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ACTIVATION-DEPENDENT INDUCTION OF T CELL ALANYL AMINOPEPTIDASE AND ITS POSSIBLE INVOLVEMENT IN T CELL GROWTH

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1. INTRODUCTION

Membrane alanyl aminopeptidase (APN, EC 3.4.11.2) is a 150-kDa metalloprotease which has been identified as the leukocyte surface differentiation antigen CD13[1]. In humans the APN gene is located on the long arm of chromosome 15 (q11-qter) [2] with the coding part of the gene encoded by 20 exons[3]. Within the haematopoetic system APN is dominantely expressed in cells of the myelo-monocytic lineage and is used, therefore, as a standard marker in the diagnosis of leukaemia. Aminopeptidase N of leukocytes is supposed to be involved in the degradation of neuropeptides[4–7] and cytokines[8,9], but its function remains to be fully elucidated. APN may function as a corona virus receptor[10–13] and seems to contribute in tumor invasion and matrix degradation[14–15]. APN is also implicated in antigen processing[16]. Furthermore, anti-CD13 monoclonal antibodies have been shown to neutralize CMV[17]. In recent years evidence accumulated showing that malignant B and T cells[18–23] as well as activated T cells[24–26] are capable of expressing APN on the cell surface. A similar mechanism of induction may underlie the CD13 surface expression of tumor infiltrating T cells[27], or T cells[28] and NK cells[29] derived from local sites of inflammation.

T cell lines H9 and HuT78 have been shown to contain both neutral aminopeptidase activity and high amounts of APN-mRNA[30]. Determination of the exact copy number of APN transcripts by competitive PCR[31] indicated that APN-mRNA represents an abundance class II mRNA-species in these cell lines.

Here we describe the induction of aminopeptidase activity, CD13 surface expression, and APN-mRNA contents in response to T cell activation. Furthermore, we show that inhibition of either aminopeptidase activity or APN-gene expression decreases T cell proliferation and alters their cytokine production.

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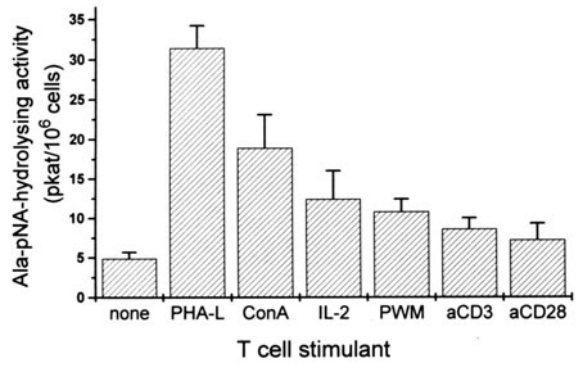

Figure 1. Capability of a panel of T cell activating agents to induce Ala-pNA-hydrolysing activity. T cells enriched by nylon wool adherence were cultured in RPMI1640 / 10% FCS in the presence of the stimulants indicated together with 10 nM PMA for 3 days. Cells were harvested and Ala-pNA-hydrolysing activity was measured in the cell lysate as described previously[30]. Concentrations of the stimulants were as follows: phytohaemagglutinine (PHA-L) and concanavalin A (ConA) 1µg/ml each, 2 µg/ml pokeweed mitogen (PWM), 50 units/ml IL-2, 100 ng/ml aCD3 or aCD28.

2. INDUCTION OF Ala-pNA-HYDROLYSING ACTIVITY

Resting peripheral T cells contain only minute amounts of Ala-pNA-hydrolysing activity (4.8 pkat/10^6 cells). However, in response to activation T cells gradually increase their neutral aminopeptidase activity reaching peak levels of up to 31 pkat/10^6 cells 3 to 4 days after activation. As shown in figure 1, different stimulating agents all induce T cell aminopeptidase activity, although with different efficiency. Ala-pNA-hydrolysing activity of activated T cells could not simply attributed to aminopeptidase N. However, support for the assumption that the increase in activity is due to an increase of APN expression comes from the observed induction of CD13 surface expression and APN-mRNA levels in response to T cell activation (see below).

