Ecto-ATP diphosphohydrolase/CD39 is overexpressed in differentiated human melanomas

Karine N. Dzhandzhugazyan, Alexei F. Kirkin, Per thor Straten, Jesper Zeuthen*

Department of Tumor Cell Biology, Institute of Cancer Biology, Danish Cancer Society, Strandboulevarden 49, Bldg. 7.1, DK-2100 Copenhagen, Denmark

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Abstract Ecto-ATPase activities of melanocytes and human melanoma cell lines differing in the stage of progression were compared. A dramatic increase in ecto-ATPase activity above the level of normal melanocytes was demonstrated in the differentiated melanomas and was followed by a gradual decrease with tumor progression. The characteristics of this enzymatic activity were consistent with CD39/ecto-ATP diphosphohydrolase (ATPDase) which was found to be the major ecto-ATP-hydrolysing enzyme in melanomas. Indeed, the expression of CD39 and the level of CD39 mRNA followed a similar pattern. Since CD39 is known to regulate homotypic adhesion and, supposedly, affects the disaggregation step, we suggest that overexpression of CD39 may enable tumor cells to reduce contacts with T-lymphocytes and escape from immunological recognition.

Key words: CD39; Ecto-ATPase; Adhesion; Differentiation marker; Immune escape mechanism; Human melanoma

1. Introduction

Sporadic cutaneous melanoma is a cancer form with high mortality, despite of its capacity to induce a specific immune response [1]. Analysis of the genetic defects leading to disruption of the mechanisms of the intracellular control of the mammalian cell cycle [2,3] or defects on the antigen presentation pathway potentially enabling immune escape of tumor cells [4] are of importance for understanding the melanoma pathogenesis. Several common features of tumorigenesis, such as impaired cell-cell communication, adhesion and signal transduction, often result from changes in the components exposed on the cell surface.

Extracellular ATP is becoming recognized as a cell-cell communication molecule which acts via purinergic receptors [5] and various widely distributed nucleotide-hydrolysing ectoenzymes [6]. CD39, originally described as a marker of activated immunocompetent cells and somehow involved in homotypic adhesion [7], was recently shown to be one of numerous extracellular nucleotide-hydrolysing enzymes, ecto-ATP diphosphohydrolase (ATPDase, 3.6.1.5) [8], thus, exhibiting both ATPase and ADPase activities. Here, we report that (1) differentiated, but not progressed melanomas show an increase in ecto-ATPase activity above the level found in normal melanocytes; (2) CD39 is the major ecto-ATP-hydrolysing enzyme in melanomas and follows the same pattern of expression as does the enzymatic activity; (3) the level of CD39 mRNA transcripts follows both the enzymatic activity and CD39 expression, pointing to transcriptional regulation of expression of CD39 in relation to the state of tumor progression. CD39 might therefore be considered a marker of melanoma differentiation. A hypothesis associating the overexpression of CD39 with the escape of tumor cells from immunological effector mechanisms at early steps of tumor progression is proposed.

*Corresponding author. Fax: +45 (35) 25 77 01.
E-mail: jz@bio.cancer.dk

Abbreviations: ATPDase, ATP diphosphohydrolase; CTL, cytotoxic T-lymphocytes; RT-PCR, reverse transcriptase-coupled polymerase chain reaction; mABs, monoclonal antibodies

2. Materials and methods

2.1. Cell lines

Normal melanocytes were purchased from Clonetics (USA). Melanoma cell lines were established as has been described previously [9] using tumor samples obtained from patients who underwent surgery for primary and metastatic melanoma at the Department of Plastic Surgery, Rigshospitalet (Copenhagen, Denmark). All melanoma cells (except FM3 sublines) were used at early passages in culture (no more than 10). The FM3 sublines FM3.D (differentiated) and FM3.P (progressed) differing in their state of differentiation have been described previously [10]. K562 erythroleukemic cells and EBV-transformed JY lymphoblastoid cells were used as controls with known low and high levels of CD39 expression, respectively [7]. All cells were grown in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% heat inactivated fetal calf serum. The mean cell size was determined using a Coulter Multisizer II (Coulter Electronics Ltd., Northwell Drive, England).

2.2. Antibodies and flow cytometric analysis

Unconjugated monoclonal antibodies (mABs) against CD39 (clone A1) were purchased from Zymed Laboratories, Inc. (San Francisco, CA, USA). Goat anti-mouse FITC-labeled $F(ab')_2$ fragments were used as the secondary antibodies. Flow cytometric analyses were carried out using a FACScan flow cytometer (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA).

