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TUMOR DE WALKER 256: UM ESTUDO SOBRE CARACTERÍSTICAS BIOQUÍMICAS E EXPRESSÃO DAS ECTONUCLEOTIDASES

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Resumo

Nucleotídeos da adenina e adenosina estão envolvidos em uma variedade de processos patofisiológicos em diferentes células. O metabolismo extracelular destes nucleotídeos gerando nucleosídeos tem uma importante função regulatória no controle da homeostase, principalmente por regularem a agregação plaquetária, via receptores purinérgicos P2. Além disso, uma associação destes nucletídeos e nucleosídeo em processos neoplásicos tem sido sugerida. Enquanto ATP tem demonstrado exibir atividade anticâncer em uma grande variedade de células animais e humanas, estudos sugerem que adenosina possui ações promotoras de tumor. Muitos estudos também demonstram que antiinflamatórios não esteroidais, como o ácido acetil salicílico (AAS), podem proteger contra o desenvolvimento do câncer. As ações induzidas pela sinalização purinérgica são reguladas pelas ectonucleotidases, que incluem membros da família das ecto-nucleosídeo trifosfato difosfoidrolase (E-NTPDase) e ecto-nucleotídeo pirofosfatases/fosfodiesterases (E-NPPs), e ecto-5'-nucleotidase (ecto-5'N). Assim, no presente estudo nós investigamos o efeito do AAS na hidrólise do ATP até adenosina, bem como estudamos as características bioquímicas e expressão das ectonucleotidases no tumor de W256. A hidrólise dos nucleotídeos da adenina por plaquetas de ratos foi significativamente inibida por AAS, tanto para ATP quanto para ADP, mas não houve alteração na hidrólise do AMP. Em ratos submetidos ao tumor de W256, a hidrólise dos nucleotídeos da adenina em plaquetas e soro, obtidos 6, 10 e 15 dias após a indução subcutânea do tumor, foi significativamente reduzida. Em células tumorais de W256 (forma ascítica do tumor), a análise cinética indicou que várias ecto-nucleotidases estão envolvidas nessa cascata enzimática. Quanto às propriedades bioquímicas das E-NTPDases e ecto-5'-nucleotidase, para a hidrólise de ATP, ADP e AMP, o pH ótimo ficou entre 6,5-8,0 e também foi observado um requerimento de cátions divalentes (Mg^{2+} > Ca^{2+}). Uma significativa inibição na hidrólise do ATP e ADP foi observada em presença de altas concentrações de azida sódica e de 0,5 mM de cloreto de gadolínio. Ainda, a análise de mRNA por PCR identificou a presença das NTPDases 2 e 5, e também da ecto-5'-nucleotidase nas células tumorais de W256.

Com o objetivo de investigar as enzimas envolvidas no catabolismo dos nucleotídeos da adenina durante o crescimento do tumor, nós avaliamos a expressão destas enzimas bem como o padrão de degradação dos nucleotídeos extracelulares no tumor de W256, 6, 10 e 15 dias após a indução subcutânea. Os mRNAs de todas as ectonucleotidases estudadas (NTPDase 1, 2, 3 5 e 6 e ecto-5'-nucleotidase), foram identificados por RT-PCR. A análise quantitativa, realizada por real-time PCR, apresentou como genes dominantes expressos durante o crescimento do tumor, as NTPDases 1 e 2 e ecto-5'-nucleotidase. O padrão de hidrólise do ATP extracelular determinado por HPLC, embora com alguma diferença entre os tempos estudados, se manteve similar. As células do tumor de W256 rapidamente hidrolisaram ATP levando à formação transitória de ADP, que foi completamente hidrolisado a AMP. A participação das NPPS 1, 2 e 3 na forma ascítica do tumor e

durante o desenvolvimento do tumor subcutâneo de W256 também foi investigada. Nas duas formas do tumor o gene dominante foi o da enzima NPP3. Considerando que o ATP é reconhecido como um composto citotóxico em células tumorais, e que adenosina tem sido considerada promotora de tumor, é possível sugerir que, na circulação, a substancial redução na hidrólise dos nucleotídeos da adenina em plaquetas e soro após a indução do tumor pode representar um mecanismo de proteção contra o desenvolvimento tumoral. Por outro lado, nas células tumorais de W256, a elevada expressão de enzimas potencialmente envolvidas na hidrólise do ATP e do AMP, bem como a rápida hidrólise do ATP, pode representar um mecanismo que facilita a proliferação e invasão do tumor.

Abstract

Adenine and adenosine nucleotides are involved in a variety of pathophysiological process in different cells. The extracellular metabolism of these nucleotides creating nucleosides has an important regulatory function in controlling the homeostasis, mainly for regulate the platelets aggregation, via purinergic P2 receptors. Besides this, an association of these nucleotides and nucleoside in neoplasics process has been suggested. While ATP has shown to exhibit anticancer activity in a great variety of animals and human cells, studies suggest that adenosine presents tumor-promoting actions. Many studies have also demonstrated that nonsteroidal anti-inflamatory, such as acetylsalicylic acid (ASA) can protect against the cancer development. The induced actions from purinergic signalization are ruled by the ectonucleotidases, that include members of the ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) and ecto-nucleotide pyrophosphatases/phosphodiesterases (E-NPPs) families, and ecto-5'-nucleotidase (ecto-5'N). Then, in the present study we have investigated the effect of the ASA on the ATP hydrolysis to adenosine, such as studied the biochemical characteristics and expression of the ectonucleotidases in W256 tumor. The hydrolysis of the adenine nucleotides by rat platelets was significantly inhibited by ASA, both for ATP and ADP, but there was no alteration on AMP hydrolysis.

In rats submitted to W256 tumor, hydrolysis of the adenine nucleotides by platelets and serum, obtained 6, 10 and 15 days after tumor's subcutaneous induction, was significantly reduced. In W256 tumor cells (ascitic form of tumor), the kinetic analysis indicated that various ectonucleotidases are involved in this enzymatic cascade. Regarding the biochemistry properties of E-NTPDases and ecto-5'-nucleotidase, for the ATP, ADP e AMP hydrolysis, the optimum pH was reached among 6,5-8,0 and it was also observed a requirement of divalent cations ($Mg^{2+} > Ca^{2+}$). A significant inhibition on ATP and ADP hydrolysis was observed in the presence of high concentrations of sodium azide and 0.5 mM of gadolinium chloride. Also, the mRNA by PCR analysis identified the presence of NTPDases 2 and 5, and also the ecto-5'-nucleotidase in W256 tumor cells.

With the purpose of investigating the enzymes involved in the catabolism of adenine nucleotides during the tumor's growth, we evaluated the expression of these enzymes as well as the pattern degradation of extracellular nucleotides in W256 tumor, 6, 10 and 15 days after the tumor's subcutaneous induction. The mRNAs of all ectonucleotidases studied (NTPDase 1, 2, 3 5 e 6 and ecto-5'-nucleotidase) were identified by RT-PCR. The quantitative analysis, done by real-time PCR, presented as dominant genes expressed during the tumor's growth, the NTPDases 1 and 2 and ecto-5'-nucleotidase. The pattern of ATP extracellular hydrolysis determined by HPLC, despite a small difference between the times studied, kept similar. The W256 tumor's cells quickly hydrolyzed ATP, leading to the transitory formation of ADP, which was completely hydrolyzed to AMP.

Considering that ATP is recognized as a citotoxic compound in tumor cells, and that adenosine has been considered tumor's promoter, it is possible to suggest that, in the circulation, the substantial

reduction on adenine nucleotides hydrolysis in platelets and serum after tumor's induction may represent a mechanism of protection against the tumor's development. The participation of NPPs 1, 2 and 3 in the ascitic form of tumor and during the subcutaneous W256 tumor's growth was also investigated. In the two forms of the tumor, the dominant gene expressed was the NPP3 enzyme.
On the other hand, in W256 tumor cells, the high expression of enzymes potentially involved in ATP and ADP hydrolysis, as well as the fast ATP hydrolysis, may represent a mechanism that facilitate the proliferation and invasion of tumor.

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AAS - ácido acetil salicílico

ACR - apyrase Conserved Regions

ADK – Adenosina quinase

AINE - antiinflamatório não esteroidal

CD39 - antígeno de ativação celular linfóide

CD73- ecto-5'-nucleotidase

COX - ciclooxigenase

EBV - vírus Epstein Barr

Ecto-ADA - ecto-adenosina deaminase

Ecto-ATPDase - ecto-ATP diphosphohydrolase

E-NPP family - ectonucleotide pyrophosphatase/phosphodiesterase family

E-NTPDase family - ectonucleoside triphosphate diphosphohydrolase family

GPI - glicosil-fosfatidilinositol

HB6 - human brain E-type ATPase clone

NTPDase - nucleoside triphosphate diphosphohydrolase

P2X - receptor purinérgico ionotrópico

P2Y - receptor purinérgico metabotrópico

p-nitrophenyl-TMP - p-nitrophenyl-5'-thymidine-monophosphate

RT-PCR - reverse transcriptase-polimerase chain reaction

W256 - Walker 2

1 INTRODUÇÃO

1.1 Ácido acetil salicílico como agente anti-tumoral

O ácido acetil salicílico (AAS) é um fármaco analgésico, antipirético e antiinflamatório não esteroidal (AINE), da classe dos salicilatos. Sua ação primária é a inativação por acetilação irreversível da enzima ciclooxigenase, que catalisa a primeira fase da biossíntese das prostaglandinas a partir do ácido araquidônico (Korolkovas, 1999). Existem dois tipos de ciclooxigenase até agora descritas, conhecidas como ciclooxigenase-1 (COX-1) e ciclooxigenase-2 (COX-2). A primeira é encontrada, de forma constitutiva, nos vasos sangüíneos, estômago e rins, enquanto a COX-2 é induzida por processos inflamatórios (no local da inflamação), por citosinas e mediadores da inflamação. O AAS modifica covalentemente as duas enzimas resultando assim em uma inibição irreversível da atividade das ciclooxigenases (Gilman et al., 1996).

Esta classe de medicamentos além das diversas atividades terapêuticas apresenta vários efeitos colaterais, entre eles o bloqueio da agregação plaquetária, por inibição da síntese de tromboxanos. O AAS pode ser detectado na circulação sangüínea apenas por pouco tempo, em virtude de sua hidrólise no plasma, fígado e eritrócitos. As concentrações plasmáticas desta droga são sempre baixas e em raras situações ultrapassam 20 μg/mL depois do uso de doses terapêuticas convencionais (Gilman et al., 1996).

Várias observações clínicas, estudos epidemiológicos e experimentais sugerem os AINES, incluindo o AAS, como drogas promissoras para o tratamento

do câncer (Thun et al., 1993; Dikshit et al., 2006). O uso prolongado de AINES tem reduzido o risco de câncer de colón, bem como de mama, pulmão e próstata (Gupta & DuBois 1998; Rao & Reddy, 2004). Ainda, o AAS tem diminuído o crescimento de células de glioma de ratos (Aas et al., 1995), além de inibir a produção de fatores de invasão celular (Jiang et al., 2001).

Alguns estudos também demonstram que o AAS pode inibir a atividade de enzimas envolvidas no desenvolvimento do câncer, tais como a via da COX-2, enzima cuja expressão está aumentada em muitos tipos de câncer (Williams et al., 1999; Jiang et al., 2001), outros sugerem que esta inibição pode promover efeitos quimiopreventivos e quimioterápicos contra carcinoma de próstata (Hussain et al., 2003), bem como em linfoma de células B humanos (Phipps et al., 2004). O AAS também pode inibir a atividade da metaloproteinase-2, uma enzima envolvida no desenvolvimento de mestástases (Jiang et al., 2001). Os efeitos anti-tumorais do AAS também podem estar relacionados com a indução de apoptose em várias linhagens de células tumorais (Dikshit et al., 2006). Entretanto, o mecanismo pelo qual o AAS reduz o desenvolvimento e risco do câncer ainda não está totalmente esclarecido.

1. 2 Plaquetas

As plaquetas são originadas da maturação dos megacariócitos na medula óssea. São anucleadas e delimitadas por uma membrana que contém glicoproteínas, incluindo integrinas, essenciais para adesão e agregação requeridas para manter a homeostase. As invaginações da membrana plasmática

aumentam a área de superfície para a absorção das proteínas da coagulação. A ativação das plaquetas resulta na geração de tromboxano A_2 e liberação do conteúdo estocado nos grânulos alfa e densos (Nash et al., 2002). Os grânulos alfa contêm glicoproteínas, fatores da coagulação e muitos fatores pró e antiangiogênicos (Nash et al., 2001). Os grânulos densos consistem de íons cálcio, serotonina, ATP e ADP, requeridos para a agregação plaquetária (Nash et al., 2002).

A função primária das plaquetas é de iniciar o reparo a um dano na parede dos vasos sangüíneos, no sentido de evitar a perda de sangue e manter a homestase. Quando há um dano no endotélio, elas se ligam a proteínas adesivas, tornam-se ativadas, formam agregados e secretam o conteúdo de seus grânulos. A ativação de plaquetas pode também ter conseqüências patológicas importantes, levando a trombose (Levy-Toledano, 1999; Olas et al., 2001).

1.2.1 Plaquetas e câncer

Nos últimos 30 anos, tem aumentado o número de evidências de que as plaquetas têm uma função importante no desenvolvimento de metástases. A correlação clínica entre disfunção plaquetária e progressão de tumores está baseada em dados experimentais onde se observa que a depleção de plaquetas por vários mecanismos reduz o número de metátases no pulmão e medula óssea em modelos de tumor (Karpatkin & Pearlstein, 1981).

Em pacientes com câncer avançado, a expressão de moléculas de adesão em plaquetas está aumentada, indicando um estado de ativação plaquetária (Nash et al., 2002). Tecidos neoplásicos secretam direta ou indiretamente uma série de

substâncias potencialmente responsáveis pela ativação da coagulação sangüínea. As células tumorais também podem ativar as plaquetas por vários mecanismos. O ADP, um potente agonista de ativação, é liberado por plaquetas ativadas pelo tumor ou diretamente pelas células tumorais, resultando em agregação plaquetária. Além disso, a atividade procoagulante de alguns tumores leva à produção de trombina, outro importante ativador das plaquetas (Goad & Gralnick, 1996). A ativação das plaquetas também pode ser desencadeada por contato direto com moléculas presentes na superfície das células tumorais (Falanga & Rickles, 1999).

As plaquetas podem contribuir para a formação de metástases por acumularem em células tumorais embólicas, e desta forma protegê-las do sistema imune (Nieswandt et al., 1999). Além disso, as plaquetas facilitam a adesão de células cancerosas a leucócitos e células endoteliais, que pode promover o extravasamento de metástases (Karpatkin & Pearlstein, 1981). Uma vez que as células tumorais saem da circulação e invadem os tecidos, fatores derivados das plaquetas ativadas são abeis a induzir angiogênese, capacitando o crescimento no sítio metastático (Trikha & Nakada, 2002).

Os múltiplos mecanismos pelos quais as plaquetas facilitam o desenvolvimento de metástases estão representados na figura 1.

1.3 Nucleotídeos Extracelulares

A existência de um sistema purinérgico de sinalização, utilizando nucleotídeos e nucleosídeos purínicos como mensageiros extracelulares, foi proposta pela

primeira vez cerca de 30 anos atrás (Burnstock, 1972; Burnstock 1978). Burnstock (1972), demonstrou que além da transmissão colinérgica e noradrenérgica até então conhecidas, existe uma transmissão purinérgica em sistema nervoso autônomo, onde o ATP atua como o principal neurotransmissor.

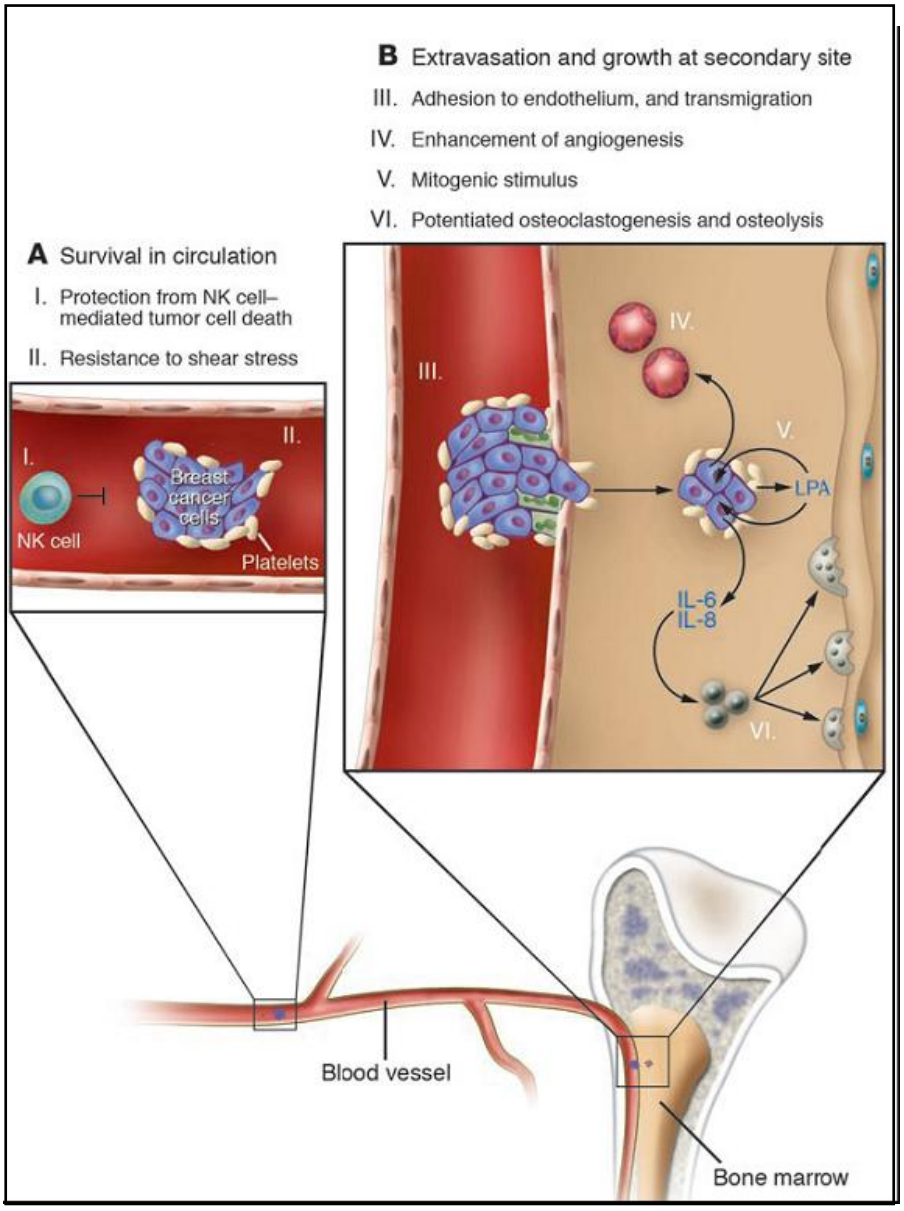

Figura 1: Ilustração dos vários mecanismos pelos quais as plaquetas podem facilitar metástases. Gupta & Massague (2004).

Os nucleotídeos extracelulares purínicos (ATP, ADP e o nucleosídeo adenosina) e pirimidínicos (UTP e UDP) são moléculas sinalizadoras importantes, as quais induzem diversos efeitos biológicos via receptores celulares, denominados purinoreceptores. Os efeitos promovidos por essas moléculas incluem contração do músculo liso, neurotransmissão no sistema nervoso central (SNC) e periférico, secreção exócrina e endócrina, resposta imune, inflamação, agregação plaquetária, dor, modulação da função cardíaca, proliferação, diferenciação, apoptose, entre outras (Ralevic e Burnstock, 1998; Burnstock, 2006). Além disso, uma atividade anticâncer dos nucleotídeos da adenina foi primeiramente descrita por Rappaport (1983) e desde então, muitos estudos têm sugerido um potencial terapêutico do ATP e outros nucleotídeos extracelulares no tratamento do câncer (Burnstock, 2002; White & Burnstock, 2006).

1.3.1 Receptores

Os efeitos dos nucleotídeos extracelulares são mediados por receptores presentes na superfície das membranas celulares. Os receptores que ligam nucleotídios e nucleosídios são divididos em receptores de adenosina ou P1, e receptores P2, ativados por ATP, ADP, UTP e UDP. Os receptores P1 se subdividem em A_1, A_{2A}, A_{2B} e A_3 e são ativados por adenosina, com o potencial agonista na ordem adenosina > AMP > ADP> ATP. Enquanto os receptores A_1 e A_3 são acoplados a proteína G_i e inibem a adenilato ciclase, ambos os receptores A_{2A}, e A_{2B} são acoplados à proteína G_s e estimulam a adenilato ciclase (Ralevic e Burnstock, 1998; Czajkowski e Baranska, 2002).

A classificação dos receptores P2 foi inicialmente baseada em critérios farmacológicos (Burnstock & Kennedy, 1985), e posteriormente reforçada através de técnicas de clonagem e expressão em sistemas heterólogos (Evans et al., 1995). Estes receptores podem ser classificados em duas famílias: receptores P2X e P2Y. Os receptores P2X atuam como canais ionotrópicos ativados por ATP e estão divididos em sete subtipos ($P2X_{1-7}$). Os receptores metabotrópicos P2Y são acoplados a proteínas G e estão divididos nos subtipos $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$ (Ralevic e Burnstock, 1998; Communi et al., 2001; Hollopeter et al., 2001; von Kugelgen & Wetter, 2000).

Diferentes subtipos de receptores P2 têm sido identificados em muitos tipos de câncer, tanto em amostras de tecido tumoral de humanos quanto em linhagens celulares (White & Burnstock, 2006).

1.3.2 O nucleotídeo ATP

A demonstração da liberação de ATP em nervos sensoriais, em 1954, foi a primeira evidência da função neurotransmissora do ATP (Holton & Holton, 1954). Posteriormente, Burnstock (1972) demonstrou que além da transmissão colinérgica e noradrenérgica até então conhecidas, existe uma transmissão purinérgica em sistema nervoso autônomo, onde o ATP atua como o principal neurotransmissor. Por seu importante papel no metabolismo celular e como fonte de energia o conceito do ATP como uma molécula sinalisadora extracelular levou muito tempo para ser aceito (Burnstock, 1997). Atualmente sabe-se que a sinalização purinérgica está envolvida, não somente em processos de sinalização rápida, como a neurotransmissão, mas

também em uma ampla variedade de outros tecidos e processos biológicos, que incluem proliferação celular, diferenciação e apoptose (Burnstock & Knight, 2004).

O ATP exerce influência sobre o sistema vascular, onde pode interferir no processo de agregação plaquetária, mediar vasoconstrição via receptores do subtipo $P2X_1$ (predominantemente expressos em tecido muscular liso) e promover proliferação de células musculares lisas e células endoteliais (Ralevic & Burnstock, 2003).

Além de respostas fisiológicas, o ATP extracelular também pode desencadear respostas patológicas dependendo do receptor ativado. A ativação dos receptores $P2Y_2$ e $P2Y_4$, desencadeia processos como proliferação celular, já a ativação do receptor $P2X_7$, está relacionada com morte celular (Harada et al., 2000).

A indução de apoptose parece exercer uma importante função na atividade anticâncer descrita para o ATP (Abbracchio & Burnstock, 1998). Alguns autores têm proposto que este nucleotídeo permeabiliza a membrana celular e induz morte celular programada em vários sistemas de células tumorais *in vitro*, incluindo seu envolvimento na morte de células tumorais mediada via linfócitos T ativados (Di Virgilio et al., 1990). Com base nestes estudos, o ATP pode ser considerado um mediador citotóxico, podendo ser secretado por linfócitos, ativados por algum estímulo, e estes mesmos linfócitos poderiam se proteger da morte celular induzida por ATP, aumentando a expressão de ecto-nucleotidases na membrana celular (Filippini et al., 1990). Ainda, o ATP extracelular, dependendo da concentração, tem se mostrado citotóxico ou mesmo um inibidor do crescimento para várias linhagens de células de mamíferos tais como fibroblastos transformados de ratos (Weisman et

al., 1988), células de linfomas, células leucêmicas (Spranzi et al., 1993), células tumorais pancreáticas, mamárias e epiteliais (Rapaport, 1983). O mecanismo molecular pelo qual o ATP extracelular exerce seus efeitos de toxicidade ainda não está bem definido, mas acredita-se que sejam mediados por ativação de purinoreceptores $P2X_7$. Desta forma, já que o ATP exibe efeitos citotóxicos em células tumorais, pode ser considerado um composto importante para a modulação do desenvolvimento de tumores e possivelmente apresenta uma aplicação como droga alternativa na terapia anticâncer.

Recentemente, um estudo clínico randomizado em pacientes com câncer de pulmão, em estágio avançado, demonstrou que infusões regulares de ATP inibem a perda de peso e melhoram a qualidade de vida nestes pacientes (Agteresch et al., 2002).

1.3.3 O nucleosídeo adenosina

Estudos de Drury e Szent-Gyorgyi (1929), forneceram as primeiras evidências de que a adenosina desempenha importantes funções fisiológicas em tecido cardíaco. A partir destes resultados, outros estudos descreveram a importância deste nucleosídeo para a manutenção de uma série de processos fisiológicos em diferentes tecidos.

A formação de adenosina intracelular pode se dar por duas vias principais que são: a clivagem da S-adenosil-homocisteína pela enzima S-adenosil-homocisteína hidrolase, e pela degradação de AMP por ação de uma 5'-nucleotidase citosólica (Patel & Tudball, 1986). Depois de formada, a adenosina

pode passar através da membrana plasmática por difusão facilitada, através de transportadores de nucleosídeos. Estes transportadores são bidirecionais e equilibram os níveis intracelular e extracelular de adenosina (Dunwiddie & Masino, 2001). Além de ser liberada como tal para o meio extracelular, a adenosina também pode ser formada no espaço extracelular, através da hidrólise do AMP extracelular por ação de uma ecto-5'-nucleotidase (Zimmermann, 1992; Dunwiddie & Masino, 2001).

Após interagir com receptores específicos, a ação da adenosina pode ser finalizada através da enzima ecto-adenosina deaminase (ecto-ADA), ou ainda através de fosforilação até 5'-AMP catalisada pela enzima adenosina quinase (ADK) (Brundege & Dunwiddie, 1997; Dunwiddie & Masino, 2001).

Dentre os efeitos fisiológicos da adenosina que foram primeiramente demonstrados nos estudos de Drury e Szent-Gyorgi (1929), está incluído o de vasodilatação coronária. Posteriormente, outros estudos descreveram a vasodilatação coronária mediada por este nucleosídeo durante hipóxia como uma tentativa de aumentar o fluxo sanguíneo e restaurar o suprimento de oxigênio ao tecido cardíaco (Ralevic & Burnstock, 2003). Sabe-se que a vasodilatação induzida pela adenosina é resultado da ativação dos receptores A_2, que são expressos em praticamente todo o sistema vascular de mamíferos (Ralevic & Burnstock, 2003).

A adenosina também atua como um inibidor da agregação plaquetária, sendo por este motivo, também conhecida como uma molécula antitrombogênica (Kitakaze et al., 1991; Kawashima et al., 2000).

Por estimular a proliferação celular, a adenosina tem sido considerada como uma substância promotora de tumores (Rathbone et al., 1992; Morrone et al., 2003). Como resultado de uma alta proliferação celular e comprometimento vascular, os tumores sólidos apresentam áreas de hipóxia, aumento do consumo de glicose e liberação de lactato. Estas condições levam a um aumento dos níveis de adenosina, que em altas concentrações, e estimula o crescimento dos tumores e a angiogênese; bem como inibe a síntese de citocinas, a adesão das células imunes à parede do endotélio e a função de células-T, macrófagos e células NK (Figura 2). Entretanto, os mecanismos pelos quais a adenosina se acumula no câncer e os resultados específicos desse acúmulo são ainda contraditórios e não são completamente entendidos (Spychala, 2000; Mujoomdar et al., 2003; Spychala et al., 2004).

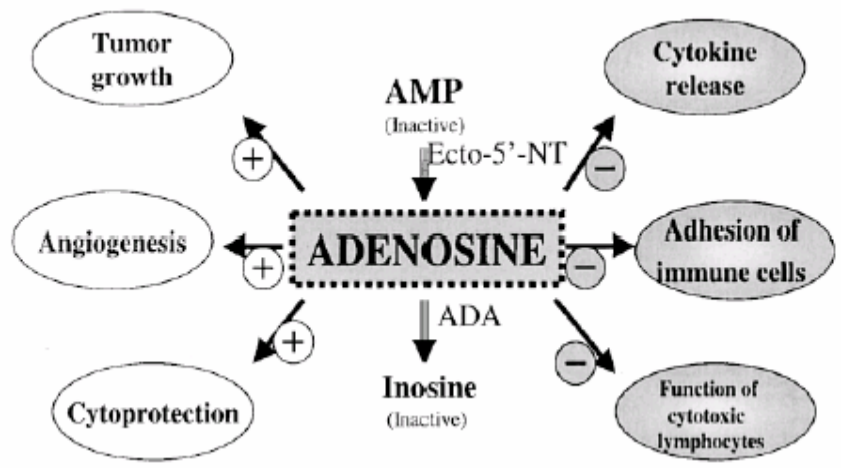

Figura 2. Ilustração geral dos efeitos da adenosina. Incluindo funções promotoras (à esquerda) e funções imunosupressoras (à direita). Spychala (2000).

