

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
DEPARTAMENTO DE BIOQUÍMICA PROFESSOR TUISKON DICK
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

**SISTEMA PURINÉRGICO E A PROGRESSÃO DOS GLIOMAS: AVALIAÇÃO
DE PARÂMETROS PROLIFERATIVOS E INFLAMATÓRIOS**

ELIZANDRA BRAGANHOL

Orientadora

DRA. ANA MARIA OLIVEIRA BATTASTINI

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas –
Bioquímica, como requisito parcial à obtenção do grau de Doutor.

Porto Alegre

2010

DEDICO

Àqueles que sempre me apoiaram:

meus pais Waldir e Cedina,

meu marido Jose Emilio.

*”Feliz aquele que transfere o que sabe
e aprende o que ensina.”*

Cora Coralina

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SUMÁRIO

LISTA DE ABREVIATURAS	IX
LISTA DE FIGURAS	XIV
LISTA DE TABELAS	XV
RESUMO	XVI
ABSTRACT	XVII
APRESENTAÇÃO	XVIII
1. INTRODUÇÃO	1
1.1. Gliomas	1
1.1.1 Aspectos gerais.....	1
1.1.2 Glioblastoma multiforme	4
1.1.3. Alterações moleculares	5
1.1.4 Gliomas e a inflamação	7
1.2. Sistema Purinérgico	11
1.2.1 Nucleotídeos extracelulares.....	11
1.2.2 Receptores purinérgicos	14
1.2.3 Família das ectonucleotidasés	18
1.2.3.1 E-NTPDases	19
1.2.3.2 Ecto-5'-nucleotidase/CD73	22
1.2.4 Sistema purinérgico e os tumores.....	24
1.3. E-NTPDases e gliomas: a hipótese do estudo	26
2. OBJETIVOS	28
2.1. Objetivo geral	28

2.2. Objetivos específicos	28
3. RESULTADOS	29
3.1 Artigo 1: A comparative study of ectonucleotidases and P2 receptor mRNA profiles in C6 cell line cultures and C6 <i>ex vivo</i> glioma model.....	29
3.2 Artigo 2: Selective NTPDase2 expression modulates <i>in vivo</i> rat glioma growth.....	40
3.3 Artigo 3: Selective NTPDase2 overexpression in gliomas drives inflammation, platelet activation and metastasis.....	50
3.4 Artigo 4: Extracellular nucleotides control IL-8 and MCP-1 secretion in U251MG glioma cell line.....	81
3.5 Artigo 5: Study of plasma membrane bound NTPDases and ecto-5'-nucleotidase/CD73 in rat and human brain tumors.....	118
4. DISCUSSÃO.....	146
5. CONCLUSÕES.....	167
5.1. Conclusão geral	167
5.2. Conclusões específicas	167
6. PERSPECTIVAS	169
7. REFERÊNCIAS BIBLIOGRÁFICAS.....	171
8. ANEXOS.....	195
8.1 Outros artigos realizados em coautoria durante o periodo do doutorado	195
8.2. Instruções das revistas aos autores para submissão de artigo.....	198
8.2.1 Artigo 3: Cancer Science.....	199
8.2.2 Artigo 4: European Journal of Immunology	207
8.2.3 Artigo 5: BMC Cancer	214

LISTA DE ABREVIATURAS

AKT/PKB - proteína cinase B (*Protein Kinase B*)

ADP – adenosina difosfato (*Adenosine diphosphate*)

AMP – adenosina monofosfato (*Adenosine monophosphate*)

ATP - adenosina trifosfato (*Adenosine Triphosphate*)

Bcl-2 - linfoma de células B 2 (*B-cell lymphoma 2*)

CCND1 – ciclina D1 (*Cyclin D1*)

CCND2 – ciclina D2 (*Cyclin D2*)

CCND3 - ciclina D3 (*Cyclin D3*)

CDKN2A (p14^{ARF}; p16^{INK4A}; INK4A): inibidor da ciclina dependente de quinase 2A (*cyclin-dependent kinase inhibitor 2A*)

CDK4 - ciclina dependente de cinase 4 (*Cyclin-Dependent Kinase 4*)

CDK6 - ciclina dependente de cinase 6 (*Cyclin-Dependent Kinase 6*)

CDP – citidina difosfato (*Cytidine diphosphate*)

c-Myc – oncogene viral v-myc mielocitomatose (*v-myc myelocytomatosis viral oncogene homolog*)

CNTF- fator de crescimento neurotrófico ciliar (*ciliary neurotrophic factor*)

CTP – citidina trifosfato (*cytidine triphosphate*)

DAB - 3,3'-tetra-hidrocloreto de diaminobenzidina (*3,3'-diaminobenzidina tetrahydrochloride*)

E-ATPases – ecto-ATPases

EC₅₀ - é definida como a concentração de um agonista capaz de produzir metade da resposta máxima que aquele agonista pode gerar numa determinada estrutura biológica.

Ecto-5'-NT/CD73- ecto-5'-nucleotidase

EGF- fator de crescimento epidermal (*Epidermal Growth Factor*)

EGFR - receptor de fator de crescimento epidermal (*Epidermal Growth Factor Receptor*)

E-NPPS – ecto- nucleotídeo pirofosfatase/fosfodiesterase (*Ectonucleotide pyrophosphatase/phosphodiesterase*)

E-NTPDase- ecto-nucleosídeo trifosfato difosfohidrolase

E-NTPDase1/CD39 - ecto-nucleosídeo trifosfato difosfohidrolase 1

ERK- MAPK regulada extracelularmente (*extracellular regulated MAPK*)

F-ATPase- F₀F₁ –ATPase

FA- fosfatase alcalina (*Alcaline phosphatase*)

FGF- fator de crescimento de fibroblasto (*Fibroblast growth factor*)

FasL – Ligante Fas (*Fas ligand; TNF superfamily, member 6*)

GBM - Glioblastoma Multiforme

G-CSF - fator de estimulação de colônias de granulócitos (*Granulocyte - Colony Stimulating Factor*)

GDP – guanosina difosfato (*guanosine diphosphate*)

GFAP - proteína glial fibrilar ácida (*Glial Fibrillary Acidic Protein*)

GPI- glicosil-fosfatidilinositol (*glycosil phosphatidylinositol*)

GRO α - oncogene alfa regulador do crescimento (*Growth regulated oncogene-alpha*)

GTP - guanosina trifosfato (*guanosine triphosphate*)

HDM2 (ou MDM2)- regulador negativo da p53 (*human double-minute 2*)

HGF – fator de crescimento de hepatócitos (*hepatocyte growth factor*)

HIF-1 – fator 1 induzido por hipóxia (*hypoxia inducible factor 1*)

IDP – inosina difosfato (*inosine diphosphate*)

IGF-1 - fator de crescimento semelhante a insulina (*insulin-like growth factor 1*)

IL-2 - interleucina-2 (*Interleukin-2*)

IL-6 - interleucina-6 (*Interleukin-6*)

IL-4- interleucina-4 (*Interleukin-4*)

IL-8/CXCL8- interleucina-8 (*Interleukin-8*)

IL-10- interleucina-10 (*Interleukin-10*)

IL-12- interleucina-12 (*Interleukin-12*)

IL-14- interleucina-14 (*Interleukin-14*)

IL-1 β - interleucina-1 beta (*Interleukin-1beta*)

