-cells was found to be CD13-positive. As with the Ala-pNA-hydrolysing activity, PHA or ConA respectively, together with PMA, provoked a dramatic increase in the surface expression of CD13 on T-cells (on a maximum of 51 % positive cells). Other stimulants were found to be less effective. Secondly, T-cell activation is simultaneous with a marked increase in APN mRNA levels. The clear effects of ConA/PMA and PHA-L/PMA on APN mRNA content make it reasonable to assume that the other stimulants found capable of inducing Ala-pNA-hydrolysing activity and surface expression of CD13 act in the same way on the APN mRNA levels.

The activation-dependent increase in Ala-pNA-hydrolysing activity and surface expression of CD13 of peripheral T-cells is accompanied by, and is therefore likely to be based on, the increases in APN mRNA content, which have been observed in the dot-blot hybridization and the RPA experiments. Interestingly, the exposure of T-cells to cycloheximide in the course of activation provoked a further elevation of their APN mRNA content. Therefore APN gene transcription seems to be repressed by a negative transcription factor, as is well known for interferon γ , for example. The induction of aminopeptidase B in response to T-cell activation has also been reported to be significantly enhanced in the presence of cycloheximide [24]. A recent report convincingly demonstrated the repression of APN gene expression in leukaemic B cells by stromal cells [26]. Active repression might therefore be the general mechanism underlying the regulation of APN gene expression in human tissues, and in particular in lymphocytes.

With the RPA we investigated not only peripheral T-cells but also several T-cell lines and one case of T-CLL. Interestingly,

significant amounts of APN mRNA were detected in all cases, and they did not differ significantly from that of the myeloid U937 cell line, which is highly CD13-positive and was included in the experiments as a reference. The finding that leukaemic T-cells contain APN mRNA amounts comparable to those in U937 cells is quite unexpected. We therefore conclude that enhanced T-cell proliferation, achieved either by T-cell activation or by malignant transformation as seen in T-cell leukaemias or derived cell lines respectively, is accompanied by or even caused by an induction of APN gene expression.

We have shown that actinonin, bestatin and probestin suppress DNA synthesis of PHA-stimulated peripheral blood mononuclear cells and of T-cells stimulated with IL-1 or IL-2 [13]. Other groups also reported on the inhibiting properties that bestatin exerts on the growth of human leukaemic cell lines, i.e. HL-60, K562, MT-1, MT-2, Molt-4 and Raji [27], or peripheral B- and T-cells [28,29]. Probestin and actinonin are supposed to be highly specific inhibitors of APN [30]. Therefore the suppression of T-cell proliferation by bestatin, actinonin and probestin is likely to be due to the inhibition of APN. The context provided by the results on the induction of APN gene and surface expression supports this view and may give first clues to the role of APN in the process of T-cell activation.

Our results therefore demonstrate the induction of the APN (CD13) gene and surface expression in the course of T-cell activation. To our knowledge this is the first report to demonstrate clearly, both enzymically/immunologically and on the mRNA level, that human peripheral T-cells markedly increase APN gene and surface expression in response to activation.

In conclusion, our results support the assumption [15] that APN is an activation marker of human T-cells. In this regard APN performs like the dipeptidyl peptidase IV (CD26), another ectopeptidase whose expression is significantly up-regulated in the course of T-cell activation [31,32].

The stimulation system used in our study properly reflects the situations found *in vivo* at local inflammatory sites and might prove a suitable system *in vitro* for exploring the role of APN induction on T-cells as well as the underlying mechanisms. These key issues need to be addressed in future studies.

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