2.3. Determination of ecto-ATPase activity

Cells from semi-confluent monolayers were harvested by trypsin-EDTA treatment, to which ecto-ATPase activity was shown to be resistant. Cells were washed twice with assay medium consisting of 20 mM HEPES, 10 mM glucose, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.5, and suspended in the same medium at a concentration of 0.5–1.0×10^6 cells/ml. Aliquots were withdrawn for cell counting in Coulter Multisizer II enabling also to evaluate viability of the cells. Routinely, duplicate samples of 0.1 ml of cell suspension were added to the assay medium containing ATP, so that the final substrate concentration was 1 mM. Samples were incubated for 30 min at 37°C. Subsequently the cells were sedimented, and the amount of P_i released from ATP was determined in the supernatants using two colorimetric procedures [11,12], modified as described previously [13]. Two values – non-enzymatic P_i release from ATP into assay medium during incubation without cells, and P_i content in samples incubated without ATP – were subtracted from the total P_i release giving values for the enzymatic ATP hydrolysis. KH_2PO_4 was used as P_i standard. The specific activity was expressed as $mU/10^6$ cells, where mU is release of one nmol P_i per min at 37°C. The same procedure was employed, when other nucleotide triphosphates and ADP were used as sub-

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strates. Intactness of the cells was evaluated by measuring Na^+,K^+-ATPase activity [14] detectable only in disrupted cells. Melanoma or EBV-transformed B cells (approximately 2.5×10^5) were incubated for 1.5 h at 37°C in 0.2 ml of growth medium in absence or in the presence of 1 μg mABs either to CD39 or to unrelated antigen biotin. Subsequently, cells were sedimented, washed with assay medium and, after cell counting the enzymatic activity was determined as described above. All the reagents were from Sigma Chemical Co. (St. Louis, MO, USA) if not indicated otherwise.

2.4. RT-PCR

RNA was extracted using the Purescript Isolation Kit (Gentra). cDNA synthesis was carried out using 1–3 μg of total RNA with oligo-dT and M-MLV SuperScript II reverse transcriptase (Gibco-BRL, Life Technologies Inc., Gaithersburg, MD, USA) as described [4]. Serial dilutions of cDNA were amplified in the PCR amplification containing the following in 1×PCR buffer (50 mM KCl, 20 mM Tris pH 8.4, 2.0 mM $MgCl_2$, 0.2 mM cresol, 12% sucrose, 0.005% (w/v) BSA (Boehringer-Mannheim, Mannheim, Germany): 40 μM of dNTPs (Pharmacia LKB, Uppsala, Sweden), 2.0 units of AmpliTaq polymerase (Perkin Elmer Cetus Corporation, Emeryville, CA, USA) and 2.5 pmol of each primer. The primers used amplify the cDNA fragments of glyceraldehyde-3-phosphate dehydrogenase, GADPH (5′-AGGGGGGAGCCAAAAGGG-3′ and 5′-GAGGAGTGGGTGTCGCTGTTG-3′; 540 bp) and of CD39 (5′-TTTGGAGCTTTGGACCTTGG-3′ and 5′-GGGGGTCTTGTAAAGGTCACTTA-3′; 275 bp). The parameters used for the amplification were 94°C for 30 s, 60°C for 30 s and 72°C for 30 s for 26 or 30 cycles (GAPDH and CD39 respectively).

3. Results

Characteristics of the sublines FM3.D and FM3.P derived from the melanoma cell line FM3 have been described previously [10]. According to these characteristics, the FM3.D and FM3.P sublines differ in their stage of tumor progression. FM3.D is a differentiated variant of FM3 melanoma cells, while the FM3.P subline has all the characteristics of progressed melanoma cells. Subline FM3.D/40 is a differentiated FM3.D subline between passages 40 and 50. This subline has lost some of the differentiation properties (less pigmented) and acquired a high proliferation rate, but the other characteristics are similar to those of FM3.D. The mean sizes of melanocytes and melanoma FM3.D and FM3.D/40 sublines, as determined by Coulter Multisizer II, were 16.17 μm, 18.81 μm and 18.09 μm, respectively, and did not change further during progression to FM3.P cells. Fig. 1 illustrates the hydrolysis in Ca^{2+}-containing medium of extracellularly added ATP by normal melanocytes and three FM3 melanoma sublines. Values for the enzymatic activity exhibited by cultured normal melanocytes (1.4 ± 0.15 mU/10^6 cells) were rather low, consistent with the observation in vivo, that in epidermal layers only the Langerhans cells exhibit histochemically detectable ecto-ATPase activity [15]. A drastic increase of ecto-ATPase activity was observed in the FM3.D subline, representing the most differentiated variant of FM3 melanoma cells. The ecto-ATPase activity gradually declines to the initial level of normal melanocytes or lower during melanoma progression. The characteristics of the enzymatic activity are similar in all cell