1.4 Ectonucleotidases

Os nucleotídeos da adenina, após sua liberação no meio extracelular, exercem seus efeitos através da interação com receptores específicos localizados na membrana celular e posteriormente são metabolizados através da ação de ecto-enzimas, que convertem estes nucleotídeos até adenosina (figura 3).

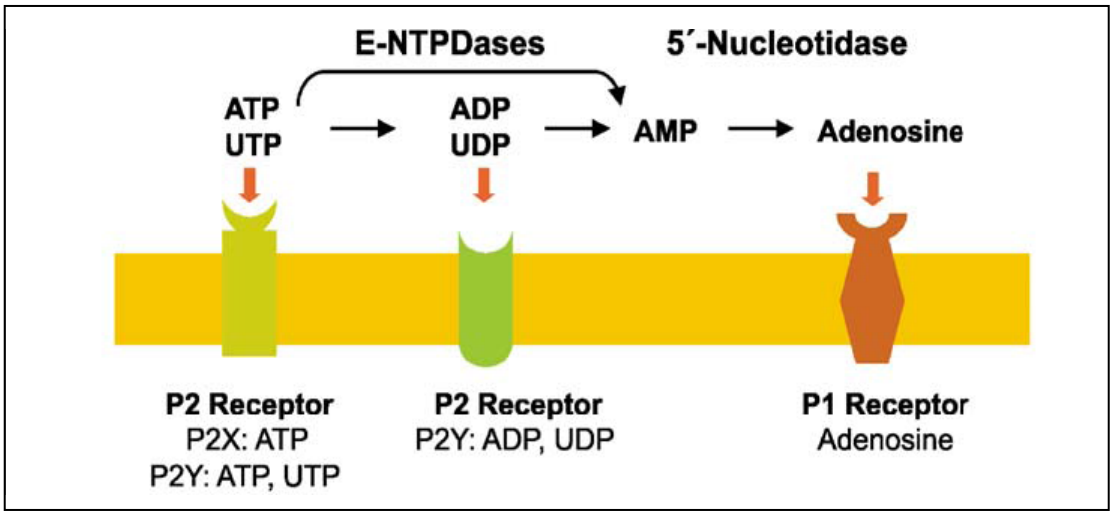

Figura 3. Catabolismo dos nucleotídeos extracelulares na superfície celular e potencial de ativação de receptores para nucleotídeos (P2) e adenosina (P1). Robson et al. (2006).

A degradação seqüencial de ATP extracelular pela "via das ectonucleotidases", pode resultar na inativação da sinalização mediada pelo ATP via receptores P2, e contribuir para a sinalização mediada pela adenosina, através dos receptores P1 (Richardson et al., 1987; Sebastião et al., 1999). Desta forma, as ecto-nucleotidases constituem um eficiente mecanismo de controle dos níveis de nucleotídeos e nucleosídeos no espaço extracelular (Zimmermann, 1996;

Zimmermann, 2001). Este processo envolve uma variedade de enzimas, dentre as quais podemos citar: (a) os membros da família das E-NTPDases (ecto-nucleosídeo trifosfato difosfoidrolase), para a hidrólise de nucleotídeos di- e trifosfatados, e (b) a ecto-5'-nucleotidase, principal enzima responsável pela hidrólise de nucleotídeos monofosfatados (Zimmermann, 2001).

As ecto-apirases, juntamente com as ecto-ATPases, fazem parte de uma família de enzimas denominada de ATPases do tipo E ou E-NTPases. Uma nova nomenclatura para as ecto-ATPases do tipo E, incluindo também os membros da família das fosfodiesterases, foi sugerida em 1999, em Diepenbeek na Bélgica, durante o Segundo Workshop Internacional sobre "Ecto-ATPases e Ecto-nucleotidases relacionadas". A nova nomenclatura teve inspiração na sistemática utilizada para os receptores P2X e P2Y, com o intuito de prover uma ordem sistemática das várias enzimas, que indicasse a ordem de clonagem e a caracterização funcional. Assim uma nova nomenclatura classificou duas famílias de enzimas: as E-NTPDases (Ectonucleoside triphosphate diphosphohydrolases) e as E-NPPs (ectonucleotide pyrophosphatase/phosphodiesterases) como envolvidas na degradação dos nucleotídeos extracelulares (Figura 4) (Zimmermann, 2001).

1.4.1 NTPDases

Fisiologicamente, as NTPDases estão envolvidas em muitas funções importantes, incluindo contração do músculo liso, percepção da dor e modulação da agregação plaquetária (Plesner, 1995). Nestes processos, estas enzimas modulam a

relação [nucleotídeo]/[nucleosídeo] no espaço extracelular, regulando desta forma, as respostas nos receptores purinérgicos.

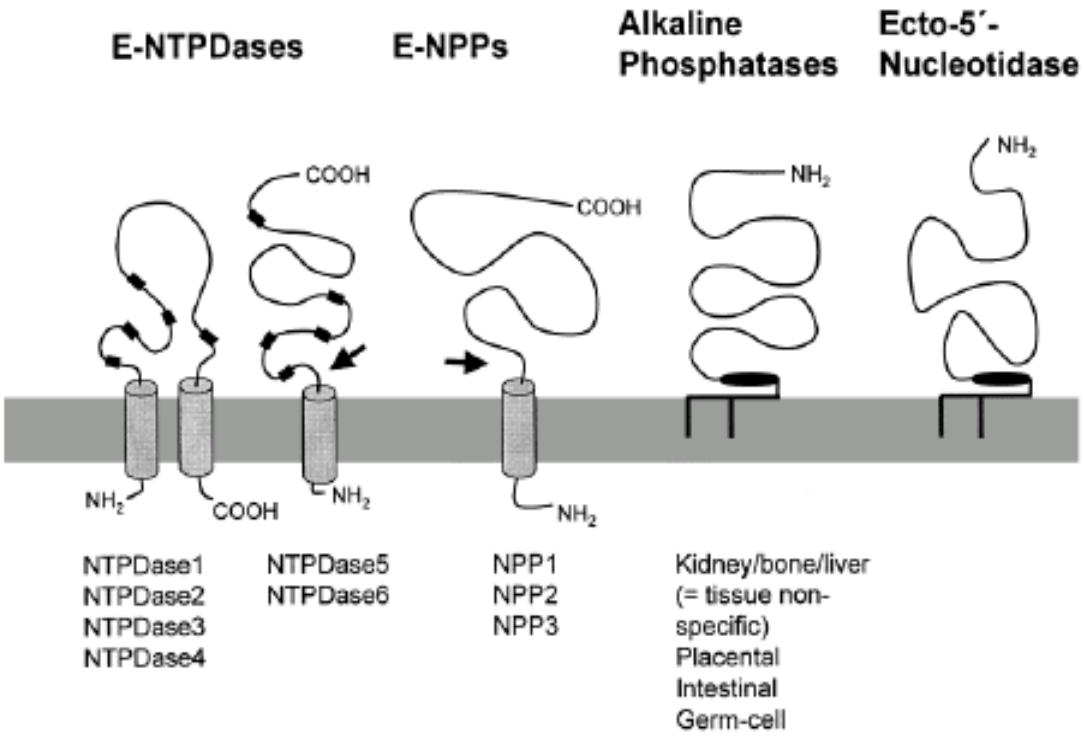

Figura 4. Topografia de membrana proposta para as ectonucleotidases. As NTPDases de 1 a 4 são ligadas à membrana plasmática por dois domínios transmembrana, N e C-terminal. As NTPDases 4, 5 e 6 estão localizadas intracelularmente. NTPDase 5 e NTPDase 6 não possuem o domínio transmembrana C-terminal e podem ser clivadas próximo ao domínio N-terminal para formar uma proteína solúvel liberada (seta). Os quadros escuros na sequência das NTPDase 1 a 6, representam as regiões conservadas das apirases (ACR). Todas as ectonucleotidases representadas são glicoproteínas. Zimmermann (2001).

A família das ecto-NTPDases é um grupo de enzimas responsável pela hidrólise dos fosfatos β e γ de tri e difosfonucleosídeos extracelulares (Meyerhof,

1945). Em mamíferos, as E-NTPDases foram primeiramente encontradas associadas à grânulos de zimogênio em pâncreas de porco (LeBel et al., 1980). Desde então, membros desta família têm sido localizados em todos os sistemas de mamíferos: sistema cadiovascular (Sévigny et al., 1997a), sistema respiratório (Sévigny et al., 1997b), sistema digestório (Sévigny et al., 1997b; Leclerc et al., 2000), sistema nervoso (Schadeck et al., 1989; Sarkis & Salto, 1991; Zimmermann & Braum, 1996; Wang & Guidotti, 1998), sistema imune (Maliszewski et al., 1994) e no sistema reprodutor/ excretor (Valenzuela et al., 1989; Lemmens et al., 2000).

As E-NTPDases, anteriormente identificadas como ecto-ATPases, ecto-ATPDases ou CD39 , são enzimas que compartilham as seguintes características: (1) um sítio de hidrólise de nucleotídeos voltado para o espaço extracelular, (2) subunidade catalítica glicosilada, (3) atividade dependente de cátions divalentes (principalmente cálcio e/ou magnésio), (4) insensibilidade a inibidores específicos de ATPases do tipo P (ex. Na^+K^+ATPase), tipo F (ex. ATPase mitocondrial), tipo V (ex. bomba de prótons vacuolar) e (5) habilidade para hidrolisar uma ampla variedade de nucleotídeos púricos e pirimídicos tri e difosfatados (Plesner., 1995; Zimmermann et al., 1998). Além disso, após a análise das sequências de diversas ecto-ATPDases e ecto-ATPases, foi demonstrado que estas enzimas compartilham cinco regiões altamente conservadas chamadas de “regiões conservadas da apirase”, ACR (ACR 1 - 5 apyrase conserved regions) (Handa e Guidotti, 1996; Schulte et al., 1999). A existência desses sítios conservados pode estar relacionada com a formação do sítio catalítico das enzimas e/ou com a integridade estrutural das E-NTPDases (Grinthal & Guidotti, 2000).

Baseado em sua estrutura e propriedades catalíticas, particularmente na relação ATP/ADP, a família das NTPDases em mamíferos, pelo menos até o momento, está constituída de oito membros clonados e funcionalmente caracterizados (Figura 5): NTPDase 1 (CD39), NTPDase 2 (CD39L1), NTPDase 3 (CD39L3, HB6), NTPDase 4 (UDPase), NTPDase 5 (CD39L4) e NTPDase 6 (CD39L2), NTPDase 7 e NTPDase 8. Essa família de genes também tem membros em invertebrados, plantas, fungos e protozoários. As enzimas de mamíferos hidrolisam nucleosídeos di e tri fosfatados, com diferenças consideráveis na preferência pelos substratos. Enquanto as NTPDases 1, 2, 3 e 8 estão localizadas na superfície celular, com sítios catalíticos voltados para o meio extracelular, as NTPDases 5 e 6 apresentam localização intracelular e sofrem secreção depois de expressão heteróloga. Já as NTPDases 4 e 7 têm localização totalmente intracelular com o sítio ativo voltado para o lúmem das organelas intracelulares (Zimmermann, 2001; Robson et al., 2006).

A NTPDase 1 (CD39, ecto-apirase, ecto-ATPdifosfohidrolase) hidrolisa ATP e ADP igualmente bem, sendo a proporção de velocidade de hidrólise destes dois substratos de 1:1 (Heine et al., 1999; Wang & Guidotti, 1996), e tem sido a mais estudada dos membros da família das E-NTPDases.

Os primeiros estudos sobre apirases baseavam-se em conhecer sua distribuição e definir suas características cinéticas. Estas enzimas têm apresentado ampla distribuição em tecidos vegetais (Krishnan, 1949; Valenzuela et al., 1989), insetos (Ribeiro et al., 1984; Ribeiro et al., 1989; Sarkis et al., 1996), aves (Carl & Kirley, 1997) e tecidos de mamíferos, como preparações de SNC e periférico (Battastini et al., 1991; Bruno et al., 2002; Sarkis & Saltó, 1991), aorta bovina (Coté

et al., 1992), plasma humano (Holmsen & Holmsen, 1971), secreções seminais (Rosenberg et al., 1988), vasos umbilicais humanos (Yagi et al., 1992), pulmão bovino (Picher et al., 1993), plaquetas e soro de ratos (Frasseto et al., 1993; Oses et al., 2004), células neoplásicas humanas (Dzhandzhugazyan et al., 1998), dentre outros.

Em 1994, Maliszeswski e colaboradores caracterizaram o antígeno de ativação celular linfóide, CD39, originalmente definido como um marcador de superfíce de células B transformadas pelo EBV, e mais tarde em um trabalho realizado por Handa & Guidotti (1996) de purificação e clonagem de uma ATPdifosfoidrolase solúvel de batata, foi sugerido haver uma grande similaridade entre as seqüências de aminoácidos desta enzima e do antígeno celular linfóide CD39. Evidências como a similaridade entre seqüência de aminoácidos da apirase clonada e CD39, levaram os autores a obterem a confirmação de que o antígeno CD39 e a ecto-apirase constituem a mesma proteína (Wang & Guidotti, 1996).

Estudos recentes em nosso laboratório revelaram que a ecto-apirase presente em diferentes preparações de tecido cerebral e cultura de astrócitos pode ser identificada como uma fosfoproteína, sugerindo ser este um dos mecanismos responsáveis pela regulação da mesma em diferentes situações fisiológicas e patológicas (Wink et al., 2000).

Em melanomas diferenciados de humanos foi observado um aumento na expressão da ecto-apirase/39, sugerindo que esta proteína pode ser um marcador de diferenciação tumoral, por apresentar gradual diminuição com a progressão do tumor (Dzhandzhugazyan et al., 1998).

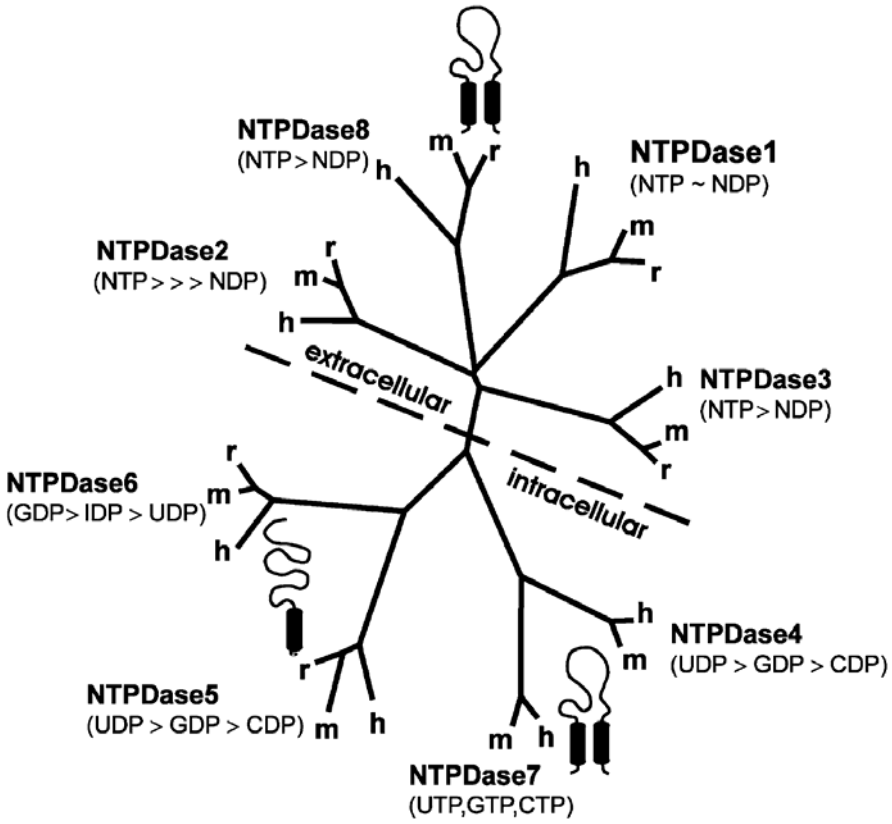

Figura 5. Árvore filogenética hipotética para os membros da família das NTPDases (NTPDase 1 a 8) de rato (r), humano (h) e camundongo (m). O comprimento das linhas indica as diferenças entre as seqüências de aminoácidos. O gráfico representa a separação entre NTPDases localizadas na superfície (superior) e intracelular (inferior). A preferência por substrato para cada subtipo e a topografia na membrana para cada grupo de enzimas está também representada (um ou dois domínios transmembrana, indicados por cilindros). Robson et al. (2006).

A NTPDase 1 tem sido extensivamente estudada em células endoteliais e em plaquetas, possuindo um papel bem descrito na regulação do fluxo sanguíneo e trombogênese. A enzima presente na superfície das células endoteliais e plaquetas converte o ADP, pró-agregante plaquetário, em adenosina, anti-agregante, limitando

a extensão da agregação plaquetária intravascular (Frassetto et al., 1993; Pilla et al., 1996; Kaczmarek et al., 1996; Marcus et al., 1997; Imai et al., 1999; Koziak et al., 1999).

Estudos prévios demonstraram que ratos deficientes de CD39/ATP difosfoidrolase apresentam problemas relacionados com hemostasia e trombogênese (Enjyoji et al., 1999). Assim, formas solúveis e ligadas à membrana da NTPDase1/CD39 são potenciais agentes terapêuticos para a inibição de processos trombogênicos (Gayle et al., 1998) e podem representar uma nova geração de moléculas cardioprotetivas (Marcus et al., 2005).

O tratamento de linhagens de células B positivas para NTPDase 1 com anticorpos monoclonais anti-NTPDase 1 é capaz de induzir adesão, sugerindo uma função desta enzima na adesão celular (Zimmermann, 2001). Além disso, estudos com uma variedade de inibidores também sugerem que esta ecto-enzima pode controlar a função de linfócitos incluindo reconhecimento de antígenos e/ou ativação das células T citotóxicas (Dombrowski et al., 1995).

A enzima NTPDase 2 (CD39L1, ecto-ATPase) possui uma preferência 30 vezes maior pela hidrólise do ATP em comparação com a hidrólise do ADP (Kirley, 1997). Devido a esta preferência, esta enzima pode inativar nucleotídeos trifosfatados e agir como um produtor extracellular de nucleotídeos difosfatados (Zimmermann, 2001).

As ecto-ATPases podem influenciar vários processos fisiológicos, por exemplo, hidrolisando ATP e outros nucleotídeos extracelulares, e desta forma modulando a concentração de ligantes em receptores purinérgicos P2 (Knowles & Chiang, 2003). Recentemente, a clonagem do cDNA de ambas ecto-ATPases

humana e de camundongos de células tumorais indicou que esta enzima é altamente expressa em alguns tumores (Gao et al., 1998; Knowles & Chiang, 2003).

A NTPDase 3 (CD39L3) é considerada um intermediário funcional entre a NTPDase 1 e a NTPDase 2, pois hidrolisa o ATP e o ADP em uma razão de 3:1. Muito do conhecimento sobre a NTPDase 3 está relacionado a sua estrutura, entretanto muito pouco é sabido a respeito do seu papel fisiológico, bem como sua distribuição na natureza. Permanece também a ser elucidado a sua exata contribuição e relação com os outros membros relacionados, NTPDase 1 e NTPDase 2 nos diversos tecidos onde há sobreposição de suas funções.

As NTPDases 4 (UDPases), embora possuam a mesma estrutura geral das NTPDases 1 a 3, diferem principalmente em relação à localização celular. NTPDases 4 de humanos estão localizadas no complexo de Golgi (NTPDase 4β) ou em vacúolos lisossomais (NTPDase 4α) (Wang & Guidotti, 1998). Ambas enzimas hidrolisam nucleotídeos di e trifosfatados, mas possuem uma baixa preferência por ATP e ADP. Elas diferem em sua preferência por nucleotídeos e também na dependência de cátions divalentes. A NTPDase 4α tem alta preferência por UTP e TTP, enquanto que CTP e UDP são os melhores substratos da NTPDase 4β.

Já as NTPDases 5 (CD39L4) e NTPDases 6 (CD39L2), encontram-se ancoradas na membrana celular somente pela porção NH_2 terminal e possuem uma larga região COOH terminal extracelular. Ambas enzimas são ativadas por cátions divalentes e apresentam uma maior preferência por nucleotídeos difosfatados. A NTPDase 5 tem maior preferência para hidrolizar principalmente GDP e UDP,

enquanto a NTPDase 6, hidroliza preferencialmente GDP e IDP. Acredita-se que estas enzimas sejam liberadas da membrana e então secretadas para o meio extracelular, indicando tratar-se de enzimas na forma solúvel. A NTPDase 5 está localizada no retículo endoplasmático, enquanto a NTPDase 6, encontra-se no complexo de Golgi (Zimmermann, 2001). A recente identificação e caracterização bioquímica da NTPDase 5 (Mulero et al., 2000), não permitiu ainda um completo entendimento da função fisiológica da CD39L4. Foi demonstrado recentemente que a NTPDase 5 é idêntica ao HCPH, produto de um proto-oncogene descoberto depois de sua ativação por tratamento com um agente carcinogênico (Paez et al., 2001). Devido a sua clara preferência por nucleosídeos di-fosfatados, acredita-se que o seu papel fisiológico seja reduzir os níveis circulantes do ADP e não do ATP.

As NTPDases 7 e 8 preferem como substratos nucleosídeos trifosfatados, entretanto a NTPDase 7 está localizada em vesículas intracelulares, enquanto que a NTPDase 8 foi recentemente clonada, caracterizada e descrita como uma ecto-enzima de membrana expressa em fígado, rins e intestino de camundongos com uma razão de hidrólise de aproximadamente 2:1 (Bigonnesse et al., 2004; Zimmermann, 2001). Recentemente, uma NTPDase 8 também foi clonada e caracterizada em fígado humano, e uma forma solúvel desta enzima foi gerada por expressão de seu domínio extracelular em células embrionárias de rim humano (HEK293) (Knowles & Li, 2006).

1.4.2 Ecto-5'-nucleotidase

A ecto-5'-nucleotidase ("lymphocyte surface protein CD73") é uma enzima ancorada à membrana plasmática por glicosil-fosfatidilinositol (GPI) (Figura 4), que representa um marcador de maturação para os linfócitos T e B, sendo ausente nas células imaturas (Airas et al. 1997). O ancoramento da enzima pode ser clivado por uma fosfolipase C específica para GPI, dando origem às formas solúveis da enzima (Zimmermann, 1992). A ecto-5'N pode exercer uma ampla variedade de funções dependendo de sua expressão tecidual e celular. Ela encontra-se presente na maioria dos tecidos e sua principal função é a hidrólise de nucleosídeos monofosfatados extracelulares, tais como AMP, GMP ou UMP, a seus respectivos nucleosídeos (Sträter, 2006).

O principal papel fisiológico atribuído a ecto-5'-nucleotidase, é a formação de adenosina a partir do AMP extracelular e a subsequente ativação dos receptores P1, que em sistema nervoso, resulta principalmente na inibição da liberação de neurotransmissores excitatórios (Brundege & Dunwidie, 1997), enquanto que em sistema vascular, resulta em vasodilatação e na inibição da agregação plaquetária (Kawashima et al., 2000). Além disso, a ecto-5'-nucleotidase pode estar envolvida na adesão celular (Zimmermann, 2001). Estudos com linfócitos humanos identificaram a proteína 2 de adesão linfócito-vascular como sendo CD73, que medeia a adesão dos linfócitos ao endotélio (Fenoglio et al., 1997).

A atividade desta enzima em células tumorais também tem sido descrita e é bastante variável. Uma atividade elevada desta enzima foi encontrada em carcinoma de mama (Canbolat et al., 1996), câncer gástrico (Durak et al., 1994),

câncer pancreático (Floke & Mannherz, 1991) em glioblastoma (Fenoglio, 1997), entre outros. Ainda, estudos demonstram que a alta expressão da ecto-5'-nucleotidase em diferentes células de melanomas está associada com um fenótipo altamente invasivo. Além de gerar adenosina, ela pode ter outras funções em melanomas, tais como adesão e interação com componentes da matriz extracelular (Sadej et al., 2006).

1.4.3 A família E-NPP

Os membros da família E-NPP (Nucleotide pyrophosphatase/phosphodiesterase) possuem uma ampla distribuição tecidual e incluem a NPP1(PC-1), NPP2 (PD-Iα, autotaxina), NPP3 (PD-Iβ, B10, gp130^{RB13-6}), NPP4, NPP5, NPP6 e NPP7 (esfingolielinase alcalina). Exceto para a NPP2, que é secretada no meio extracelular, todos os demais membros são ligados à membrana por um único domíno transmembrana N-terminal e apresentam um domínio para clivagem proteolítica, sugerindo que possam ocorrer como enzimas solúveis (Zimmermann, 2001; Stefan et al., 2005; Stefan et al., 2006). Três dos sete membros desta família, as NPPs 1-3 são conhecidas por hidrolizar nucleotídeos (Stefan et al., 2006).

Essas enzimas apresentam atividade de fosfodiesterase alcalina bem como atividade nucleotídeo pirofosfatase, hidrolisando uma grande variedade de substratos, entre eles purinas e pirimidinas. O p-nitrofenil-5'-timidina-monofosfato (*p*-nitrophenyl-TMP) tem sido usado como um substrato artificial, específico para as E-NPPs (Sakura et al., 1998).

A catálise por NPPs afeta processos como proliferação e motilidade celular, angiogênese, mineralização óssea e digestão. Estão também envolvidas na patofisiologia do câncer, resistência à insulina e alterações na calcificação (Stefan et al., 2005).

Até o momento, as NPPs mais bem estudadas e caracterizadas são as ecto-enzimas de mamíferos, NPP1, 2 e 3. O camundongo deficiente em NPP1 revelou uma produção excessiva de tecido ósseo, sugerindo que esta enzima desempenhe um papel essencial no controle da mineralização óssea, pela produção de PP_i. A NPP2 é uma proteína secretada que se acumula em fluidos corporais tais como plasma e fluido cerebroespinhal. Esta enzima estimula a proliferação, contração e migração celular (Stefan et al., 2005; Stefan et al., 2006). Possui também uma atividade estimulante de motilidade, tendo sido identificada como um fator estimulante de motilidade tumoral secretado por melanomas (Goding et al., 2003). Uma função importante da NPP2 em metástases também tem sido descrita. A expressão da NPP2 está aumentada em muitos tipos de câncer incluindo câncer de mama e de pulmão, e sua presença está relacionada com a invasividade das células tumorais (Stefan et al., 2005). A NPP3 possui um papel importante em alergias, sendo definida como um marcador de ambos basófilos e mastócitos (Stefan et al., 2005). Ainda, uma participação da NPP3 no desenvolvimento e transformação tumoral tem sido descrita (Goding et al., 2003).

1.5 Tumor de Walker 256

Em 1928 George Walker observou o aparecimento de uma neoplasia de crescimento espontâneo, na região da glândula mamária de uma rata albina prenhe, que regredia, totalmente, durante o período de lactação mas que voltava a crescer, após o desmame da prole. Naquela ocasião, aquele tumor foi denominado como tumor de Walker 256 (W256), o qual foi classificado histologicamente como carcinoma e posteriormente, testes de transplantabilidade mostraram a capacidade de fragmentos do tumor original crescerem em ratos receptores (Earle, 1935). As características de transplantabilidade deste tumor, bem como técnicas de crioconservação e de cultura de tecidos, permitiram manter esta linhagem até a atualidade, sendo descritas variantes morfológicas como sarcoma, carcinossarcoma e carcinoma (Iwana et al., 1973).

Desde então, diversas vias de inoculação foram utilizadas para administração das células tumorais de W256, como a via subcutânea, intramuscular, intraperitoneal, intrapleural, intracardíaca, intraesplênica, sangüínea arterial e venosa e tecido ósseo (Iwama et al., 1973). Quando a via subcutânea (*sc*) é utilizada há o desenvolvimento de tumores sólidos, inicialmente firmes a palpação, encapsulados e de forma arredondada, que provocam infiltração da pele e da musculatura adjacente (Earle, 1935; Iwama, 1979). Por outro lado, a inoculação destas células tumorais através da via intraperitoneal (*ip*), ocasiona o desenvolvimento da forma ascítica do tumor de W256 (Iwama et al., 1973).