INF γ - interferon gama (*interferon gamma*)

ITP – inosina trifosfato (*inosine triphosphate*)

LOH - Perda de heterozigidade (*loss of heterozygosity*)

LPS – lipopolissacarídeo (*Lipopolysaccharide*)

MAPK - proteína cinase ativada por mitógenos (*Mitogen-Activated Protein Kinase*)

MCP-1 – proteína quimiotática de monócitos (*monocyte chemotactic protein 1*)

MEK - proteína cinase ativada por mitógenos ativadora de ERK (*Mitogen-Activated ERK-activating Kinase*)

MIF – fator inibitório de migração de macrófagos (*macrophage migration inhibitory factor*)

MIP – proteína inflamatória de macrófagos (*macrophage inflammatory protein*)

NF1 - neurofibromina (*neurofibromin 1*)

NFAT - fator nuclear de células T ativadas (*Nuclear Factor of Activated T cells*)

NF-κB- fator nuclear kappa (*nuclear factor-kappaB*)

OMS - Organização Mundial da Saúde

P-ATPase- E₁-E₂- ATPase

P1- receptor purinérgico metabotrópico para adenosina, dividido em quatro subtipos: A₁, A_{2a}, A_{2b} e A₃.

P2X- receptor purinérgico ionotrópico

P2Y- receptor purinérgico metabotrópico

p53- gene supressor tumoral (*tumor protein p53*)

PDGF- platelet-derived growth factor, fator de crescimento derivado de plaquetas

PDGFR- platelet-derived growth factor receptor, receptor do fator de crescimento derivado de plaquetas

PGE₂ – prostaglandina E₂ (*prostaglandin E2*)

PI3K - fosfatidilinositol 3-quinase (*phosphatidylinositol 3-kinase*)

PLA₂ - fosfolipase A₂ (*phospholipase A2*)

PLC- fosfolipase C (*phospholipase C*)

PLD - fosfolipase D (*phospholipase D*)

PTEN - homólogo fosfatase e tensina deletado do cromossomo 10 (*Phosphatase and tensin homologue deleted on chromosome 10*)

PI3K- fosfatidilinositol 3-quinase

PKC- proteína quinase C (*protein kinase C*)

RAF- raf protooncogene (*serine/threonine protein kinase*)

RAS- família de proteínas envolvidas no controle do crescimento celular (*RAS protein*)

RB- proteína retinoblastoma (*retinoblastoma susceptibility protein*)

RhoA – família de genes homólogos a Ras, membro A (*ras homolog gene family, member A*)

SNC- Sistema Nervoso Central

TGF- α - fator alfa de transformação do crescimento (*Transforming growth factor alpha*)

TGF- β - fator beta de transformação do crescimento (*Transforming growth factor beta*)

TLR – receptores similares a *Toll* (*Toll-like receptors*)

TNF- α - fator de necrose tumoral- alfa (*Tumor Necrosis Factor alpha*)

UDP – uridina difosfato (*uridine diphosphate*)

UTP - uridina trifosfato (*uridine triphosphate*)

V-ATPases – ATPases vacuolares (*vacuolar -ATPase*)

VEGF - fator de crescimento vascular endotelial (*Vascular Endothelial Growth Factor*)

LISTA DE FIGURAS

Figura 1. Relação entre sobrevivência média, características histológicas e alterações genéticas associadas a cada tipo de tumor.....	3
Figura 2. Principais vias de sinalização envolvidas na patogênese dos gliomas.....	7
Figura 3. Possíveis interações entre células neoplásicas, macrófagos e microglia nos tumores cerebrais.....	8
Figura 4. As vias de transdução de sinal dos receptores TLR3 e TLR4 e a consequente produção de citocinas.....	9
Figura 5. Modelo de como as células tumorais “educam” as células imunes a desempenhar funções de estímulo a progressão tumoral.....	11
Figura 6. Receptores purinérgicos.....	17
Figura 7. Topografia de membrana das diferentes famílias de enzimas que compõem o grupo das ectonucleotidases.....	18
Figura 8. Metabolismo extracelular do ATP e o controle da ativação dos receptores purinérgicos.....	24

LISTA DE TABELAS

Tabela 1. Nomenclatura e preferência por substratos dos membros da família E-NTPDase em vertebrados.....	22
Tabela 2. Localização cromossomal dos genes que codificam a expressão das NTPDase1-3 e da ecto-5'-NT/CD73 e a possível correlação com a expressão de genes envolvidos com o câncer.....	166

RESUMO

Os nucleotídeos extracelulares modulam uma variedade de ações biológicas via ativação de receptores purinérgicos. Esses efeitos são controlados pela ação de ectonucleotidases, tais como as E-NTPDases e a ecto-5'-NT/CD73, as quais hidrolisam o ATP até adenosina no meio extracelular. Alterações no sistema purinérgico podem estar envolvidas na progressão dos gliomas. O ATP induz a proliferação celular dos gliomas e, ao contrário dos astrócitos, gliomas apresentam baixa expressão das E-NTPDases, particularmente da NTPDase2. Assim, a restauração da atividade ATPásica nesses tumores poderia ser uma estratégia para prevenir as ações mediadas pelo ATP. De acordo com essa idéia, a coinjeção de apirase/NTPDase1, uma enzima depletora de ATP, e gliomas diminuiu a progressão tumoral *in vivo*. O objetivo desse estudo foi aprofundar a participação dos receptores purinérgicos e das E-NTPDases na progressão dos gliomas. A análise das culturas de linhagem de glioma C6 e de C6 *ex vivo* revelou similaridade na expressão de receptores purinérgicos e das E-NTPDases, sugerindo que alterações nessa via estariam presentes em culturas primárias de gliomas. Ao contrário do esperado, o restabelecimento da expressão da NTPDase2 nos gliomas resultou em aumento da malignidade tumoral. A superexpressão da NTPDase2 aumentou a adesão das células C6 em cultura e em experimentos *in vivo* promoveu dramático aumento do crescimento tumoral e presença de alterações histológicas nos pulmões sugestivas de metástase. Esses eventos foram relacionados ao acúmulo de ADP extracelular, recrutamento de plaquetas para a área tumoral e pulmonar, aumento de angiogênese e da resposta inflamatória, denotado pela presença de infiltrado leucocitário nos gliomas e aumento sérico das citocinas IL-1 β , TNF- α e IL-6. Além disso, os receptores purinérgicos modularam a secreção espontânea e estimulada por TLR de IL-8 e MCP-1, bem como a proliferação celular induzida por LPS em linhagem de glioma humano. Finalmente, a análise da expressão das ectonucleotidases em gliomas de ratos e de humanos revelou ausência das NTPDase1 e 2, baixa expressão da NTPDase3 e expressão elevada da ecto-5'-NT/CD73, indicando que a alteração no padrão de expressão das ectonucleotidases também é uma característica de tumores humanos. Os resultados dessa tese reforçam a hipótese do envolvimento do sistema purinérgico na progressão dos gliomas e sugerem esse sistema como um alvo interessante no combate dos tumores cerebrais.

