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Short Communication

Evidence for Coordinated Induction and Repression of Ecto-5´-Nucleotidase (CD73) and the A2a Adenosine Receptor in a Human B Cell Line

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In the human B cell line P493-6 two mitogenic signals, the Epstein-Barr virus nuclear antigen 2 (EBNA2) and myc, can be independently regulated by means of an estrogen receptor fusion construct or an inducible expression vector, respectively. Shut off of EBNA2, either in the presence or absence of myc, leads to a significant increase in enzymatic activity and surface expression of ecto-5´-nucleotidase (CD73) as well as an increased adenosine receptor response in cyclic AMP formation. Shut off of myc expression has a small additional positive effect on CD73 activity. Among the four different subtypes of adenosine receptors, the A2a receptor exclusively is subject to regulation in this system, which is substantiated by pharmacologic data (specific agonists and inhibitors), as well as on the mRNA level. With up-regulated CD73 and A2a, cells also respond to 5´-AMP with increased cyclic AMP formation. Turn on of EBNA2 has the reverse effect of repression of CD73 and A2a expression. The time course of both induction and repression of CD73 and A2a is rather slow.

Key words: A2a mRNA / 5´-AMP / c-myc / Cyclic AMP / EBNA2.

Excretion of ATP into the extracellular space under physiological conditions and its subsequent breakdown by a cascade of ecto-nucleotidases has been well established in various tissues and cell types. The enzymes involved, among others an ecto-apyrase (CD39) and the ecto-5´-nucleotidase CD73, have been studied on molecular, structural and functional levels (Zimmermann, 1992; Wang and Guidotti, 1996; Resta *et al.*, 1998; Knöfel and Sträter, 1999; Mulero *et al.*, 1999). The end product of this breakdown, adenosine, may serve either the re-uptake by carrier-mediated diffusion – *i.e.* the salvage of the purine – or more specifically as a ligand for P1 purinergic receptors, the adenosine receptors. Various combinations of CD73 and one of the four subtypes of these receptors (A1, A2a, A2b and A3) have been described in different tissues involving either autocrine or paracrine stimulation, for example in intestinal epithelia (Strohmeier *et al.*, 1997), the microvascular endothelial bed (Lennon *et al.*, 1998), kidney (LeHir and Kaissling, 1993), central nervous system (Richardson *et al.*, 1987) and the immune system (Resta *et al.* 1998). Because of such close cooperation one might speculate that expression of CD73 and the cooperating subtype of the adenosine receptor could be coordinately regulated, especially in situations where both are expressed on the surface of the same cell.

Regulation of CD73 expression – presumably always on the transcriptional level – was suggested by earlier reports on rat mesangial cells (IL-1ß/TNFα; Savic *et al.*, 1990), chicken myoblasts (signaling *via* extracellular laminin; Méhul *et al.*, 1993) and HL60 cells (phorbol-myristate-acetate mediated induction of differentiation; Spychala *et al.*, 1997) and could be studied in more detail after sequencing and characterization of the promoter region of the human CD73 gene (Hansen *et al.*, 1995; Spychala *et al.*, 1999). A cross-regulation of CD73 expression mediated by adenosine and adenosine receptor signaling (Stefanovic *et al.*, 1993; Narravula *et al.*, 2000) seems to be particularly interesting.

Expression of CD73 has been studied extensively in the human lymphoid system (for a review see Resta *et al.*, 1998). Expression in B cells is regulated at several stages of differentiation. Maturation from the stage of cord blood or fetal spleen is followed by an increase in CD73 expression during the first six months of life until B cells achieve the capacity for IgG antibody responses. Seventy-five percent of adult peripheral blood, spleen and lymph node B cells express CD73. Usually B cells in primary follicles and resting memory B cells are CD73^{+} while germinal center B cells are CD73^{-}. Among B cell lines, lymphoblastoid lines (LCLs) are characterized by varying degrees of CD73 expression, whereas the typical Burkitt lymphoma line is usually devoid of CD73 activity (Gutensohn and Jahn, 1988).