O tumor de W256 tem sido amplamente usado nos estudos de fisiopatologia do câncer (Toal et 1960; Morrison et 1971; Guaitani et 1982; Rettori

et al., 1995; Ventrucci et al., 2001; He et al., 2003; Ikeda et al., 2004; Mao-Ying et al., 2006). Já foram observadas duas linhagens morfologicamente e estruturalmente diferentes de células tumorais de W256, nomeadas como WS e WR (Simpkins et al., 1991). Outros autores obtiveram também duas variantes deste tumor descritas, previamente, como W256 A e B (Guaitani et al., 1983). Recentemente, foi demonstrado que várias passagens intra-peritoniais da variante A do tumor de W256, leva à geração de uma variante imunogênica regressiva denominada AR, caracterizada por aumento da fragilidade osmótica das hemácias e uma hipertrofia no baço do hospedeiro (Schanoski et al., 2004).

2 OBJETIVOS

Este trabalho está apresentado na forma de capítulos constituídos por artigos científicos publicados e/ou submetidos, que visaram cumprir os seguintes objetivos:

Capítulo 1- Investigar a influência do AAS sobre as enzimas responsáveis pela hidrólise de ATP, ADP e AMP até adenosina em plaquetas de ratos adultos e discutir seu significado e sua relação com o câncer.

Capítulo 2- Estudar e comparar o efeito do tumor de W256 na hidrólise dos nucleotídeos da adenina em soro e plaquetas de ratos 6, 10 e 15 dias após a inoculação subcutânea do tumor.

Capítulo 3- Identificar e caracterizar os membros da família das E-NTPDases e ecto-5'-nucleotidase como parte de um sistema enzimático responsável pelo metabolismo dos nucleotídeos da adenina, em células tumorais de W256 na forma ascítica.

Capítulo 4- Avaliar a expressão dos membros da família das E-NTPDases e ecto-5'-nucleotidase e estudar o metabolismo extracelular do ATP no tumor de W256, obtido de ratos 6, 10 e 15 dias após a inoculação subcutânea do tumor.

Anexo - Constitui-se de dados preliminares de um manuscrito em preparação, onde o objetivo é estudar a expressão e caracterização das NPPs em células tumorais de W256, como complemento do estudo das ectonucletidases envolvidas no controle dos níveis de nucleotídeos extracelulares ao redor do tumor.

3 CAPÍTULOS - ARTIGOS CIENTÍFICOS

3.1 Capítulo 1

Acetylsalicylic acid inhibits ATP diphosphohydrolase activity by platelets from adult rats

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Acetylsalicylic acid inhibits ATP diphosphohydrolase activity by platelets from adult rats

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Abstract

Background and methods: The in vitro effect of the nonsteroidal anti-inflammatory drug, acetylsalicylic acid (ASA), on the extracellular adenine nucleotide hydrolysis by intact rat blood platelets was studied. *Results*: Our results demonstrate that aspirin, at final concentrations of 2.0 and 3.0 mM, inhibits ATP extracellular hydrolysis in vitro by approximately 17% and 21%, respectively. Aspirin, at a final concentration of 3.0 mM, also inhibited in vitro extracellular ADP hydrolysis by approximately 41%. The same concentrations of this drug, however, did not alter AMP hydrolysis by intact rat blood platelets under similar assay conditions. The kinetic analysis demonstrated that the inhibition of ADP and ATP hydrolysis by aspirin in rat platelets is of the uncompetitive type. *Conclusion*: In this study, we demonstrated an inhibitory effect of ASA upon E-NTPDase 3 activity of platelets from adult rats and discussed the significance of our findings.

Keywords: Aspirin; ATP diphosphoydrolase/CD39; Cancer; Metastasis; NTPDase; Platelets

1. Introduction

Adenine nucleotides and nucleosides may play a role in the regulation of vascular tone and in platelet aggregation, since ATP and ADP are vasoactive and platelet-active nucleotides [1,2], respectively, and adenosine, the final product of the nucleotide hydrolysis, is a vasodilator [3] and an inhibitor of platelet aggregation [4]. The most relevant ecto-enzymes involved in adenine extracellular nucleotide hydrolysis are E-NTPDase, or Ectonucleoside triphosphate diphosphohydrolase (including the enzyme also named as ecto-ATPDase, ATP-diphosphohydrolase or apyrase, CD39), and ecto-5′-nucleotidase (CD73) [5].

In the blood, E-NTPDase 3 sequentially converts extracellular purine nucleotides, such as adenosine triphosphate (ATP) and adenosine diphosphate (ADP), to the monophosphate form (AMP) [6], which may generate adenosine through the action of an ecto-5′-nucleotidase [7]. These enzymes have been characterized in intact rat blood platelets [8].

In addition to the various physiological and pathologic properties already described for adenine nucleotides, an anticancer activity has been suggested [9,10]. Studies have indicated that ATP permeabilizes cell

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membranes and induces programmed cell death in several tumor cell systems in vitro [11]. This nucleotide has also been proposed to be involved in tumor-cell killing, mediated by activated T-lymphocytes [12]. Moreover, ATP has been shown to be growth inhibitory or cytotoxic for several mammalian cell lines such as in mouse leukemia cells [13] and lymphoma cells as well as in human cell lines such as leukemia cells [14], pancreatic carcinoma cells [15], breast cancer cells [16] and skin carcinoma cells [17]. Recently, another randomized clinical study, in advanced lung cancer patients, reported that regular infusions of ATP inhibit loss of body weight and increased life quality in these patients [18]. Since extracellular ATP can display cytotoxic activity, it may be considered a potential compound for the modulation of tumoral cell development and, thus, have an application as an alternative anticancer therapy.

Conversely, adenosine, an immediate catabolite of adenine nucleotides, produced by the action of ecto or soluble enzymes, has been shown to possess a tumor-promoting action [19,20]. Adenosine released by solid tumors, as a result of tissue hypoxia, provides a supportive environment that benefits malignancy and may include protection against ischemia, stimulation of growth and angiogenesis, and suppression of immune responses [20]. The levels of adenosine in such tumors are sufficient to interfere with the anti-tumor immune response by suppressing T cell activation [21] and the interaction of T lymphocytes with tumor cells [22]. Thus, given the strong immunosuppressive function of this nucleoside, it is likely that adenosine may constitute an important part of the ''immunological barrier'' that is responsible for the failure of the immune system response towards malignant cells [20].

Hence, we suggest that an inhibition in E-NTPDase 3 activity of platelets, may represent a mechanism of protection against tumoral-cell development, since the levels of ATP in the circulation would be increased, and ATP breakdown by dephosphorylation (main source of the extracellular adenosine production), would be inhibited.

On the other hand, ATP diphosphoydrolase/CD39 (an enzyme able to promote ATP hydrolysis to adenosine in association with a 5′-nucleotidase) is a marker of activated immunocompetent cells and somehow involved in adhesion [23]. Studies in neural cells have demonstrated that NCAM adhesion molecules are able to bind the cells and promote the hydrolysis of extracellular ATP [24]. Moreover, platelets, express glycoproteins (including integrins) on their surface, which are essential for adhesion and aggregation and have recently been shown to play a role in metastasis [25].

Thus, circulating blood platelets may possess an ecto-enzyme (ecto-ATP diphosphohydrolase) involved in cancer in two manners. In first place, platelets may possess an enzyme that modulates the ATP (cytotoxic) and adenosine (tumor-promoting) levels in the circulation. Alternatively, an enzyme may exist to modulate the adhesion between platelets and tumoral and endothelial cells, consequently, controlling metastasis. Platelets have been suggested to contribute to the metastatic spread by accumulating on embolic tumor cells, thus protecting them from clearance by the immune system [26].

The understanding of the mechanisms that control metastasis and invasion is critical for the identification of new targets for drug development. Aspirin, a nonsteroidal anti-inflammatory drug, has been shown to reduce the risk of development of many cancer types [27,28], and it seems that this effect is not due to the inhibition of prostaglandin synthesis [27].

In the present study, we evaluate whether aspirin can modulate in vitro extracellular adenine nucleotide hydrolysis by platelets from adult rats and discuss the significance of our findings.

2. Materials and methods

2.1. Chemicals

Nucleotides were obtained from Sigma (St. Louis, MO, USA). Acetylsalicylic Acid (ASA) was obtained from Gerbras (Germany). Sepharose 2B gel was obtained from Pharmacia and was de-aerated in a vacuum flask before packing in a polyethylene column. All other reagents were of analytical grade. Polyethylene or siliconized labware was used for all platelet isolation and incubation procedures.

2.2. Platelet isolation

Male Wistar rats, weighing approximately 250 g, from our own breeding stock were maintained on

a 12-h light/12-h dark cycle at constant room temperature.

Platelets were isolated exactly as described previously by Hantgan [29]. In an effort to obtain preparations of normal, undamaged platelets free of non-adsorbed plasma constituents, we separated intact platelets from plasma by means of gel filtration on a 1.5 × 7.0-cm Sepharose 2B column [30]. The column was equilibrated with a buffer consisting of 140 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 5.5 mM dextrose, 0.2 mM EGTA and 0.05% azide, pH 6.8 (Ca^{2+}-free Tyrode's Buffer). Platelets were eluted with the same buffer at room temperature; 0.5-ml fractions were collected and the tubes containing the maximum platelet count (determined visually) were used for subsequent experiments.

2.3. Isolation of blood serum fraction

We used male Wistar rats of approximately 60 days old, weighting around 250 g. Blood samples were drawn after decapitation, as described by Yegutkin [31], and were soon centrifuged in plastic tubes at 5000 × *g* for 5 min at 20 °C. The serum samples obtained were then stored on ice and immediately used in the experiments.

2.4. Assays of E-NTPDase 3 and ecto-5′-nucleotidase activities

For platelets, unless otherwise stated, the reaction medium used to assay Ca^{2+}-ATPase and Ca^{2+}-ADPase activity contained 120 mM NaCl, 5.0 mM KCl, 60 mM glucose, 5.0 mM $CaCl_2$ and 50 mM Tris–HCl buffer, pH 7.5, in a final volume of 200 μl. About 20 μl of platelet preparation (20–30 μg of protein) was added to the reaction medium and preincubated for 30 min at 37 °C. ASA was added from a water solution to a final concentration of 1.0, 2.0 and 3.0 mM in the test tubes. The enzyme reaction was started by the addition of ATP, ADP or AMP to a final concentration of 0.5 mM, and incubated for 60 min. For blood serum fraction, ATP, ADP and AMP hydrolysis were determined using a modification of the method described by Yegutkin [31]. The reaction medium was preincubated for 30 min at 37 °C. ASA was added from a water solution to a final concentration of 1.0, 2.0 and 3.0 mM in the test tubes. The reaction mixture containing ATP, ADP or AMP as substrate to a final concentration of 3 mM, 112.5 mM Tris–HCl, pH 8.0, was incubated with approximately 1.0 mg of serum protein at 37 °C for 40 min in a final volume of 200 μl. The reaction for the platelets and blood serum fraction assay, was stopped by the addition of 200 μl of 10% trichloracetic acid (TCA). The samples were chilled on ice and the amount of inorganic phosphate (Pi) liberated was measured by the method of Chan et al. [32]. The samples of blood serum fraction were centrifuged at 5000 × *g* for 5 min to eliminate precipitated protein and the supernatant was used for the colorimetric assay. Incubation times and protein concentration (for both fractions) were chosen to ensure the linearity of the reaction. In order to correct non-enzymatic hydrolysis, we performed controls by adding the platelets or blood serum fraction after the reaction was stopped with TCA. All samples were run in triplicate. Enzyme activities were generally expressed as nmol Pi released/min/mg of protein.

For platelet ecto-5′-nucleotidase assay, we used the same procedure and conditions as we used for the E-NTPDase 3 assay, except that AMP (0.5 mM final concentration) instead of ATP or ADP was used as substrate and 5.0 mM $CaCl_2$ was replaced by 5.0 mM $MgCl_2$. Other procedures used were the same as those of the ATP and ADP hydrolysis procedures.

2.5. Protein determination

Protein was measured by the Coomassie Blue method using bovine serum albumin as standard [33].

2.6. Statistical analysis

Data were analyzed by one-way ANOVA, followed by the Duncan multiple range test. $P < 0.05$ was considered to represent a significant difference in the statistical analysis used. All analyses were performed with an IBM compatible computer using the SPSSPC software.

2.7. Ethics

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

3. Results

The anti-inflammatory drug, ASA, was found to inhibit in vitro ATP hydrolysis in experiments using intact platelets obtained from adult rats. The inhibition was observed at a final concentration of 2.0 and 3.0 mM, when compared to control enzyme activity (17% and 21% inhibition, respectively, $P<0.05$). The final concentration of 1.0 mM did not have any effect on the ATP hydrolysis (Fig. 1A).

The ADP extracellular hydrolysis by rat platelets was also efficiently inhibited by ASA, as shown in Fig. 1B. The enzyme activity was reduced only in the presence of 3.0 mM aspirin (41% inhibition, when compared to control enzyme activity, $P<0.05$). ASA, at a final concentration of 1.0–3.0 mM, did not significantly alter the hydrolysis of AMP by rat platelets in the same conditions (data not shown).

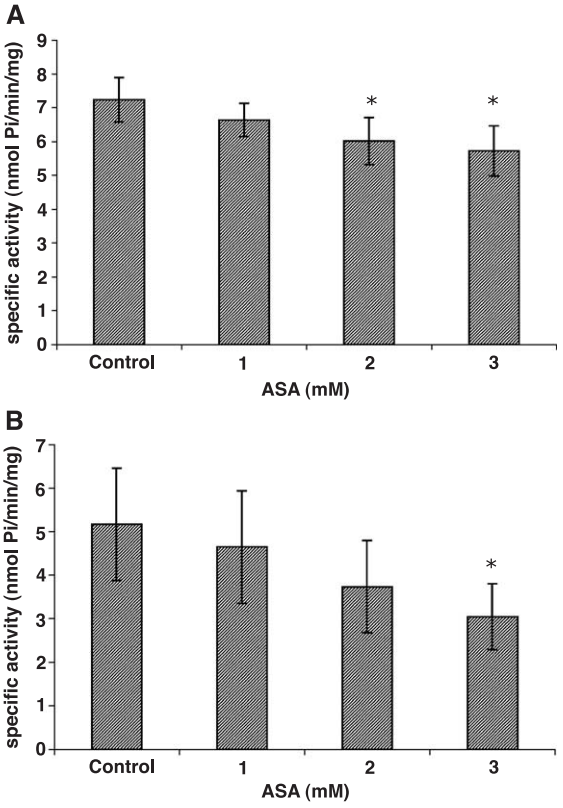

Fig. 1. Effect of ASA on ATP (A) and ADP (B) hydrolysis by intact rat platelets. Bars represent mean ± S.D. for four independent experiments. Results are expressed as nmol Pi released/min/mg of protein. *Indicates significant difference from control enzyme activity ($P<0.05$). Data were analyzed statistically by one-way analysis of variance, followed by Duncan's multiple range test.

The results demonstrated that ATP and ADP hydrolysis have different sensitivities to the anti-inflammatory drug, ASA (with regard to inhibitor concentration). This effect may be explained considering one or two different active sites as involved in nucleotide hydrolysis.

On basis of these results, the kinetics of the interaction of ASA with the ATPase and ADPase activities in adult rat platelets was determined. The Lineweaver–Burk double-reciprocal plot was analyzed over a range of ATP (Fig. 2A) or ADP (Fig. 2B) concentrations (50–150 μM) in the absence and in the presence of aspirin 3.0 mM for ATP and ADP hydrolysis. The data indicated that the type of inhibition is uncompetitive for ASA for both ATP and ADP hydrolysis, in rat platelets.

Other studies from our laboratory described an NTPDase (ATP diphosphohydrolase) in addition to a Phosphodiesterase (PDase) as an enzyme that may be involved in the hydrolysis of triphospho- and diphosphonucleosides in rat blood serum. Based on this result, we postulated the co-existence of a NTPDase and a PDase that together with a 5′-nucleotidase are constituents of an enzymatic chain able to promote ATP–ADP hydrolysis to adenosine in the circulation [34]. For this reason, we also investigated the in vitro effect of the ASA on the extracellular hydrolysis of adenine nucleotides by rat blood serum fraction, in the concentration range of 1.0–3.0 mM. The results (data not shown) demonstrated no significant changes on the hydrolysis of ATP, ADP and AMP, and that nucleotidases of the platelets and blood serum fraction respond in different way in the presence of ASA.

4. Discussion

Vascular E-NTPDase 3 is a plasma membrane-bound enzyme that hydrolyses extracellular ATP and ADP to AMP. In the coagulation cascade, the enzymes ATP diphosphohydrolase/CD39 and 5′-nucleotidase have an important function in the regulation of platelet aggregation [35,36]. Extracellular ATP is involved in the modulation of platelet aggregation by ADP in stimulated platelets, thereby inhibiting further

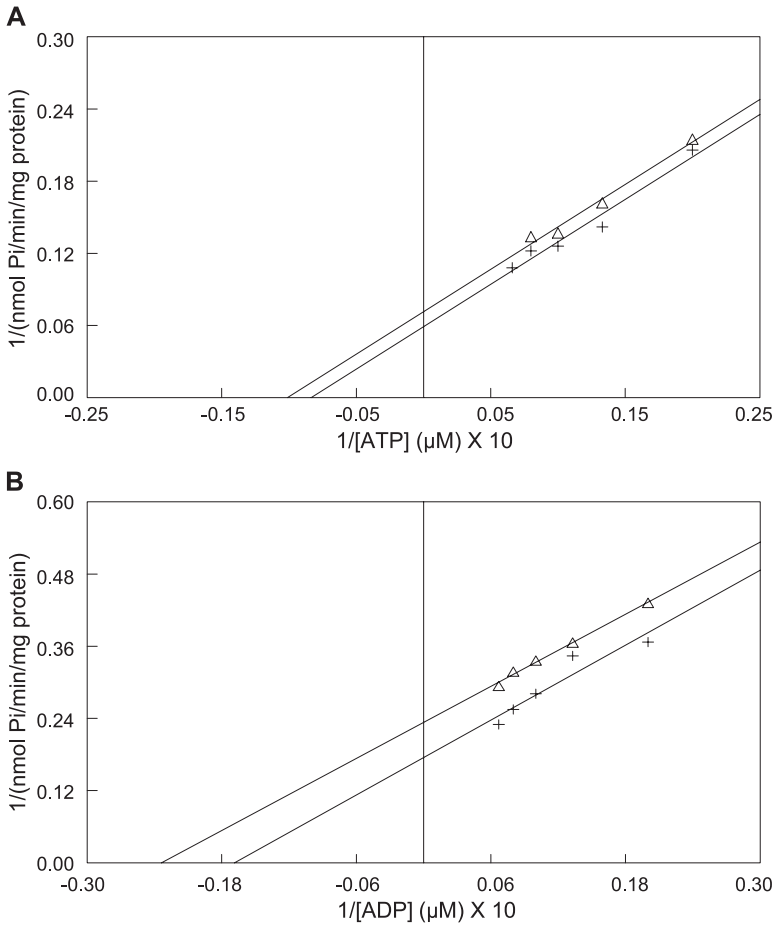

Fig. 2. Kinetic analysis of the inhibition of ATP (A) and ADP (B) hydrolysis by ASA in intact platelets from rats. In (A), the results show ATPase activity in the absence (+) and in the presence (△) of 3.0 mM ASA. In (B), the results demonstrate ADPase activity in the absence (+) and in the presence (△) of 3.0 mM ASA. Both ATPase and ADPase activities were determined in the range 50–150 μM of substrate. The calcium/nucleotide ratio used in experiments was always 10. Plots are representative of four independent experiments for each nucleotide.

platelet activation and recruitment [37,38]. The final product of the concerted action of the two enzymes (ATP diphosphohydrolase and 5′-nucleotidase) on ATP is adenosine, a very important compound or metabolite in the circulation, since it has anti-aggregatory properties with respect to platelets.

Adenosine accumulates in solid tumors at high concentrations and it has been shown to stimulate tumor growth angiogenesis and to inhibit cytokine synthesis, adhesion of immune cells to the endothelial wall, and the function of T-cells, macrophages and natural killer cells [20]. On the other hand, studies have also reported that extracellular ATP exhibits anticancer activity, in vivo, and suggest a possible application of this nucleotide as a chemotherapeutic metabolite or compound for the treatment of cancer patients [9,17]. If this is true, a drug with an inhibitory effect on ATP hydrolysis must be considered as an important therapeutic agent for using in anticancer chemotherapy. It is, therefore, tempting to suggest a possible involvement of the platelet ectonucleotidase cascade (ecto-ATP diphosphohydrolase and ecto-5′-nucleotidase) in modulating the ATP and adenosine levels in the circulation, consequently, modulating tumor growth.

Recently, an ATPase activity has been found in association with the neural cell-adhesion molecule (NCAM) [24]. Cellular adhesion molecules (CAMs)

are critical components in processes such as tumor metastasis [39]. In patients with advanced cancer, the expression of adhesion molecules on platelets is increased, indicating an activated state. Platelets may enhance tumor-cell adhesion to endothelial cells during hematogenous metastasis formation [40]. Studies have shown that an increase in circulating activated platelets may improve metastatic efficiency at any stage and that this point represents an opportunity to interrupt the cancer spread [41]. In addition, studies with human lymphocytes have identified a lymphocyte-vascular protein 2 as a 5′-nucleotidase (CD73) that mediates lymphocyte adhesion to endothelium [42]. Considering that there is doubt if the ATPase described as the NCAM molecule has an activity like an ATP diphosphohydrolase and that the enzyme ecto-ATP diphophohydrolase/CD39 from platelets could have the adhesion function, it is tentative to think in a cancer therapy on basis on the inhibition of the last enzyme by ASA. Also respect the ecto-5′-nucleotidase (another ecto-enzyme present in platelets), a role as an adhesive molecule, has been proposed to promote tumor invasiveness in human glioblastoma [43].

If the platelet adhesion molecules function facilitating metastasis [41] and if one of such molecule with hydrolytic activity had ATP diphosphohydrolase characteristics, the inhibition by ASA could impede the spread of the tumor in the organism. Then, this effect of ASA may be an additional effect in the sense to control the tumor growth via the ATP diphosphohydrolase/CD39 enzyme.

Numerous studies have suggested that the use of non-steroidal anti-inflammatory drugs, including ASA, can protect against the development of cancer [44], however, the mechanisms by which these drugs reduce the risk of cancer are not entirely clear. Jiang et al. [45] demonstrated that ASA can modulate the production of cell invasive regulatory factors including metalloproteinase-2 and E-cadherin and also inhibit the invasion of tumor cells.

Our current study showed that ASA significantly inhibited in vitro ATP and ADP hydrolysis by intact rat platelets. At final concentrations of 2.0 and 3.0 mM, the ATP hydrolysis was inhibited by about 17% and 21%, respectively. For the ADP hydrolysis, a different sensitivity was observed since the inhibition appeared only at 3.0 mM. An inhibition by aspirin of about 41% was found for this nucleotide as a substrate.

The kinetic analysis of the effects of ASA upon ATP and ADP hydrolysis indicated an uncompetitive inhibition (occurs when the inhibitor binds only to the enzyme-substrate complex). This type of inhibition frequently occurs with enzymes that have an ordered sequence of substrate binding, such as E-NTPDase 3.

Based on these results, it may be suggested that ATP and ADP hydrolysis by rat platelets is inhibited in different manners. Two enzymes, ecto-ATP-diphosphohydrolase and ecto-ATPase, may be co-expressed in rat platelets, as occurs in many other rat tissues [46–48]. Another possibility to explain the different sensitivity with regard to ATP and ADP hydrolysis, may be the presence of just one enzyme, an E-NTPDase 3, able to hydrolyze both substrates. This enzyme may, however, be more sensitive to ASA when hydrolyzing ATP. Conversely, ASA did not change the ATP, ADP and AMP hydrolysis by serum fraction. Then a soluble form (serum) of ATP diphosphohydrolase presents a different behavior when compared with one enzyme not soluble also present in the circulation (platelets) as an ecto-enzyme.

In this study, we demonstrated an inhibitory effect of ASA upon ATP diphosphoydrolase activity by platelets from adult rats. We propose that our results could partially explain, how ASA could modulates tumorogenesis. In addition, the effect described herein is important, since it provides further evidence to support the use of ASA as a coadjuvant drug together with ATP in cancer treatment. In the presence of aspirin, levels of ATP, a molecule cytotoxic to tumoral cells, will be higher. As a consequence of the ATP diphosphohydrolase inhibition, the ATP molecule will not be degraded to adenosine, which is involved in promoting tumor growth.

Studies in tumor rat models are in progress (without conclusions) in our laboratory to verify a possible involvement of this enzyme in the growth and spread cancer. The mechanism by which the inhibitory effect of aspirin upon ATP diphosphohydrolase/CD39 may interfere in these processes is also being investigated. Since adhesion molecules demonstrate nucleotide hydrolysis activities, we intended to evaluate how an alteration in nucleotide metabolizing enzyme activities may be related to adhesion and metastasis.

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3.2 Capítulo 2

Diminution in adenine nucleotide hydrolysis by platelets and serum from rats submitted to Walker 256 tumour

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Diminution in adenine nucleotide hydrolysis by platelets and serum from rats submitted to Walker 256 tumour

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Abstract

Extracellular adenine nucleotide hydrolysis in the circulation is mediated by the action of an NTPDase (CD39, apyrase) and of a 5′-nucleotidase (CD73), presenting as a final product, adenosine. Among other properties described for adenine nucleotides, an anti-cancer activity is suggested, since ATP is considered a cytotoxic molecule in several tumour cell systems. Conversely, some studies demonstrate that adenosine presents a tumour-promoting activity. In this study, we evaluated the pattern of adenine nucleotide hydrolysis by serum and platelets from rats submitted to the Walker 256 tumour model. Extracellular adenine nucleotide hydrolysis by blood serum and platelets obtained from rats at, 6, 10 and 15 days after the subcutaneous Walker 256 tumour inoculation, was evaluated. Our results demonstrate a significant reduction in ATP, ADP and AMP hydrolysis in blood serum at 6, 10 and 15 days after tumour induction. In platelets, a significant reduction in ATP and AMP hydrolysis was observed at 10 and 15 days after tumour induction, while an inhibition of ADP hydrolysis was observed at all times studied. Based on these results, it is possible to suggest a physiologic protection mechanism against the tumoral process in circulation. The inhibition in nucleotide hydrolysis observed probably maintains ATP levels elevated (cytotoxic compound) and, at the same time, reduces the adenosine production (tumoor-promoting molecule) in the circulation. (Mol Cell Biochem **281:** 189–195, 2006)

Key words: adenine nucletides, blood serum, cancer, metastasis, NTPDase, platelets, Walker 256 tumour

Introduction

Extracellular nucleotides can be hydrolysed by a variety of enzymes that are located on the cell surface, or may be soluble in the interstitial medium or within body fluids [1]. The most important ecto-enzymes involved in adenine extracellular nucleotide hydrolysis are named E-NTPDases, or Ectonucleoside triphosphate diphosphohydrolases (including the enzyme also named as ecto-ATPDase, ecto-ATP-diphosphohydrolase or ecto-apyrase, CD39), and they act together with ecto-5′-nucleotidase (CD73) [1]. E-NTPDase enzymes sequentially convert extracellular purine nucleotides, such as adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to the monophosphate form (AMP) [2], which may generate adenosine through the action of an ecto- 5′-nucleotidase [3]. In a previous study, these enzymes were characterised in intact rat blood platelets [4]. Other studies have described an NTPDase (ATP diphosphohydrolase),

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in addition to a NPP (pyrophosphatase/phosphodiesterase), as enzymes that may be involved in the hydrolysis of triphospho- and diphosphonucleosides by rat blood serum [5]. Based on this result, we postulated the co-existence of an NTPDase and a NPP that, together with a 5′-nucleotidase, are constituents of an enzymatic chain able to promote ATP–ADP hydrolysis to adenosine in the circulation [5].

The extracellular metabolism of adenine nucleotides generating nucleosides plays an important regulatory role in the control of adequate hemostasis, mainly by regulating the platelet aggregation status [6]. ATP, via activation of $P2X_1$ receptors, is vasoconstrictor to smooth muscle cells, while ADP causes platelet aggregation via activation of $P2Y_{12}$ receptors [7]. In contrast, adenosine produced by ATP/ADP degradation is a vasodilator and an inhibitor of platelet aggregation [7, 8], acting on A_2 receptors [7]. Moreover, the anti-cancer activity of adenine nucleotides against a wide variety of animal and human tumour cells has been suggested [9, 10]. Studies have indicated that ATP permeabilises cell membranes and induces programmed cell death in several tumour cell systems *in vitro* [11, 12], suggesting that induction of apoptosis is able to play an important role in ATP anti-cancer activity. This nucleotide has also been proposed to be involved in tumour-cell killing, mediated by activated T-lymphocytes [13]. Moreover, ATP has been shown to be growth inhibitory or cytotoxic for several mammalian cell lines such as mouse leukemia cells [14] and lymphoma cells as well as in human cell lines such as leukemia cells [15], pancreatic carcinoma cells [16], breast cancer cells [17] and skin carcinoma cells [18]. Recently, another randomised clinical study, including advanced lung cancer patients, reported that regular infusions of ATP inhibit loss of body weight and increase the life quality in these patients [19]. Since extracellular ATP can exhibit cytotoxic activity *in vivo* and *in vitro*, it may be considered a potential compound for the modulation of tumoural cell development and, thus, have a possible application as a drug for alternative anticancer therapy.

