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Differential expression of nucleotide pyrophosphatase/phosphodiesterases by Walker 256 mammary cancer cells in solid tumors and malignant ascites

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ABSTRACT

Aims: Expression of ectoenzymes responsible for nucleotide phosphohydrolysis to form adenosine may represent a mechanism that facilitates the proliferation and spread of malignancy. In this study, we have identified and characterized the ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP) family members expressed during the subcutaneous tumor growth and in the ascitic form of Walker 256 mammary tumor cells.
Main methods: The biochemical characteristics in ascitic forms and expression of NPP 1, 2, and 3 in both solid and ascitic forms of Walker 256 tumor were investigated using RT-PCR and real-time PCR.
Key findings: Walker 256 tumor cells demonstrate E-NPP activities that are associated with extracellular hydrolysis of p-Nph-5′-TMP, and define the biochemical characteristics. The K_m and maximal velocity for the hydrolysis of p-Nph-5′-TMP in the ascitic tumor cells were in accordance with the NPP reaction. The mRNA expression in the cells of the ascitic form of Walker 256 tumor revealed transcripts for NPP2 and NPP3, whereas elevated expression of NPP3 was observed in solid tumor, after 6, 10, and 15 days of inoculation. The dominant gene expressed in both forms of the tumor was the NPP3 enzyme. However, this enzyme was expressed more during tumor development in vivo, when compared with the ascitic cells.
Significance: We have previously demonstrated that Walker 256 tumor cells express mRNA for ecto-5′-nucleotidase and E-NTPDases. Thus, coexistence with NPP3 suggests an ectonucleotidase "enzyme chain" that is responsible for the sequential hydrolysis of ATP to adenosine, which may be an important therapeutic target in anticancer therapy.

Introduction

Extracellular adenine nucleotides and nucleosides act as signaling molecules involved in a wide spectrum of biological effects. A variety of pathophysiological responses to ATP have been described, including the long-term (trophic) roles in cell proliferation and growth (Burnstock 2006), induction of apoptosis, and anticancer activity (Bours et al. 2006; White and Burnstock 2006). Moreover, the expression of ectonucleotidases at the cell surface has recently been found to be associated with certain pathological states, in particular cancer (Stefan et al. 2006; Buffon et al. 2007a,b).

There are important external mechanisms that control the extracellular concentration of the nucleotides, thus regulating the P2-receptor-mediated effects. Members of several families of ectonucleotidases are able to hydrolyze extracellular nucleotides. The most important ectonucleotidases, including members of ectonucleotide pyrophosphatases/phosphodiesterases (E-NPPs) and ectonucleoside triphosphate diphosphohydrolases (E-NTPDases, also referred to as ecto-ATPDase, ecto-ATP-diphosphohydrolase, ecto-apyrase, CD39) families, as well as the ecto-5′-nucleotidase/CD73 (Robson et al. 2006), are involved in adenine extracellular nucleotide hydrolysis.

The family of E-NPPs consists of seven structurally related enzymes that are located at the cell surface, and are either expressed as transmembrane proteins or secreted enzymes. These proteins hydrolyze pyrophosphate or phosphodiester bonds of the molecules, such as nucleotides and dinucleotides (NPP1–3), (lyso) phospholipids (NPP2), and choline phosphate esters (NPP6 and NPP7). The E-NPPs hydrolyze a broad range of substrates, such as UDP-galactose, NAD+,

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[1] In memoriam.

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cAMP, ATP, and ADP (Stefan et al. 2005, 2006). The p-nitrophenyl 5′-thymidine monophosphate (p-Nph-5′-TMP) has been used as an artificial substrate marker for E-NPPs characterization, generating p-nitrophenol as a final product (Sakura et al. 1998).

Catalysis by NPPs also affects processes, such as cell proliferation, cell motility, and angiogenesis, and hold great promise as they are easily accessible therapeutic targets (Stefan et al. 2005). Much evidence points to a role for NPP2 (autotaxin, autocrine motility factor) in cancer progression, and hence, this compound has recently emerged as an attractive target for the development of anticancer chemotherapeutics. NPP2 is secreted by various cancer cells, including skin, lung, and breast cancer cells. Tumor growth and motility effects have been primarily attributed to the production of lysophosphatidic acid from lysophosphatidylcholine (Moolenaar 2002). Moreover, in a coupled reaction with 5′-nucleotidase, NPP2 has the potential to hydrolyze ATP (which is cytotoxic for tumors) to adenosine, which is a tumor-growth promoting factor and stimulator of angiogenesis (Stefan et al. 2006).