Regulation of the expression of the A2a adenosine receptor had not yet been studied in conjunction with ecto-nucleotidases. A2a receptor expression in the pheochromocytoma cell line PC12 is stimulated either by hypoxia (Kobayashi and Millhorn, 1999) or *via* nerve growth factor

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signaling (Malek *et al.*, 1999). Transcription of the A2a receptor gene in a neuroblastoma cell line (SH-SK5Y) is increased by phorbol ester *via* protein kinase C (Peterfreund *et al.*, 1997).

Table 1 Phenotypes of P493-6 Cells.

State	EBNA 2	c-myc	Phenotype
1	+	+	Lymphoblastoid cell line (LCL)
2	+	–	Lymphoblastoid cell line (LCL)
3	–	+	Burkitt lymphoma group I
4	–	–	‘Resting’

P493-6 cells were grown as previously described (Pajic *et al.*, 2000, 2001) in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, in an atmosphere containing 5% CO_2. Occasionally, cultures were subjected to selection by hygromycin B (150 µg/ml final concentration) for the maintenance of the episomal myc expression vector. To activate the estrogen receptor-EBNA2 fusion protein, the medium was supplemented with 1 µM ß-estradiol. For the suppression of myc expression 1 µg/ml tetracycline was added. For the removal of estradiol and/or tetracycline, cells were washed 3 times in RPMI 1640 and re-suspended in complete medium without hormone and/or tetracycline. Viability of the cells as checked by the trypan blue exclusion test varied between different cultures (65 – 90%) but was independent of the state.

Here we present evidence for coordinated regulation of the expression of CD73 and the A2a adenosine receptor in a B cell line. In this line (P493-6) two mitogenic signals, the Epstein-Barr virus nuclear antigen 2 (EBNA2) and myc, can be independently regulated *via* an estrogen receptor fusion construct (EBNA2) and *via* expression from a stably-transfected vector with the Tet off system (myc), respectively (Pajic *et al.*, 2000, 2001). Depending on which of the two mitogenic signals is turned on or off, the P493-6 cells show different phenotypes as given in Table 1. Cells change from a more lymphoblastoid-like (with both mitogens or with EBNA2 alone active) to a Burkitt lymphoma-like phenotype (with only myc active) and finally stop dividing (when both mitogens are shut off). For convenience the four different states will be numbered in the following according to this scheme.

When the 4 different states of P493-6 cells are compared in their enzymatic (CD73) activity (tested on intact cells) and in their response to stimulation with the A2 adenosine receptor-specific agonist N-ethyl-carboxamido-adenosine (NECA), the results given in Figure 1 are obtained. Whereas we see a more steady increase in the enzymatic activity going from state 1 to 4, the most pronounced effect on the adenosine receptor response, as measured by cyclicAMP formation, is caused by the shut off of EBNA2 (going from state 2 to 3). Statistically signif-