On the other hand, adenosine, the degradation product of ATP, produced by the action of ecto or soluble enzymes, has been shown to possess a tumour-promoting action [6, 20]. In solid cancers, this purine nucleoside is produced by adenine nucleotide degradation, as a result of tissue hypoxia and necrosis [6, 20]. The release of adenosine in the extra-cellular fluid of such tumours, provides a supportive environment that benefits malignancy and may include protection against ischemia, stimulation of growth [6] and tumour cell migration [21] and angiogenesis [22]. Also adenosine can promote suppression of immune responses [23], as well as an increase intratumoral blood flow [24]. The levels of adenosine in such tumours are sufficient to interfere with the anti-tumour immune response by suppressing T cell activation [23] and the interaction of T lymphocytes with tumour cells [25]. Thus, given the strong immunosuppressive function of this nucleoside, it may constitute an important part of the "immunological barrier" that is responsible for the failure of the immune system response towards malignant cells [6].

In the present study, we evaluated the extracellular adenine nucleotide hydrolysis by blood serum and platelets obtained from rats at, 6, 10 and 15 days after the subcutaneous Walker 256 tumour inoculation. We also suggest that an inhibition in extracellular adenine nucleotide hydrolysis of blood serum and platelets in rats, after the Walker 256 tumour inoculation, may represent a mechanism of protection against tumoral-cell development. In this condition, the levels of ATP (cytotoxic) in the circulation would be increased, and the levels of adenosine (involved in promoting tumour growth) would be reduced, decreasing the tumour development.

Materials and methods

Materials

Nucleotides were from Sigma Chemical Co. (St. Louis, MO). Sepharose 2B gel was from Pharmacia and was deaerated in a vacuum flask before packing in a polyethylene column. All other reagents were of analytical grade. Polyethylene or siliconised labware was used for all platelet isolation and incubation procedures.

Walker 256 tumour

The Walker 256 carcinosarcoma [26, 27], (originally from the National Cancer Institute Bank, Cambridge, MA, USA), donated by Dr T.C. Cavalcanti and Dr O. Rettori, from the Laboratory of Biochemical Research, CAISM/UNICAMP, is currently being maintained in our laboratory via intraperitoneal or subcutaneous passages in rats. Male Wistar rats, weighing approximately 250 g, from our own breeding stock maintained on a 12 h light/12 h dark cycle (lights on at 7.00 a.m.) at constant room temperature, were divided into tumour-bearing and control groups. A Walker 256 tumour cell 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 tumour-bearing group. Cell suspensions with 98% of viability estimated by trypan blue were obtained from the ascitic fluid of a donor rat. The control rat group received an inoculation of the vehicle only.

Platelet isolation

Platelets of the tumour-bearing and control group rats were isolated 6, 10 and 15 days after tumour or vehicle inoculation, exactly as described previously by Hantgan [28].

In an effort to obtain preparations of normal, undamaged platelets free of non-adsorbed plasma constituents, we separated intact platelets from plasma by means of gel filtration on a 1.5 × 7.0 cm Sepharose 2B column [29]. The column was equilibrated with a buffer consisting of 140 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 5.5 mM dextrose, 0.2 mM EGTA and 0.05% azide, pH 6.8 (Ca^{2+}-free Tyrode's Buffer). Platelets were eluted with the same buffer at room temperature; 0.5 ml fractions were collected and the tubes containing the maximum platelet count (determined visually) were used for subsequent experiments. The integrity of the platelet preparation was verified by measuring platelet LDH (lactate dehydrogenase) activity, as described in a previous study by our laboratory [4].

Isolation of blood serum fraction

Blood samples were drawn after decapitation of the tumour-bearing and control group rats, as described by Yegutkin [30], 6, 10 and 15 days after tumour cells or vehicle inoculation, and were centrifuged soon after in plastic tubes at 5000 × *g* for 5 min at 20 °C. The serum samples obtained were then stored on ice and immediately used in the experiments.

Assays of E-NTPDase and ecto-5′-nucleotidase activities

The reaction medium used to assay ATP, ADP and AMP hydrolysis by rat platelets and serum preparations were essentially as described previously by Frassetto *et al.* [4] and Oses *et al.* [5], respectively. For platelets, unless otherwise stated, the reaction medium used to assay Ca^{2+}-ATPase and Ca^{2+}-ADPase activity contained 120 mM NaCl, 5.0 mM KCl, 60 mM glucose, 5.0 mM $CaCl_2$ and 50 mM Tris–HCl buffer, pH 7.5, in a final volume of 200 μl. A sample of 20 μl of platelet preparation (20–30 μg of protein) was added to the reaction medium and preincubated for 10 min at 37 °C. The enzyme reaction was started by the addition of ATP or ADP to a final concentration of 0.5 mM, and incubated for 60 min. For blood serum fraction, ATP or ADP hydrolysis were determined using a modification of the method described by Yegutkin [30]. The reaction medium was preincubated for 10 min at 37 °C. The reaction mixture containing ATP or ADP as substrate to a final concentration of 3 mM, 112.5 mM Tris–HCl, pH 8.0, was incubated with approximately 1.0 mg of serum protein at 37 °C for 40 min in a final volume of 200 μl. The reaction for the platelets and blood serum fraction assay was stopped by the addition of 200 μl of 10% trichloracetic acid (TCA). The samples were chilled on ice and the amount of inorganic phosphate (Pi) liberated was measured by the method of Chan *et al.* [31]. The samples of blood serum fraction were centrifuged at 5000 × *g* for 5 min to eliminate precipitated protein and the supernatant was used for the colorimetric assay. Incubation times and protein concentration (for both fractions) were chosen to ensure the linearity of the reaction. In order to correct non-enzymatic hydrolysis, we performed controls by adding the platelets or blood serum fraction after the reaction was stopped with TCA. All samples were run in triplicate. Enzyme activities were generally expressed as nmol Pi released/min/mg of protein. For the platelet ecto-5′-nucleotidase assay, we used the same procedure and conditions as we used for the E-NTPDase assay, except that AMP (0.5 mM final concentration) instead of ATP or ADP was used as substrate and 5.0 mM $CaCl_2$ was replaced by 5.0 mM $MgCl_2$.

Protein determination

Protein was measured by the Coomassie blue method using bovine serum albumin as standard [32].

Statistical analysis

Statistical analysis of differences between control and tumour-bearing groups, for each day and each nucleotide, were expressed by means ± standard error and analysed by Student's *t*-test.

To compare the significance between 6, 10 and 15 days after the tumour inoculation, for each nucleotide, the data were analysed by one-way-ANOVA, followed by the Duncan multiple range test. $P < 0.05$ was considered to represent a significant difference in the statistical analysis used. All analyses were performed with an IBM compatible computer using the SPSSPC software.

Ethics

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

Results

Hydrolysis of adenine nucleotides by the blood serum of rats after tumour inoculation

The effect of Walker 256 tumour inoculation on hydrolysis of ATP, ADP and AMP was evaluated in the rat blood serum. As shown in Fig. 1, our results demonstrate a significant decrease in adenine nucleotide hydrolysis at 6, 10 and 15 days after tumour induction, to ATP (60, 58 and 67%, respectively), ADP (39, 58 and 78%, respectively) and AMP (54, 59 and 76%,

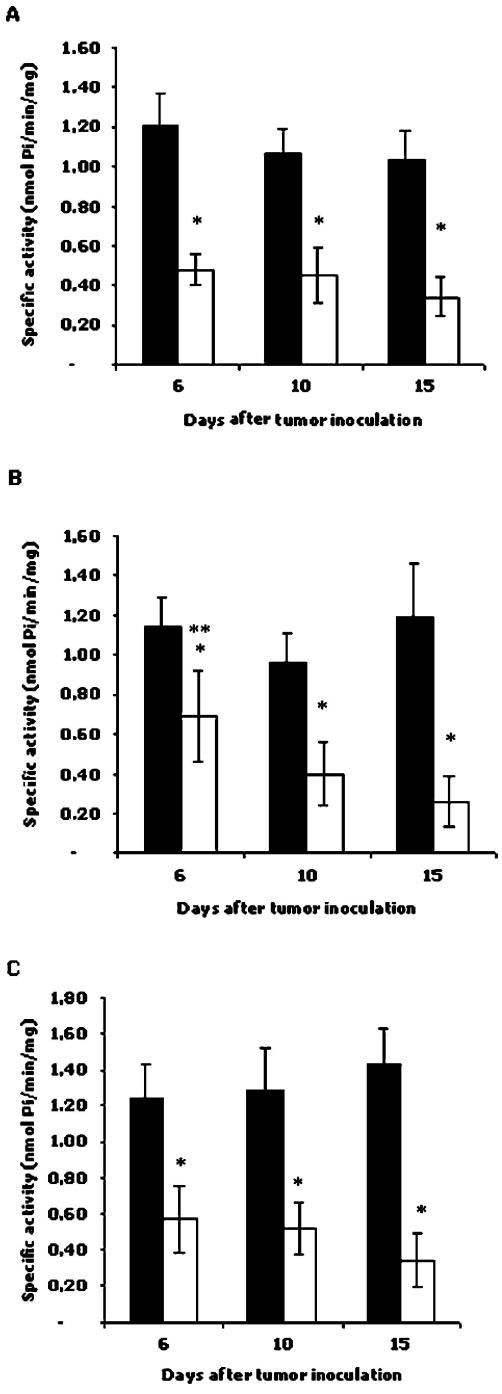

Fig. 1. ATP (A), ADP (B) and AMP (C) hydrolysis by rat blood serum, 6, 10 and 15 days after tumor inoculation. Bars of the control (■) and tumor-bearing (□) groups represent mean ± S.D. for four independent experiments. Results are expressed as nmol Pi released/min/mg of protein. *Significantly different from the control enzyme activity (Student's *t*-test, $p < 0.05$). **Indicates that ADP hydrolysis activity of the tumor bearing-rats in rat blood serum 6 days after the tumor inoculation is significantly different to that at 15 days (one-way-analysis of variance, followed by Duncan's multiple range test, $p < 0.05$).

respectively), when compared to respective control groups (Student's *t*-test, $p < 0.05$). The most relevant decrease was observed 15 days after the tumour inoculation, remaining at 33, 22 and 24% of control, for ATP, ADP and AMP, respectively (Fig. 1).

When comparing tumour-bearing groups at different times after inoculation, the ADP hydrolysis by rat blood serum obtained 6 days after tumour inoculation, was significantly different only from the 15-days (one-way-analysis of variance, followed by Duncan's multiple range test, $p < 0.05$). No statistically significant alterations were observed, however, in the ATP or AMP hydrolysis by rat blood serum when compared between tumour-bearing groups, at the times studied (one-way-analysis of variance, followed by Duncan's multiple range test, $p < 0.05$).

Hydrolysis of adenine nucleotides in the platelets of rats after tumour inoculation

The hydrolysis of adenine nucleotides was also affected in experiments using intact platelets obtained from adult rats. A significant decrease was observed only 10 and 15 days after subcutaneous tumour inoculation for ATP (31 and 21%, respectively) and AMP (50 and 54%, respectively) hydrolysis, when compared to control groups, as shown in Fig. 2A and C (Student's *t*-test, $p < 0.05$). The hydrolysis of these nucleotides by rat platelets was not significantly affected 6 days after tumour inoculation when compared to control groups (Fig. 2A and C) (Student's *t*-test, $p < 0.05$). The ADP extracellular hydrolysis by rat platelets was also efficiently inhibited, and this inhibition was observed at 6, 10 and 15 days after tumour inoculation (44, 45 and 46%, respectively), when compared to the respective control groups (Fig. 2B).

The results did not reveal any statistically significant alterations in the ATP or ADP hydrolysis for platelets when compared only between tumour-bearing groups, for the three period studied (one-way-analysis of variance, followed by Duncan's multiple range test, $p < 0.05$). However, AMP hydrolysis by rat platelets 6 days after tumour inoculation, was significantly different from hydrolysis at 10 and 15 days (one-way-analysis of variance, followed by Duncan's multiple range test, $p < 0.05$).

Similarly to blood serum, nucleotide hydrolysis by platelets was affected in the same direction when inoculated with Walker 256 tumour, indicating that there is a common mechanism that turn on in this condition.

Discussion

The present study was performed to investigate the extracellular adenine nucleotide hydrolysis by blood serum and

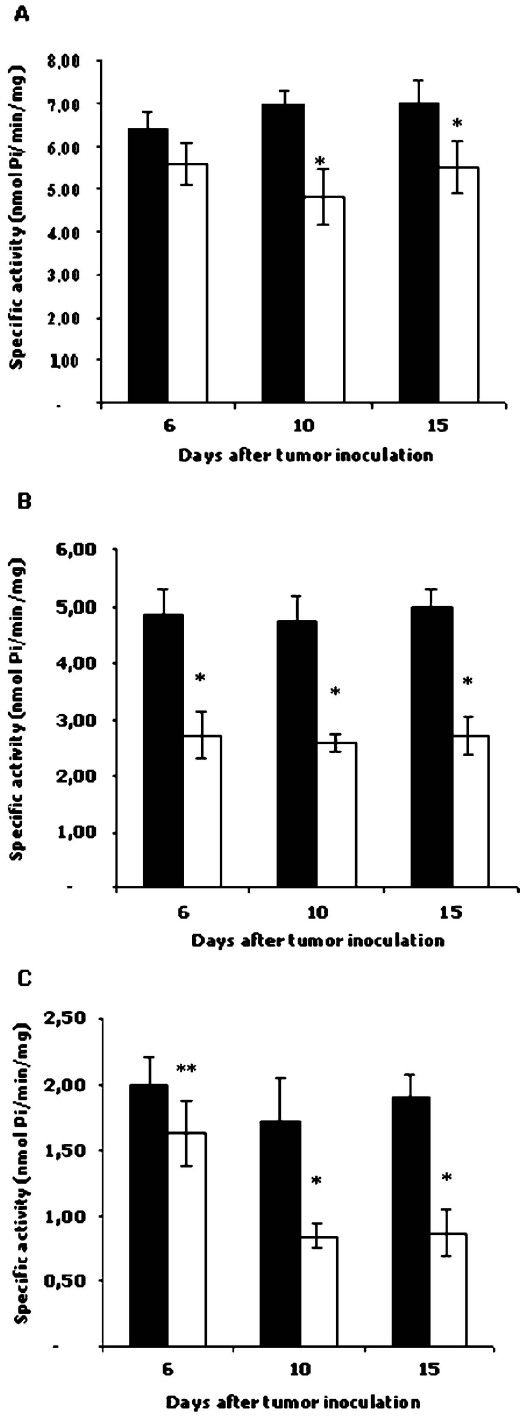

Fig. 2. ATP (A), ADP (B) and AMP (C) hydrolysis by rat intact platelets, 6, 10 and 15 days after tumor inoculation. Bars of the control (■) and tumor-bearing (□) groups represent mean ± S.D. for four independent experiments. Results are expressed as nmol Pi released/min/mg of protein. *Significantly different from the control enzyme activity (Student's *t*-test, $p < 0.05$). **Indicates that AMP hydrolysis activity of the tumor bearing-rats in platelets 6 days after the tumor inoculation is significantly different to those at 10 and 15 days (one-way-analysis of variance, followed by Duncan's multiple range test, $p < 0.05$).

platelets from rats, 6, 10 and 15 days after Walker 256 tumour inoculation. Data presented here demonstrate a significant reduction in ATP, ADP and AMP hydrolysis by serum (Fig. 1) and platelets (Fig. 2) of rats submitted to the Walker 256 tumour model, a carcinosarcoma which characteristically causes immunosuppression and cachexia [33]. Changes in the activity of the ectonucleotidases have been reported in several pathological conditions, such as human melanoma cells [34] and glioma cell lines [35], demonstrating the important role for these proteins in cancer. Additionally, the anti-cancer activity of adenine nucleotides has been described in several tumour cell lines [9, 11, 13] and an aspect of the potential clinical utility of the anti-cancer activities of these nucleotides is their inhibition of host weight loss, when the tumours become progressively larger [9].

The levels of exogenous ATP may be increased in various inflammatory and shock conditions, mainly as a consequence of nucleotide release from platelets, endothelial and blood vessel cells [28, 36, 37]. It has also been reported that extracellular ATP exhibits anticancer activity, *in vivo*, and was demonstrated to be synergistic with anti-cancer agents in mouse tumour cells [38] suggesting a possible application of this nucleotide as a chemotherapeutic compound for the treatment of cancer patients [9, 18]. Taking this finding into consideration, a drug with an inhibitory effect on ATP hydrolysis should be considered as an important therapeutic agent for use in anticancer chemotherapy.

In contrast, despite results are still debatable, adenosine, a purine nucleoside, has been shown to have tumour-promoting activities [6]. Hypoxic conditions in solid tumours are known to cause accumulation of this nucleoside, which has been shown to stimulate tumour growth angiogenesis and to inhibit cytokine synthesis, adhesion of immune cells to the endothelial wall, and the function of T-cells, macrophages and natural killer cells [6]. Hence, an increased activity of ecto-5′-nucleotidase, which converts AMP to adenosine, might promote the growth of breast cancer cells [39]. It is, therefore, tempting to suggest a possible involvement of the nucleotidase cascade (ATP diphosphohydrolase and 5′-nucleotidase), ecto enzymes (platelets) or soluble (serum), in modulating the ATP and adenosine levels in the circulation, consequently, modulating tumour growth.

Many studies have focused on the mechanisms involved in the pathogenesis of cancer, including the platelets assistance in cancer growth and spread [40], and the existence of a prothrombotic or hypercoagulable state associated with disease [41]. A malignant tissue secretes directly or indirectly a number of substances potentially responsible for the activation of the coagulation system. Tumour cells may also activate platelets by several mechanisms. ADP, a potent activation agonist, is released by some tumour cells, resulting in platelet aggregation [42]. Vascular E-NTPDase-1 is a plasma membrane-bound enzyme that

hydrolyses extracellular adenine nucleotides [2]. In the coagulation cascade, this enzyme and 5′-nucleotidase have an important function in the regulation of platelet aggregation [43, 44], modulating ATP and ADP levels. These two are vasoactive and platelet-active nucleotides, respectively, and adenosine, the final product of the nucleotide hydrolysis, is a vasodilator [7] and an inhibitor of platelet aggregation [8]. Thus, the reduction of the NTPDase and 5′-nucleotidase activities in the circulation, following Walker 256 tumour inoculation, shown in this study, could also contribute to the activation of the coagulation system by maintaining the ATP and ADP levels in the blood stream.

In patients with advanced cancer, the expression of adhesion molecules on platelets is increased, indicating an activated state. Platelets may enhance tumour-cell adhesion to endothelial cells during hematogenous metastasis formation [45]. It has been proposed that ATP diphosphohydrolase (CD39) may be a negative regulator of cell adhesion, capable of reducing the strength of cell contacts during binding or hydrolysis of ATP [34]. An ATPase activity has also been found in association with the neural cell-adhesion molecule (NCAM) [46]. In addition, studies with human lymphocytes have identified a lymphocyte-vascular protein 2 as a 5′-nucleotidase (CD73) that mediates lymphocyte adhesion to endothelium [47]. Thus, if platelet adhesion molecules function facilitating metastasis [40] and if such a molecule with hydrolytic activity has ATP diphosphohydrolase characteristics, the inhibition observed in this study could contribute to impede the spread of the tumour in the organism. This effect may be an additional mechanism for the control of tumour growth via the ATP diphosphohydrolase/CD39 enzyme.

Since soluble nucleotidases can act together with ectonucleotidases in the circulation to produce adenosine, by ATP and ADP hydrolysis, and this process is reduced in the blood serum and platelets of rats, following Walker 256 tumour inoculation, we suggest a mechanism of protection against tumoural-cell development. As a consequence of this reduction, ATP, a molecule that is cytotoxic to tumoral cells, would not be degraded to adenosine (involved in promoting tumour growth) with a consequent increase in ATP levels in the circulation. Howerver, we cannot eliminate the possibility that the diminution observed can be a result of a defect in platelet maturation, since Walker cells are known to metastasize and develop in bones, where platelets are produced. If this is true, metastasis to the bone could explain the diminution which would mean that the diminution in activity is a consequence of cancer instead of an adaptation limiting tumour growth.

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3.3 Capítulo 3

Nucleotide metabolizing ecto-enzymes in Walker 256 tumor cells: molecular identification, kinetic characterization and biochemical properties

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Nucleotide metabolizing ecto-enzymes in Walker 256 tumor cells: molecular identification, kinetic characterization and biochemical properties

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Keywords: Walker 256 tumor cells; adenine nucleotides; cancer; NTPDases; 5'-nucleotidase.

Abstract

In this study we describe the molecular identification, kinetic characterization and biochemical properties of an E-NTPDases and an 5´-nucleotidase in Walker 256 cells. For the ATP, ADP and AMP hydrolysis there were optimum pH in the range 6.5-8.0, and absolute requirement for divalent cations (Mg^{2+} > Ca^{2+}). A significant inhibition of ATP and ADP hydrolysis was observed in the presence of high concentrations of sodium azide and 0.5 mM of Gadolinium chloride. These activities were insensitive to ATPase, adenylate kinase and alkaline phosphatase classical inhibitors. The K_m values were 464.2 ± 86.6 μM (mean ± SEM, n=4), 137.0 ± 31 μM (mean ± SEM, n=5) and 44.8 ± 10.2 μM (mean ± SEM, n=4), and V_{max} values were 655.0 ± 94.6 (mean ± SEM, n=4), 236.3 ± 27.2 (mean ± SEM, n=5) and 177.6 ± 13.8 (mean ± SEM, n=5) nmol of inorganic phosphate.min^{-1}.mg of $protein^{-1}$ for ATP, ADP and AMP, respectively. Using RT-PCR analysis we identified the mRNA of two members of the ecto-nucleoside triphosphate diphosphohydrolase family (NTPDase 2 and 5) and a 5'-nucleotidase. The presence of NTPDases and 5'-nucleotidase enzymes in Walker 256 tumor cells may be important to regulate the ratio adenine nucleotides/adenine nucleoside extracellularly, therefore motivating tumor growth.

Introduction

The presence of a variety of enzymes in the plasma membrane of different tumors have been correlated with proliferative and metastatic activities (Kittel et al., 2002; Vannoni et al., 2004; Spychala et al., 2004). In Walker 256 carcinoma, a preliminary investigation of enzymes functioning at the cell surface, such as Na^+,K^+-ATPase, 5'-nucleotidase, alkaline phosphatase and phosphodiesterase activities were described by Clark and Goodlad (1993). The Walker 256 rat tumor has been extensively used in studies of cancer pathophysiology (Guaitani et al., 1982; Rettori et al., 1995; He et al., 2003; Piffar et al., 2003; Ikeda et al., 2004), and initially arose spontaneously in the mammary gland of a pregnant albino rat. This tumor was transplanted and grew with the morphology of a carcinosarcoma, which exhibited 2–3 cell types forming independent patterns (Stewart et al., 1959).

The anticancer activity of adenine nucleotides against animal and human tumor cells has been suggested (Agteresch et al., 1999; Burnstock, 2002). Studies have indicated that ATP permeabilizes cell membranes and induces programmed cell death in several tumor cell systems in vitro (Beyer and Steinberg, 1991; Wen and Knowles, 2003), suggesting that induction of apoptosis may play an important role in ATP anticancer activity. Recently, another randomized clinical study regarding advanced lung cancer patients, reported that regular infusions of ATP inhibit loss of body weight and increase the life quality in these patients (Agteresch et al., 2002). On the other hand, adenosine has been shown to hold a tumor-promoting action (Mujoomdar et al., 2003; Spychala et al., 2004). This purine nucleoside is present in the extracellular fluid of solid tumors as a result of tissue

hypoxia (Mujoomdar et al., 2003; Spychala et al., 2004). It provides a supportive environment that benefits malignancy and may include protection against ischemia, stimulation of tumor growth (Spychala et al., 2004), tumor cell migration (Woodhouse et al., 1998), angiogenesis (Barcz et al., 2000), as well as suppression of immune responses (Hoskin et al., 1994).

There are important regulatory mechanisms that control the external concentration of nucleotides and hence regulate P2-receptor mediated effects. The actions induced by extracellular ATP and adenosine are directly correlated to the activity of a variety of ectoenzymes that are either located on the cell surface, or in a soluble form in the interstitial medium, or within body fluids (Zimmermann, 2001). The most important ecto-enzymes involved in adenine extracellular nucleotide hydrolysis are the ecto-nucleotidases, including members of ecto-nucleotide pyrophosphatases/phosphodiesterases (E-NPPs) and ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases counting here the enzyme also named as ecto-ATPDase, ecto-ATP-diphosphohydrolase, ecto-apyrase, CD39) families as well as the ecto-5'-nucleotidase/CD73 (Zimmermann, 2001). E-NTPDase enzymes sequentially convert extracellular purine nucleotides, such as adenosine triphosphate (ATP) and adenosine diphosphate (ADP), to the monophosphate form (AMP) (Kaczmarek et al., 1996; Bigonnesse et al., 2004), which may generate adenosine through the action of an ecto- 5'-nucleotidase (Zimmermann, 1992).

Members of the E-NTPDase/CD39 family, previously classified as E-type ATPases, constitute a class of ecto-enzymes characterized by their capacity to hydrolyze nucleoside tri- and diphosphates, strict dependence upon divalent

cations and insensitivity to the classical inhibitors of the P-, F-, and V-type ATPases (Plesner, 1995). In mammals, there are eight related and homologous enzymes that share five apyrase-conserved regions (ACRs), named NTPDase1-8 that have been cloned and characterized. These enzymes are NTPDase1 (CD39, ecto-apyrase), NTPDase2 (ecto-ATPase), NTPDase3, NTPDase4, NTPDase5, NTPDase6, NTPDase7 and NTPDase8 (Zimmermann, 2001; Paez et al., 2001; Bigonnesse et al., 2004; Kukulski et al., 2005).

In this paper, we report the molecular identification, kinetic characterization and biochemical properties of E-NTPDases and ecto-5'-nucleotidase activities in Walker 256 cells, as part of a complex system to control the ratio of nucleotides/nucleoside in the tumor cells environment.

Materials and methods

Materials

Nucleotides were from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Walker 256 cells

The Walker 256 cells line A (originally from the Christ Hospital Line, National Cancer Institute Bank, Cambridge, MA, USA), donated by Laboratory of Biochemical Research, CAISM/UNICAMP, was maintained in our laboratory within adult Male Wistar rats as an ascites tumor in vivo, via intraperitoneal passages. Male Wistar rats, weighing about 250g, from our breeding stock were maintained

on a 12 h light/ 12 h dark cycle at a constant temperature room. For the experiments, tumor ascites fluids were harvested in Ringer-lactate solution, and the contaminating erythrocytes removed by two or three cycles of osmotic lyses. The Walker 256 cells were resuspended in a buffer containing 50 mM HEPES, 5.0 mM KCl, 135 mM, NaCl and 10 mM dextrose, pH 7.5. Cell suspensions with 98% of viability estimated by trypan blue were obtained from the ascitic fluid.

Assays of E-NTPDase and ecto-5´-nucleotidase activities

The reaction medium used to assay ATP, ADP and AMP hydrolysis by Walker 256 cells contained 50 mM HEPES, 5.0 mM KCl, 135 mM, NaCl and 10 mM dextrose, pH 7.5, in a final volume of 200 µl. A 40 µl cell suspension sample (approximately 30 µg of protein) was added to the reaction medium and preincubated for 2 min at 37ºC. The enzyme reaction was started by the addition of ATP, ADP or AMP to a final concentration of 2.0 mM, and incubated for 5 min. The reaction was stopped by the addition of 200 µl of 10% trichloracetic acid (TCA). The samples were chilled on ice and centrifuged at 14000 *g* for 5 minutes at 4ºC to precipitate protein. The supernatant was used to measure the amount of inorganic phosphate (Pi) liberated by the method of Chan et al. (1986). Incubation times and protein concentration were chosen to ensure the linearity of the reaction. In order to correct non-enzymatic hydrolysis, we performed controls by adding the cell suspensions after the reaction was stopped with TCA (5% final concentration). All samples were run in triplicate. Enzyme activities were generally expressed as nmol Pi released/min/mg of protein.

Divalent cation dependence

In order to verify the divalent cation dependence for ATP, ADP and AMP hydrolysis, Mg^{+2} or Ca^{+2}, in the range of 1.0 to 6.0 mM, was added to the reaction medium containing 50 mM HEPES, 5.0 mM KCl, 135 mM NaCl, 10 mM dextrose, and 3.0 mM EGTA pH 7.5. For control groups, EGTA was not added to the reaction medium.