ABSTRACT

Extracellular nucleotides modulate a variety of biological actions via purinergic receptor activation. These effects are modulated by ectonucleotidases, such as E-NTPDases and ecto-5'-NT/CD73, which hydrolyze ATP to adenosine in the extracellular milieu. Disruption on purinergic signaling has been involved in glioma progression. ATP induces glioma cell proliferation and, in opposite to astrocytes, gliomas have low expression of all NTPDases, particularly NTPDase2. Therefore, the restoration of the ATPase activity to gliomas could be a strategy to overcome the ATP mediated effects. Accordingly, the co-injection of apyrase/NTPDase1, an ATP scavenger, with gliomas decreases the glioma progression *in vivo*. The objective of this study was to better evaluate the purinergic receptor and E-NTPDase participation on glioma progression. The analysis of C6 cell line and C6 *ex vivo* cultures revealed similarity in the purinergic receptor and E-NTPDase expression, suggesting that disruption in the purinergic pathway is a characteristic exhibited by primary glioma cell cultures. Surprisingly, the NTPDase2 restoration to C6 gliomas resulted in increased tumor malignity. The NTPDase2 over-expression increased the C6 cell adhesion in culture and in *in vivo* experiments it promoted dramatic glioma growth and induced histological alterations in lung tissue suggestive of metastasis. These events were related to extracellular ADP accumulation, platelets recruitment to glioma and lung tissues, increase of angiogenesis and inflammation. Additionally, it was observed an increase of leukocyte infiltration in the implanted tumors and in IL-1 β , TNF- α e IL-6 serum levels. Moreover, the purinergic receptors modulated the basal and TLR stimulated IL-8 and MCP-1 release and the LPS induced cell proliferation in a human glioma cell line. Finally, the analysis of ectonucleotidases expression in rat and human gliomas showed the absence of NTPDase1 and 2, low expression of NTPDase3 and high expression of ecto-5'-NT/CD73, indicating that alterations in the ectonucleotidase expression pattern is a characteristic also exhibited by human tumors. In conclusion, these results reinforce the hypothesis of purinergic signaling involvement in glioma progression and point to this pathway as an interesting target for glioma treatment.

APRESENTAÇÃO

Esta tese está organizada em seções dispostas da seguinte maneira: Introdução, Objetivos, Resultados, Discussão, Conclusões, Perspectivas, Referências Bibliográficas e Anexos.

A Introdução apresenta o embasamento teórico que nos levou a formular as propostas da tese, as quais estão determinadas na seção Objetivos.

A seção Resultados contém os artigos científicos publicados e os trabalhos a serem submetidos, assim como os materiais, os métodos e as referências bibliográficas específicas de cada artigo.

A seção Discussão contém uma interpretação geral dos resultados obtidos nos diferentes artigos.

A seção Conclusões aborda as conclusões gerais obtidas na tese.

A seção Perspectivas aborda as possibilidades de desenvolvimento de projetos a partir dos resultados obtidos, dando continuidade a essa linha de pesquisa.

A seção Referências Bibliográficas lista as referências utilizadas na Introdução e Discussão da tese.

A seção Anexos contém uma lista de outros trabalhos realizados em co-autoria durante todo o período de desenvolvimento do doutorado e as instruções das revistas aos autores para a preparação e submissão de artigos.

1. INTRODUÇÃO

1.1 Os Gliomas

1.1.1 Aspectos Gerais

Os gliomas constituem um grupo de tumores cerebrais geneticamente e histologicamente heterogêneo, associado à significativa mortalidade e morbidade (Prados and Levin, 2000). Aproximadamente 18.000 novos casos são registrados anualmente nos Estados Unidos da América (USA) com aproximadamente 13.000 mortes/ano (Jemal *et al.*, 2006). Apesar da prevalência em adultos, os gliomas são os tumores sólidos mais comuns em crianças (Maher and Raffel, 2004). Infelizmente, a incidência desses tumores têm aumentado ao longo dos últimos 30 anos (Jemal *et al.*, 2006) e o prognóstico para os pacientes permanece muito ruim (Konopka and Bonni, 2003). Apesar de intensos esforços em desenvolver novas terapias, agentes efetivos ainda não estão disponíveis, tornando o tratamento limitado à cirurgia, quando possível, seguido de radio/quimioterapia (Butowski *et al.*, 2006). A sobrevida média após o diagnóstico é de até cinco anos, porém, pacientes diagnosticados com glioma de alto grau sucumbem à doença em apenas 6-12 meses (Huncharek and Muscat, 1998).

Os gliomas apresentam características morfológicas e expressão gênica similares à glia, astrócitos e oligodendrócitos, suportes funcionais dos neurônios (Chintala *et al.*, 1999). Ao longo dos anos, muitas investigações têm sido desenvolvidas com o objetivo de identificar os mecanismos celulares e moleculares envolvidos na patogênese dos gliomas. Embora a transformação neoplásica de células gliais diferenciadas seja tradicionalmente assumida como um mecanismo da gliomagenese, recentemente foi demonstrado que células-tronco neurais podem estar envolvidas nesse processo (Kondo, 2006). As células-tronco neurais são reguladas pelas mesmas vias de sinalização que estão ativas em tumores

cerebrais. Conseqüentemente, elas são capazes de exibir comportamento característico aos gliomas, incluindo elevada motilidade, associação com vasos sanguíneos, desenvolvimento de fenótipos antigênicos imaturos como a expressão de nestina e do marcador de células tronco CD133, além da ativação de vias sinalizadoras de crescimento e proliferação celular (Shoshan *et al.*, 1999; Palmer *et al.*, 2000; Doetsch *et al.*, 2002). Além disso, as células-tronco neurais potencialmente podem se diferenciar em células neuronais, astrogliais e oligodendrogliais, gerando gliomas compostos por mais de um tipo celular como por exemplo, o oligoastrocitoma. Dessa forma, os gliomas são constituídos por populações de células diferenciadas e uma minoria de células tumorigênicas indiferenciadas multipotentes que varia em torno de 1-30%. Embora ambas contenham mutações oncogênicas que poderão contribuir para a tumorigênese, somente as células-tronco neurais têm capacidade de se autorenovar, sendo possivelmente as responsáveis pela sustentação e propagação do tumor (Sanai *et al.*, 2005).

Uma das características mais marcantes do GBM é o seu elevado grau de proliferação e invasibilidade. Como conseqüência, pacientes com esse tipo de tumor apresentam um grande comprometimento do tecido nervoso periférico ao tumor com o desenvolvimento de sintomas que incluem cefaléia, mudanças cognitivas, papiloedema e convulsões (Girolami, 2000). Gliomas são usualmente detectados por tomografia computadorizada e por ressonância magnética e são usualmente classificados histologicamente, imunistologicamente ou ultraestruturalmente como astrocitomas, oligodendrogliomas ou oligoastrocitoma. O grau de malignidade dos gliomas é determinado seguindo o sistema de classificação de tumores cerebrais da Organização Mundial da Saúde (OMS) que utiliza quatro graus para descrever os gliomas, baseados na malignidade celular, nas características invasivas e na capacidade de desenvolver necrose

(Chintala *et al.*, 1999). Os tumores de grau I (astrocitoma pilocítico) são biologicamente benignos e podem ser eliminados por ressecção cirúrgica; os tumores de grau II (astrocitoma de baixo grau) constituem malignidades de baixo grau, porém tendem a infiltrar-se difusa e rapidamente no parênquima cerebral ao redor do tumor, tornando difícil a sua remoção completa por ressecção cirúrgica; os tumores de grau III (astrocitoma anaplástico) exibem aumento das características anaplásicas e proliferativas, podendo progredir rapidamente a glioblastoma; os tumores de grau IV (GBM) são letais e exibem características de extrema malignidade, incluindo proliferação vascular, necrose e resistência a radio/quimioterapia. A figura 1 mostra a relação entre tipo tumoral, sobrevida e características histológicas e genéticas associadas.