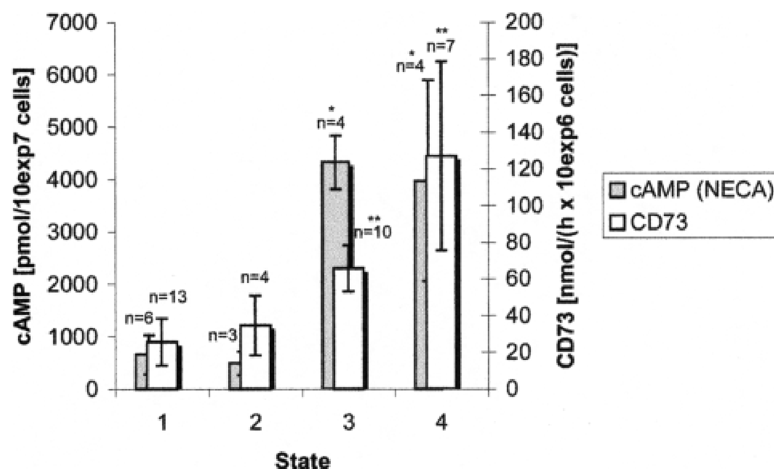

Fig. 1 Enzymatic Activity of CD73 and Adenosine Receptor Response of P493-6 Cells with Different Phenotypes.
CD73 activity (5´N, white bars) and cyclicAMP (cAMP) production in response to 5 µM NECA (gray bars). 1: EBNA2+/myc+; 2: EBNA2+/myc-; 3: EBNA2-/myc+; 4: EBNA2-/myc-. The number of independent experiments using different cultures is given. Statistically significant differences (** = 1% level, * = 5% level) with respect to state 1 are indicated (Mann-Whitney U-test). CD73 activity was measured on intact cells under isotonic conditions at 37 °C using [8-^{14}C]5´-AMP as a substrate. After stopping the reaction with trichloroacetic acid the substrate and the products (adenosine and inosine, derived from adenosine in a second reaction catalyzed by adenosine deaminase) were separated by thin layer chromatography on PEI cellulose and quantified by scintillation counting. The part of total 5´-nucleotidase activity (between 65 – 90%) that is inhibitable by 200 µM α,ß-methylene-adenosinediphosphate (AOPCP) is defined as CD73. As shown previously, detergent extraction does not liberate additional CD73 activity (Gutensohn *et al.*, 1980). On the other hand, viability has no influence on the activity of this membrane enzyme. Values given are the average of duplicate determinations performed, each in the presence and absence of AOPCP. For the adenosine receptor response 5.4×10^6 cells in a total volume of 300 µl RPMI 1640 were incubated in the presence of the phosphodiesterase inhibitor Ro 20 – 1724 (final concentration 175 µg/ml) and the indicated concentrations of ligand [N-ethyl-carboxamido-adenosine (NECA), CGS 21680, or 5´-AMP] for 10 min at 37 °C. After a short centrifugation (5 000 *g*) the supernatant was removed and the cell sediment resuspended in 110 µl 0.1 M HCl. After a further short centrifugation (10 000 *g*) 100 µl of the supernatant were taken and its cyclicAMP (cAMP) content determined using the ‘correlate-EIA direct cyclic AMP enzyme immunoassay kit’ (AssayDesigns Inc., Frankfurt, Germany). Values were calculated by comparison with a standard curve established in each individual experiment. cAMP production, corrected for background in the absence of ligand, was related to the number of viable cells in the sample. Values given are the average of duplicate determinations.

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icant differences with respect to state 1 are indicated in Figure 1, in addition CD73 activity in state 4 is different from that in state 3 on the 5% level. Such a difference between states 3 and 4, however, is not seen in the adenosine receptor response. In a preliminary experiment, surface expression of the CD73 molecule was monitored by impulse cytofluorimetry using two different monoclonal antibodies, CD73.5-IFH-5N2 and CD73.6-IFH-5N4 (CD workshop; Thompson *et al.*, 1995), and corresponds to the enzymatic activity. Within the same experiment – as a control for the phenotype of the cells in the four different states – down-regulation of CD21 and up-regulation of CD38 (data not shown) were seen as previously described (Pajic *et al.*, 2001). The increases in either CD73 activity or adenosine receptor response vary considerably between experiments (which is evident by the rather wide error margins in Figure 1); however, when compared with each other within the same experiments they are correlated with $r = 0.78$.