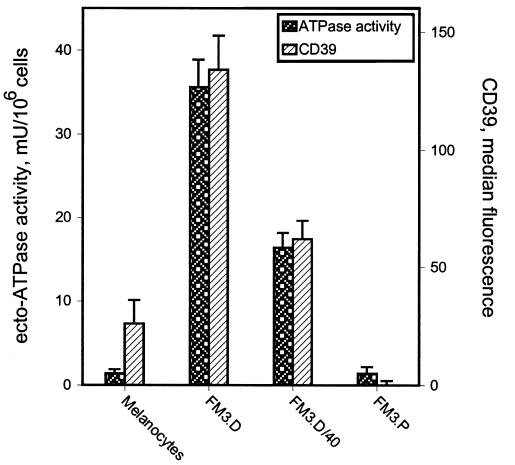

Fig. 1. Ecto-ATPase activity and CD39 expression of melanocytes and FM3 sublines differing in their state of progression. Ecto-ATPase activity of intact cells was determined at 37°C in 20 mM HEPES, pH 7.5, containing 10 mM glucose, 150 mM NaCl, 2 mM $CaCl_2$ and 1 mM ATP, and enzymatic release of P_i from ATP during incubation with the cells was measured. CD39 expression was analyzed by flow cytometry and shown as difference between mean fluorescence intensity of bound FITC-labeled secondary antibodies in the presence and absence of the primary antibodies. Ecto-ATPase activity was determined in five independent experiments, in three of them CD39 expression was analyzed simultaneously. Mean values ± S.D. (bars) are shown.

Table 1
Nucleotide specificity and the effect of some inhibitors on ecto-ATPDase activity on FM3.D/40 melanoma cell subline

Substrate	Inhibitors	Nucleotide-hydrolysing activity, % of control with ATP
Ca^{2+}-containing medium		
ATP		100
UTP		109.1 ± 4.7
CTP		95.6 ± 3.3
GTP		91.0 ± 1.4
ADP	0.2 mM AP_5A	57.4 ± 5.2
ADP	0.2 mM AP_5A+10 mM NaN_3	30.1 ± 2.2
ATP	10 mM NaN_3	65.5 ± 2.5
ATP	1 mM levamisole	96.0 ± 5.2
ATP	0.1 mM vanadate+1 mM ouabain+10 μM oligomycin	89.4 ± 2.6
ATP	control mABs	94.2 ± 3.4
ATP	mABs to CD39	71.0 ± 2.3
Mg^{2+}-containing medium		
ATP	0.1 mM vanadate+1 mM ouabain+10 μM oligomycin	94 ± 3.1
ATP	5 mM EDTA	2.5 ± 0.4

Assay medium contained 20 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$ or $MgCl_2$, pH 7.5, substrates at concentration 1 mM and inhibitors at the indicated concentrations. Values are means ± S.D. of five independent experiments. Specific activity in Ca^{2+}-containing medium with ATP as a substrate, 18.4 mU/10^6 cells, corresponds to 100%.

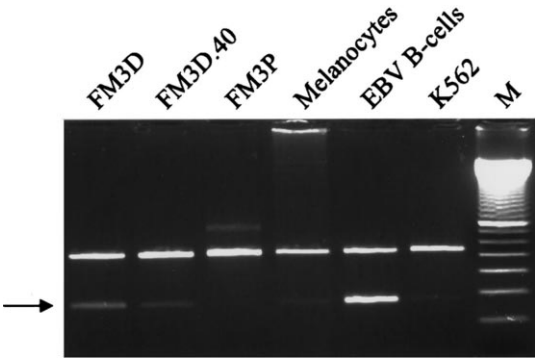

Fig. 2. CD39 RT-PCR products from melanoma FM3 cell sublines. The arrow indicates the position of the CD39 RT-PCR product. EBV-B and K562 cells were used as positive and negative controls for CD39 expression, respectively. GAPDH (upper band) was included as an internal control. M, 100 bp ladder.