E-NPP3 is also associated with malignant subversion and invasive properties. Prior studies have suggested that E-NPP3 is associated with carcinogenesis, and that serum E-NPP3 might serve as a circulating tumor marker for human colon cancer and bile duct carcinoma (Yano et al. 2003; Yano et al. 2004).

The Walker 256 tumor is a model that has been extensively used in cancer research (Guaitani et al. 1982; He et al. 2003; Piffar et al. 2003; Ikeda et al. 2004, Buffon et al. 2007a,b). We have been using this model to evaluate the role of purinergic system in tumor biology. Recently, we kinetically characterized the E-NTPDases and ecto-5′-nucleotidase activities present in Walker 256 tumor of adult rats, at a molecular level (Buffon et al. 2007a,b). In this work, we have identified and characterized the E-NPP family members during subcutaneous tumor growth and in the ascitic form of Walker 256 tumor cells, and have suggested a complex system to control the ratio of nucleotides/nucleosides in the immediate tumor environment.

Materials and methods

Walker 256 tumor cells

The Walker 256 cell line A (National Cancer Institute Bank, Cambridge, MA, USA) was maintained in our laboratory via intraperitoneal or subcutaneous passages in rats. Male Wistar rats, weighing approximately 250 g, from our own breeding stock were maintained on a 12-h light/12-h dark cycle (lights on at 7.00 a.m.) at constant room temperature. The cell suspensions with 98% of viability, estimated by trypan blue, were obtained from the ascitic fluid of a donor rat. Tumor ascite fluids were harvested in Ringer-lactate solution, and the contaminating erythrocytes were removed by 2 or 3 cycles of osmotic lyses. The Walker 256 cells were resuspended in a buffer containing 50 mM of HEPES (pH 7.5), 5.0 mM of KCl, 135 mM of NaCl, and 10 mM of dextrose. The cell suspensions with 98% viability were obtained from the ascitic fluid (Buffon et al. 2007a). A Walker 256 tumor cell ascitic suspension (5×10^6 cells in 0.25 ml of Ringer-lactate solution) was inoculated at a single dorsal subcutaneous site in the dorsolumbar region of the tumor-bearing group.

Assay of ectonucleotide pyrophosphatase/phosphodiesterase activity

The phosphodiesterase activity in ascitic cells of Walker 256 tumor cells was assessed using p-Nph-5′-TMP as the substrate, as previously outlined (Sakura et al. 1998). The reaction medium containing 50 mM Tris–HCl buffer, 5 mM KCl, 10 mM glucose, 135 mM NaCl, and pH 8.9, was preincubated for 10 min at 37 ºC, with 40 µl of cell suspension sample (approximately 30 µg of protein). The enzyme reaction was started by the addition of p-Nph-5′-TMP to a final concentration of 0.5 mM. After 10 min of incubation, 200 µl of NaOH of 0.2 N was added to the medium to stop the reaction. An incubation time of 10 min was chosen to ensure linearity of the reaction. The amount of p-nitrophenol released from the substrate was measured at 400 nm using a molar extinction coefficient of 18.8×10^{-3}/M/cm.

To investigate the cation dependency for E-NPP activity, we tested the hydrolysis rate in the presence or absence of divalent cations (Ca^{2+} and Mg^{2+}) or EDTA. In the same order, the kinetic parameter was measured by incubating the cells with 0.01–1.3 mM of p-Nph-5′-TMP and the apparent Michaelis–Menten constants (K_m, app) and the calculated maximum velocities (V_{max}, app) were obtained using the Eadiee–Hofstee plot. Controls to correct for nonenzymatic substrate hydrolysis were prepared by adding cells suspension in preparation after the reaction had been stopped with NaOH, as described earlier. All the samples were prepared in duplicates. The enzyme activities were expressed as nmol of p-nitrophenol released per minute per milligram of protein.