3. INDUCTION OF CD13 SURFACE EXPRESSION

Freshly isolated peripheral T cells are CD13-negative by cytofluorimetric anlysis or other techniques of similar sensitivity, however, when activated by PHA-L/PMA or ConA/PMA T cells rapidly become CD13-positive. The increase in CD13 surface expression is detectable as early as 20 hours after activation and is reaching peak levels after 48 to 72 hours. Although the CD13-density per cell is rather weak, about half of the activated cells are positive by cytofluorimetric analysis and nearly 100 % of cells are CD13-positive when analyzed by fluorescence microscopy as shown in figure 2. Obviously, there is an induction of both intracellular and surface-associated CD13 in response to T cell activation.

4. ACTIVATION-DEPENDENT INCREASE OF APN-mRNA CONTENTS

Activation of T cells goes along with a dramatic increase of APN-mRNA contents, as shown recently by both RNase protection assay and dot-blot hybridization[25]. Studying the time course of APN-mRNA induction by RT-PCR we found that trace amounts only of

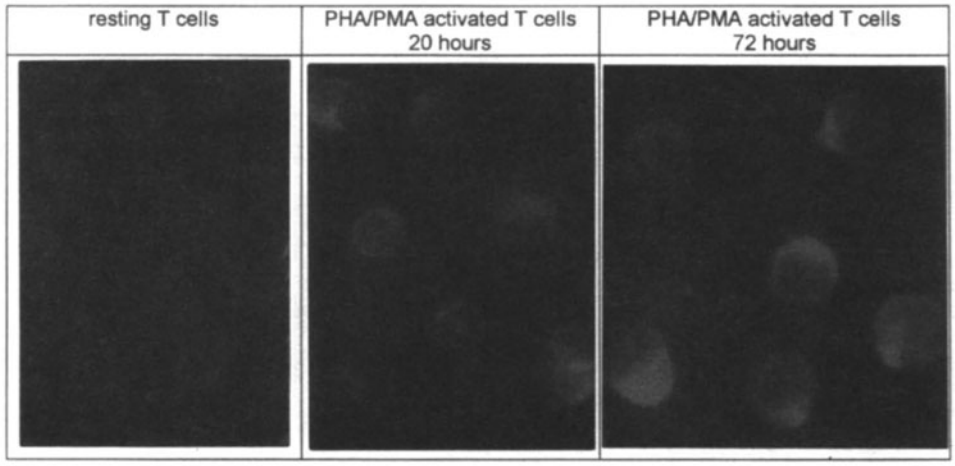

Figure 2. CD13-immunostaining of resting and activated T cells. T cells were cultured as described in legend to figure 1. Resting cells were analyzed after one day of culture in medium only. PHA-L/PMA-activated T cells were analyzed 20 or 72 hours after stimulation. Fifty microliters of cell suspension ($5x10^6$ cells/ml) were used for immunostaining of internal or membrane associated CD13 by means of the antiCD13 mab (clone WM15, Dianova) and the cell permeabilization kit (Dianova), essentially as recommended by the supplier. Normal goat serum was included to block unspecific binding of the secondary antibody, goat-anti-mouse conjugated to texas red). Controls lacking the primary antibody showed no fluorescence (not shown). Resting T cells (left) showed low CD13-immuno-reactivity, which increased gradually in response to T cell activation. Middle: 20 hours, right: 72 hours after activation. Cells were analyzed by video fluorescence microscopy (Axiovert 135 TV (Zeiss); Fluar x100 oil immersion, filter set 00 (Zeiss), 3CCD video camera (Sony), contron frame grabber) Integration: 250 fields for resting cells, 150 fields for cells activated 20 hours, 50 fields for cells activated 72 hours.

APN-mRNA are detectable until 24 hours after stimulation by PHA-L/PMA, but maximum APN-mRNA levels occur 3 days after activation and represent an 8-fold increase, approximately (figure 3).