Differential effects of some compounds on ATP and ADP hydrolysis

The effects of 2.0 μg/ml oligomycin, 1.0 mM ouabain, 0.1 mM orthovanadate, 1.0 mM levamisole, 0.1 mM lanthanum, 1.0 mM NEM (*N*-ethylmaleimide), 1.0, 5.0, 10, 20 and 30 mM sodium azide, and 0.05, 0.1 and 0.5 mM gadolinium chloride were tested on ATP and ADP hydrolysis. Incubation times, protein and substrate concentrations were used exactly as described before for assays of E-NTPDase and ecto-5´-nucleotidase activities. Results are expressed as percentage of control enzyme activity and data analyzed by one-way ANOVA, followed by Student-Neumann-Keuls post hoc test ($P<0.05$; $P<0.01$).

RT-PCR analysis

Total RNA from Walker 256 cells was isolated with the RNA Mini Kit (Qiagen) in accordance with 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 using both oligo (dT) primer and random hexamers in accordance with 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 units of Ex *Taq*DNA 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 for rat *Entpd1* (*Cd39*), *Entpd2* (*Cd39L1*), *Entpd3* (*Cd39L3*), *Entpd5* (*Cd39L4*) *Entpd6,* (*Cd39L2*) RT-PCR were described by Vollmayer et al. (2001). For rat ecto-5'-nucleotidase (CD73) and β-actin RT-PCR were described by Wink et al. (2003a), and for NTPDase 8 were 5'-AGGTGCCTTTGGTTGGATC-3' and 5' – GGTAGCTGTGAGTGTAGAC-3'. Oligonucleotides were obtained from Invitrogen. Negative controls were performed with water as template and positive controls were plasmids with cDNA sequences for mouse *Entpd1*, rat *Entpd2* (Kegel et al., 1997; Sevigny et al., 2002), and for human *ENTPD3* (Smith and Kirley, 1998), ENTPD5 (Mulero et al., 1999) and ENTPD6 (Yeung et al., 2000), ecto-5'-nucleotidase/CD73 and β-actin cDNA C6 rat glioma cells (Wink et al., 2003a).

Protein determination

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

LDH measurement

The Lactate dehydrogenase (LDH) activity in the Walker cells was measured by the kinetic approach using a Cobas Mira Plus Aparattus (Roche

Diagnostics®), after the washing procedure and also after the enzymatic reaction in the presence of the substrate, ATP 2 mM. The LDH activity measured in the supernatant of incubation medium (in the incubation times 0 and 10 minutes) was compared with that found in the cells solubilized with 1% Triton X-100 (Casali et al., 2001).

Ethics

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

Results

Cellular integrity

The activity of LDH was used as a marker of cell integrity. The LDH activity was measured in two conditions: a) in the cells after the washing procedure and the results point to approximately 90% of the cells in as intact condition (data not shown); b) the integrity of cells after the incubation was performed by LDH assay and confirmed by microscopic observations (data not shown). In this last condition no significant difference was found in the measurement of LDH activity after the incubation times 0 and 10 minutes, showing that approximately 90% were intact after the incubation (data not shown).

ATP, ADP and AMP hydrolysis as a function of time and protein concentration

In this study, we first investigated the ATP, ADP and AMP hydrolysis by Walker 256 cells, as a function of time and protein concentration in order to determine the correct assay conditions. To ensure the linearity of the enzymatic reaction as a function of time, the Walker 256 cellular suspension was incubated as described in Materials and Methods with 2.0 mM of ATP, ADP or AMP. We observed that the hydrolysis was linear up to 20 minutes (data not shown). Conversely, the protein curve demonstrated that the incubation with 2.0 mM ATP, ADP or AMP for 20 min was linear when the amount of protein used in the assay was 50 µg or less of protein (data not shown).

pH dependence

To evaluate pH dependence, the pH range assayed was between 4.5 and 9.0 for ATP and ADP using the buffers 50 mM Tris-HCl and 50 mM HEPES (applied in a range from pH 6.5 to pH 9.0), and 50 mM citric acid (applied in a range from pH 4.5 to pH 5.5). For AMP the range of pH was between 6.5 and 9.0, using the buffers 50 mM Tris-HCl and 50 mM HEPES. The optimum pH for the three nucleotides reached between 6.5 - 8.0 (Fig.1), and the pH 7.5 was chosen for the subsequent experiments.

Cation dependence

In order to investigate the possibility of cation dependence for the Walker 256 cell enzymes, we tested the hydrolysis rate for ATP, ADP and AMP in the

presence or absence of divalent cations, or EGTA, as indicated in Materials and Methods. As shown in Fig. 2, in the presence of 3.0 mM EGTA, ATP, ADP and AMP hydrolysis was less when compared to control (without the addition of divalent cations). On the other hand, the ATP, ADP and AMP hydrolysis were greatly stimulated by 1.0 mM Mg^{+2}, when compared to the control group without cation addition. Increasing concentrations of divalent cations added to the reaction mixture containing 3.0 mM EGTA recovered NTPDase and 5'-nucleotidase activities. Thus, we can conclude that the major enzymes responsible for the hydrolysis of ATP, ADP and AMP in Walker 256 cells are cation dependent. No additive effects were observed when the two divalent cations were added at the same time to the reaction medium, suggesting that both Ca^{2+} and Mg^{2+} were competing for the same activation site (Fig. 2). On basis in these results, we established the ratio of 2.0 mM / 4.0 mM for nucleotides/divalent cation as optimal conditions for measuring the ecto-nucleotidase activity. In this manner, the ecto-nucleotidase activities were measured in a physiological range of divalent cation (using Mg^{2+}) and nucleotides.

Kinetic Parameters

Mg^{+2}-ATP and Mg^{+2}-ADP hydrolysis were determined at ATP and ADP concentrations ranging from 0.075 to 3.0 mM and 0.075 to 2.0 mM, respectively. Mg^{+2}–AMP hydrolysis was determined at AMP concentrations ranging from 0.05 to 1.0 mM. The results (Fig. 3, insets) indicated that all enzymatic activities increased with increasing nucleotide concentration until saturation. The Eadie-Hofstee plot for the hydrolysis of ATP, ADP and AMP is shown in Fig.3. The apparent Michaelis-

Menten constants (K_m, app) calculated by linear regression were 464.2 ± 86.6 μM (mean ± SEM, n=4), 137.0 ± 31 μM (mean ± SEM, n=5) and 44.8 ± 10.2 μM (mean ± SEM, n=4) for ATP, ADP and AMP, respectively. The calculated maximum velocities (V_{max}, app) were 655.0 ± 94.6 (mean ± SEM, n=4), 236.3 ± 27.2 (mean ± SEM, n=5) and 177.6 ± 13.8 (mean ± SEM, n=5) nmol of inorganic phosphate.min^{-1}.mg of protein^{-1} for ATP, ADP and AMP, respectively.

Single Active Site

ATP and ADP hydrolysis could be catalyzed by a single enzyme or by a combination of ecto-enzymes that are able to mimic apyrase activity. To investigate if ATP and ADP hydrolysis occurs either by only one active site that is able to hydrolyze both substrates, or by independent catalytic sites, we used the Chevillard competition plot enzyme (Chevillard et al., 1993). To assay the combination of substrate in a Chevillard competition plot we chose concentrations at which the rate of hydrolysis was the same when either ATP or ADP (from Fig. 3, insets) was used as substrate. From the chosen concentrations we determined combinations as described for this method. The *P* values ranged from 1 to 0. The horizontal straight-line obtained in the competition plot (Fig.4) indicates a constant hydrolysis rate at all substrate combinations tested.

Differential effects of some inhibitors on ATP and ADP hydrolysis

In order to evaluate the sensitivity of the enzymatic activity for ATP and ADP hydrolysis described in this study, and to distinct compounds that were reported to

affect nucleotide hydrolysis, we have tested ATPase and alkaline phosphatase inhibitors, and gadolinium chloride, an inhibitor of membrane-bound and soluble E-NTPDases (Escalada et al., 2004) (Table 1). The following inhibitors had no effect on ATP and ADP hydrolysis in Walker tumor cells: (a) the Na^+/K^+-ATPase inhibitor, ouabain; (b) orthovanadate, an inhibitor for transport ATPases, acid phosphatases and phosphotyrosine phosphatases; (c) levamisole, a specific alkaline phosphatase inhibitor (d) the Ca^{2+}, Mg^{2+}-ATPase inhibitors, N-ethylmaleimide (NEM) and lanthanum; and (e) the mitochondrial inhibitors, oligomycin and sodium azide (1.0, 5.0 and 10 mM). However, 20 and 30 mM sodium azide, an inhibitor of ATP diphosphohydrolase from several sources (Plesner, 1995; Knowles and Nagy, 1999), inhibited ATP (14 and 27%, respectively) and ADP (29 and 45%, respectively) hydrolysis (Table 1). In the final concentration of 0.05 and 0.1 mM, gadolinium chloride had no effect on ATP and ADP hydrolysis in Walker tumor cells. However, when tested at a final concentration of 0.5 mM, inhibited both ATP (14%) and ADP (20%) hydrolysis.

Ecto-nucleotidase mRNAs in Walker 256 cells

We investigated by RT-PCR the mRNA expression of *Entpd* 1-3, 5,6 and 8 and ecto-5'-nucleotidase/CD73 in Walker 256 cells (Fig 5). Using specific primers, oligonucleotide fragments were amplified and analyzed on agarose gels. mRNAs for *Entpd*2, *Entpd5,* and ecto-5'-nucleotidase/CD73 were detected in Walker 256 cells (Fig. 5). However, the *Entpd1, 3 and 6* signal in Walker 256 cells was almost undetectable. The NTPDase 8 mRNA was absent (data not shown).

The length of the oligonucleotide fragments obtained for the samples was comparable to that obtained for the positive controls and corresponded to the expected size.

The NTPDases 4 and 7 were not investigated in this study as these proteins are of exclusively intracellular localization and thus cannot be responsible for nucleotide degradation at the surface of the cells (Biederbick et al., 2000; Shi et al., 2001).

Discussion

Evidence that extracellular nucleotides affect cell growth and cell migration (Agteresch et al., 1999; Stracke et al., 1997), brings up the relationship between ectonucleotidase expression and carcinogenesis. In the present study, we describe an E-NTPDase and an ecto-5'-nucleotidase, both activities were characterized and at the same time we identified the enzyme expression from Walker 256 cells. The optimum pH for the three nucleotides, ATP, ADP and AMP was reached among 6.5 - 8.0 (Fig.1) and the pH 7.5 was chosen to perform the enzyme assays. These pH values are in accordance with those previously described for E-NTPDases (Zimmermann, 2001; Knowles et al., 2002; Leal et al., 2005). The enzyme activities studied here are cation-dependent. Mg^{2+} is the best activator for ATP, ADP and AMP hydrolysis in Walker 256 cells, and divalent-cation dependence was confirmed by the decrease of the enzyme activity in presence of EGTA (Fig. 2).

Regarding the inhibitors of nucleotide hydrolysis, we previously evaluate the effect of gadolinium chloride. Although its mode of action remains unclear, gadolinium has been described as the most potent inhibitor for both membrane-

bound (Escalada et al; 2004) and soluble forms of E-NTPDases (Escalada et al., 2004; Furstenau et al., 2006). This last reference regards to gadolinium chloride inhibition of NTPDase in mammals. In this work, we demonstrate that ATP and ADP hydrolysis were significantly inhibited by 0.5 mM gadolinium chloride. Additionally, ATP and ADP hydrolysis were reduced in a parallel manner by higher sodium azide concentrations (20 and 30 mM). This compound is known to be a classical ATPase inhibitor at 0.1 mM, and at high concentrations (5.0, 10 and 20 mM) can also act as an E-NTPDase inhibitor (Oses et al., 2004). Both results, in the presence of gadolinium chloride as well as in the presence of sodium azide are in accordance with the occurrence of E-NTPDase in Walker 256 cells. Moreover, we used the Chevillard competition plot (Fig.4), which is a method to determine if two substrates are hydrolyzed at the same active site of an enzyme (Chevillard et al., 1993). The horizontal straight-line obtained in the competition plot indicates a constant hydrolysis rate at all substrate combinations tested. Notably, this same protocol was used to demonstrate the presence of an NTPDase enzyme in human platelets (Pilla et al., 1996), in human lymphocytes (Leal et al., 2005) and in slices obtained from the central nervous system (Bruno et al., 2002). Considering the real knowledge about different forms for NTPDases and our results in the competition plot it is possible to postulate two different interpretations for the result showing a straight-line. First, it is attractive to think in a dominant enzyme in the enzyme activity determination, a rationale that can explain why we have a straight-line and at the same time we detect more than one NTPDase using RT-PCR analysis. Second, the hydrolysis can occur at more than one active site, but all the sites presenting a pattern of competition between ATP and ADP. If we consider this

possibility we can have also a straight line as a sum of different active sites, all hydrolyzing ATP and ADP. Both interpretations presented are possible, considering the results obtained in the competition plot and the results found in the RT-PCR analysis.

Using RT-PCR analysis, we investigated the mRNA expression of *Entpd* 1, 2, 3, 5,6 and 8 and ecto-5'-nucleotidase. The mRNAs of *Entpd*2 and *Entpd5,* typical members of the E-NTPDase family, and also of ecto-5'-nucleotidase were identified. The hydrolysis ratio for ATPase/ADPase is 2.77 at the surface of Walker 256 cells. Recently, NTPDase5 was shown to be identical to PCPH a human proto-oncogene product that was discovered after its activation upon treatment with a chemical carcinogen (Paez et al., 2001). The enzyme, which has been characterized as a soluble E-NTPDase, can be extracellularly released from mammalian cells and exhibit higher affinity for nucleoside diphosphates than for nucleoside triphosphates (Mulero et al., 1999). Our results show that ADPase activity in Walker 256 cells are stimulated mostly by Mg^{+2} than by Ca^{+2}. This is in accordance with studies already described for human NTPDase5 (Mulero et al., 1999). We did not disregard the possibility that this enzyme can be secreted from Walker cells and also its contribution for extracellular hydrolysis of nucleoside diphosphates.

Previously published data have shown that the expression of ecto-ATPases is increased during cancer progression (Saphner et al., 1991; Rathbone et al., 1992). Recent cloning of both mouse and human ecto-ATPase cDNA from tumor cells (Gao *et al*., 1998; Mateo *et al*., 1999; Knowles and Chiang, 2003) indicates that NTPDase2 is highly expressed in some tumors and its expression may be

result of tumorigenesis (Knowles and Chiang, 2003). Moreover, an ATPase activity has been found in association with the neural cell-adhesion molecule (NCAM) (Skladchikova et al., 1999). Cellular adhesion molecules (CAMs) are critical components in processes such as tumor metastasis (Cavenagh et al., 1998). Considering that extracellular ATP, a substrate for NTPDase2, exerts cytotoxic effects in a variety of cell systems via activation $P2X_7$ receptors (Beyer and Steinberg, 1991; Wen and Knowles, 2003), this enzyme in Walker 256 tumor cells would terminate actions induced by this nucleotide, protecting the tumoral cells from ATP-mediated apoptosis. In this study we demonstrate that E-NTPDase members are expressed in Walker 256 tumor cells, and suggest that they may play a role in controlling nucleotide levels around the tumor. Our data indicates that in Walker cells, ATP may be hydrolyzed to AMP by a set of enzymes that includes, at minimum NTPDase2 (ecto-ATPase) and NTPDase5 or even by the ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs) that may be present on the Walker cell surface.

AMP, the final product of ATP and ADP hydrolysis by an ecto-ATP diphosphohydrolase/NTPDase is the substrate in Walker 256 cells for an ecto-5´-nucleotidase activity. This last enzyme was characterized with regard to cation dependence, kinetic parameters and RT-PCR analysis, as shown in our results (Fig.2, 3 and 5). The ecto-5´-nucleotidase is usually considered to be Mg^{+2} dependent (Zimmermann, 1992), and this is in accordance with our results. This enzyme play an important role in the generation of extracellular adenosine, a purine nucleoside, that, acting through G-protein coupled receptors, has been demonstrated to have tumor-promoting activities (Spychala, 2000). Adenosine

may be an important metabolite released by cancer cells that elicits physiological responses that promote tumor progression (Spychala et al., 2004). Hence, the involvement of ecto-5'-nucleotidase in cancer has been suggested. Also, a role as an adhesive molecule has been proposed in promoting tumor invasiveness in human glioblastoma (Fenoglio et al., 1997). An increase in its activity was found in glioma cell lines (Wink et al., 2003b) and might promote the growth of breast cancer cells (Canbolat et al., 1996).

Taken together, and not disregarding the presence of others nucleotide metabolizing enzymes such as E-NPPs, our data demonstrate that these cells contain all components of the enzymatic cascade, which are necessary for the complete metabolism of extracellular nucleotides to nucleosides. Considering that ATP can exhibit cytotoxic activity, the ecto-ATPase may control tumor proliferation by limiting the mechanisms of extracellular ATP-induced apoptosis, and the NTPDase 5 may be responsible for ADP hydrolysis, therefore producing AMP. By the action of ecto-5'-nucleotidase, AMP can be hydrolyzed to adenosine, a stimulant of tumor growth. The co-existence of these enzymes is very important for their participation in an "enzyme chain" for the sequential hydrolysis of ATP to adenosine, and for the control of the nucleotide/nucleoside ratio in the tumoral cells, and may be important therapeutic targets for anticancer therapy.

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Table 1: Effects of inhibitors on ATP and ADP hydrolysis by Walker 256 tumor cells

Compound	Concentration (mM)	% Control enzyme activity	
		ATPase	ADPase
Oligomycin	2.0μg/ml	102 ± 2.2	100 ± 5.7
Ouabain	1.0	101 ± 1.2	102 ± 5.6
Orthovanadate	0,1	108 ± 7.6	102 ± 3.0
Levamisole	1.0	105 ± 5.2	100 ± 3.3
Lanthanum	0.1	109 ± 3.6	107 ± 1.7
NEM	1.0	108 ± 5.6	112 ± 5
Sodium Azide	1.0	106 ± 3.2	107 ± 2.5
	5.0	97 ± 7.6	87 ± 5.5
	10	92 ± 4.2	88 ± 3.2
	20	86 ± 2.3*	71 ± 6.9*
	30	73 ± 2.5**	55 ± 6.9**
Gadolinium chloride	0,05	104 ± 2.5	105 ± 2.6
	0.10	98 ± 7.5	102 ± 2.6
	0.50	84 ± 7.7*	80 ± 5.1*

Table 1: Differential effect of distinct compounds on ATP and ADP hydrolysis by Walker 256 tumor cells. The inhibitors were added to the reaction medium and preincubated for 2 min at 37ºC, and the enzyme reaction was started by the addition of ATP and ADP. Results are expressed as mean ± SD (n=3). Data was analyzed statistically by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test. ***** represents difference from control enzyme activity (100%) (P<0.05). ****** represents difference from control enzyme activity (100%) (P<0.01). Control enzyme activity was 623.0 ± 38.8 and 149.33.3 ± 13.2 nmol Pi min^{-1} mg $protein^{-1}$ (mean ± SD) for ATP and ADP hydrolysis, respectively, indicating a control activity of 100% and the results were expressed as percentages of control activity.

Legends

Figure 1: Effect of pH on ATP (◆), ADP (■) and AMP (▲) hydrolysis. The pH values tested were 4.5, 5.5, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 for ATP and ADP and 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 for AMP. Buffers used were 50 mM Tris-HCl and 50 mM Hepes (applied in a range from pH 6.5 to pH 9.0), and 50 mM Citric acid (applied in a range from pH 4.5 to pH 5.5). Conditions are described in Material and Methods. 0.1 mg of protein corresponds to $4.6x10^6$ cells. Data represents a typical experiment.

Figure 2: Divalent cation dependence on ATP (A), ADP (B) and AMP (C) hydrolysis. Hydrolysis of ATP, ADP and AMP by Walker 256 cells was analyzed in the absence of cations (c), in the presence of 1.0 mM Mg^{+2} (Mg^{+2} 1.0), 1.0 mM Ca^{+2} (Ca^{+2} 1.0). EGTA 3 mM was used to inhibit enzyme activity. The activity was determined in the presence of 3.0 mM EGTA plus a cation concentration (1.0 - 6.0 mM calcium or magnesium) as shown in the figure. Bars represent mean ± SD for three independent experiments. Results are expressed as nmol Pi released/min/mg of protein.

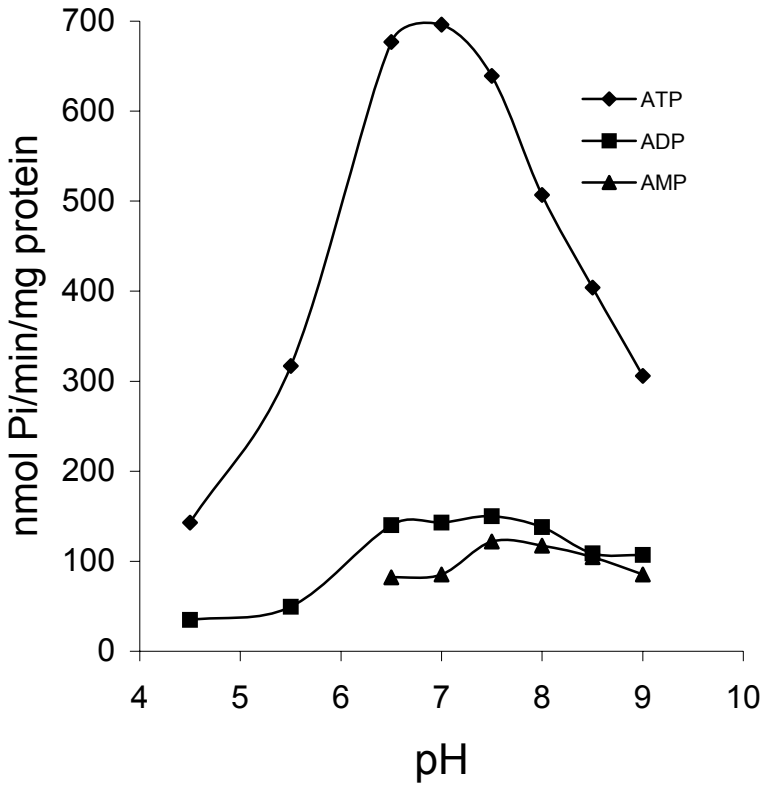
Figure 3: Eadie-Hofstee plots for extracellular hydrolysis of ATP (A), ADP (B) and AMP (C). Reaction rate was measured by released Pi, as described in Material and Methods. Results were obtained with a nucleotide concentration ranging from 0.075 to 3.0 mM for ATP, 0.075 to 2.0mM for ADP and 0.05 to 1.0mM for AMP, plus 4.0mM Mg^{+2}, as described in Material and Methods. The insets show

activation of the enzyme for all substrates. Best-fit analysis indicated a linear relationship. Data represents a typical experiment.

Figure 4: Competition Plot for extracellular hydrolysis of ATP and ADP. The concentration at which the velocities were the same for ATP and ADP was chosen for the Chevillard plot. Assay conditions are described in Material and Methods. The incubation time was 5 min, the concentration of substrate A (ADP) at *P*=0 was 0.180 mM and substrate B (ATP) at *P*=1 was 0.075 mM. Bars represent mean ± SD for three independent experiments. No significant difference was found between different points. The R^2 for a linear regression was 0.060.

Figure 5: RT-PCR analysis of *Entpds* and 5'-nucleotidase gene expression by Walker 256 cells. Total RNA was isolated from Walker 256 cells and the cDNA was analyzed by PCR with primers for *Entpds* and 5'-nucleotidase as described in Experimental Procedures. Plasmids containing the sequences of all *Entpd*s and 5'-nucleotidase were used as positive controls (PC) (the cDNA was replaced by plasmids encoding the NTPDases sequences). The length (bp) of the PCR products obtained with each pair of primers is given; (NC) negative control (the cDNA was replaced by water); (WT) Walker tumor.

Fig. 1


700
600
500
400
300
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nmol Pi/min/mg protein
4
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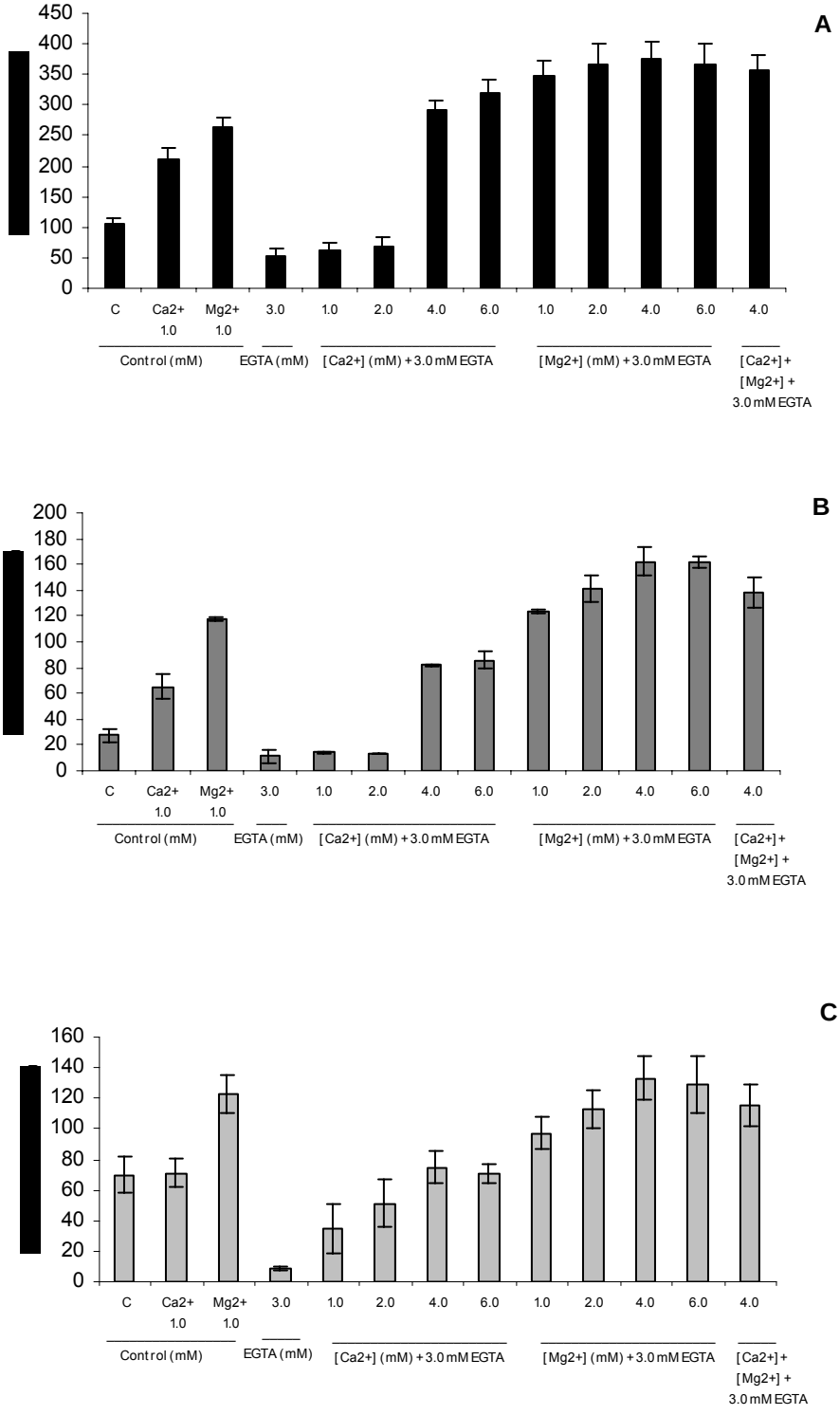
pH
ATP
ADP
AMP

Fig.2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40
20
0
160
140
120
100
80
60
40
20
0
C
Ca2+ 1.0
Mg2+ 1.0
3.0
1.0
2.0
4.0
6.0
1.0
2.0
4.0
6.0
4.0
Control (mM)
EGTA (mM)
[Ca2+] (mM) + 3.0 mM EGTA
[Mg2+] (mM) + 3.0 mM EGTA
[Ca2+] + [Mg2+] + 3.0 mM EGTA