RT-PCR analysis

The total RNA from malignant ascites and Walker tumor-bearing cells at 6, 10, and 15 days after tumor inoculation was isolated using RNA Mini Kit (Qiagen), according to the manufacturer's instructions. The cDNA species were synthesized with Super-Script II (Life Technologies) from 5 µg of total RNA in a total volume of 20 µl, with both oligo (dT) primer and random hexamers, according to the manufacturer's instructions. One microliter of the RT reaction mix was used as a template for PCR in a total volume of 20 µl, using a concentration of 0.5 µM of each primer (indicated below) and 0.5 U of Ex TaqDNA polymerase (Takara Bio Inc., Japan). The PCR was run for 35 cycles and the cycling conditions were as follows: 1 min at 95 °C, 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C, and a final 10 min extension at 72 °C. Ten microliters of the PCR reaction was analyzed on a 1.3% agarose gel. The primer sets used were: for NPP1 and NPP3: as described by Vollmayer et al. (2001); for NPP2: 5′-GAAAATGCCTGTCACTGCTC-3′ and 5′-GCTGTAATCCATAGCGGTTG-3′; and for rat β-actin: as described by Wink et al. (2003). The oligonucleotides were obtained from Invitrogen. Negative controls were prepared with water as the template, and positive controls were plasmids with cDNA for NPPs, as described by Wink et al. (2006).

Real-time PCR

The total RNA and cDNA were generated as described in RT-PCR analysis. SYBR Green I-based real-time PCR was carried out on MJ Research DNA Engine Opticon™ Continuous Fluorescence Detection System (MJ Research Inc., Waltham, MA) as described by Zerbini et al. (2003). All the PCR mixtures contained the following: PCR buffer (final concentration of 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 2.0 mM $MgCl_2$, and 0.1% Triton X-100), 250 µM deoxy-NTP (Roche), 0.5 µM of each PCR primer, 0.5× SYBR Green I (Molecular Probes), 5% DMSO, and 1 U of Taq DNA polymerase (Promega, Madison, WI) with 2 µl of cDNA in a 25 µl of final volume reaction mix. The samples were loaded into the wells of Low Profile 96-well microplates. After an initial denaturation step for 1 min at 94 °C, conditions for cycling were 35 cycles of 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C. The fluorescence signal was measured right after incubation for 5 s at 79 °C following the extension step, which eliminates the possible primer dimer detection. At the end of the PCR cycles, a melting curve was generated to identify the specificity of the PCR product. For each run, serial dilutions of human β-actin plasmids were used as standards for quantitative measurement of the amount of amplified DNA. Also, for normalization of each sample, rat β-actin primers were used to measure the amount of β-actin cDNA. All the samples were run in triplicate, and the data were presented as the ratio of enzymes/β-actin. The primers used for real-time PCR were described in RT-PCR analysis.

Protein determination

Protein concentration was measured by the Coomassie Blue method, according to Bradford (1976), using bovine serum albumin as standard.

Animal welfare

The study was performed in accordance with the University IACUC Guidelines for experiments with animals.

Results

Biochemical properties of p-Nph-5′-TMP hydrolysis by E-NPPs

To ensure linearity as a function of enzymatic reaction time, the ascitic cell suspension was incubated as described in Materials and methods, with 0.5 mM of p-Nph-5′-TMP, and the amount of p-nitrophenol released was measured colorimetrically. The Walker 256 ascite fluid cells promoted p-Nph-5′-TMP hydrolysis that was linear up to 30 min (data not shown). The hydrolysis of p-Nph-5′-TMP was linear up to 40 μg of protein per tube (data not shown), and subsequently, the amount of protein used in the assays was 30 μg. Cellular integrity was determined by measuring the activity of the cytosolic enzyme, lactate dehydrogenase (LDH), described in a previous study where we demonstrated that approximately 90% of the Walker 256 cells remained intact (Buffon et al. 2007a).