5. INHIBITION OF APN ENZYMATIC ACTIVITY OR APN-TRANSCRIPTION

5.1. Aminopeptidase Inhibitors

The potent aminopeptidase inhibitor bestatin has been reported to influence the proliferation of a wide variety of cells[32–35]. Bestatin strongly inhibits both leucyl-aminopeptidase and soluble arginyl-aminopeptidase at sub-micromolar concentrations and partially inhibits APN and probably a number of other aminopeptidases at higher concentrations as well. The aminopeptidase inhibitors probestin and actinonin appear to be more specific, at concentrations up to 10 μM both effectors predominantely inhibit APN[36–38]. Therefore, the effects of bestatin on the DNA synthesis of activated T cells were compared to those observed with probestin and actinonin. As it is shown in figure 4, all inhibitors provoked a significant reduction of DNA synthesis at micromolar concentrations in a dose-dependent manner. At 5 μM probestin caused a 50% reduction of DNA synthesis, whereas bestatin and actinonin reduced DNA synthesis to less than 35% of control. Actinonin and bestatin were equally effective in inhibiting DNA synthesis suggesting that this effect is indeed due to the inhibition of APN.

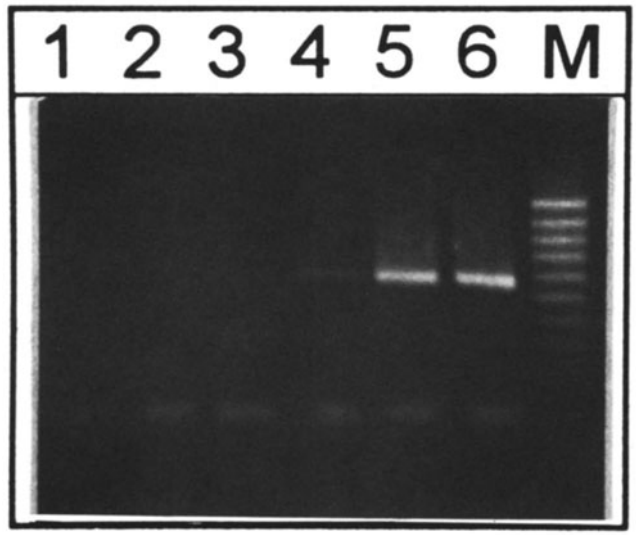

Figure 3. Induction of APN-mRNA in the course of T cell activation. RT-PCR. was performed using 500 ng total RNA from T cells harvested after 1, 3, 5, 24, 48, or 72 hours (lanes 1 to 6) of activation by PHA-L/PMA. In a 20 µl -volume RNA was transcribed by 20 units of AMV reverse transcriptase (Boehringer Ingelheim) in the supplied buffer with the addition of 0.5 mmol/l dNTP, 10 mmol/l DTT, 50 mmol/l random hexanucleotides (Boehringer Mannheim) and 50 units of placenta RNase inhibitor (Ambion) during a 1 hr incubation at 37°C. The enzyme was inactivated by a 10 min incubation at 65°C and then one tenth of the reverse transcription reaction was used as the template for the amplification reaction. Twenty five cycles were performed in an Autogene II (CLF) in 50 µl reaction buffer containing 0.5 units Goldstar Taq-polymerase (Eurogentec), 0.5 mmol/l dNTP, and 100 ng of APN-specific primers (forward: gtctactgcaacgctatcg; reverse: gatggacacatgtgggcaccttg; yielding a 573 bp product). The initial denaturing step was for 1.5 min at 95°C. Each cycle consisted of annealing for 0.7 min at 60°C, elongation for 72°C for 1.0 min, and denaturing at 96°C for 0.3 min. The final elongation step was extended to 3.0 min. Ten microliters of each reaction mixture was loaded on a 1.9% agarose gel and electrophoresed at 5V/cm in 1xTBE buffer and then stained with ethidium bromide.