Fig.3

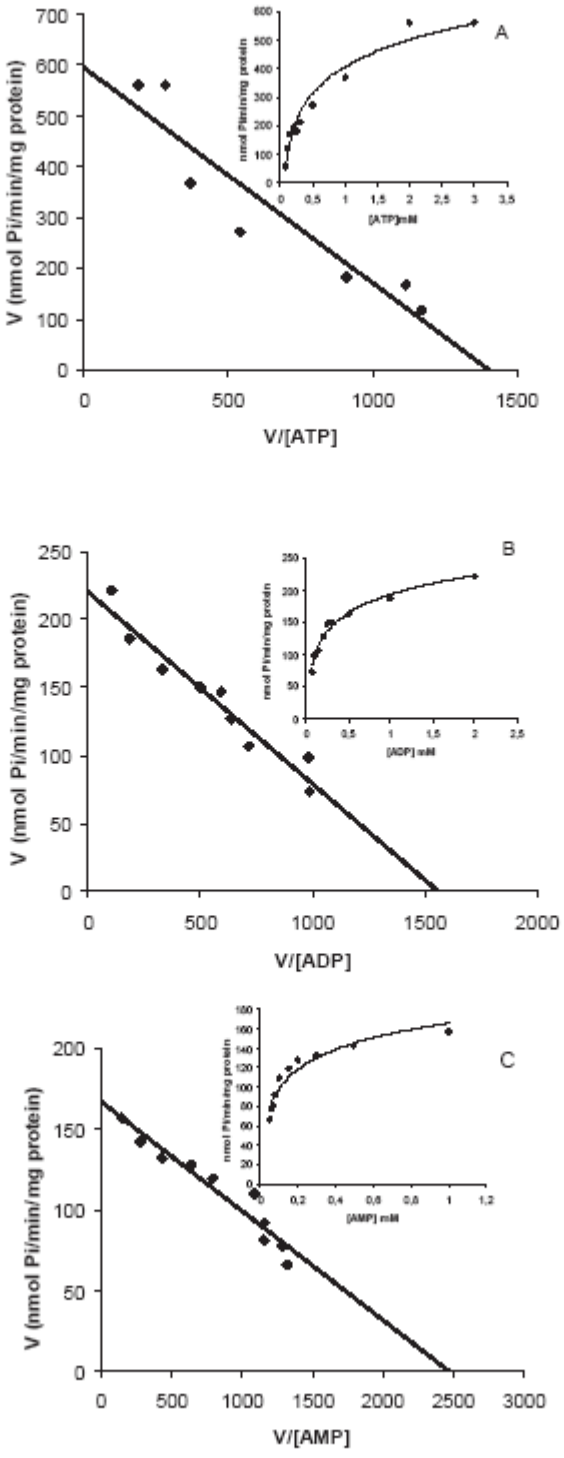
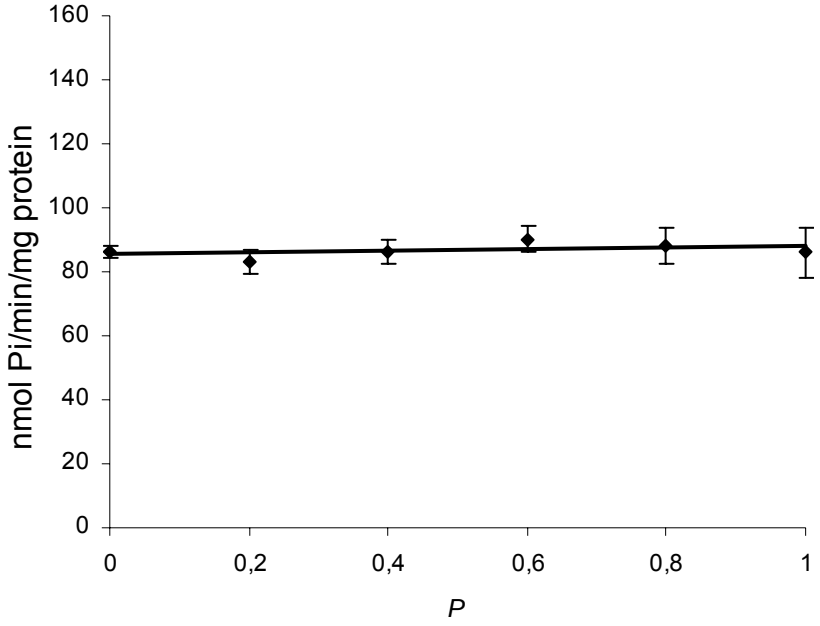

A
V (nmol Pi/min/mg protein)
V/[ATP]
nmol Pi/min/mg protein
[ATP]mM
B
V (nmol Pi/min/mg protein)
V/[ADP]
nmol Pi/min/mg protein
[ADP] mM
C
V (nmol Pi/min/mg protein)
V/[AMP]
nmol Pi/min/mg protein
[AMP] mM

Fig. 4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nmol Pi/min/mg protein
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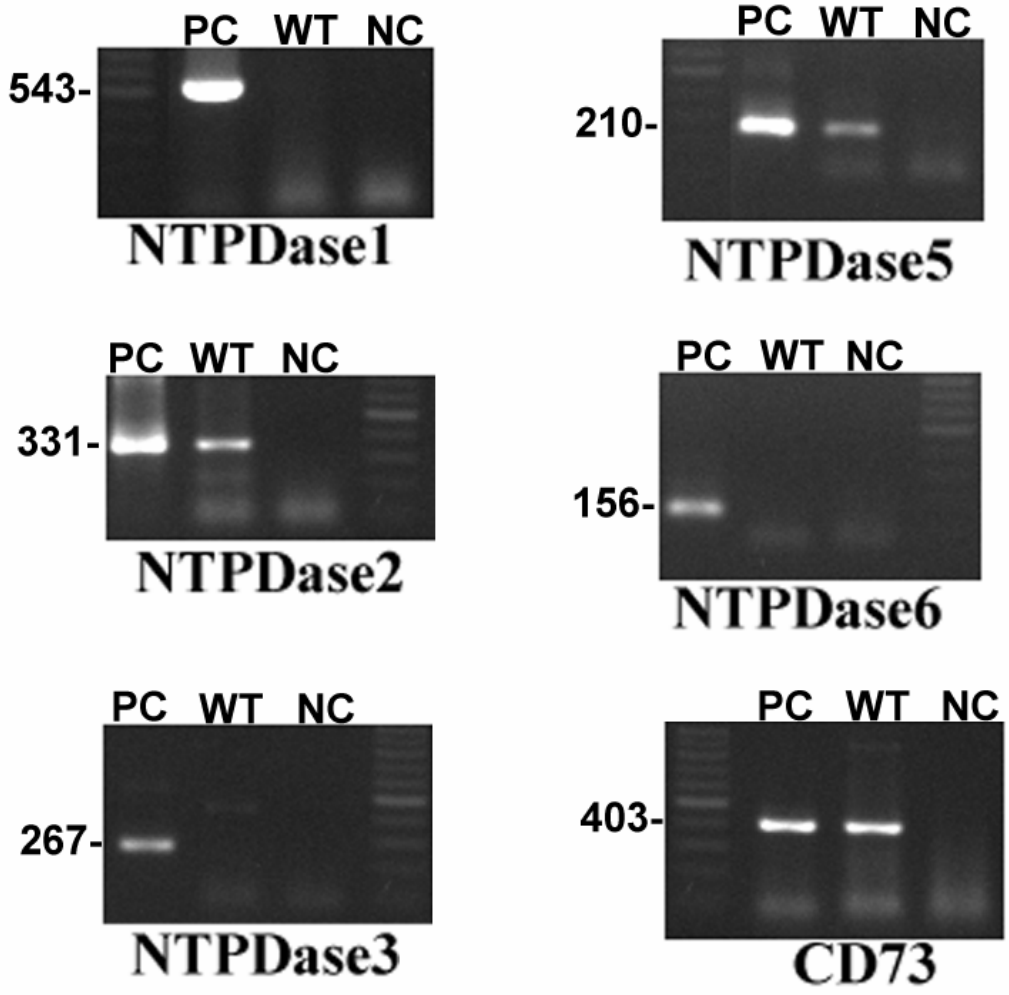
Fig. 5


PC WT NC
543-
NTPDase1
PC WT NC
210-
NTPDase5
PC WT NC
331-
NTPDase2
PC WT NC
156-
NTPDase6
PC WT NC
267-
NTPDase3
PC WT NC
403-
CD73

3.4 Capítulo 4

NTPDase and 5' ecto-nucleotidase expression profiles and the pattern of extracellular ATP metabolism in the Walker 256 tumor.

Paper a ser submetido ao jornal

Biochimica et Biophysica Acta – Molecular Basis of Disease

NTPDase and 5' ecto-nucleotidase expression profiles and the pattern of extracellular ATP metabolism in the Walker 256 tumor.

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Abstract

In this study, we evaluated the NTPDase and 5' ecto-nucleotidase expression profiles and the pattern of adenine nucleotide hydrolysis in rats submitted to the Walker 256 tumor model, 6, 10 and 15 days after the subcutaneous inoculation. Using RT-PCR analysis, we identified mRNA for all of the members of the ecto-nucleoside triphosphate diphosphohydrolase family investigated and a 5'-nucleotidase. By quantitative real-time PCR, *Entpd1* (*Cd39*) and *Entpd2* (*Cd39L1*) and CD73 were identified as the dominant genes expressed by the Walker 256 tumor, at all times studied. Extracellular adenine nucleotide hydrolysis by the Walker 256 tumor was estimated by HPLC analysis. Rapid hydrolysis of extracellular ATP by the tumor cells was observed, leading to the formation of adenosine and inosine in cells obtained from solid tumors at 6 and 10 days after inoculation. Cells obtained from solid tumors at 15 days of growth presented high levels of AMP and presented adenosine as a final product after 90 min of incubation. Results demonstrate that the presence of NTPDases and 5'-nucleotidase enzymes in Walker 256 tumor cells may be important for regulation of the extracellular adenine nucleotides/adenine nucleoside ratio, therefore leading to tumor growth.

Keywords: Walker 256 tumor; NTPDases; 5'-nucleotidase; adenine nucleotides; cancer.

Introduction

Signaling events, induced by extracellular adenine nucleotides, are controlled by the action of ectonucleotidases, including members of the Ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDases) and Ecto-nucleotide pyrophosphatases/phosphodiesterase (E-NPPs) families, as well as the ecto-5'-nucleotidase/CD73. These ecto-enzymes constitute a highly organized enzymatic cascade for the regulation of nucleotide-mediated signaling, controlling the rate, amount and timing of nucleotide (e.g. ATP) degradation and, ultimately, nucleoside (e.g. adenosine) formation.

Members of the E-NTPDase/CD39 family, previously classified as E-type ATPases, constitute a class of ecto-enzymes characterized by their capacity to hydrolyze nucleoside tri- and diphosphates; these have a strict dependence upon divalent cations and insensitivity to the classical inhibitors of the P-, F-, and V-type ATPases [1]. In mammals, there are eight related and homologous enzymes, sharing five apyrase-conserved regions (ACRs), named NTPDase1 to 8 that have been cloned and characterized. NTPDases 1-3 and 8 share a common membrane topography with two transmembrane domains at the N- and C-terminus and a catalytic site facing the extracellular compartment [2]. However, these NTPDases differ regarding the specific preferences for nucleotides. While NTPDases 1, 3 and 8 hydrolyse nucleoside tri- and diphosphates, NTPDase2 preferentially hydrolyses nucleoside triphosphates by 10- to 40-fold [3, 4, 5, 6, 7,8, 9, 10,11].

The family of ecto-nucleotide pyrophosphatases/ phosphodiesterases (E-NPPs) consists of seven structurally-related enzymes that are located at the cell surface,

either expressed as transmembrane proteins or as secreted enzymes. These proteins hydrolyze pyrophosphate or phosphodiester bonds of molecules such as nucleotides and dinucleotides (NPP1-3), (lyso) phospholipids (NPP2) and choline phosphate esters (NPP6 and NPP7). The actions of NPPs modulate a wide diversity of physiological, as well as pathophysiological events, e.g. cancer, insulin resistance and calcification diseases [12].

The expression of E-NTPDases at the cell surface has recently been associated with the development of certain pathologies, more particularly the proliferation of cancer cells [13,14,15,16]. Moreover, studies have indicated the anticancer activity of adenine nucleotides against animal and human tumor cells [17,18]. Inhibitory effects of extracellular ATP on tumor growth have been described in cells and tissues, including the human histiocytic leukemia cell line U-937 [19], pancreatic cancer cells [20], endothelial cells [21], pulmonary artery endothelial cells [22], colorectal carcinoma cells [23], prostate carcinoma cells [24](Janssens 2001) and esophageal carcinoma cells [25]. ATP is able, via activation of $P2X_7$ receptors, to permeabilize cell membranes and induces programmed cell death in several tumor cell systems *in vitro* [26,27], suggesting that induction of apoptosis may constitute an attractive target for cancer therapy. In addition, a synergistic action of ATP, when administered with established chemotherapeutic agents or radiotherapy, has been demonstrated [28,29]. On the other hand, there is some evidence to indicate that adenosine, acting through G-protein coupled receptors may be an important metabolite released by cancer cells [16,30]. This purine nucleoside elicits physiological responses that promote tumor

progression. Thus, ATP may inhibit tumor development, whilst adenosine may increase tumor development.

In the present study, we evaluated the expression profiles of the NTPDases and ecto-5'-nucleotidase and the pattern of extracellular ATP metabolism in Walker 256 tumors obtained from rats at, 6, 10 and 15 days after subcutaneous inoculation. The expressions of these enzymes, potentially involved in the extracellular hydrolysis of ATP to adenosine, may be important for the control of tumor progression and, consequently, for the development of new anti-tumoral drugs.

Materials and methods

Walker 256 tumors

The Walker 256 cell line A (originally from the National Cancer Institute Bank, Cambridge, MA, USA), donated by Dr. T.C. Cavalcanti and Dr. O. Rettori, from the Laboratory of Biochemical Research, CAISM/UNICAMP, is currently being 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. A Walker 256 tumor cell suspension ($5x10^6$ 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. Cell suspensions with 98% viability, as estimated by trypan blue, were obtained from the ascitic fluid of a donor rat.

RT-PCR analysis

Total RNA from tumor-bearing rats 6, 10 and 15 days after tumor inoculation was isolated with the RNA Mini Kit (Qiagen) in accordance with 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 20μl with both oligo (dT) primer and random hexamers in accordance with 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 units of Ex *Taq*DNA 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 were analyzed on a 1.3% agarose gel. The primer sets used for rat *Entpd1* (*Cd39*), *Entpd2* (*Cd39L1*), *Entpd3* (*Cd39L3*), *Entpd5* (*Cd39L4*) and *Entpd6,* (*Cd39L2*) and for rat ecto-5`-nucleotidase (CD73) and β-actin, as previously used by Wink et al. [31,32]. Oligonucleotides were obtained from Invitrogen. Negative controls were performed with water as template and positive controls were plasmids with cDNA for NTPDases, as previously used [31].

Real time PCR

Total RNA and cDNA were generated as described in RT-PCR analysis. SYRB Green I-based real-time PCR was carried out on MJ Research DNA Engine OpticonTM Continuous Fluorescence Detection System (MJ Research Inc.,Walthan, MA), as described [33]. All PCR mixtures contained: PCR buffer (final

concentration; 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2 mM $MgCl_2$, and 0.1% Triton X-100), 250 μM deoxy-NTP (Roche), 0.5 μM of each PCR primer, 0.5x SYBR Green I (Molecular Probes), 5% DMSO, and 1U taq DNA polymerase (Promega, Madison, WI) with 2 μl cDNA in a 25 μl final volume reaction mix. The samples were loaded onto 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 sec at 94°C, 30 sec at 56°C, 1 min at 72°C. The fluorescence signal was measured right after incubation for 5 sec at 79°C following the extension step, which eliminates possible primer dimer detection. At the end of the PCR cycles, a melting curve was generated to identify specificity for 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. In addition, for normalization of each sample, rat β-actin primers were used to measure the amount of β-actin cDNA. All samples were run in triplicate and the data were presented as the ratio of enzymes/β-actin. The primers used for real time PCR are described in RT-PCR analysis.

Analysis of extracellular ATP metabolism by HPLC

The solid tumor was removed 6, 10 and 15 days after inoculation, and a tissue sample of 3g was minced and dissociated with HBSS supplemented with 10ml of collagenase I-S (0.1 mg/ml) during 60 min at 37°C. The cellular suspension obtained had the contaminating erythrocytes removed by three cycles of osmotic lyses [34]. The cellular suspension was washed three times with phenol red free

modified HBSS containing 136.9 mM NaCl, 5.37 mM KCl, 0.81 mM $MgSO_4$, 0.34 mM Na_2PO_4, 0.44 mM KH_2PO_4, 0.42 mM $NaHCO_3$, 1.26 mM $CaCl_2$, 5.56 mM glucose and 15.1 mM HEPES (pH 7.4). Total protein content was measured by the Comassie Blue method [35] and samples of intact cells (30 mg of protein) were incubated in the same modified HBSS medium described above. The reaction was started by the addition 0.2 mM of ATP (final concentration), at 37°C. After the incubation time (0, 5, 10, 30, 60 and 90 min), the reaction was stopped and the samples maintained on ice. The incubation medium was centrifuged at 1°C for 30 min at 16 000 *g*. Aliquots of 30 μl were applied to a reversed-phase HPLC system (Shimadzu, Japan) using a C_{18} Shimadzu column of 12 cm x (Shimadzu, Japan) at 260 nm with a mobile phase containing 60 mM KH_2PO_4, 5.0 mM tetrabutylammonium chloride, pH 6.0, in 30% methanol, according to a previously described method [36]. All peaks were identified by their retention time and by comparison with standards. The results were expressed as nmoles of the different purinergic compounds per mg of protein in the incubation time. All incubations were carried out in triplicate and the controls to correct for non-enzymatic hydrolysis of nucleotides were performed by measuring the peaks present into the same reaction medium incubated without cells. The control for cellular purine secretion was performed by incubating the cells without substrate under the same conditions described above.

Cellular integrity

The cellular disruption was determined by the Trypan blue method and the cellular integrity estimated in 98% before and after the incubation.

Results

Ectonucleotidases mRNAs expression in the Walker 256 tumor

Since adenine nucleotides can be metabolized by different enzymes, we investigated the mRNA expression of *Entpd* 1-3, 5 and 6 and ecto -5'-nucleotidase at 6, 10 and 15 days after the subcutaneous inoculation of the Walker 256 tumor. Using specific primers, oligonucleotide fragments were amplified and analyzed on agarose gels. mRNAs for all members of the *Entpd* family investigated and ecto-5'-nucleotidase/CD73 were detected, at different levels of expression in Walker 256 tumors (Fig. 1). However, the *Entpd3* signal at 6 and 10 days and the *Entpd5* signal at 15 days after tumor inoculation were almost undetectable. The length of the oligonucleotide fragments obtained for the samples was comparable to that obtained for the positive controls, corresponding to the expected size.

Quantitative real-time PCR analysis

To investigate the major nucleotide degradation enzymes expressed by the tumor and whether they could present different expression patterns during the tumor growth, we performed quantitative real-time RT-PCR analysis of E-NTPDase family and ecto-5'-nucleotidase/CD73 genes. The tumor analyzed at 6, 10 or 15 days after inoculation, expressed the *Entpd1* (*Cd39*) and *Entpd2* (*Cd39L1*) as the major NTPDases genes (Fig. 2). The CD73 gene was also considerably

expressed, showing that these tumors have all the genes necessary to express the enzymes responsible for the complete degradation of ATP to adenosine. However, the *Entpd5* was expressed at low levels by the Walker tumor at all times studied, when compared with *Entpd1* and *Entpd2*, and the *Entpd3* and *Entpd6* were not detected.

The NTPDases that were most expressed by the tumor tissue presented differences in relation to their expressions during growth. At day 10, NTPDases 1, 2 and ecto-5'-nucleotidase/CD73 enzymes were clearly expressed, suggesting the importance of nucleotides in the control of tumor proliferation and invasion at this stage. The increase in mRNAs of NTPDases 1 and 2, observed at 10 days, may result in a major protein expression after this period. This finding is in accordance with the more rapid extracellular ATP hydrolysis, presented by cells obtained from solid tumors at 15 days of growth (Figure 3C). Probably, this is a critical period in the tumor's development, since after this time, there is a notable decrease in mRNA expression of all the enzymes expressed, as shown by real-time PCR and depicted in Fig. 2.

Metabolism of extracellular ATP in the Walker 256 tumor

We also investigated the pattern for extracellular ATP metabolism in Walker 256 tumor cells, 6, 10 and 15 days after the inoculation, by HPLC analysis over a period of 90 minutes. As shown in Fig.3, although there were some minor differences between the days studied, their main pattern of substrate hydrolysis remained similar. The Walker 256 cells quickly hydrolyzed extracellular ATP, leading to the transient formation of ADP in the medium that was hydrolyzed only

when ATP levels were significantly decreased (Fig 3). After 90 min, ADP was completely hydrolyzed to AMP in the three times studied (Fig 3 A, B and C). The extracellular ATP cascade produced high quantities of AMP and ended with adenosine and inosine production in cells obtained from solid tumors after 6 and 10 days of *in vivo* growth (Fig. 3A and B). The cells obtained from solid tumors of 15 days growth, hydrolyzed extracellular ATP more quickly than cells obtained at 6 and 10 days, producing high levels of AMP and having adenosine as final product after 90 min of incubation (Fig. 3C).

Our data regarding extracellular ATP hydrolysis are in agreement with the quantitative real-time PCR analysis. The expression of the ecto-5'-nucleotidase gene was lower in cells obtained from solid tumors of 15 days growth when compared with 6 and 10 days and presented high levels of AMP after 90 min of incubation. Hence, the rapid extracellular ATP hydrolysis is in accordance with the high expression of the *Entpd1* (*Cd39*) and *Entpd2* (*Cd39L1*) genes observed at all times studied. The sequential ATP degradation to AMP, with the accumulation of ADP as an intermediate product, shows that this pattern cannot be attributed only to the NTPDase 1 expression, considering that in this case ATP would be hydrolysed directly to AMP. Thus, ATP appears to be metabolized by a combination of NTPDase 1 and 2. These results are very similar to those obtained with double-transfected CHO cells with rat ecto-ATPase and ecto-apyrase [7], and are in agreement with the real-time PCR, which showed that the NTPDases 1 and 2 are the most expressed genes in Walker tumors.

Discussion

Studies have indicated that adenine nucleotides and nucleosides have an important role in tumor growth [16,18]. Moreover, the presence of enzymes in the plasma membrane of different tumors, such as ectonucleotidases, has been correlated with proliferative and metastatic activities [15,16,37]. This investigation describes the NTPDases and ecto-5'-nucleotidase expression profiles and extracellular ATP metabolism in Walker 256 tumors, obtained from rats at, 6, 10 and 15 days after subcutaneous inoculation. RT-PCR analysis showed that the Walker 256 tumor, at all times studied, expresses multiple ecto-nucleotidases with the potential to hydrolyze nucleotides to their respective nucleosides. By quantitative real-time RT-PCR analysis, we demonstrated that the tumors express the *Entpd1* (*Cd39*), *Entpd2* (*Cd39L1*) and ecto-5'-nucleotidase as the major genes. The pattern of product formation following extracellular ATP hydrolysis, determined by HPLC, revealed a rapid extracellular ATP hydrolysis, producing high quantities of AMP. This result is in agreement with the high expression NTPDases 1 and 2, two important enzymes involved in the extracellular ATP hydrolysis, as shown by quantitative real-time RT-PCR. Therefore, since the accumulation of ADP cannot be accounted for by NTPDase 1 alone, another enzyme with properties corresponding to NTPDase 2 should be co-expressed in Walker cells. The ATP hydrolysis pattern obtained by us is similar to that obtained with the coexpression of rat ecto-ATPase and ecto-apyrase in CHO cells, suggesting that the hydrolysis of ATP by ecto-apyrase directly produces AMP with no significant production of ADP as an intermediate product [7]. In contrast, ADP is released from ecto-ATPase and accumulates in the medium. It may be postulated, on basis of our

results, that a combination of both enzymes in CHO cells permits a pattern of ATP degradation followed by the sequential appearance of ADP, AMP and adenosine in the medium [7]. This result was expected, due to the coexpression of both enzymes during the Walker tumor's growth.

The Walker 256 carcinoma is a tumor model that has been extensively used in studies of cancer pathophysiology [38,39,40,41,42]. 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 [39]. It is important to note that, mainly at 10 days, NTPDase and ecto-5'-nucleotidase genes were more expressed, suggesting a possible relationship between the enzymes' expressions and the beginning of these changes in the tumor growth. Moreover, an increase in the expressions of the *Entpd1* (*Cd39*), *Entpd2* (*Cd39L1*) and ecto-5'-nucleotidases could suggest an important role for these enzymes in controlling nucleotide levels around the tumor, favoring the tumor implantation and survival.

Previously published data have shown that the expression of ecto-ATPases is increased during cancer progression [43,44]. Recent cloning of both mouse and human ecto-ATPase cDNA from tumor cells [14,45,46] indicates that NTPDase2 is highly expressed in some tumors and its expression may be the result of tumorigenesis [14]. These observations agree with the results obtained by us (Fig. 1).

NTPDase1, one of the most intensively studied members of the ecto-NTPDase enzyme family, is considered to play an important role in purinergic signaling, in

thromboregulation, and in cell-protective processes by converting ATP released by damaged cells to AMP. Moreover, changes in the activity and distribution of this protein have been reported in several pathological conditions such as cancer. NTPDase1 is overexpressed in human melanoma cell lines, in rat ovarian tumor cells, in multidrug-resistant breast carcinoma cells (MCF-7-AdR), and in cancerous human pancreas [13,15,47,48]. The expressions of several NTPDases, with overlapping roles and activities, are not well understood and suggest a complexity of the purinergic system.

The expression of enzymes with the capacity to hydrolyze nucleotides such as ATP, may be advantageous to the tumoral cells. Elevated levels of extracellular ATP have been observed in many tumors. One of the release mechanisms may utilize overexpressed P-glycoprotein, for example, cytotoxic lymphocytes that have been shown to release ATP upon T cell activation [13]. Furthermore, Walker 256 tumor cells induce destruction of the cellular barrier and promote death of normal tissue [49], probably allowing the release of intracellular ATP. Thus, since extracellular ATP, a substrate for NTPDase1 and 2, exerts a cytotoxic effect in a variety of cell systems via activation of $P2X_7$ receptors [26,50], these enzymes that are highly expressed in Walker 256 tumor cells, would terminate actions induced by this nucleotide, protecting the tumoral cells from ATP-mediated apoptosis, possibly constituting a natural defense of the tumor to promote its own growth.

Ecto-5'-nucleotidase is an essential enzyme in the extracellular pathway, since it generates adenosine from AMP and is expressed in many different tissues [51]. Although it is highly expressed in many tumor cells, its specific function during tumorigenesis is unclear. Studies have found that up regulated expression of ecto-

5'-nucleotidase is associated with a highly invasive phenotype [52], drug resistance and tumor-promoting functions [30,53]. Furthermore, in addition to generating adenosine, ecto-5'-nucleotidase may have independent roles in adhesion and interact with extracellular matrix components [52]. Adenosine is reported to be present at high concentrations in solid tumors, with accumulation in the extracellular tumor microenvironment, and is reported to stimulate tumor growth, angiogenesis and to have immunosuppressive effects [30,54]. Since ecto-5'-nucleotidase is essential for extracellular adenosine formation, high expression of this enzyme in the Walker 256 tumor could help the rapid growth of the tumor by generation of adenosine from extracellular AMP. Again, as proposed for NTPDase, these enzymes may promote tumoral growth.

We have recently shown a significant reduction in ATP, ADP and AMP hydrolysis by serum and platelets in rats submitted to the Walker 256 tumor, 6, 10 and 15 days after subcutaneous inoculation. We hypothesized that an inhibition of extracellular adenine nucleotide hydrolysis (by soluble and ecto-enzymes), in circulation, may represent a mechanism for protecting the animal against tumoral cell development. The levels of ATP (cytotoxic) in the circulation would be increased, and the levels of adenosine (involved in promoting tumor growth) would be reduced, leading to a decrease in tumor development [55]. The data presented here suggest that, in tumor cells, the opposite may occur. The high NTPDase 1, 2 and ecto-5'-nucleotidase expression, as well as the rapid ATP hydrolysis by tumor-producing adenosine, may represent an important mechanism to facilitate high proliferation and invasion of tumoral cells.

Taken together, our results demonstrate that the Walker 256 tumour contains all the components of the enzymatic cascade necessary for the complete metabolism of extracellular nucleotides to nucleosides. The tumors presented NTPDases 1 and 2, as the major NTPDase family genes, a finding that was in accordance with the nucleotide metabolism pattern demonstrated by HPLC. A difference in relation to the enzymes expression during the growth time was also noted. An elevated expression of NTPDase 1 and 2 and ecto-5'-nucleotidase was observed at all times studied, but the major expression of these enzymes was at 10 days of growth. It is, therefore, tempting to suggest a possible involvement of the nucleotidase cascade in the modulation of ATP and adenosine levels in the tumor that, consequently, modulates tumor growth. Thus, this study allows us to understand one of the possible mechanisms that tumoral cells may use for their protection and, possibly, identify targets for the development of a new class of drugs to control tumoral growth.