Cation dependence

To investigate the possibility of cation dependence for the ascitic Walker 256 tumor enzyme, we tested the hydrolysis rate of p-Nph-5′-TMP in the presence or absence of divalent cations or EDTA (cation chelator), as indicated in the Materials and methods section. Crescent concentrations of calcium and magnesium were tested in the range of 2–8 mM, in the presence of 0.5 mM of substrate. As expected, at the concentration of 0.5 mM, EDTA greatly reduced the catalytic activity, indicating the presence of a cation-dependent enzyme (Fig. 1). However, p-Nph-5′-TMP hydrolysis in ascitic cells of Walker 256 tumor was not further stimulated by the addition of crescent concentrations of cations, when compared with the activity of the controls. The catalytic rates were similar in the presence of Ca^{2+} or Mg^{2+}, when compared with the control group without added cation (Fig. 1).

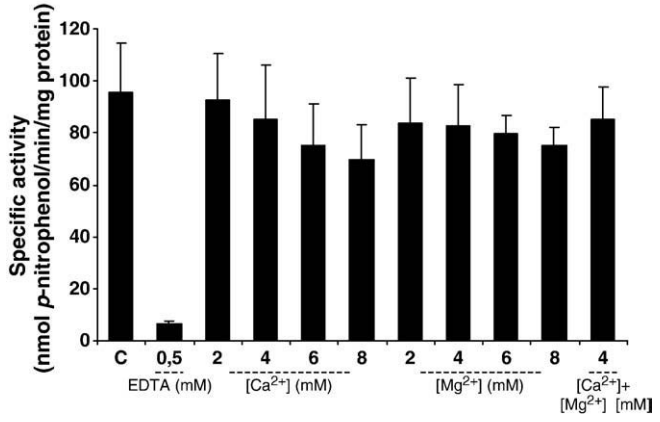

Fig. 1. Divalent cations' dependence on p-Nph-5′-TMP hydrolysis in ascitic Walker 256 tumor cells. Hydrolysis of p-Nph-5′-TMP was analyzed in the absence of cations (C), in the presence of 0.5 mM EDTA, and in the presence of 2.0–8.0 mM Ca^{2+} or Mg^{2+}. Bars represent mean ± SD of four independent experiments. Results are expressed as nmol of p-nitrophenol/min/mg of protein.

Kinetic parameters

Michaelis constant, K_m, and V_{max} values in the ascitic cells of Walker 256 tumor were calculated from the Eadie–Hofstee plot (Fig. 2) with p-Nph-5′-TMP as the substrate. The substrate concentrations tested ranged from 0.01 to 1.3 mM. The K_m value corresponded to 101 ± 40 μM and the V_{max} value calculated was 122.53 ± 26.7 nmol of p-nitrophenol released/min/mg of protein (mean ± SD, $n = 4$). This result is in accordance with the NPP reaction that was characterized by a K_m of 50–500 μM with a V_{max} of 5–300 μmol/min/mg enzymes (Kelly et al. 1975; Hosoda et al. 1999; Vollmayer et al. 2003).

mRNA expression and quantitative analysis of E-NPPs in Walker 256 cells and solid tumor

The NPPs mRNA expression in cells from ascitic form and during the development of the Walker 256 tumor cells was investigated using RT-PCR analysis and real-time PCR (Fig. 3). As demonstrated by mRNA expression, in the ascitic cells (Fig. 3A), NPP1 was not detected. The RT-PCR analysis also suggested a higher expression of NPP3 in relation to NPP2. The expression of the NPPs 1, 2, and 3 during the development of the Walker 256 solid tumor in rats was also investigated by RT-PCR, 6, 10, and 15 days after the subcutaneous inoculation of the ascitic tumor cells (Fig. 3B). The mRNA detection of these enzymes by RT-PCR revealed that the NPP3 was expressed during all the days studied. NPP 1 and 2 were expressed only on days 6 and 10, whereas by 15 days of tumor growth, the signal for both became almost imperceptible.