The increases in CD73 enzymatic activity and the adenosine receptor response as seen in states 3 and 4 also render the cells more susceptible to stimulation with 5′-AMP. In several experiments the cAMP response to 5 μM 5′-AMP increased up to 10-fold from state 1 to states 3 or 4, respectively, of the cells, whereas the background cAMP production in the absence of 5′-AMP remained at a low level (100–200 pmol/10^7 cells). In this situation the nucleotide serves as a precursor of the ligand adenosine. Given the K_m value of CD73, the activity of 5.4×10^6 cells in state 3 should be sufficient to convert 5′-AMP (5 μM) almost quantitatively to adenosine within the 10 min incubation period. This shows that the two components, enzyme and receptor, when more highly expressed on the surface of the same cell do in fact cooperate in signaling starting from extracellular nucleotides.

The increase in the adenosine receptor response seen in states 3 and 4 is due to the activity of the A2a subtype as shown by two sets of data: (i) the same responses as shown in Figure 1 for the general agonist NECA can be achieved with the A2a-specific agonist CGS 21680 (5 μM). The response to NECA in states 3 and 4 is inhibited by 75% with the A2a-specific inhibitor chlorostyrylcaffeine (5 μM) and is not influenced by the A2b-specific inhibitor alloxazine (5 μM). (ii) When tested by semi-quantitative RT-PCR, out of the four different adenosine receptor subtypes only the mRNA level of the A2a subtype shows a steady and consistent, albeit slow, increase within 24 h after shut down of EBNA2 (in the absence of myc; going from state 2 to 4). A representative experiment is shown in Figure 2. This suggests that the up-regulation of the A2a receptor expression takes place on the transcriptional level.

A reverse effect, *i.e.* down-regulation of CD73 expression and activity as well as of the adenosine receptor response to NECA after turn on of EBNA2 in the presence of myc (going from state 3 to 1), could also be observed in several experiments and this too is a rather slow process as shown by representative examples in Figure 3A and 3B. Figure 3B, in addition, demonstrates the effect on the adenosine receptor response of a change in both directions (state 3 to 1 and 1 to 3, respectively). Thus, in this cell line A2a receptor expression also corresponds to the state of the mitogenic stimuli.

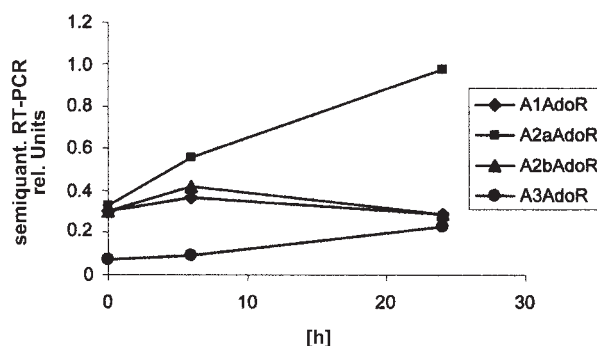

Fig. 2 Time Course of mRNA Expression in P493-6 Cells after a Change in Phenotype.
Semi-quantitative RT-PCR. mRNA levels of the 4 different subtypes of adenosine receptors after a switch from state 2 to state 4 (shut off of EBNA2 in the absence of myc).
A cDNA pool was synthesized by reverse transcription of total RNA with AMV reverse transcriptase (Promega) using a poly-$(dT)_{18}$-primer. The semi-quantitative RT-PCR is based on a principle first published by Gilliland *et al.* (1990). In short, the target cDNA and a known amount of a heterologous competitor DNA are allowed to compete for an identical pair of primers within the same reaction. The competitor is constructed in order to yield an amplification product differing slightly in length from the target product. The products are separated, signals registered digitally using the NIH Image 1.61 or the Scion Image 4.0.2 program and quantified densitometrically. After correction for background the ratio of target and competitor products was calculated in relative units. These were standardized in relation to values for the housekeeping gene pgk1 obtained from the same cDNA pool by the same procedure. The primers used in the RT-PCR were chosen in order to allow a clear distinction between the 4 different subtypes of adenosine receptors:
A1: Forward 5′ TTCTGCTTCATCGTGTCGC 3′ (133)
Reverse 5′ CTTCTTGTTGAGCTGCTTGC 3′ (641)
A2a: Forward 5′ TCCTCGGTGTACATCACG 3′ (16)
Reverse 5′ AAGAGACAGGCCACTTGGC 3′ (502)
A2b: Forward 5′ GACTTCTACGGCTGCCTCTTC 3′ (219)
Reverse 5′ GCAGCTTTCATTCGTGGTTC 3′ (497)
A3: Forward 5′ GGCCAATGTTACCTACATCACC 3′ (28)
Reverse 5′ GATGTCAAGATAGATGGCGC 3′ (599)
pgk1: Forward 5′ ATGATTATTGGTGGAATGGCT 3′
Reverse 5′ TCATCCATGAGAGCTTTGGTTCC 3′
Numbers in parentheses give the 5′ nucleotide positions of the primers in the open reading frame of the respective cDNA.