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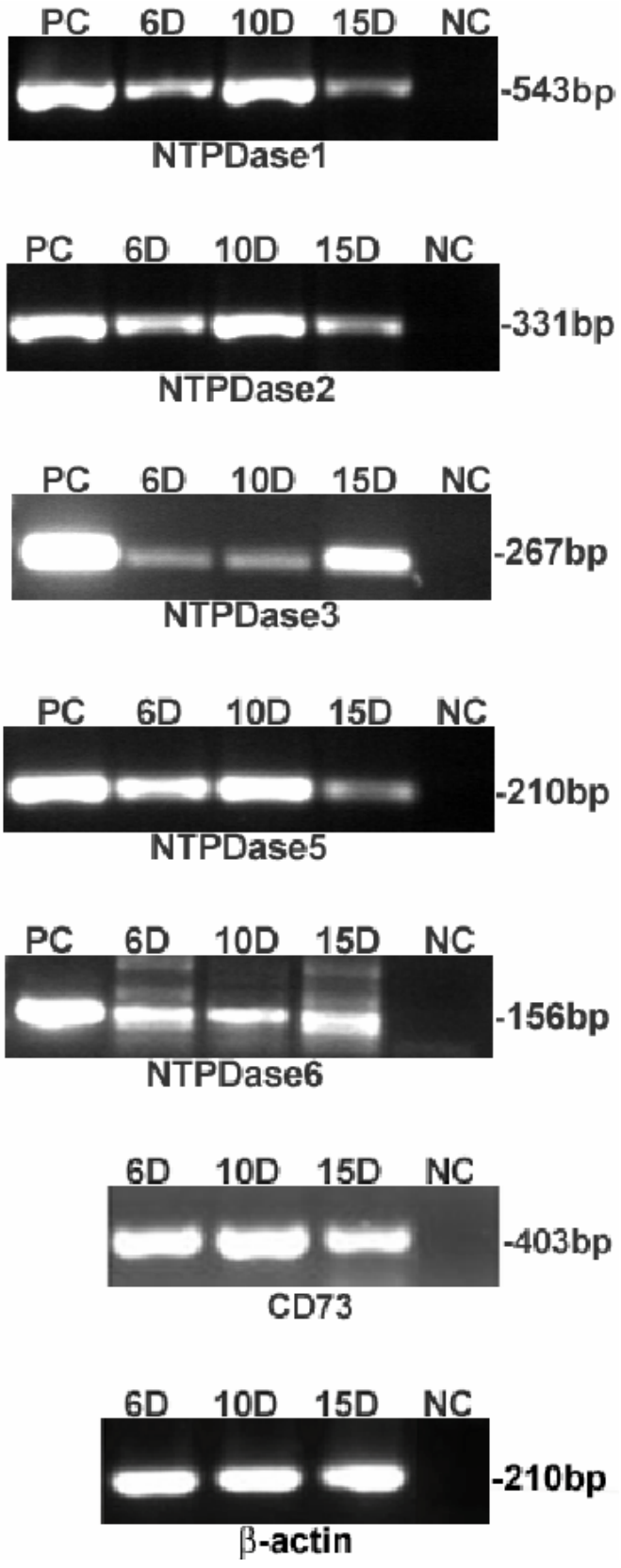
Legends

Fig. 1: RT-PCR analysis of NTPDases and CD73 expression by the Walker 256 tumor. Total RNA was isolated from tumor-bearing rats 6, 10 and 15 days after tumor inoculation and the cDNA were analyzed by PCR with primers for the CD39 family and CD73, as described in Material and Methods. Plasmids containing the sequences of all NTPDases were used as positive controls (PC). The length (bp) of the PCR products obtained with each pair of primers is given in each figure.

Fig. 2: Comparison of NTPDases and CD73 expression in the Walker 256 tumor by quantitative real-time RT-PCR analysis. The expression of NTPDase members and CD73 in Walker 256 tumor 6, 10 and 15 days after tumor inoculation, was quantitatively analyzed by real-time PCR, as described in Material and Methods. Results are presented as the ratio of cDNA enzymes/β-actin. Bars represent mean ± SD for two experiments.

Fig. 3: Hydrolysis of ATP and product formation by Walker 256 tumor. Cells were incubated, as described in Materials and Methods, containing 500 μM ATP. Nucleotides were analyzed by HPLC 6 (A), 10 (B) and 15 (C) days after tumor inoculation. Values are representative of three different experiments: mean ± S.E.M. (n=3) of one typical experiment.

Fig. 1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NTPDase2
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-210bp
NTPDase5
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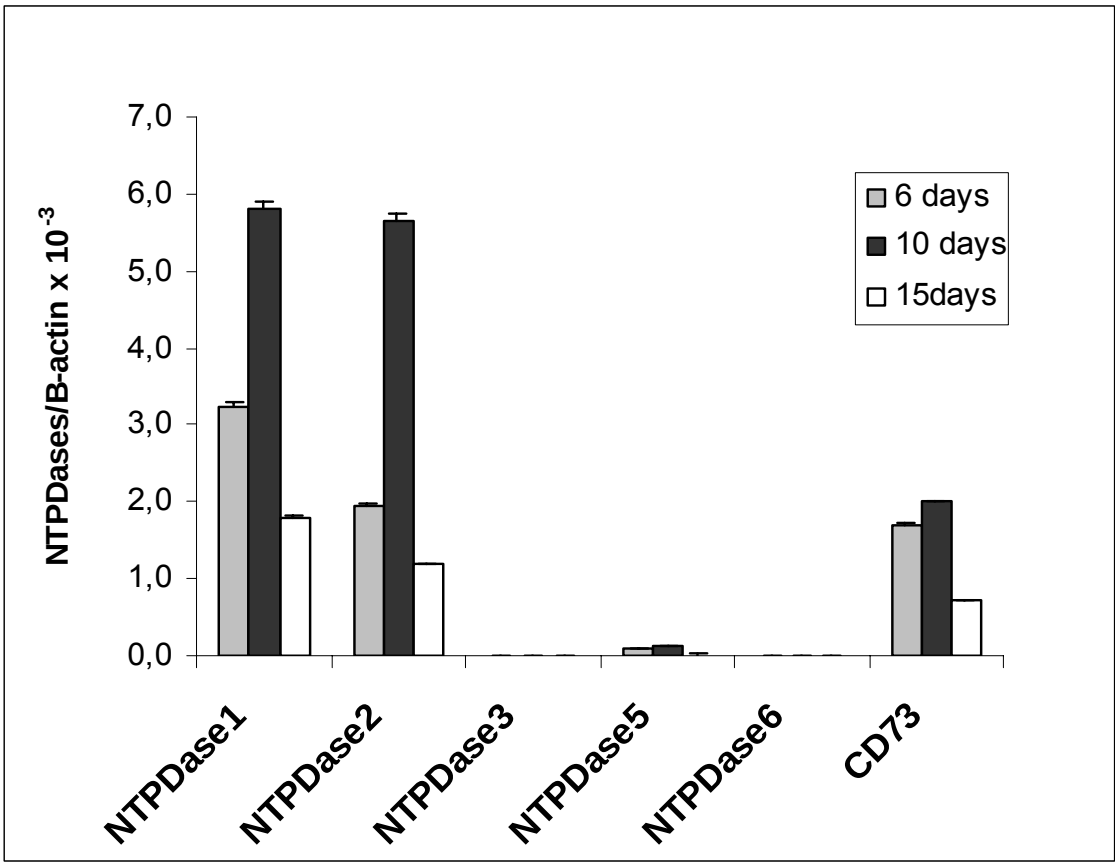
6D 10D 15D NC
-210bp
β-actin

Fig. 2


NTPDases/B-actin x 10-3
7,0
6,0
5,0
4,0
3,0
2,0
1,0
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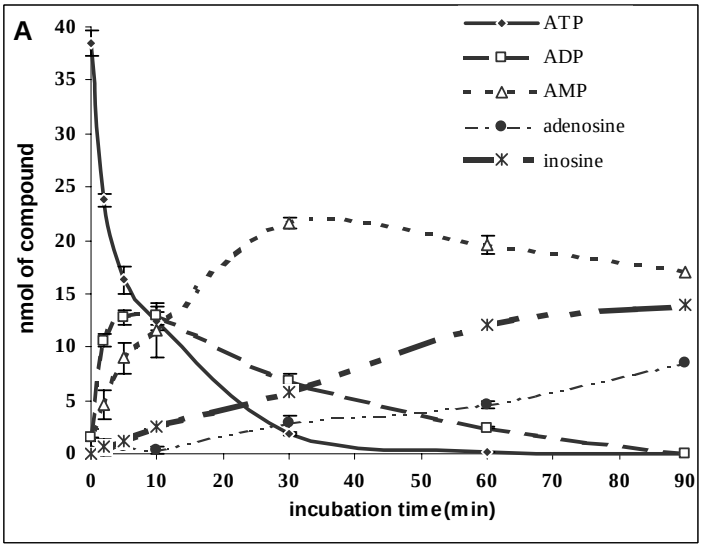
6 days
10 days
15days
NTPDase1
NTPDase2
NTPDase3
NTPDase5
NTPDase6
CD73

Fig. 3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ATP
ADP
AMP
adenosine
inosine
nmol of compound
incubation time(min)
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10
15
20
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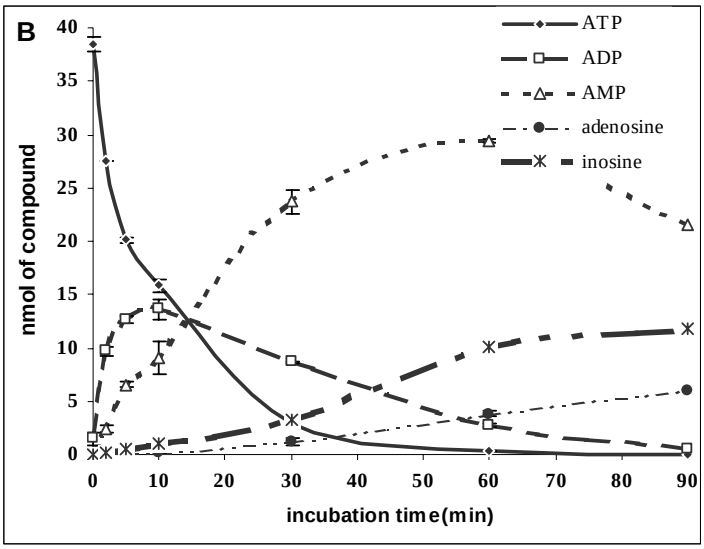
20
30
40
50
60
70
80
90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ADP
AMP
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inosine
nmol of compound
incubation time(min)
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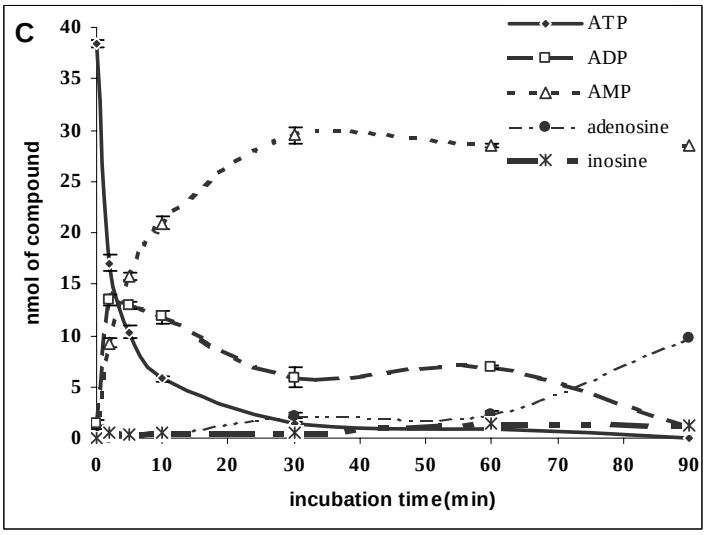
50
60
70
80
90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AMP
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inosine
nmol of compound
incubation time(min)
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5
10
15
20
25
30
35
40
0
10
20
30
40
50
60
70
80
90

3.5 Anexo

Resultados obtidos durante o doutorado e que serão finalizados após o término dos experimentos de caracterização bioquímica, para preparação do paper.

NPP3 is the dominant Nucleotide pyrophosphatase/phosphodiesterase family member in Walker tumor cells

Materials and Methods

RT-PCR Analysis

Total RNA from Walker tumor cells (ascitic liquid) and tumor-bearing rats 6, 10 and 15 days after tumor inoculation was isolated with RNA Mini Kit (Qiagen) in accordance with 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 in accordance with 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 units of Ex *Taq*DNA 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 PCR products of the cycle curve were analyzed in the 20, 23, 25, 28, 30 and 33 cycles. 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). Oligonucleotides were obtained

from Invitrogen. Negative controls were performed with water as template and positive controls were plasmids with cDNA for NPPs as described (Wink et al., 2006).

Real time PCR

Total RNA and cDNA were generated as described in RT-PCR analysis. SYRB Green I-based real-time PCR was carried out on MJ Research DNA Engine OpticonTM Continuous Fluorescence Detection System (MJ Research Inc.,Walthan, MA) as described (Zerbini et al., 2003). All PCR mixtures contained: PCR buffer (final concentration 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, 0.5x SYBR Green I (Molecular Probes), 5% DMSO, and 1U taq DNA polymerase (Promega, Madison, WI) with 2 μl cDNA in a 25 μl final volume reaction mix. The samples were loaded into 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 sec at 94°C, 30 sec at 56°C, 1 min at 72°C. The fluorescence signal was measured right after incubation for 5 sec at 79°C following the extension step, which eliminates possible primer dimer detection. . At the end of the PCR cycles, a melting curve was generated to identify 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 samples were run in triplicate and the data were

presented as ratio of enzymes/β-actin. The primers used for real time PCR are described in RT-PCR analysis.

Legend to figures

Fig. 1: RT-PCR analysis of NPP1, 2 and 3 expression by Walker 256 cells. Total RNA was isolated from Walker 256 cells and the cDNA was analyzed by PCR with primers for NPPs as described in Materials and Methods. Plasmids containing the sequences of all NPPs were used as positive controls (PC). The length (bp) of the PCR products obtained with each pair of primers is given; (NC) negative control; (WT) Walker tumor.

Fig. 2: Comparison of NPP2 and NPP3 expression in Walker 256 cells by RT-PCR analysis. The PCR products of the cycle curve were separated on 1.3% agarose gel (A). The results are presented in the graphic as ratio of cDNA enzymes/β-actin (B). Representative data of two independent experiments.

Fig. 3: RT-PCR analysis of NPPs expression by Walker 256 tumor. Total RNA was isolated from tumor-bearing rats 6, 10 and 15 days after tumor inoculation and the cDNA was analyzed by PCR with primers for NPP family as described in material and methods. Plasmids containing the sequences of all NPPs were used as positive controls (PC). The length (bp) of the PCR products obtained with each pair of primers is given in each figure.

Fig. 4: Comparison of NPPs expression in Walker 256 tumor by quantitative real-time RT-PCR analysis. The expression of NPPs in Walker 256 tumor 6, 10 and 15 days after tumor inoculation, was quantitatively analyzed by real-time PCR as

described in material and methods. The results are presented as ratio of cDNA enzymes/β-actin. Bars represent mean ± SD for two experiments.

Figure 1

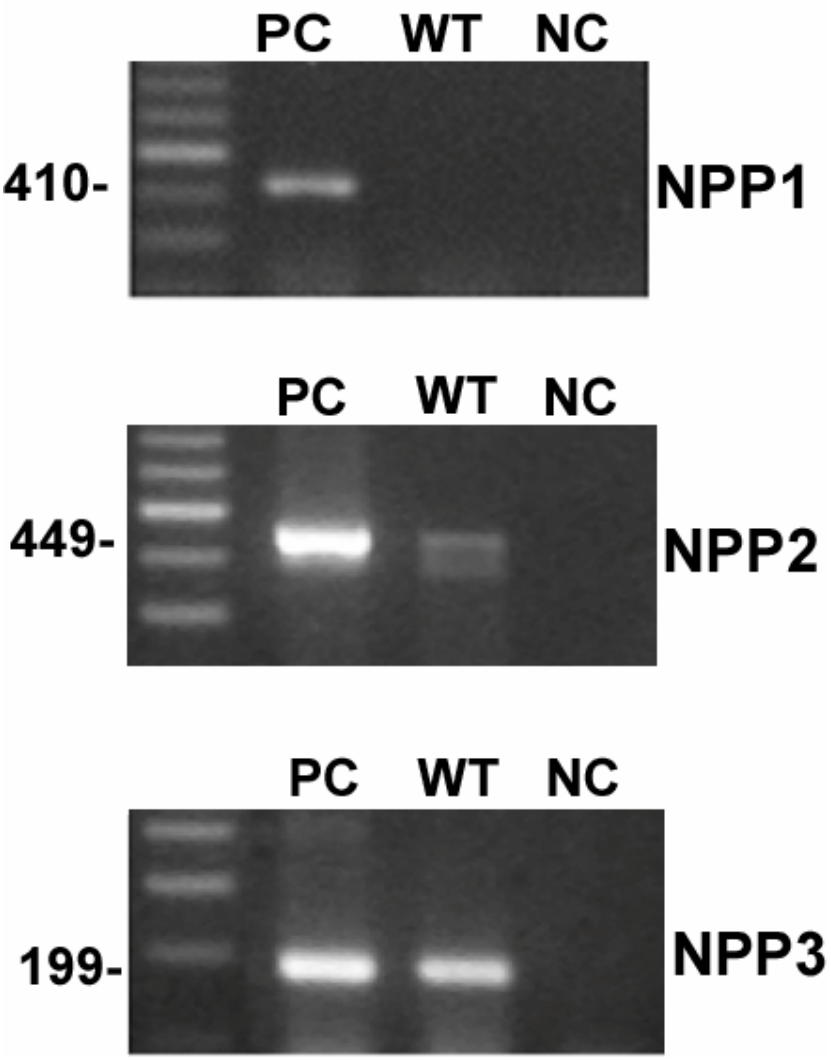


Figure 2

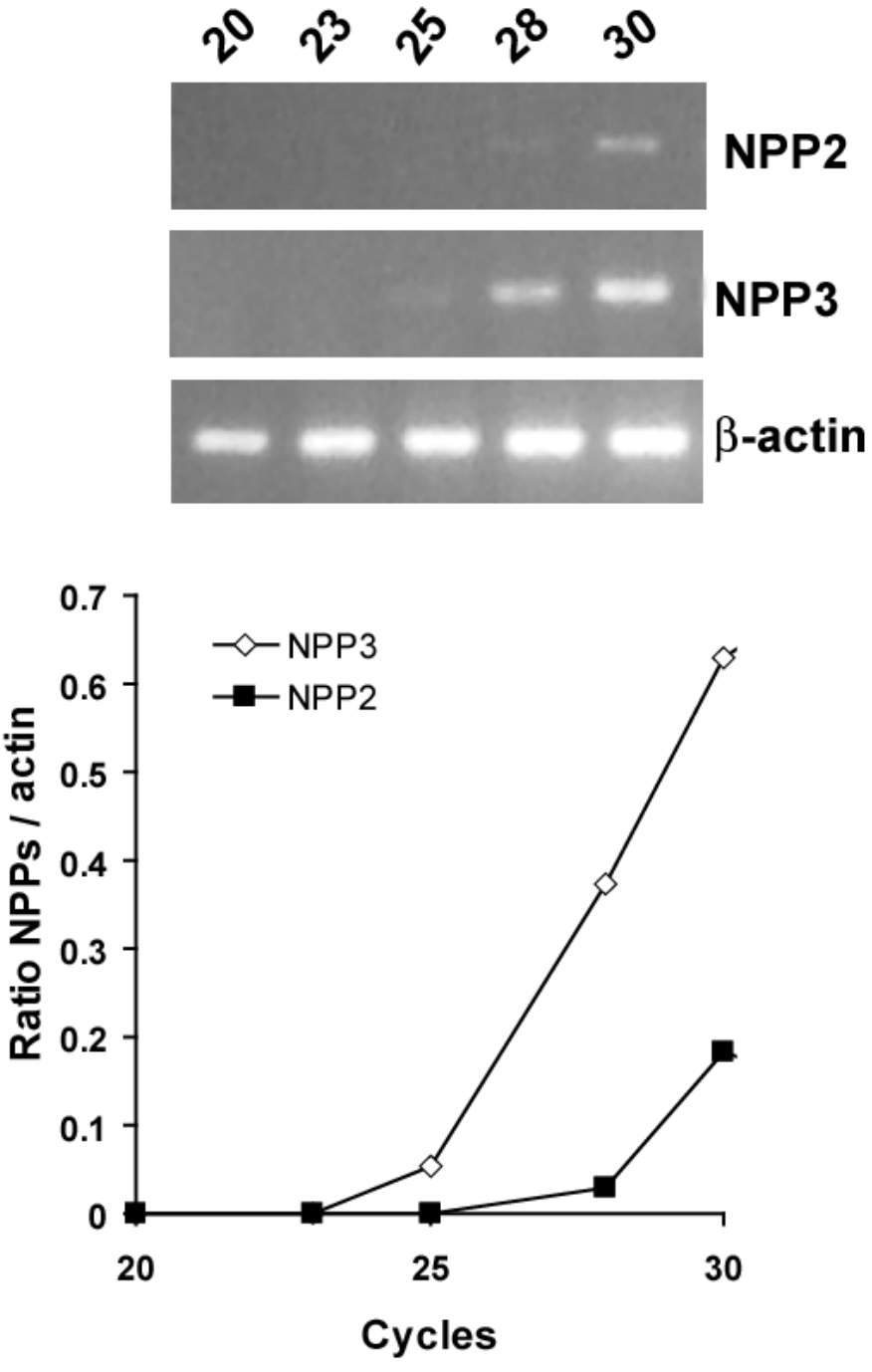
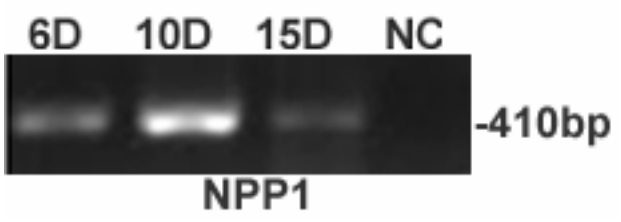

20
23
25
28
30
NPP2
NPP3
β-actin
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
NPP3
NPP2
Ratio NPPs / actin
20
25
30
Cycles

Figure 3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NC
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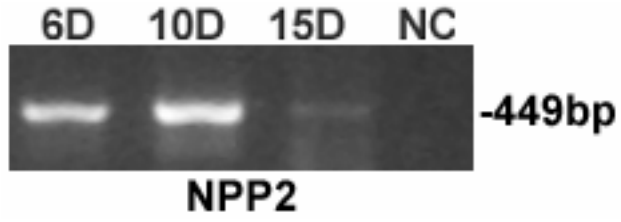
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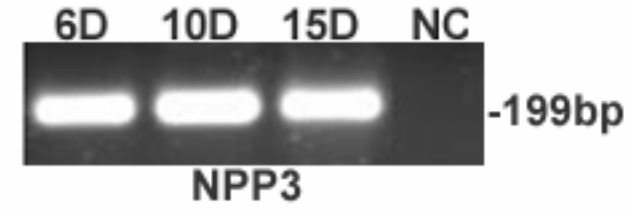
NPP2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NPP3

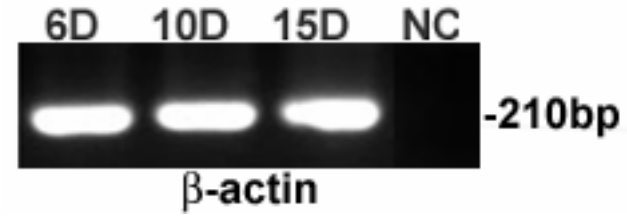
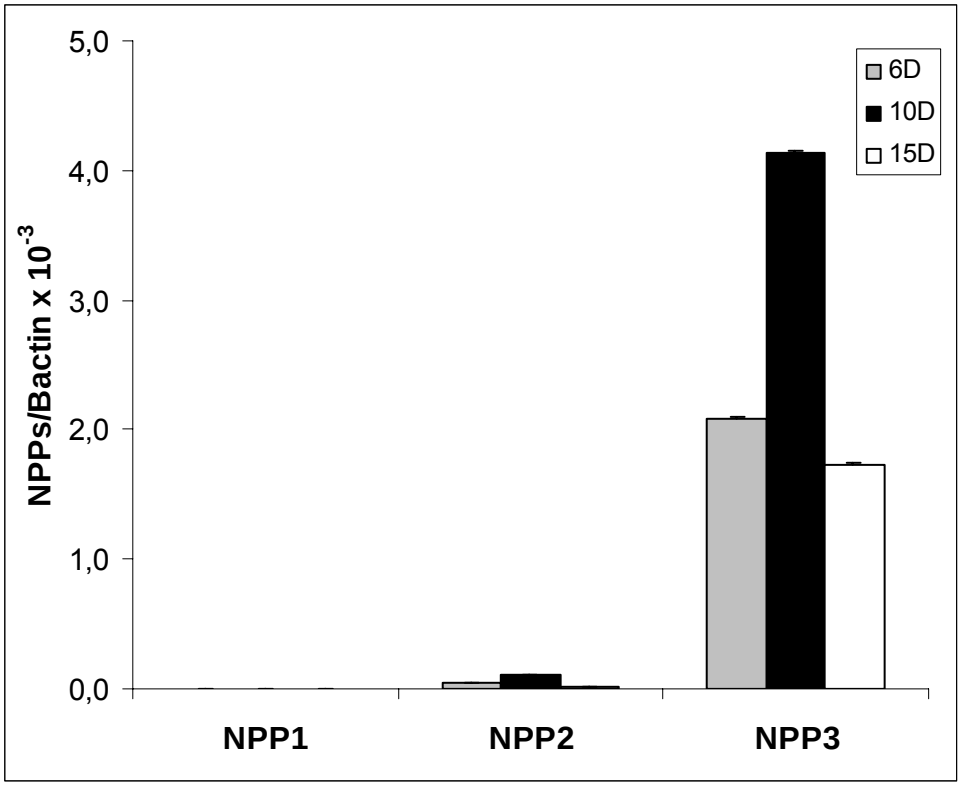

6D
10D
15D
NC
-210bp
β-actin

Figure 4



4 Discussão

4.1 Efeito *in vitro* do ácido acetil salicílico na hidrólise dos nucleotídeos da adenina em plaquetas de ratos

As funções dos nucleotídeos da adenina e seu nucleosídeo adenosina na circulação estão bem estabelecidas. O ATP é vasoconstritor, o ADP promove agregação plaquetária, enquanto que a adenosina é vasodilatadora e inibe a agregação plaquetária (Colman, 1990; Kitakaze et al., 1991; Soslau & Youngprapakorn, 1997; Kawashima et al., 2000). Estudos realizados em nosso laboratório demonstram a coexistência de uma NTPDase e uma fosfodiesterase (NPP) em soro de ratos (Oses et al., 2004). Além disso, as plaquetas também expressam uma ATPdifosfoidrolase e uma NPP (Frassetto et al., 1993; Furstenau et al., 2006), que juntamente com uma 5'-nucleotidase (Bergamini & Grazi, 1980) participam de uma cadeia enzimática que leva à completa hidrólise do ATP à adenosina, modulando as ações destes na circulação.

Os resultados apresentados nesta tese demonstram que o AAS é capaz de inibir significativamente a atividade de hidrólise do ATP e do ADP em plaquetas de ratos adultos. A hidrólise do ATP foi inibida em 17% e 21% nas concentrações de 2,0 e 3,0 mM de AAS, respectivamente (capítulo 1, figura 1). O AAS também inibiu significativamente a hidrólise do ADP em 41%, mas apenas na concentração de 3,0mM, demonstrando uma sensibilidade diferente do ATP e do ADP em relação ao AAS. A hidrólise do AMP, entretanto, não sofreu alterações significativas, nas concentrações de 1,0-3,0 mM de AAS. Além disso, a cinética de interação do AAS

com as atividades ATPásica e ADPásica demonstrou um inibição do tipo acompetitiva para ambos os substratos em plaquetas de ratos.

O efeito do AAS na hidrólise dos nucleotídeos da adenina em soro de ratos também foi investigado, porém nenhuma alteração significativa foi observada nas atividades de hidrólise do ATP, ADP e AMP nas concentrações 1,0, 2,0 e 3,0 mM. Este resultado indica que as nucleotidases respondem de forma diferente aos efeitos do AAS na circulação.

De acordo com vários estudos da literatura, o AAS tem sido colocado como um agente promissor no tratamento e prevenção do câncer. Alguns autores acreditam que o efeito antitumoral desta droga seja por inibição de algumas enzimas tais como a COX-2, altamente expressa em alguns tipos de tumor (Jiang et al., 2001), ou ainda por induzir apoptose em várias linhagens de células tumorais (Dikshit et al., 2006), mas o mecanismo pelo qual o AAS exerce esse efeito ainda não está claro. Os resultados obtidos nos permitem propor que a inibição na atividade da NTPDase 3 em plaquetas pelo AAS, poderia ser um mecanismo alternativo pelo qual o AAS exerce seus efeitos protetivos contra o câncer, uma vez que os níveis de ATP, descrito na literatura como uma molécula citotóxica, estariam aumentados, na circulação. Além disso, esta inibição observada na presença do AAS, não produziria adenosina, um composto que possui atividades promotoras de tumor.

Um outro aspecto a ser explorado a partir destes resultados, diz respeito a uma possível função como molécula de adesão celular que tem sido sugerida para a ATPdifosfoidrolase (Kansas et al., 1991; Skladchikova et al., 1999). Considerando que em plaquetas a presença de moléculas de adesão pode facilitar

metástases, a inibição da NTPDase 3 pelo AAS em plaquetas, poderia estar contribuindo também para a inibição da disseminação de tumores.