To confirm these results and to specifically quantify the expression levels, the genes of the NPPs 1, 2, and 3 were analyzed by real-time PCR, from ascitic cells obtained 6, 10, and 15 days after the inoculation of the tumor cells (Fig. 3C). In fact, in ascitic Walker 256 tumor cells, the dominant gene expressed was the NPP3 enzyme. During tumor development, the tumors expressed NPP3 every day, revealing that it is the dominant gene and confirming the standard of expression of the enzyme detected by RT-PCR. However, the NPP3 expression was much higher during tumor development when compared with ascitic tumor cells. Besides, over the 10-day period in which the tumor developed, there was a high-level expression of this enzyme, as previously observed for the NTPDases 1 and 2 and ecto-5′-nucleotidase in Walker 256 tumor (Buffon et al. 2007b).

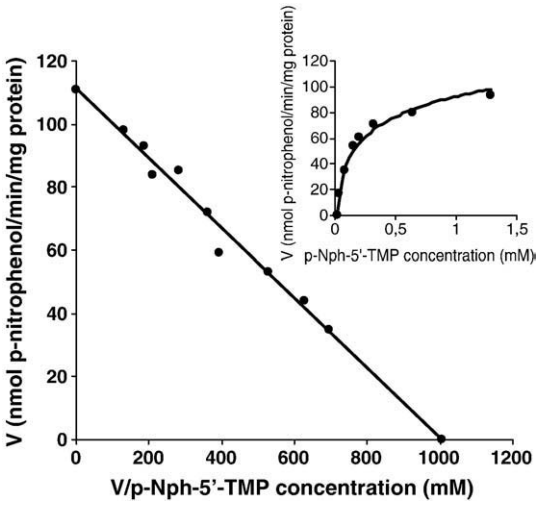

Fig. 2. Eadie–Hofstee plot for p-Nph-5′-TMP hydrolysis in ascitic Walker 256 tumor cells. The substrate concentrations tested ranged from 0.01 to 1.3 mM (inset figure). The K_m value corresponded to 101 ± 40 μM and the maximal velocity (V_{max}) value calculated was 122.53 ± 26.7 nmol of p-nitrophenol released/min/mg of protein (mean ± SD). Data are representative of four different experiments.

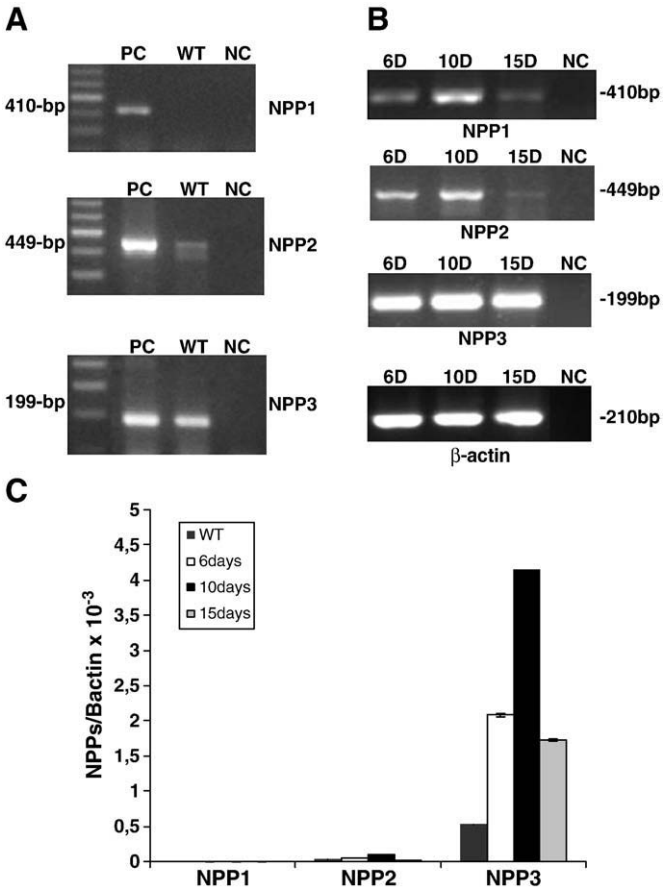

Fig. 3. RT-PCR analysis and quantitative real-time RT-PCR analysis of NPPs expression by ascitic and solid tumor Walker 256 cells. The total RNA was isolated from ascitic Walker 256 cells and tumor-bearing rats, after 6, 10, and 15 days of tumor inoculation, and the cDNA was analyzed with the primers for NPP family, as described in Materials and methods section. (A) RT-PCR analysis from ascitic Walker 256 cells. (B) RT-PCR analysis from solid tumor Walker 256 cells. (PC) Positive control. (NC) Negative control. (WT) Walker tumor cells. The length (bp) of the PCR products obtained with each pair of primers is given in each figure. (C) Real-time PCR from ascitic cells and solid tumor Walker 256 cells. The results are presented as ratio of cDNA enzymes/β-actin. Bars represent mean ± SD for three experiments.