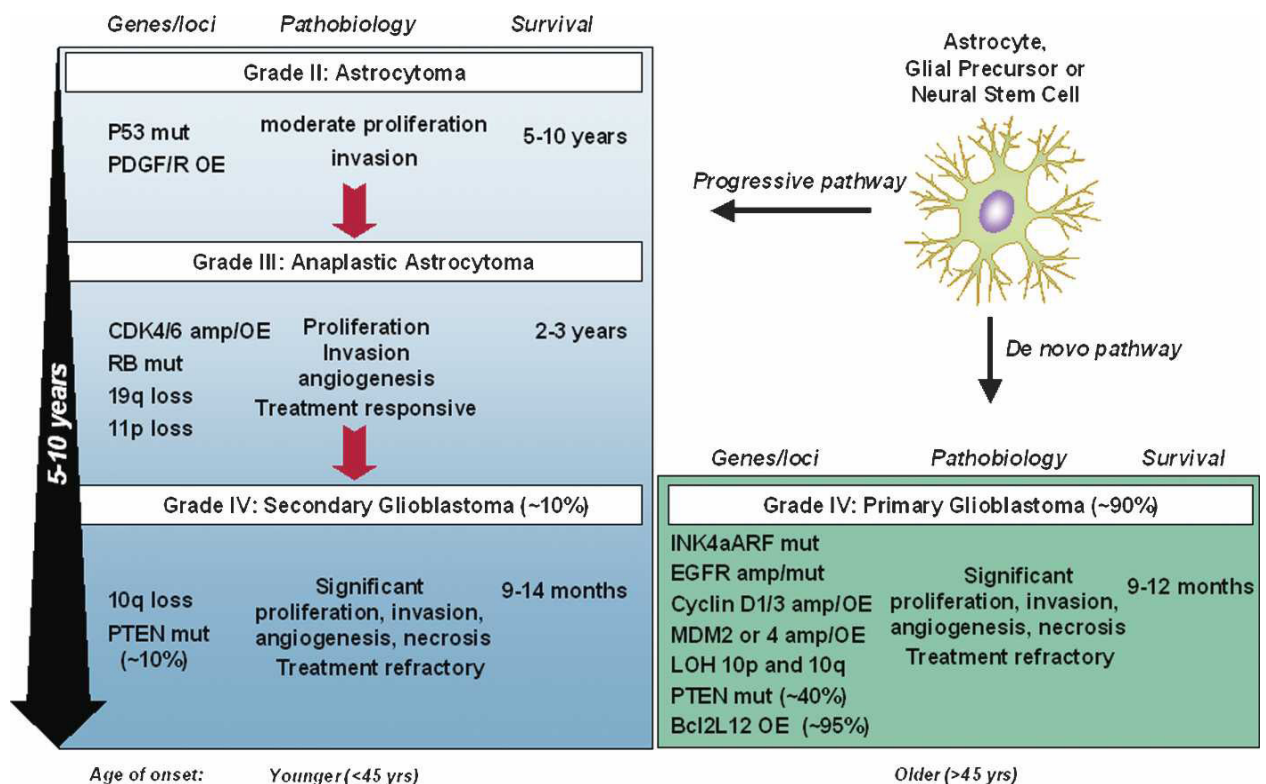


Figura 1. Relação entre sobrevivência média, características histológicas e alterações genéticas associadas a cada tipo de tumor (adaptado de Furnari *et al.*, 2007).

1.1.2 Glioblastoma multiforme

O GBM é o tumor cerebral primário mais comum e devastador (grau IV), caracterizado por descontrole da proliferação celular, infiltração difusa no parênquima cerebral, presença de necrose, angiogênese, intensa resistência a apoptose e elevada instabilidade genômica (Laws and Shaffrey, 1999).

Glioblastomas são quase sempre infiltrativos e comumente apresentam características multifocais (Dai and Holland, 2001). Além disso, esses tumores apresentam significativa heterogeneidade intratumoral nos níveis citopatológicos, transcricionais e genômicos. Microscopicamente, o tumor consiste de diferentes tipos celulares: células próprias do glioma, células endoteliais hiperproliferativas, macrófagos e células normais de áreas do cérebro que estão sendo invadidas pelo glioma. Essa complexidade, combinada a presença de uma subpopulação de células tronco tumorais e a variabilidade das alterações genéticas envolvidas na patogênese dos GBM, tem contribuído para tornar esse câncer um dos mais difíceis de entender e de tratar (Furnari *et al.*, 2007). Entre as características histológicas mais comuns do GBM, incluem-se regiões de necrose rodeadas por células de arquitetura pseudopaliçada; vasos sanguíneos hipertrofiados no interior e em áreas adjacentes ao tumor; grande variabilidade de tamanho e formato de núcleo (pleiomorfismo nuclear). O GBM pode tanto ter origem *de novo*, como ser resultado da progressão de um glioma de baixo grau. Apesar do GBM ser um tumor extremamente angiogênico e invasivo, esses tumores raramente fazem metástases, apresentando uma incidência menor que 2% dos casos (Smith *et al.*, 1969; Piccirilli *et al.*, 2008). Entre as possíveis razões para a baixa propensão dos GBMs em metastizar para órgãos distantes do SNC estão incluídas a ausência de circulação linfática no cérebro e a falta de comunicação entre a barreira hematoencefálica e os espaços intra- e extracraniais.

O tratamento inicial para esses tumores é a cirurgia, associada à radioterapia e seguida de quimioterapia, tendo essa última grande limitação devido às restrições impostas pela barreira hematoencefálica (Brandes *et al.*, 2000). Além disso, o tratamento cirúrgico dos gliomas fica comprometido pela natureza invasiva dessas células tumorais, sendo a infiltração no tecido cerebral adjacente um fator limitante da ressecção cirúrgica (Grobben *et al.*, 2002). Dessa forma, a recorrência do tumor é quase inevitável, uma vez que a remoção cirúrgica do tumor invariavelmente deixa no tecido normal uma população de células tumorais. Quase todos os pacientes com GBM morrem devido à sua patologia e apresentam sobrevida média de 12 meses (Dai and Holland, 2001).

1.1.3 Alterações moleculares

Alterações na expressão de muitos genes e anormalidades cromossômicas são comumente encontradas em gliomas e, na maioria dos casos, estão correlacionados com o grau clínico do tumor.

Em gliomas de menor grau, diversos fatores de crescimento, tais como: fator de crescimento derivado de plaquetas (PDGF), fator de crescimento de fibroblasto 2 (FGF2) e fator de crescimento neurotrófico ciliar (CNTF), bem como os seus respectivos receptores geralmente estão superexpressos e a p53, proteína supressora tumoral, freqüentemente mutada. Gliomas de grau III (anaplásticos), adicionalmente apresentam um descompasso do ciclo celular, devido a deleção da CDKN2A, amplificação da ciclina dependente de cinase 4 (CDK4), ou perda do gene do retinoblastoma (RB). Os gliomas de grau IV (GBM) apresentam todas as alterações já citadas anteriormente, somadas a perda da região 10q22 – 25, porção cromossômica que carrega importantes genes supressores tumorais, entre os quais a PTEN (proteína fosfatase e homóloga a tensina) (Holland, 2001).