lines (data not shown) and are exemplified in Table 1 for the cell line FM3.D/40. All tested nucleotide triphosphates were hydrolysed almost equally efficiently and ADP was also hydrolysed. Hydrolysis of ADP, in the presence of the adenylate kinase inhibitor Ap_5A (added to prevent ATP formation) was 57% of ATP hydrolysis. For comparison, specific activity of 5′-nucleotidase in FM3.D/40 cells determined with 1 mM extracellular AMP was 2.3 mU/10^6 cells, i.e. 8-fold lower than ATPase activity (not shown). Common inhibitors of P- and F_1-F_O-type membrane ATPases and some phosphatases (sodium orthovanadate, oligomycin and ouabain) did not significantly influence ecto-ATPase activities, either when used separately (not shown) or combined (Table 1). Levamisole, an inhibitor of alkaline phosphatase, also was found not to be inhibitory. Taken together, these results indicated that in intact cells there was no significant contribution to measured ecto-ATPase activity from other membrane nucleotide-hydrolysing enzymes. Among potentially inhibitory compounds, only sodium azide at a concentration of 10 mM partially inhibited extracellular hydrolysis of ATP and to a higher extent ADP, by 35% and 48%, respectively. Mg^{2+} and Ca^{2+} were equally effective in activating ecto-ATP hydrolysis, but the presence of divalent cations was obligatory since 5 mM EDTA was strongly inhibitory. All these characteristics – broad nucleotide specificity, absence of strict selectivity towards divalent cations and insensitivity to some common inhibitors (Table 1) – fits the group of ecto-, or E-type, ATP-hydrolysing enzymes [6], while the partial sensitivity to azide suggests CD39 [16] as a candidate ATPDase expressed in melanomas.

Table 2
Ecto-ATPase activity of melanoma cell lines

Cell line	Cell morphology[a]	Ecto-ATPase, mU/10^6 cells[b]
Normal melanocytes	D/S	1.38 ± 0.12
FM59	P/D	9.62 ± 0.77
FM87	D	8.23 ± 0.65
FM91.2	D/P	8.62 ± 0.60
FM91.5	D/P	14.10 ± 1.22
FM94	P/D	41.40 ± 3.30
FM97	S/D	13.14 ± 1.05
FM2	P	9.18 ± 0.71
FM6	P/S	0.81 ± 0.06
FM9	P	1.28 ± 0.10
FM28.4	P/E	7.30 ± 0.61
FM39	P	2.68 ± 0.11
FM45	P	1.92 ± 0.13
FM55p	P	1.31 ± 0.10
FM55M1	P	1.72 ± 0.14
FM57	P	0.81 ± 0.05
FM76	P	2.60 ± 0.14
FM81	P	0.21 ± 0.02

[a]D, dendritic; P, polygonal; S, spindle; E, epithelioid.
[b]mU, nmol P_i enzymatically released from ATP per 1 min at 37°C. Values are means ± S.D. from at least three independent experiments.

Flow cytometric analysis of CD39 expression in normal melanocytes and several sublines of the FM3 melanoma (Fig. 1) confirmed this assumption, since the pattern of expression of CD39 coincided with the enzymatic activity pattern. Thus, according to the enzymatic characteristics and its pattern of expression, CD39 is the major ATP- and ADP-hydrolysing ecto-enzyme in melanomas. Furthermore, the qualitative RT-PCR analysis (Fig. 2) suggests that CD39 expression is controlled at the level of transcription (compare Figs. 1 and 2). The level of CD39 mRNA is highest in the differentiated FM3.D subline, and low in normal melanocytes and in the progressed melanoma subline FM3.P.

Despite the obvious advantages of using sublines of the same tumor cell culture, this approach could cause certain artifacts based on cell culture. In order to exclude this possibility, unrelated melanoma cell lines were compared according to their morphological characteristics and expressed ecto-ATPase activity (Table 2). All melanomas having a dendritic morphology as a morphological indicator of differentiation, exhibited an ecto-ATPase activity which was higher than 8 mU/10^6 cells, while the majority (9/11) of the progressed melanomas exhibited a low enzymatic activity, ranging between 0.2–2.7 mU/10^6 cells (Table 2). Taken together, these results demonstrate that overexpression of ecto-ATPDase/CD39 is a marker of differentiation in melanomas.