It is important to note that the results obtained from the analysis using real-time PCR, which demonstrated a higher expression of NPP3 in the development of the solid tumor, are in accordance with the data found for the ascitic form of tumor cells. In these cells also, the enzyme NPP3 was found to be the most expressed among the three studied members of the NPP family. These results suggest an important role of these ectonucleotidases in controlling the pericellular levels of nucleotides around the tumor during this phase.

Discussion

The Walker 256 carcinoma is a tumor model used in studies of cancer pathophysiology (Guaitani et al. 1982; He et al. 2003; Piffar et al. 2003; Ikeda et al. 2004; Buffon et al. 2007a,b). In rats inoculated with these cells, the tumor grows without causing apparent physiological disturbances for a certain period of time (usually about 5–8 days). This period is suddenly interrupted by the initiation of a period of rapid tumor growth and marked metabolic changes in the host (Rettori et al. 1995). We have proposed that this change is associated with differential NPP and other ectonucleotidase expression patterns.

NPP1–3 have been detected in almost all tissues, and aberrant expression of E-NPP family members has been observed in a number of disease states, including pathological mineralization, crystal depositions in joints, invasion and metastasis of cancer cells, and type 2 diabetes. However, the physiological mechanisms of E-NPP action are still unclear (Goding et al. 2003). In this study, we investigated the altered expression of NPPs during Walker 256 tumor growth and its development to a widespread form.

The p-Nph-5′-TMP hydrolysis observed in ascitic cells, resulting in the formation of p-nitrophenol in vitro, suggests the existence of E-NPP activity in Walker 256 cells. The hydrolysis of p-Nph-5′-TMP by NPPs was determined in the presence of divalent cations, and the results indicated that the enzyme is cation-dependent, considering that increasing concentrations of EDTA greatly reduced the catalytic activity.

In this study, we demonstrated, for the first time, that NPP family expression is affected by tumor establishment and growth. The analysis of these enzymes by RT-PCR and real-time PCR showed important differences in relation to the ascitic cells and solid tumor, as well as the days studied after inoculation of ascitic cells. Interestingly, the NPP1 expression that was not detected in cells of ascitic form appeared in solid tumor, mainly at 10 days of growth. Our results also demonstrated an elevated expression of NPP3 in solid tumor, at 10 days of growth, suggesting the importance of extracellular nucleotides in the control of tumor proliferation and invasion at this stage. Besides, in the ascitic form as well as during the development of the solid tumor, the dominant gene expressed was NPP3. In fact, this enzyme seems to be important for tumor development, once it is more expressed in solid tumor when compared with the ascitic form. Therefore, it can be suggested that there may be a possible involvement of NPP3 in modulating the adenine nucleotides in the tumor, consequently, modulating its growth.

The involvement of NPPs in the pathophysiology of cancer, ectopic mineralization, and insulin resistance, which when combined with their extracellular action, makes them suitable targets for therapeutic intervention. Our data demonstrated that Walker 256 cells express all three members of the NPP family. The NPP1 expression and localization have been investigated in inflammatory and neoplastic bile duct diseases (Yano et al. 2004). However, the participation of this enzyme in cancer is poorly studied. The expression of NPP1 in tumoral cells can be stimulated by TGF-1 (Huang et al. 1994; Stefan et al. 1999) whereas the level of NPP1 in FAO hepatoma cells increases with cell density (Stefan et al. 1998), and increases during hepatic growth phase in the first weeks after birth, in rats (Stefan et al. 1998, 1999). NPP1 also has been implicated in purinoceptor-mediated signaling in various cell lines, converting ATP into adenosine, an activator of the P1 receptor family. In Walker 256 carcinoma, an increase in the phosphodiesterase activity was observed in solid tumor, when compared with cells in ascitic form (Clark and Goodlad 1993). This supports the expression of NPP1 observed in the solid form of Walker 256 tumor.