A perda da expressão da PTEN resulta em ativação constitutiva da AKT, uma das vias centrais de sobrevivência celular (Holland, 2001). Tumores que expressam maiores níveis de PTEN estão correlacionados com um melhor prognóstico para os pacientes, o que sugere que a PTEN ocupa um papel decisivo na gliomagenese (Lin *et al.*, 1998). A amplificação e ativação de mutações no gene que codifica o receptor do fator de crescimento epidermal (EGFR), também têm sido observadas. Quase todas essas mutações resultam em desregulação da parada do ciclo celular.

Outra característica importante que difere o GBM dos tumores de menor grau é a extensiva angiogênese. Muitas evidências sugerem que a angiogênese associada aos tumores não é somente uma adaptação à hipóxia, mas sim é o resultado de mutações genéticas que ativam o programa transcricional da angiogênese, que é posteriormente modulado pela disponibilidade de oxigênio na área tumoral (Folkman, 2007). Entre as mutações mais relevantes estão incluídas os genes que codificam *PTEN*, *EGFR* e *CMYC* e essas alterações têm como principal consequência a estabilização do HIF-1 e dos seus efetores como VEGF, PDGF ou IL-8 (Blum *et al.*, 2005; Phung *et al.*, 2006; Shchors *et al.*, 2006).

A soma dos efeitos provocados por alterações das vias de controle de proliferação e sobrevivência celular, invasão e angiogênese contribui para a biologia desses tumores (Holland, 2001; Fig. 2).

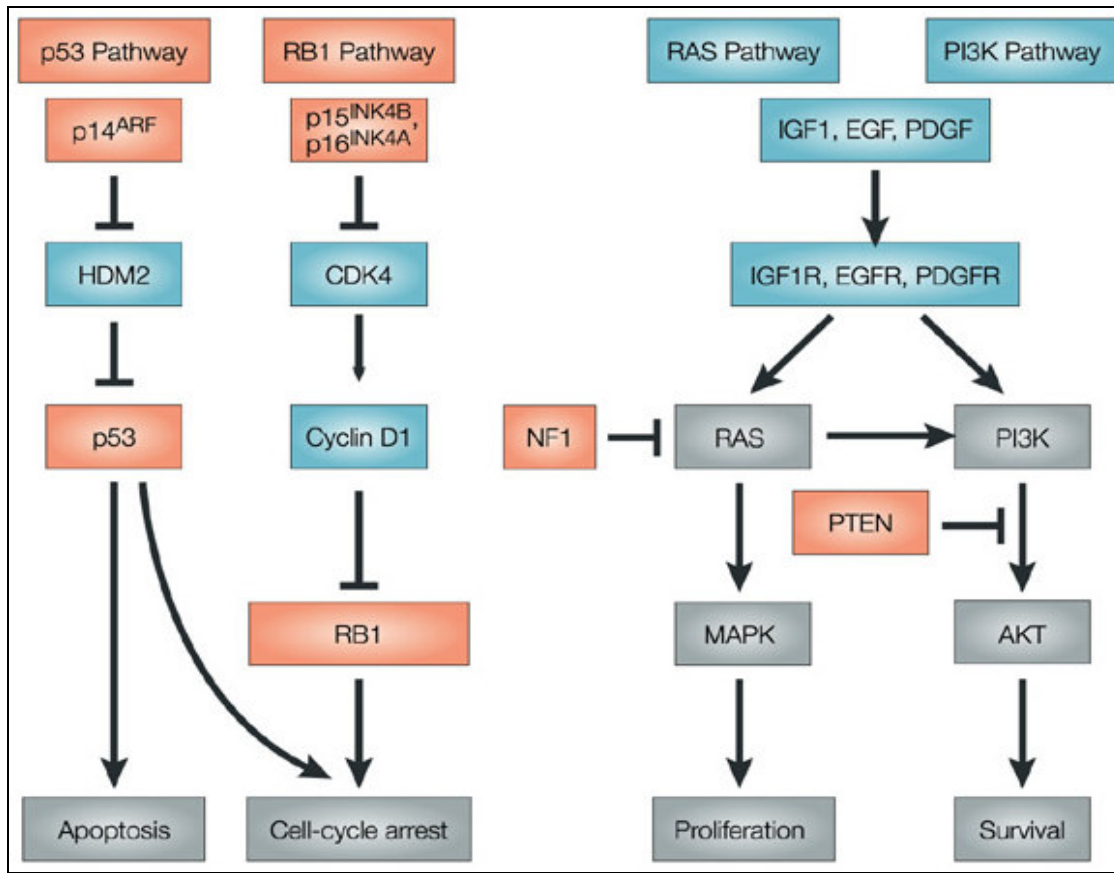


Figura 2. Principais vias de sinalização envolvidas na patogênese dos gliomas. Elementos em vermelho estão frequentemente inativados por mutação, deleção ou metilação do promotor; elementos em azul estão ativados através de superexpressão ou amplificação gênica; elementos em cinza são as vias que sofrem modulação dos elementos marcados em azul e em vermelho, constituindo as efetoras diretas das ações protumorais (adaptado de Rich and Bigner, 2004).

1.1.4 Gliomas e inflamação

A progressão dos gliomas consiste de numerosas alterações genéticas e fisiológicas que afetam as interações entre células tumorais, neurônios, glia, vasculatura e sistema imune (Demuth and Berens, 2004). Diversos tipos de câncer são originários de sítios de infecção, irritação crônica ou de inflamação. Em gliomas, a presença de infiltrado inflamatório está diretamente correlacionada com o grau de malignidade do tumor e, apesar da função das

células imunes não estar completamente elucidada, evidências sugerem que a presença de leucócitos, particularmente microglia e macrófagos, no microambiente tumoral é componente indispensável nos processos de proliferação, migração e sobrevivência celular (Fig. 3; Watters *et al.*, 2005).