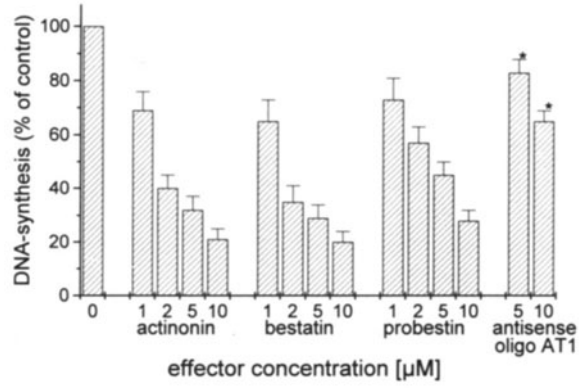

Figure 4. Inhibition of DNA-synthesis of ConA/PMA-activated peripheral T cells by amino-peptidase inhibitors and APN-specific antisense oligodesoxyribonucleotides. T cells were grown as described in legend to fig.2. Aminopeptidase inhibitors were applied simultaneously with the activation and oligodesoxyribonucleotides were given one day prior to the activation. The final concentration of the effectors was as indicated at the bottom axis. After 3 days cells were pulsed with ^{3}H-methyl-thymidine (0.2µCi per well; Amersham) for six hours. Cells were harvested onto glass fiber filters, lysed, washed with TCA and the TCA-unsoluble fraction of the incorporated radioactivity was determined by scintillation spectrometry (betaplate, LKB Pharmacia, Sweden). Given is the mean of 6 experiments ± sd. * In the case of the oligonucleotides the antisense data were compared to the sense-control.

5.2. APN-Specific Antisense Desoxyribonucleotides

Further support for the assumption that APN contributes in the cell proliferation machinery comes from the observation, that APN-specific antisense desoxyribonucleotides are capable of significantly reducing the DNA-synthesis of activated T cells. Pre-incubation of T cells for one day in the presence of 5 or 10 µM of the antisense desoxyribonucleotide AT1 (5′-thio-CACCACTGACAGTGCGATG) followed by activation with ConA/PMA reduced DNA-synthesis measured 3 days after activation to 83% or 65% of the corresponding sense control AT2 (5′-thio-CATCGCACTGTCAGTGGTG) (figure 4). Similar results were obtained using the T cell line H9 instead of peripheral T cells (not shown).

5.3. APN-Specific Antisense-Vector

As outlined above, both aminopeptidase inhibitors and APN-specific antisense desoxyribonucleotides interfer with T cell DNA synthesis. This work has been extended by showing that H9 cells transfected with an APN-antisense vector also grow at a markedly reduced rate, compared to H9 cells transfected with the corresponding sense-vector.

The vector was constructed by inserting a 2785 bp HindIII-fragment of the APN-cDNA (kindly provided by T.A.Look[1]) into pREP4 (Invitrogene). The cDNA-fragment was inserted adjacent to the RSV-promoter in either sense or antisense orientation.

Transfected H9 cells were grown in RPMI 1640 medium containing 10% fetal calf serum for two days and then DNA-synthesis was measured as described earlier[29].

Figure 5 shows a 75% decrease of DNA-synthesis in the H9 cells transfected with the APN-antisense vector compared to the sense control.

Inhibition of APN expression in H9 cells by an APN-antisense vector not only changed the proliferation rate of H9 cells, but was also found to alter the production of IL1-RA. Whereas in the supernatant of wild type H9 cells or sense transfectants less than 2 pg/ml of IL1-RA was detectable, that of H9 cells transfected with APN-antisense plasmid contained about 10 pg/ml IL1-RA, representing an fivefold increase in secreted IL -