The NPP3 has been described as a promoter of invasion, and it is associated with carcinogenesis of the human colon carcinoma (Yano et al. 2003). Besides, it is involved in the infiltration of neoplastic bile duct carcinoma (Yano et al. 2004). The increase in the levels of the soluble form of this enzyme in the serum of patients with these two pathologies suggests a possible role of a tumor marker (Yano et al. 2003, 2004).

NPP2 has been detected in many tumors, including hepatoma, neuroblastoma, prostatic carcinoma, and lung cancer (Goding et al. 2003). This enzyme was first identified in a conditioned environment of melanoma cells, like a stimulating protein of the tumor cell mobility, called autotaxin (Stracke et al. 1992). NPP2 increases the invasive and metastatic potential of *ras*-transfected NIH3T3 cells (Nam et al. 2000), in addition to promoting the angiogenic properties (Nam et al. 2001). This enzyme is still secreted in several types of tumor cells, including skin, lung, and breast cancer. The stimulatory effects of the NPP2 in the growth and spread of cancer have been attributed to lysophosphatidic acid production (Stefan et al. 2006). However, these effects can also be owing to nucleotide hydrolysis. Solid tumors release adenine nucleotides, including ATP, an inhibitor of the tumoral proliferation (Agteresch

et al. 1999), which can be hydrolyzed by NPP2. Acting together with other ectonucleotidases as well as ecto-5′-nucleotidase, previously described by us in Walker 256 tumor (Buffon et al. 2007a,b), the NPPs could participate in ATP hydrolysis to adenosine. ATP is released by solid tumors and it is described as an inhibitor of the tumoral proliferation (Agteresch et al. 1999). Thus, these enzymes in Walker 256 tumor would terminate the actions induced by this nucleotide, protecting the tumoral cells from ATP-mediated cytotoxic effects, facilitating solid tumor growth and producing adenosine, which has been shown to have a tumor-promoting action (Spychala 2000; Spychala et al. 2004).

Previously, we have also shown that Walker 256 tumor expresses mRNA for ecto-5′-nucleotidase/CD73 and E-NTPDases (Buffon et al. 2007a,b). The presence of several ectoenzymes sharing the capability to hydrolyze nucleotides with overlapping enzymatic functions is poorly understood. But, the co-existence of several members of NTPDase and NPP families has been observed in cancer cells. Ectonucleotidases seem to have functions that are not related to its catalytic activity, being also involved in adhesion (Kansas et al. 1991; Airas et al. 1995). The CD39/NTPDase1 is a marker of activated immunocompetent cells and somehow involved in adhesion (Kansas et al. 1991). Besides, the overexpression of this enzyme in human melanomas was related to the escape of tumor cells from immunological effector mechanisms at early steps of tumor progression, by regulating homotypic adhesion (Dzhandzhugazyan et al. 1998). Then, considering that cell adhesion molecules are critical components in processes such as tumor growth and metastasis (Cavenagh et al. 1998), the co-expression of NTPDases and NPPs could be due to their functions on the surface of the cell not related to their catalytic activity.

Conclusion

The data presented in this study suggest the participation of the NPP2 and NPP3 enzymes on Walker 256 tumoral cells. In addition, the data demonstrate that the NPPs, mainly NPP3, can be important for solid tumor growth, once these enzymes are expressed more during tumor development, when compared with ascitic cells.

Taken together, these results demonstrate that Walker tumor cells contain all the components for the enzymatic cascade necessary for the complete metabolism of extracellular nucleotides to nucleosides. Subsequently, by considering that either ATP or adenosine has been implicated in tumor biology, these ectonucleotidases may be controlling the rate of proliferation and invasion of Walker 256 tumor cells. Hence, the co-existence of these enzymes is very important for their participation in an “enzyme chain” for the sequential hydrolysis of ATP to adenosine.

Thus, the ability of the ectonucleotidases to control the extracellular nucleotide/nucleoside ratios in the immediate tumor environment may provide important therapeutic targets for anticancer therapy.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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