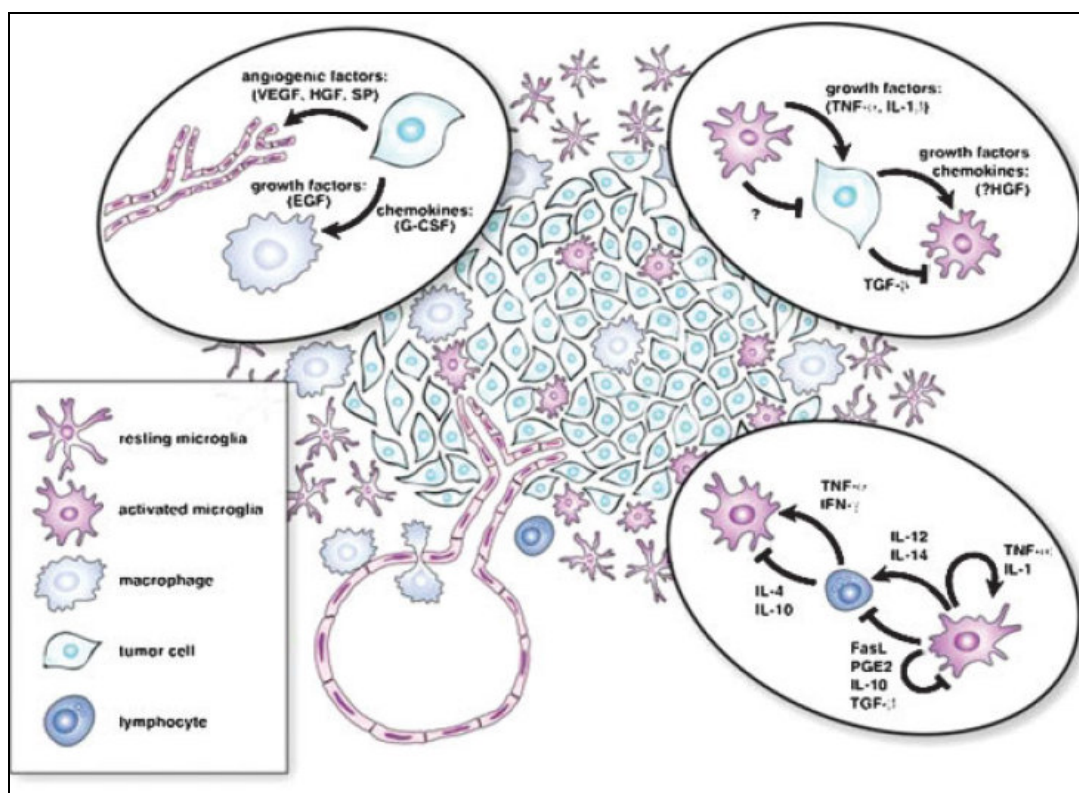


Figura 3. Possíveis interações entre células neoplásicas, macrófagos e microglia nos tumores cerebrais (adaptado de Watters *et al.*, 2005).

Os mecanismos que correlacionam infecção, imunidade inata, inflamação e câncer ainda são pouco claros, porém estudos apontam que a produção de citocinas pelas células imunes ativadas e a ativação de *toll-like receptors* (TLR) presentes nas células tumorais podem mediar a comunicação entre os diferentes sistemas. De fato, os TLRs são um componente da resposta imune inata e a sua expressão tem sido relacionada com o aumento da malignidade e da resistência a quimio/radioterapia em diversos tipos tumorais (Chen *et*

al., 2008). Entre os diferentes subtipos de TLRs, estudos sugerem que o TLR4 desempenha função importante no avanço dos gliomas (Hoelzinger *et al.*, 2007). TLRs localizados na membrana plasmática reconhecem ligantes externos exógenos, como o LPS, ou endógenos, como células em processo de apoptose, glutamato e ATP, os quais são responsáveis por desencadear uma resposta inflamatória asséptica (Chen *et al.*, 2008; Pineau and Lacroix, 2009). Em geral, a ativação de TLRs culmina na indução de NF- κ B e consequente produção de citocinas pró-inflamatórias que estimulam o avanço tumoral (Shishodia and Aggarwal, 2002; Pikarsky *et al.*, 2004). A figura 4 mostra as vias envolvidas na ativação dos receptores TLR3 e TLR4 e a consequente produção de citocinas promotoras tumorais.

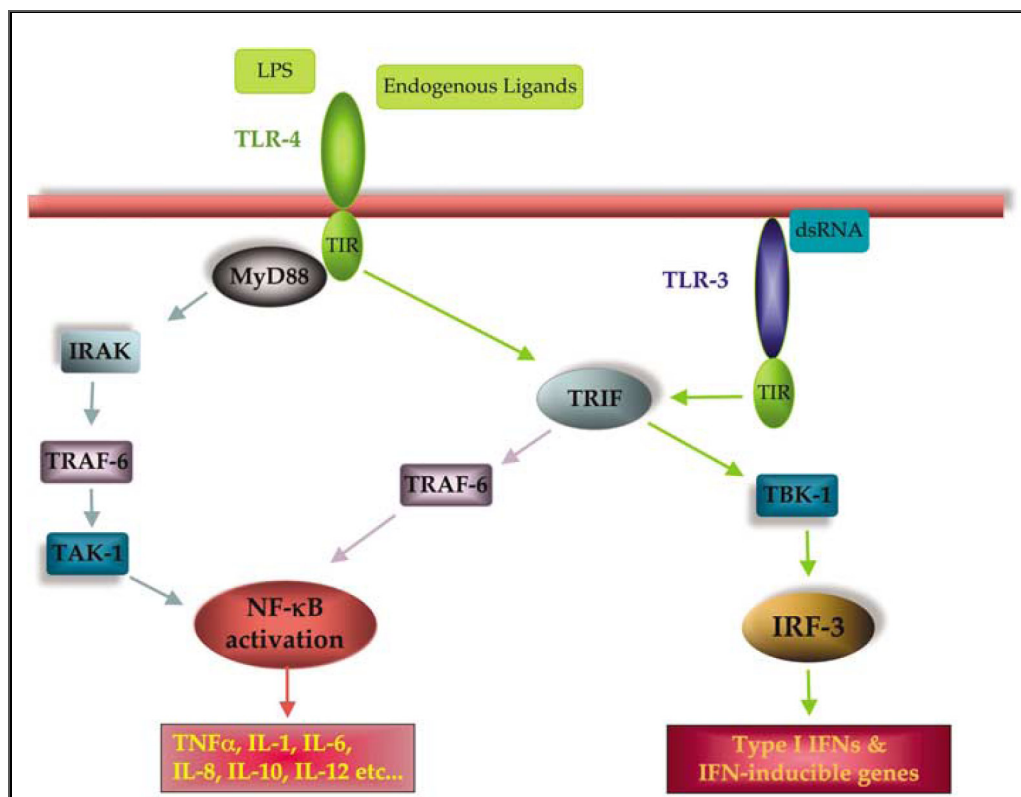


Figura 4. As vias de transdução de sinal dos receptores TLR3 e TLR4 e a consequente produção de citocinas (adaptado de Chen *et al.*, 2008).

A influência das citocinas na angiogênese durante a oncogênese é extremamente importante. Por exemplo, em diversos tumores incluindo os gliomas, a expressão de CXCL8/IL8 foi correlacionada com o aumento da angiogênese (Desbaillets *et al.*, 1997) e da proliferação celular (de la Iglesia *et al.*, 2008). Além disso, existe uma proposta que postula que células tumorais podem modular a resposta imune ao seu favor por meio da secreção de citocinas. O modelo proposto por Chen e colaboradores (2008), é composto por 3 etapas: (1) Recrutamento: via produção de citocinas (MCP-1, GRO α e IL-8), as células tumorais recrutam células imunes para o microambiente tumoral; (2) Educação: via a secreção de citocinas que regulam diferenciação celular (IL-6, TNF α e MIF), as células tumorais induzem as células imunes a desempenhar funções de suporte a progressão tumoral e (3) Resposta: as células imunes diferenciadas produzem citocinas, hormônios e fatores de crescimento que promovem crescimento tumoral e desenvolvimento de tolerância imune. Evidências indicam que a via do NF- κ B é importante em determinar o balanço das propriedades pró- e antitumoral dos macrófagos, e coloca essa via de sinalização como alvo para “reeducar” os macrófagos promotores tumorais (fenótipo M2) para desempenharem funções antitumorais (fenótipo M1). Assim, por meio de diferentes vias de sinalização, as citocinas participam ativamente na iniciação, na promoção e na progressão tumoral. O controle dessas vias pode constituir uma nova forma de controle da progressão dos gliomas. A figura 5 ilustra como as células tumorais por meio da secreção de citocinas poderiam suprimir as funções citotóxicas das células imunes e induzir um fenótipo prótumoral.