A cooperation of CD73 and one of the adenosine receptors and therefore a coordinate regulation of the functional expression of these components – as outlined above– would make sense in the context of a signaling system. However, experimental evidence for such a coordinate regulation, as presented here for the B cell line P493-6, will not necessarily reveal the underlying mechanism nor will it tell us anything about the benefits of the signaling for the cell in this particular situation.

In the P493-6 system, in order to keep the estrogen receptor-EBNA2 fusion protein in an active state, the medi-

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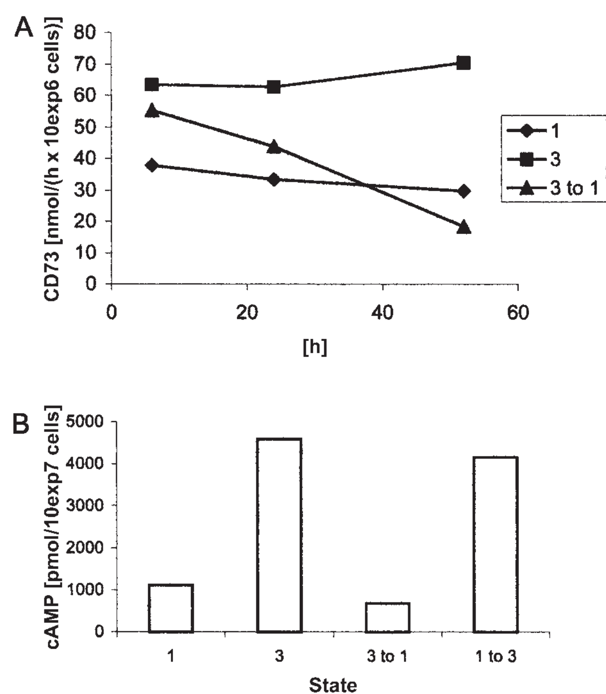

Fig. 3 Time Course of CD73 Enzyme Activity and Adenosine Receptor Responses in P493-6 Cells after a Change in Phenotype.

(A) CD73 activity after a switch from state 3 to state 1 (turn on of EBNA2 in the presence of myc). Unchanged states 1 and 3 served as controls.

(B) cAMP production in response to 5 μM NECA 72 hours after a switch from state 3 to 1 or 1 to 3, respectively (*i.e.* turn on or shut off, respectively, of EBNA2 in the presence of myc). Unchanged states 1 and 3 served as controls. CD73 activity and cAMP production were determined as described in the legend to Figure 1.