4.2 Tumor de Walker 256 e sistema vascular

Para investigar o envolvimento das ectonucleotidases em soro sangüíneo e plaquetas de ratos durante o desenvolvimento do tumor de W256, a hidrólise dos nucleotídeos da adenina foi analisada 6, 10 e 15 dias após a indução do tumor. Os resultados obtidos demonstram que o tumor de W256 é capaz de influenciar o metabolismo dos nucleotídeos extracelulares da adenina em plaquetas e soro de ratos, ao longo do desenvolvimento do tumor. A presença do tumor de W256 reduziu de forma significativa e em todos os dias avaliados a hidrólise do ATP, ADP e AMP em soro sangüíneo. Em plaquetas, uma significativa redução na hidrólise do ATP e AMP (31% e 21%, respectivamente), 10 e 15 dias após a indução do tumor. Porém, nenhuma alteração significativa foi observada em 6 dias. Já a hidrólise do ADP em plaquetas foi significativamente reduzida em presença do tumor de W256, nos 6, 10 e 15 dias de desenvolvimento do tumor (Capítulo 2, Figura 2). Estes resultados demonstram um padrão similar de redução na hidrólise dos nucleotídeos da adenina, em plaquetas e soro de ratos submetidos ao tumor de W256, indicando que há um mecanismo em comum que estabelece esta condição.

As atividades enzimáticas das ectonucleotidases são capazes de regular as concentrações dos nucleotídeos extracelulares, bem como a formação dos nucleosídeos, modulando assim seus efeitos biológicos. As NTPDases 1 e 2 bem

como a ecto-5'-nucleotidase, são as enzimas dominantes expressas no sistema vascular. A NTPDase 1 hidrolisa tri- e difosfonucleosídeos e bloqueia a agregação plaquetária induzida pelo ADP. Já a NTPDase 2 ativa as plaquetas por converter preferencialmente ATP em ADP, enquanto que a ecto-5'-nucleotidase regula as concentrações de adenosina (Atkinson et al., 2006), um nucleosídeo que inibe a agregação plaquetária (Kitakaze et al., 1991; Kawashima et al., 2000). Assim, alterações nas atividades que metabolizam nucleotídeos na superfície das plaquetas podem estar envolvidas na ativação plaquetária.

Numerosos trabalhos têm sido dedicados ao estudo das ectonucleotidases em condições fisiológicas e patológicas, incluindo o câncer (Dzhandzugazyan et al., 1998; Knowles and Chiang, 2003; Wink et al., 2003). Também é atribuída uma atividade anticâncer aos nucleotídeos da adenina, e efeitos inibitórios no crescimento de tumores tem sido descritos para o ATP extracelular, na maioria dos tecidos e células estudados até o momento (Hopfner et al., 2001; Janssens & Boeynaems, 2001; Yamada et al., 2002; Wang et. al., 2005; White & Burnstock, 2006). A adenosina, por outro lado, tem sido descrita exercer respostas fisiológicas que promovem a progressão de tumores (Sychala 2000; Spychala et al., 2004). As atividades anti-inflamatória e imunosupressora da adenosina podem inibir as respostas imunológicas contra o tumor, e de acordo com estes dados, um estudo mostrou que linfócitos T antitumor são inibidos por adenosina extracelular, via ativação de receptores A2A (Ohta et al., 2006). A alteração nas atividades das ectonucleotidases em plaquetas e soro permite sugerir um possível mecanismo de proteção contra o desenvolvimento do tumor de W256. A redução nas atividades de hidrólise dos nucleotídeos da adenina levaria a um aumento do ATP (um

composto citotóxico para as células tumorais) na circulação, uma vez que este não seria degradado a adenosina (molécula promotora de crescimento tumoral).

A participação das plaquetas no desenvolvimento e disseminação do câncer também tem sido bastante investigada (Nash et al., 2002; Jurasz et al., 2004). As células tumorais interagem com as plaquetas na circulação sangüínea, resultando em agregação plaquetária, e esta habilidade está relacionada ao potencial metastático do tumor. Além disso, o ADP liberado durante a ativação plaquetária pelo tumor é um dos compostos responsáveis pelo estado protrombótico observado em muitos tipos de câncer (Jurasz et al., 2004). Na corrente sangüínea, as plaquetas normalmente liberam ATP, ADP e UTP. Assim, o metabolismo dos nucleotídeos extracelulares por NTPDases e ecto-5'-nucleotidase tem uma importante função regulatória no controle da homeostase, principalmente na agregação plaquetária (Kas-Deelen et al., 2001). A redução nas atividades de hidrólise do ATP, ADP e AMP na circulação, durante o desenvolvimento do tumor de W256 observado nesta tese, poderia contribuir para a ativação do sistema da coagulação, por manter os níveis de ATP e ADP na corrente sangüínea, e diminuir a formação de adenosina.

Outra possibilidade que não pode ser excluída é esta alteração na atividade das ectonucleotidases na circulação estar relacionada a um papel como moléculas de adesão. As plaquetas, por expressarem moléculas de adesão celular na sua superfície, facilitam a adesão das células tumorais ao endotélio vascular e liberam um grande número de fatores que podem ser usados pelas células tumorais para seu crescimento (Jurasz et al., 2004). A participação das ectonucleotidases como moléculas de adesão, tem sido descrita. Neste sentido, a NTPDase1/CD39 tem

mostrado estar envolvida em adesão homotípica de linfócitos (Kansas et al., 1991) e uma atividade ATPásica tem sido associada com a molécula de adesão neural NCAM (Skladchikova et al., 1999). Ainda, o papel da ecto-5'-nucleotidase (CD73) como molécula de adesão em linfócitos humanos também tem sido relatado (Fenoglio et al., 1997). Então, já que moléculas de adesão em plaquetas têm uma importante participação em metástases e estas moléculas podem ter características de uma ATPdifosofidrolase, a inibição nas atividades ectonucleotidásicas observadas na circulação pode também contribuir para impedir a disseminação do tumor de Walker.

A análise da degradação de nucleotídeos no sistema vascular de ratos submetidos ao tumor de W256 6, 10 e 15 dias mostrou um padrão alterado de hidrólise dos nucleotídeos extracelulares da adenina que pode estar relacionado a três condições: aumento dos níveis de ATP, como um mecanismo de defesa do organismo, visto o efeito citotóxico desta molécula em células tumorais; contribuição para um estado pró-coagulante; e ainda a participação de moléculas de adesão em plaquetas que devido à inibição observada, poderiam limitar a disseminação do tumor.

4.3 Ectonucleotidases e Tumor de W256

O carcinoma de W256 é um modelo experimental de tumor em ratos, que tem sido amplamente utilizado em estudos relacionados a patofisiologia do câncer (Toal et 1960; Morrison et 1971; Guaitani et 1982; Rettori et al., 1995; Ventrucci et al., 2001; He et al., 2003; Ikeda et al., 2004; Mao-Ying et al., 2006).

Recentemente, foi demonstrado que a hidrólise extracelular dos nucleosídeos di e trifosfatados é realizada por uma família de ecto-enzimas, as E-NTPDases, composta até o momento por pelo menos 8 membros (Zimmermann, 2001; Bigonnesse et al., 2004). A investigação das NTPDases, ecto-5'-nucleotidase bem como das NPPs presentes no modelo experimental de tumor de W256 foi realizada em células mantidas em líquido de ascite e na forma subcutânea do tumor.

4.3.1 NTPDases e ecto-5'-nucleotidase em células tumorais de W256 (forma ascítica do tumor)

O estudo das propriedades bioquímicas demonstrou que as células tumorais de W256 hidrolisam ATP, ADP e AMP em um pH ótimo que varia de 6,5 a 8,0 e requerem cátions divalentes para a sua atividade de hidrólise, com uma preferência para Mg^{2+} sobre o Ca^{2+}. Em presença de azida sódica, um inibidor de ATPdifosfoidrolases de várias fontes (Plesner, 1995; Knowles and Nagy, 1999) e de cloreto de gadolínio, inibidor de E-NTPDases solúveis e ligadas à membrana (Escalada et al., 2004), uma significativa inibição foi observada na hidrólise do ATP e ADP (capítulo 3, Tabela 1). Estes resultados estão de acordo com a ocorrência de E-NTPDases nas células de W256. Ainda, o plote de competição Chevillard indicou um padrão de hidrólise constante em todas as combinações de substratos testadas (capítulo 3, figura 4).

As propriedades cinéticas também foram determinadas. Os valores de K_m foram 464.2 $\pm$ 86.6, 137.0 $\pm$ 31 and 44.8 $\pm$ 10.2 μM, e os valores de V_{max} foram

655.0 ± 94.6, 236.3 ± 27.2 and 177.6 ± 13.8 nmol de fosfato inorgânico.min^{-1}.mg de proteína^{-1} para ATP, ADP and AMP, respectivamente (capítulo 3, figura 3).

A expressão do mRNA das NTPDases 1, 2, 3, 5 e 6 e da ecto-5'-nucleotidase em células de W256 foi analisada por RT-PCR. Os genes das NTPDases 2 e 5 e a ecto-5'-nucleotidase foram detectados nas células de W256. Entretanto, para as NTPDases 1, 3 e 6 o sinal foi quase imperceptível (capítulo 3, figura 5).

A expressão da NTPDase 2 ou CD39L1 em células de W256, uma E-NTPDase caracterizada por hidrolisar preferencialmente os nucleosídeos tri-fosfatados (Kegel et al., 1997; Zimmermann, 2001), está de acordo com dados da literatura que mostram uma alta expressão desta enzima em alguns tumores (Saphner et al., 1991; Knowles and Chiang, 2003). Portanto, a presença da NTPDase 2 pode representar um eficiente meio pelo qual as células tumorais de W256 evitam os efeitos citotóxicos do ATP.

A NTPDase 5 pode estar presente na superfície da membrana plasmática e pode ser secretada como uma enzima solúvel por clivagem proteolítica, além de possuir uma maior afinidade por nucleosídeos difosfatados do que para trifosfatados (Mulero et al., 2000; Robson et al., 2006). A atividade ADPásica em células de Walker revelou uma preferência bem maior por Mg^{+2} do que para Ca^{+2}, dado este que está de acordo com características da NTPDase 5 já descritas na literatura (Mulero et al., 1999). A recente descoberta da identidade desta enzima com o proto-oncogene humano PCPH (Paez et al., 2001), sugere uma possível função para a NTPDase 5 no desenvolvimento câncer. A expressão do PCPH é

um evento que ocorre precocemente no desenvolvimento de células tumorais de testículo e possivelmente identifica esta molécula como um bom marcador molecular para neoplasias testiculares (Regadera et al., 2006). Além disso, um possível papel da NTPDase 5 (CD39L4) como uma enzima secretada, que seja responsável pela degradação de nucleotídeos difosfatados em células de W256, pode ser sugerido.

As atividades de hidrólise das NTPDases 2 e 5 podem levar à produção de AMP extracelular, substrato para a ecto-5'-nucleotidase, também expressa em células de W256. Um aumento na expressão bem como na atividade desta enzima tem sido identificado em alguns tipos de células tumorais (Spychala, 2000; Sadej et al., 2006). Além disso, a adenosina, produto da degradação do AMP pela ecto-5'-nucleotidase, tem sido descrita como promotora tumoral (Spychala, 2000) e recentemente foi mostrado que esse nucleosídeo aumenta a proliferação em linhagens de gliomas, possivelmente via receptores A3 (Morrone et al., 2003). Portanto, a formação de adenosina pode beneficiar as células tumorais de W256, devido a seus efeitos na estimulação da angiogênese, no crescimento do tumor e na inibição das respostas imunes junto ao tecido maligno. Talvez esta consideração possa ser generalizada para todos os tumores.

4.3.2 NTPDases e ecto-5'-nucleotidase no tumor subcutâneo de W256

Muitos estudos têm sugerido um importante papel para os nucleotídeos da adenina, no desenvolvimento de tumores (Burnstock, G., 2002; Spychala et al 2004). Com o objetivo de investigar as enzimas envolvidas no catabolismo dos nucleotídeos da adenina durante o crescimento do tumor de W256, foi avaliada a

expressão destas enzimas bem como o padrão de degradação dos nucleotídeos extracelulares no tumor, 6, 10 e 15 dias após a indução subcutânea. Os mRNAs de todas as ectonucleotidases estudadas (NTPDase 1, 2, 3 5 e 6 e ecto-5'-nucleotidase), foram identificados por RT-PCR, em diferentes níveis de expressão durante o desenvolvimento do tumor. Estes resultados demonstram que o tumor de W256 apresenta várias ectonucleotidases com potencial para hidrolisar nucleotídeos extracelulares a seus respectivos nucleosídeos.

A análise quantitativa, realizada por PCR em tempo real, apresentou como genes dominantes expressos durante o crescimento do tumor, as NTPDases 1 e 2 e ecto-5'-nucleotidase (capítulo 4, figura 2). Entretanto, entre os três dias estudados, o aumento mais pronunciado na expressão destas três enzimas ocorreu em 10 dias. Estes resultados indicam que 10 dias deve ser um período crítico para o crescimento do tumor e pode se refletir em uma maior expressão protéica destas enzimas em 15 dias após a indução do tumor. Esta hipótese também é reforçada por uma hidrólise mais rápida do ATP extracelular, observada em 15 dias de crescimento, confrome análise realizada por HPLC (capítulo 4, figura 3).

O padrão de hidrólise do ATP extracelular determinado por HPLC, embora com alguma diferença entre os dias estudados, foi similar. As células do tumor de W256 rapidamente hidrolisaram ATP levando à formação transitória de ADP, que foi completamente hidrolisado a AMP (capítulo 4, figura 3). Estes resultados estão de acordo com a alta expressão dos genes das NTPDases 1 e 2, como demonstrado por PCR em tempo real. Além disso, a degradação seqüencial do ATP a AMP com acúmulo transitório de ADP como produto intermediário durante o

desenvolvimento do tumor de W256, é bastante similar àquele obtido com a co-expressão das ecto-ATPase e ecto-apirase de ratos em células CHO, onde é sugerido que a hidrólise do ATP pela ecto-apirase produz diretamente AMP sem formação de ADP como produto intermediário. A expressão da ecto-ATPase em células CHO por sua vez, libera ADP que acumula no meio (Heine et. al., 1999). Desta forma, os resultados apresentados neste capítulo sugerem que o ATP é metabolizado a AMP considerando a co-expressão de ambas NTPDases 1 e 2, durante o desenvolvimento do tumor.

As ações das NTPDases são conseqüência, pelo menos em parte, de uma atividade fosfoidrolítica em nucleotídeos extracelulares e conseqüente efeito na sinalização via receptores P2, tendo impacto em vários processos pato-fisiológicos, incluindo, adesão, proliferação celular, diferenciação e apoptose (Robson et al., 2006). Além disso, alterações na atividade, distribuição e expressão das NTPDases têm sido descritas em várias condições patológicas incluindo o câncer, que por sua vez, levam à alterações nos níveis de nucleotídeos extracelulares, podendo favorecer o desenvolvimento de tumores. Além do importante papel da NTPDase 2 em processos neoplásicos já discutidos nesta tese, a participação da NTPDase 1 também tem sido descrita em vários tipos de células tumorais, tais como linhagens de melanoma humano, células tumorais de ovário de ratas, câncer de pâncreas humano e carcinoma de mama (Slivinskii et al., 1993; Dzhandzhugazyan et al., 1998; Kittel et al., 2000; Kittel et.al., 2002). A expressão de várias NTPDases com funções e atividades que se sobrepõem não é ainda bem entendida, mas sugere a complexidade do sistema purinérgico no controle de patologias como o câncer.

As NTPDases com localização na superfície celular podem ser diferenciadas quanto à sua preferência por substratos e formação de produtos. Entre as NTPDases identificadas no tumor de W256, a NTPDase 1 hidrolisa ATP e ADP igualmente bem, enquanto a NTPDase 2 tem alta preferência por nucleotídeos trifosfatados. Considerando que níveis elevados de ATP extracelular têm sido observados em muitos tipos de tumores, a ação destas duas enzimas no tumor de W256 poderia estar contribuindo para a redução dos efeitos citotóxicos e apoptóticos do ATP nas células tumorais, e produzindo grandes quantidades de AMP, substrato para a ecto-5'-nucleotidase, que por sua vez pode levar à produção de adenosina (um fator conhecido ter atividade de proliferação tumoral).

A participação da ecto-5'-nucleotidase na hidrólise de nucleotídeos no tumor de W256 também é sugerida, uma vez que esta enzima também apresentou uma elevada expressão nos três dias estudados. Além disso, a hidrólise do ATP extracelular 6,10 e 15 dias após a inoculação do tumor de W256 levou à produção de adenosina, produto da degradação do AMP pela ecto-5'-nucleotidase, e um fator promotor do crescimento tumoral e angiogênese. A geração de adenosina durante o desenvolvimento do tumor de W256 pode ser um dos mecanismos responsáveis pelo rápido crescimento do tumor, devido às atividades promotoras de tumor já descritas para este nucleosídeo.

O tumor subcutâneo de W256 quando inoculado, cresce sem causar distúrbios fisiológicos aparentes por certo período de tempo (5 a 8 dias). Este período é interrompido repentinamente pelo início de uma fase de rápido crescimento do tumor e de significativas alterações metabólicas, que podem levar à morte do animal (Rettori et al., 1995). Estes dados sobre desenvolvimento do

tumor estão de acordo com os resultados encontrados no capítulo 4 desta tese, que mostram uma maior expressão das NTPDases 1 e 2 e ecto-5'-nucleotidase em 10 dias após a inoculação subcutânea do tumor. Portanto, o aumento na expressão destas enzimas, pode levar a uma maior hidrólise dos nucleotídeos da adenina, podendo contribuir para o crescimento rápido do tumor observado neste período.

Conforme dados da análise realisada por RT-PCR, a expressão das ectonucleotidases na forma ascítca do tumor apresentou algumas diferenças em relação ao que foi encontrado no tumor sólido. Enquanto todas as NTDPases estudadas foram expressas, em diferentes níveis, durante o desenvolvimento do tumor sólido, a forma ascítica do tumor revelou a presença das NTPDases 2 e 5 somente. A expressão da ecto-5'-nucleotidase foi encontrada nas duas formas do tumor. Estes dados sugerem que quando as células em ascite são expostas ao crescimento como forma sólida do tumor de W256, estas passam a ter uma necessidade de expressar outras NTPDases que não somente as NTPDases 2 e 5, e a ecto-5'-nucleotidase, como observado pela expressão da NTPDase 1 no tumor sólido e não na forma ascítica. Isto pode ter uma relação com a proliferação das células tumorais que se desenvolvem no tecido subcutâneo.

Além disso, embora todos os membros da família das NTPDases estudados nesta tese tenham sido expressos pelo tumor sólido, não se pode descartar a possibilidade de enzimas como a NTPDase 1, serem oriundas de outros tecidos como por exemplo, vasos sanguíneos que façam parte do tumor.

Assim, em conjunto, estes resultados sugerem que um dos possíveis mecanismos que as células tumorais de W256 utilizam para seu desenvolvimento

e proteção pode ser através da expressão de ectonucleotidases, que regulem os níveis de nucleotídeos no tumor, venham a favorecer o seu crescimento.

4.3.3 NPPs no Tumor de W256

As NPPs influenciam muitos processos fisiológicos, e disfunções nestas enzimas tem mostrado estarem relacionadas com a fisiopatologia de várias doenças (Goding et al., 2003). Fazem parte de uma família de ectonucleotidases com propriedades catalíticas comparáveis e são capazes de hidrolisar 3'-5'-cAMP a AMP, ATP a AMP e PP_i, ADP a AMP e P_i, NAD^+ a AMP e nicotinamida mononucleosídeo, e diadenosina polifosfato Ap_nA a $Ap_{n-1}A$ e AMP. Também podem hidrolisar ligações fosfodiéster de ácidos nucléicos (Zimmermann, 2001). A investigação de uma possível participação das NPPs, como membros das ectonucleotidases envolvidas no controle dos níveis de nucleotídeos extracelulares nas células tumorais de W256, vem sendo realizada, e os resultados já obtidos com a análise molecular são apresentados no anexo desta tese.

O mRNA das NPP1, NPP2 e NPP3 nas células tumorais de W256 mantidas em ascite foi investigado por RT-PCR, e dos três membros da família das NPPs analisados, somente a NPP1 não foi detectada (Anexo, figura 1). A análise por RT-PCR também sugere uma maior expressão da NPP3 em relação à NPP2. Para confirmar este resultado, foi realizada uma curva de ciclos para quantificar a expressão destas enzimas (Anexo, figura 2). De fato, em células tumorais de

W256, dentre os membros da família das NPPs estudados, a NPP3 é a mais expressa.

A expressão das NPPs 1, 2 e 3 durante o desenvolvimento do tumor sólido de W256 também foi investigada 6, 10 e 15 dias após a inoculação subcutânea das células tumorais. A detecção do mRNA das enzimas por RT-PCR revelou que a NPP3 foi expressa em todos os dias estudados. As NPPs 1 e 2 foram expressas somente em 6 e 10 dias, enquanto que aos 15 dias de crescimento do tumor, o sinal foi quase imperceptível para ambas as enzimas.

Para quantificar os níveis de expressão destas enzimas durante o desenvolvimento do tumor subcutâneo de W256, e correlacionar com os dados de expressão das enzimas por RT-PCR, os genes das NPPs 1, 2 e 3 foram analisados por PCR em tempo real, 6, 10 e 15 dias após a inoculação das células tumorais. Os tumores apresentaram como dominante, o gene da NPP3 em todos os dias estudados (Anexo, figura 4), confirmando o padrão de expressão da enzima detectado por RT-PCR. Além disso, em 10 dias, período em que o tumor se desenvolve de forma mais rápida, houve uma superexpressão desta enzima, assim como observado para as NTPDases 1 e 2 e ecto-5'-nucleotidase, também estudadas durante o desenvolvimento do tumor (capítulo 4). Estes resultados sugerem um importante papel destas ectonucleotidases nesta fase, controlando os níveis de nucleotídeos ao redor do tumor e favorecendo o seu desenvolvimento.

É importante observar que os resultados obtidos com a análise através do PCR em tempo real, onde há uma maior expressão da NPP3 no desenvolvimento do tumor sólido, estão de acordo com os dados encontrados para as células tumorais na forma ascítica. Nestas células, a enzima NPP3 também é a mais

expressa entre os três membros da família das NPPs estudados, como demonstrado pela curva de ciclos.

De acordo com estes resultados, dados da literatura têm mostrado o envolvimento destas enzimas em células tumorais. A NPP3 tem sido descrita como promotora de invasão tumoral, e está associada com a carcinogênese do câncer de cólon humano (Yano et al., 2003). Além disso, está envolvida na infiltração de células neoplásicas de carcinoma ductal de bile (Yano et al., 2004). O aumento dos níveis desta enzima na forma solúvel, em soro de pacientes com estas duas neoplasias sugere um possível papel como marcador das mesmas (Yano et al., 2003; Yano et al., 2004).

A NPP2 também tem sido detectada em muitos tumores, incluindo carcinoma hepatocelular, neuroblastoma, carcinoma prostático, câncer de pulmão (Goding et al., 2003). Esta enzima foi identificada pela primeira vez em um meio condicionado de células de melanoma, como uma proteína estimulante da motilidade tumoral, foi denominada de autotaxina (Stracke et al., 1992). Em células NIH3T3 *ras*-transfectadas, a NPP2 aumenta o potencial invasivo e metastático (Nam et al., 2000) além de apresentar propriedades angiogênicas (Nam et al., 2001). Ainda, esta enzima é secretada por vários tipos de células tumorais, incluindo câncer de pele, pulmão e mama (Stefan et al., 2006).

Os efeitos estimulatórios da NPP2 no crescimento e motilidade de tumores têm sido atribuídos à sua abilidade em produzir ácido lisofisfatídico (LPA) (Stefan et al., 2006). Entretanto, estes efeitos também podem ser devido à sua abilidade de hidrolisar nucleotídeos. Tumores sólidos são conhecidos por liberarem

nucleotídeos da adenina, incluindo ATP, um inibidor da proliferação tumoral, que pode ser hidrolizado pela NPP2.

Em conclusão, os dados apresentados nesse capítulo sugerem a participação da NPP2 e principalmente da NPP3 como enzimas expressas pelas células tumorais de W256, e importantes para o desenvolvimento do tumor. Entretanto, são necessários dados complementares a respeito do papel destas enzimas como parte do conjunto de ectonucleotidases responsáveis pelo controle dos nucleotídeos extracelulares no tumor de W256. Resultados complementares estão sendo obtidos, mas ainda não estão concluídos.

4.4 Hipótese proposta para o envolvimento dos nucleotídeos da adenina na circulação e no tumor de W256 em ratos

Os resultados obtidos nos quatro capítulos e no anexo dessa tese, juntamente com dados da literatura e resultados em preparação no nosso laboratório, sugerem que o ATP extracelular e a adenosina possam ser substâncias que atuem de forma antagônica no desenvolvimento do tumor de W256. Considerando que o ATP é reconhecido como um composto citotóxico em células tumorais, e que adenosina tem sido considerada promotora de tumor e estimuladora de angiogênese, é possível sugerir que, na circulação, a significativa redução na hidrólise dos nucleotídeos da adenina em plaquetas e soro após a indução do tumor pode representar um mecanismo de proteção contra o desenvolvimento tumoral. Por outro lado, nas células tumorais de W256, a elevada expressão de ectonucleotidases envolvidas na hidrólise do ATP e do AMP, bem

como a rápida hidrólise do ATP, pode representar um mecanismo que, ao contrário do que pode estar ocorrendo na circulação, facilita a proliferação e invasão das células tumorais. Assim, este estudo nos permite também identificar possíveis alvos para o desenvolvimento de uma nova classe de drogas que possam ser utilizadas em terapias anti-neoplásicas e que auxiliem no controle do desenvolvimento de tumores.

5 CONCLUSÕES

1) A hidrólise dos nucleotídeos da adenina por plaquetas de ratos foi significativamente inibida pelo AAS, tanto para ATP quanto para ADP, mas não houve alteração na hidrólise do AMP.
2) Nenhuma alteração significativa nas atividades de hidrólise do ATP, ADP e AMP em soro de ratos foi observada em presença do AAS.
3) Ratos submetidos ao tumor de W256, apresentaram uma redução significativa na hidrólise dos nucleotídeos da adenina em plaquetas e soro, obtidos 6, 10 e 15 dias após a indução subcutânea do tumor.
4) As células tumorais de W256 na forma ascítica hidrolisam ATP, ADP e AMP em um pH ótimo que varia de 6,5 a 8,0 e requerem cátions divalentes para a sua atividade de hidrólise, com uma preferência para Mg^{2+} maior do que para Ca^{2+}. Estas células expressam o mRNA das NTPDases 2 e 5, e também da ecto-5'-nucleotidase.
5) Os mRNAs de todos os membros da família das NTPDases estudados (1, 2, 3, 4, 5 e 6) e da ecto-5'-nucleotidase/CD73 foram detectados, em diferentes níveis de expressão, durante o desenvolvimento do tumor de W256
6) Durante o desenvolvimento do tumor subcutâneo de W256, nos três tempos estudados, os genes dominantes expressos foram das NTPDases 1 e 2 e ecto-5'-nucleotidase.

7) O Tumor de W256 durante seu desenvolvimento apresenta um padrão similar do metabolismo do ATP, e compatível com a co-expressão das NTPDases 1 e 2 nas células tumorais.
8) Entre os membros da família das NPPs, a NPP3 foi a mais expressa tanto nas células da forma ascítica do tumor quanto durante o desenvolvimento do tumor subcutâneo, tendo sua maior expressão observada 10 dias após a indução tumoral.
9) A inibição na hidrólise dos nucleotídeos da adenina em plaquetas e soro de ratos submetidos ao tumor de W256, sugere que na circulação este efeito representar um mecanismo de proteção contra o desenvolvimento tumoral. Por outro lado, a expressão de ectonucleotidases envolvidas na hidrólise destes nucleotídeos e a rápida hidrólise do ATP observada durante o crescimento do tumor de W256, permitem sugerir que há um efeito contrário ao da circulação, que pode facilitar o desenvolvimento do tumor.

6 PERSPECTIVAS

1) Estudar o efeito *in vivo* do AAS, dos nucleotídeos da adenina e da adenosina no crescimento do tumor subcutâneo de W256 e na forma ascítica do tumor
2) Analisar o efeito do AAS, dos nucleotídeos da adenina e da adenosina no crescimento de culturas de células de W256
3) Determinar as propriedades cinéticas e bioquímicas das NPPs nas células de W256
4) Estudar a presença dos receptores $P2X_7$ no tumor de W256
5) Estudar marcadores de stress oxidativo em ratos submetidos ao tumor de W256.

7 PRODUÇÃO CIENTÍFICA NO DOUTORADO

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