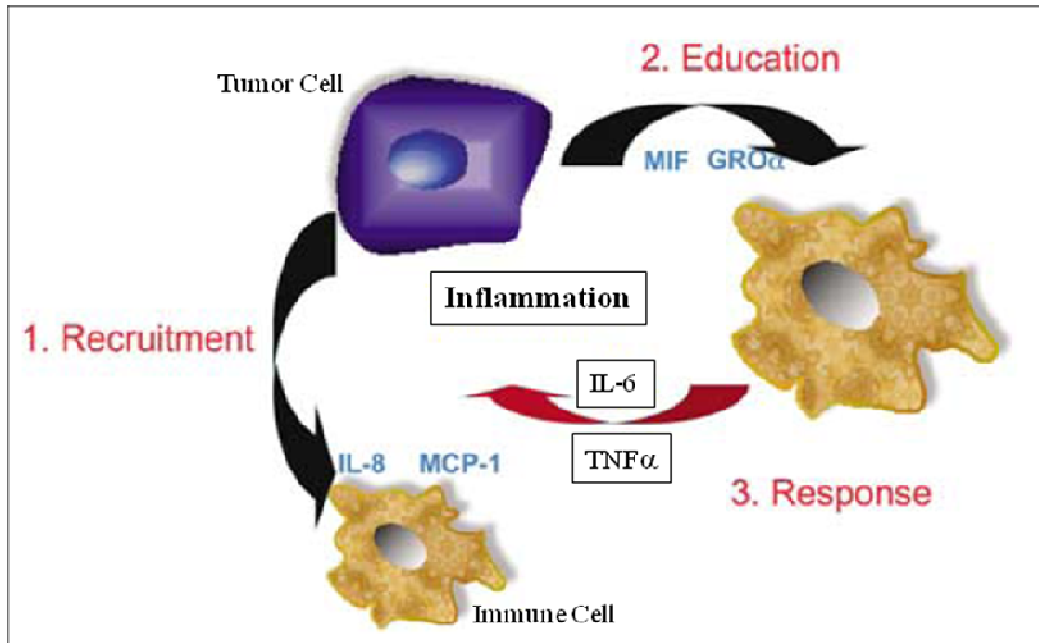


Figura 5. Modelo de como as células tumorais “educam” as células imunes a desempenhar funções de estímulo a progressão tumoral (adaptado de Chen *et al.*, 2008).

1.2 Sistema Purinérgico

1.2.1 Nucleotídeos Extracelulares

Apesar de tradicionalmente serem atribuídas aos nucleotídeos ações estritamente intracelulares, atualmente está bem estabelecido o conceito de que essas moléculas também atuam como mensageiros extracelulares, capazes de sinalizar uma variedade de efeitos biológicos no meio extracelular (Burnstock, 2006; 2007). Existe uma extensa discussão em torno das vias celulares envolvidas na liberação do ATP para o meio extracelular, porém o exato mecanismo não está completamente esclarecido (Burnstock, 2008). Além de dano celular, o ATP também pode ser liberado por células gliais em resposta à injúria mecânica ou à hipóxia. O ATP pode ser armazenado em vesículas sinápticas e ser liberado por exocitose como um co-transmissor juntamente com outros neurotransmissores como, por exemplo, a acetilcolina e o glutamato (Zimmermann, 1994). Além disso, estudos apontam

que o ATP pode ser liberado por exocitose vesicular nas células neuronais e nas células não neuronais por transportadores que ligam ATP ou via canais acoplados à conexina ou panexina (Sabirov and Okada, 2005). Uma vez liberados, os nucleotídeos interagem com receptores purinérgicos específicos, mediando eventos de neurotransmissão, resposta imune, inflamação, agregação plaquetária, entre outros (Burnstock, 2006; 2007). Essa sinalização é finalizada pela ação de ectoenzimas que hidrolisam os nucleotídeos até os seus respectivos nucleosídeos no meio extracelular (Zimmerman, 1994).

Além de exercerem papel como neurotransmissores, nucleotídeos e nucleosídeos purínicos podem atuar de diversas formas como agentes tróficos no SNC, induzindo trocas funcionais que modulam a diferenciação neuronal; a síntese e liberação de fatores tróficos; a neuritogênese e a potenciação da ação de fatores de crescimento sobre células alvo (Rathbone *et al.*, 1999). De fato, no sistema nervoso, nucleotídeos podem controlar a proliferação (Neary *et al.*, 2001; Ryu *et al.*, 2003), a migração (Scemes *et al.*, 2003) e a diferenciação (D'Ambrosi *et al.*, 2001) de astrócitos e de células neuronais. Além disso, as purinas podem ativar a via da Ras/Raf/MEK/MAPK (Tu *et al.*, 2000), indicando uma possível interação com os fatores mitogênicos neurais, como por exemplo FGF, EGF/TGF α e PDGF (Lin *et al.*, 2007). Os efeitos antagônicos mediados pelos nucleotídeos, tais como mitogênese e apoptose, podem ser induzidos dependendo do estado funcional das células, da expressão de receptores purinérgicos e da atividade das ectoenzimas que controlam a disponibilidade dos agonistas purinérgicos, como o ATP e adenosina, nessas células (Robson *et al.*, 2006).

O sistema de sinalização purinérgica também atua de forma integrada com outros sistemas no controle da resposta imune e inflamatória. Os nucleotídeos, especialmente o ATP, atuam como moléculas sinalizadoras endógenas de injúrias, desencadeando uma

resposta do sistema imune (Zhang and Mosser, 2008). Estudos revelam que o ATP está envolvido em diversas funções do sistema imune: nas células T, o ATP é importante na secreção de INF- γ e IL-2 (Langston *et al.*, 2003); nos monócitos circulantes o ATP está envolvido no recrutamento para tecidos alvo (Ventura and Thomopoulos, 1995); nas células dendríticas (DC) o ATP induz migração e diferenciação (la Sala *et al.*, 2003); nos macrófagos estimula a produção de IL-1 β (Elssner *et al.*, 2004) e TNF- α (Guerra *et al.*, 2003); nas células microgliais ATP e ADP medeiam a rápida ativação dessas células (Davalos *et al.*, 2005; Haynes *et al.*, 2006). Além da produção de mediadores solúveis de inflamação nos macrófagos, os nucleotídeos como ATP, UTP e mais recentemente o UDP estão envolvidos no processo de fagocitose (Ichinose, 1995; Koizumi *et al.*, 2007). O UDP, também está relacionado ao aumento na síntese de IL-6, quando macrófagos são ativados por LPS (Bar *et al.*, 2008). A adenosina também é considerada uma molécula sinalizadora de dano celular, porém com ações contrárias as do ATP (Bours *et al.*, 2006). Esse nucleosídeo medeia uma resposta imunossupressora para proteger os tecidos saudáveis dos ataques promovidos pelas células de defesa (Sitkovsky and Ohta, 2005). Entre as ações imunes da adenosina estão incluídas a inibição da produção de citocinas pró-inflamatórias (IL-12, INF- γ , MIPs) (Stewart and Harris, 1993; Hasko *et al.*, 2009) e a inibição da atração de linfócitos a respostas inflamatórias (Odashima *et al.*, 2005).