um is supplemented with 1 μM ß-estradiol. This applies to states 1 and 2. Could CD73 and A2a receptor expression simply depend on the presence or absence of the hormone with exclusion of EBNA2? In fact, human breast cancer cell lines expressing high levels of endogenous or transfected estrogen receptor (ER) show a strong suppression of CD73 when compared with their ER-negative counterparts (Spychala *et al.*, unpublished). Although this suppression and activation (in the ER-negative lines) is also demonstrated on the mRNA and promoter activation level, these authors interpret this as an indirect estrogen effect, since the promoter of the human CD73 gene does not contain an estrogen response element up to 1.9 kb upstream (Hansen *et al.*, 1995). The situation in B cells is less clear. A reporter (chloramphenicol acetyl transferase) containing an estrogen response element in its promoter transfected into the Burkitt lymphoma line B41 cannot be stimulated by estrogen unless ER is ectopically expressed (B. Kempkes, unpublished). Although this experiment has not been performed in the P493-6 cells, it seems less likely that these B cells express ER at levels sufficient for a hormone effect. However, even if the repression of CD73 and A2a receptor in states 1 and 2 were due directly to estradiol, the fact of a coordinate regulation of the two components – although by an unknown mechanism – would still hold. On the other hand, none of the factors discussed so far in the literature as participating in the regulation of either CD73 or A2a receptor (Hansen *et al.*, 1995; Malek *et al.*, 1999; Spychala *et al.*, 1999) seem to be components of a signaling pathway used by the viral factor EBNA2.

Whether, in addition to EBNA2, there is an effect of myc, or the shut off of myc-expression in P493-6 on the expression of CD73 and A2a is less clear. A comparison of states 3 and 4 in Figure 1 suggests that there might be a slight influence on CD73 but definitely not on the adenosine receptor response.

The core promoter region of the human CD73 gene does contain a cAMP response element (CRE site) (Hansen *et al.*, 1995). The role of this element is discussed as mediating either suppression or stimulation of promoter activity in a tissue-dependent way depending on the activity of further regulating elements (Spychala *et al.*, 1999). In other systems, like vascular endothelial cells, stimulation prevails, in this case mediated by adenosine and A2b-receptor signaling (Narravula *et al.*, 2000). If this would hold for the P493-6 cells, up-regulation of CD73 and A2a at the same time would establish an autocatalytic positive feedback loop for the expression of CD73.

Among the many different surface markers tested by immunofluorescence in the four different states of P493-6 cells, CD39 shows a slight decrease in expression when going from states 1 or 2 to 3 or 4 (Pajic *et al.*, 2001). However, since this happens on a rather high level, enough apyrase (CD39) activity should be left to ascertain sufficient breakdown of extracellular ATP by an ecto-enzyme cascade (CD39 + CD73).

As seen in Figures 2 and 3, the time course of changes in CD73 and A2a expression after turn on or shut off of EBNA 2 (in the presence or absence of myc, respectively) is rather slow. Pulse-chase experiments in cultured human chorionic cells highly expressing CD73 showed transit times of newly synthesized enzyme to the cell surface on the order of 30 min (Burgemeister *et al.*, 1990). Therefore it is less likely that a slow increase after induction (on the order of days) is regulated by post-transcriptional mechanisms. A comparably slow rise in CD73 mRNA levels after induction has in fact been described in other systems like HL60 cells (Spychala *et al.*, 1997) and vascular endothelial cells (Narravula *et al.*, 2000). Comparable data for the A2a receptor are not available in the literature. However, the IFN-γ driven up-regulation of A2b receptor expression in murine macrophages follows a similar slow time course (Xaus *et al.*, 1999). The equally slow decrease of CD73 and A2a in P493-6 after turn on of EBNA2 (Figure 3) could be explained with a rather long half-life of the molecules once expressed on the cell surface. This is supported by observations on CD73 in human chorionic cells (W. Gutensohn, unpublished) and would not be unusual for a GPI-anchored molecule. For the A2a receptor this remains speculative.

Although the effects of induction and repression of

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ecto-enzyme and receptor in this system are quite clear cut, their biological significance for the P493-6 cells cannot immediately be derived. Further studies should clarify whether ecto-enzyme-supported signaling *via* the A2a adenosine receptor would confer a survival advantage to the cells in an otherwise resting state, *i.e*. when both mitogens are inactive.

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