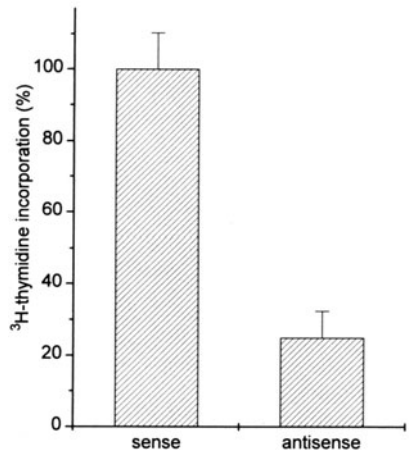

Figure 5. Inhibition of DNA-synthesis in H9-transfectants lacking APN expression. H9 cells and the derived transfectants were seeded in 96well plates at a density of 10.000 to 50.000 cells/ 200µl medium per well. After 24h cells were pulsed with ^{3}H-methyl-thymidine and DNA-synthesis was measured as described in legend to figure 4. Activities of antisense transfectants and sense transfectants were in the range of 120 - 1100 or 1100 - 3580 cpm, respectively. Given is the mean ± sd of three independent experiments, with each value determined in triplicate.

RA. IL1-RA concentrations in the supernatant were measured by ELISA (R&D Systems) according to the protocol recommended by the supplier.

6. DISCUSSION

The data presented here prove the induction of APN gene and surface expression in response to T cell activation. This induction has been shown on the level of APN-mRNA as well as on the protein and enzymatic activity levels.

With respect to CD13 surface expression and neutral aminopeptidase activity, an increase could be observed as early as 24 hours after stimulation, whereas a significant increase in APN-mRNA levels has been detected after 2 or 3 days.

The combination of PHA-L/PMA appeared to be the most efficient one capable of inducing APN/CD13 expression, but other stimulants were also effective. This suggests that there is a link between T cell activation and the expression of APN/CD13.

As it is demonstrated by fluorescence microscopy (figure 2), nearly all T cells acquire CD13 surface expression in response to T cell activation. Therefore, activation-dependent CD13 surface expression cannot be assigned to a certain T cell subset, but should be regarded as a general T cell activation marker instead.

Actinonin, bestatin and probestin have been shown recently to suppress DNA synthesis of PHA-stimulated peripheral blood mononuclear cells and of IL-1-, IL-2- or PHA/PMA-stimulated T cells[29,30]. Extending this work, we show that APN-specific antisense-oligodesoxyribonucleotides also significantly decrease DNA-synthesis of activated T cells. This led us to propose APN playing a role in regulation of cell proliferation. Further support for this assumption comes from the work on H9 cells transfected with APN antisense or sense plasmids. As seen with peripheral T cells, the T cell line H9 showed a markedly reduced DNA-synthesis when APN gene expression was inhibited by transfection of an APN-antisense plasmid. The context provided by the data on aminopeptidase inhibitors, APN-specific antisense oligodesoxyribonucleotides and the APN-antisense vector strongly implies that APN indeed contributes in the cell proliferation process. This gives a first clue on the function of APN in/on human T cells and might explain the observed activation-dependent induction of APN gene and surface expression.

Consistent with this view of APN as a regulator/modulator of cell growth is the abnormal expression on malignant lymphocytes of CD13 in cases of acute lymphocytic leukaemia (B-ALL) or chronic T cell lymphoma. It may well be, that CD13 contributes to the malignant phenotype by promoting cell proliferation. In this context it is notable that there are a few CD13-positive T cell lines existing, e.g. KARPAS (ACC31) or P12/Ichikawa (ACC34, German Collection of Microorganisms and Tissue Cultures, Braunschweig, Germany).

Unfortunately, at present the answers are largely speculative, as to how APN might affect the proliferation process. We propose, that APN proteolytically modifies peptides (and/or their precursors) involved in growth stimulation or retardation. This might also explain the changes in the level of secreted IL-1RA in the culture supernatant of H9 cells transfected with the APN-antisense plasmid.

In summary, our data demonstrate the induction of APN gene and surface expression in response to T cell activation. In addition the data are suggestive of APN playing a role in the regulation or modulation of T cell proliferation. Although the mechanisms underlying this postulated action of APN remain to be elucidated fully, one can expect APN proteolytically activating or inactivating peptides involved in T cell growth.

7. ACKNOWLEDGMENTS

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