Incubation of melanoma cells with mABs to CD39 decreased ecto-ATPase activity by 24% as compared to unrelated mABs to biotin (Table 1). Similarly, these antibodies induced decrease by approximately 30% of ecto-ATPase activity of EBV-transformed B cells (not shown). In these cells the high expression of CD39 and its involvement in the regulation of homotypic adhesion was first demonstrated [7]. Since mABs to CD39 are known to induce homotypic adhesion [7] and decrease ecto-ATPase activity, we propose that CD39/ecto-ATPDase may be a negative regulator of reversible adhesive interactions supporting the de-adhesion step.

4. Discussion

It has been noted that the specific ecto-ATPase activity in melanomas varies significantly between individual tumors [17]. The present results demonstrate that the state of tumor progression may define the level of expressed activity due to transcriptional regulation of CD39/ecto-ATPDase, while several other possible mechanisms of regulating the enzymatic activity obviously can not be excluded.

Extracellular nucleotides are released from many different cells [5], and elevated levels of extracellular ATP has been observed in many tumors, one of the release mechanisms presumably utilizing overexpressed P-glycoprotein [18,19]. An example is cytotoxic T-lymphocytes (CTL) that have been shown to release ATP upon T cell receptor activation [20].

Overexpression at the cell surface of any ATP-hydrolysing enzyme obviously can influence the process of purinergic signal transduction as well as ecto-phosphorylation. However, adhesion is another phenomenon often coupled to particular ATPDases [6] – either directly, via intrinsic ecto-ATPDase activity exhibited by some known adhesion molecules, e.g. NCAM [13], or indirectly, via both integrin-dependent and -independent adhesion pathways, as shown by homotypic adhesion triggered by monoclonal antibodies against CD39 [7]. Based on inhibitory effect of these antibodies on the enzymatic activity, we propose that CD39 may be a negative regulator of cell adhesion, capable to reduce the strength of cell contacts during binding or hydrolysis of ATP. The recently demonstrated enhancement of adhesive interactions between neutrophils and endothelial cells in the presence of suramin [21], a known inhibitor of ecto-ATPases and antagonist of purinoceptors [5,6], is in agreement with the proposed role for ecto-ATPase. Obviously this hypothesis requires direct proof, but is consistent with the observation that CTL with a lower level of CD39 expression shows higher unspecific cytotoxicity [22]. Furthermore, it has been demonstrated using melanoma FM3 sublines that the cytotoxicity of various CTL clones specific to different groups of melanoma-associated antigens increases during melanoma progression [10]. This prompted us to search for immune escape mechanisms at early steps of tumor progression, but the expression of several proteins potentially involved in the immune escape (TAP 1/2, LMP-2, LMP-7, HLA class I molecules, etc.) did not show significant differences between differentiated and progressed tumors [4,10]. However, the present results indicate that overexpression of CD39/ecto-ATPDase in differentiated melanomas might provide a mechanism enabling the escape from immune surveillance by disrupting tumor cell contacts with CTL.

The question arises if the expression pattern demonstrated here for ecto-ATPDase/CD39 is common also for expression of ecto-ATPases in other tumors. Similar observations on ecto-ATPase activity have been made in experimental carcinogenesis of rat and mouse hepatic parenchyma [23,24] and the role of elevated ecto-ATPase activity in overcoming contact inhibition has been proposed [23], but it has not been elucidated which protein was responsible for this enzymatic activity. Previously, CD39 as a major ecto-ATP-hydrolysing enzyme has been assigned to activated immune cells, endothelial cells and highly vascularized tissues [16]. To our knowledge, overexpression of ecto-ATPase activity and CD39 was reported here for the first time using human tumor cell lines.

The mechanism of down-regulation of the enzymatic activity and expression of CD39 during tumor progression is not clear. In this connection it should be noted that the genetic events involving chromosome region 10q22-10qter may be associated with tumor progression [2,3]. The established localization of CD39/ecto-ATPDase gene to the region 10q23.1-10q24.1 [25] could possibly be related to changes in CD39 expression during tumor progression. The elucidation of the role of CD39/ecto-ATPDase in immunological escape mechanisms will require further studies focused on the biological consequences of the manipulation of ecto-ATPase activity and the level of CD39 expression.

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