Finalmente, nucleotídeos extracelulares e seus receptores são componentes importantes do sistema cardiovascular e do tônus vascular (Burnstock, 2002). A estimulação dos receptores purinérgicos expressos nas plaquetas, células endoteliais e leucócitos resulta em ativação dessas células que culmina na formação de trombo vascular e de inflamação (Robson *et al.*, 2001). O ADP desempenha uma função crucial nos

processos fisiológicos de homeostase e no desenvolvimento e extensão da trombose arterial (Born, 1985). Esse nucleotídeo é um fraco e reversível agonista indutor de agregação plaquetária quando comparado à trombina e ao colágeno. Entretanto, devido a sua presença em elevados níveis nos grânulos plaquetários e a sua liberação nos sítios de injúria vascular, o ADP é chamado de agonista secundário, o qual amplifica a resposta plaquetária e contribui para a estabilização do trombo vascular (Gachet, 2008). Além dos efeitos de curta duração sobre o tônus vascular e a ativação de plaquetas, os nucleotídeos estão envolvidos em efeitos tróficos de longa duração nas células endoteliais, os quais têm importantes consequências sobre processos inflamatórios e angiogênese (Burnstock, 2002; Di Virgilio and Solini, 2002).

1.2.2 Receptores Purinérgicos

Uma característica comum dos receptores purinérgicos P1 e P2 é a heterogeneidade das suas respostas biológicas, que podem ser influenciadas por diferentes parâmetros, entre os quais: a) alteração da conformação do receptor em presença de ligantes endógenos; b) formação de um gradiente de concentração de um determinado ligante, podendo ativar simultaneamente e com distinta afinidade mais de um receptor; c) o repertório de receptores P1 e P2 expressos por um determinado tipo celular, bem como a distribuição dos mesmos na membrana celular; d) as múltiplas interações indiretas entre receptores e as interações diretas de formação de homodímeros e heterodímeros; e) presença de ectonucleotidases. Além disso, o arranjo dos receptores P1 e P2 na membrana plasmática é um processo dinâmico e varia de acordo com o desenvolvimento da célula e com a presença de fatores epigenéticos. Como consequência, o número de funções mediados por esses receptores é

amplo e varia desde proliferação e diferenciação celular, neurotransmissão até fenômenos mais complexos como fertilização, angiogênese e resposta imune (Burnstock, 2006).

Distintos purinoreceptores para adenosina e ATP, denominados de P1 e P2, respectivamente, foram originariamente identificados por Burnstock e colaboradores (Burnstock, 1976; 1978). Até o momento foram clonados e caracterizados 15 subtipos de receptores P2 (Burnstock, 2008) e 4 subtipos de receptores P1 (Fredholm *et al.*, 2001). Os receptores purinérgicos são funcionalmente classificados como: 1) metabotrópicos: acoplados à proteína-G, os quais estão incluídos os receptores do tipo P1 (adenosina) e P2Y (ATP); 2) ionotrópicos: diretamente ligados a canais iônicos, os quais estão incluídos os receptores tipo P2X (ATP) (Abbracchio *et al.*, 1994).

Os receptores metabotrópicos P1 (adenosina) são divididos em quatro subtipos A₁, A_{2A}, A_{2B} e A₃ (Abbracchio *et al.*, 1994; Fredholm *et al.*, 2001). Eles podem ser identificados pela distinta afinidade de ligação a agonistas e antagonistas e pela ativação de vias sinalizadoras acopladas à proteína-G (Palmer and Stiles, 1995). De forma geral, receptores A₁ são associados à inibição da adenil ciclase, ativação da fosfolipase C (PLC) e fosfolipase A₂ (PLA₂) e estão envolvidos na inibição da neurotransmissão excitatória (Caciagli *et al.*, 1989). Receptores A₂ são acoplados à proteína-G estimulatória, gerando aumento dos níveis de AMPc intracelulares e, assim desencadeiam ações neuronais excitatórias (Fredholm *et al.*, 2001). Foi demonstrado que receptores A₃ estão envolvidos na ativação da fosforilação da ERK 1/2 em astrócitos fetais humanos (Neary *et al.*, 1998) e em células microgliais (Hammarberg *et al.*, 2003). Adicionalmente, os receptores P1 podem modular vias de proteção e proliferação celular (A₁, A₂ e A₃), bem como apoptose (A₃) (Spychala, 2000; Latini e Pedata, 2001).

Os receptores P2 são subdivididos em duas classes: sete receptores P2X₁₋₇, ligados a canais iônicos; e oito receptores P2Y_{1, 2, 4, 6, 11-14}, acoplados à proteína-G. Os receptores P2Y diferem em sua seletividade para nucleotídeos da adenina (ATP, ADP) e da uracila (UTP, UDP), enquanto que os receptores P2X são ativados somente por ATP. A complexidade dos receptores P2 é aumentada pela formação homo- e heterodímeros entre receptores (Nakata *et al.*, 2004).

Os receptores P2X desencadeiam seus efeitos via abertura de um canal iônico na membrana celular permeáveis a Na⁺, K⁺ e Ca⁺² (Abbracchio *et al.*, 1994), não parecendo haver o envolvimento nem de proteínas-G, tampouco de segundos-mensageiros intracelulares. O ATP é o principal agonista. Outros nucleotídeos (ADP, UTP, UDP) apresentam pouca ou nenhuma afinidade pelos receptores P2X (Idelson, 2001). Os receptores P2X ocorrem normalmente como trímeros estáveis e todos eles podem ser desensibilizados, porém em escalas de tempo diferenciadas: desensibilização rápida (P2X_{1,3}) e desensibilização lenta (P2X_{2, 4-7}). Entre os receptores P2X, o subtipo P2X₇ é funcionalmente distinto dos outros, devido a sua habilidade em formar poros reversíveis na membrana em resposta a estimulação sustentada pelo ATP. A ativação desse receptor aumenta a permeabilidade a ânions, cátions e a moléculas de até 900 Da (North, 2002). A ativação desse receptor está relacionada com a ativação das vias da fosfolipase A₂, fosfolipase D, MAPK e NF-κB (North, 2002).

Os receptores P2Y usualmente são acoplados a PLC e subsequentemente modulam os níveis de cálcio intracelular. Entretanto, eles também podem se associar funcionalmente a outras proteínas G. Enquanto que os receptores P2Y_{1, 2, 4, 6} são positivamente acoplados a PLC via proteína G_{q,11}, os receptores P2Y_{12,13,14} são negativamente acoplados a adenilil

ciclase via proteína G_i . O receptor $P2Y_{11}$ é acoplado simultaneamente as proteínas $G_{q,11}$ e G_s , ativando as vias da PLC e da adenilil ciclase. Finalmente, o receptor $P2Y_{12}$ ativa a via do fosfatidilinositol 3-quinase (PI3-K) via G_{α_i} , além das vias da RhoA e RhoA quinase. A ativação dos receptores P2Y está também comumente associada a estimulação da via das MAPK, especialmente a MAPK1/2, PKC e PI3-K (Abbracchio *et al.*, 1994). A figura 6 mostra a estrutura dos receptores purinérgicos P1, P2X e P2Y.

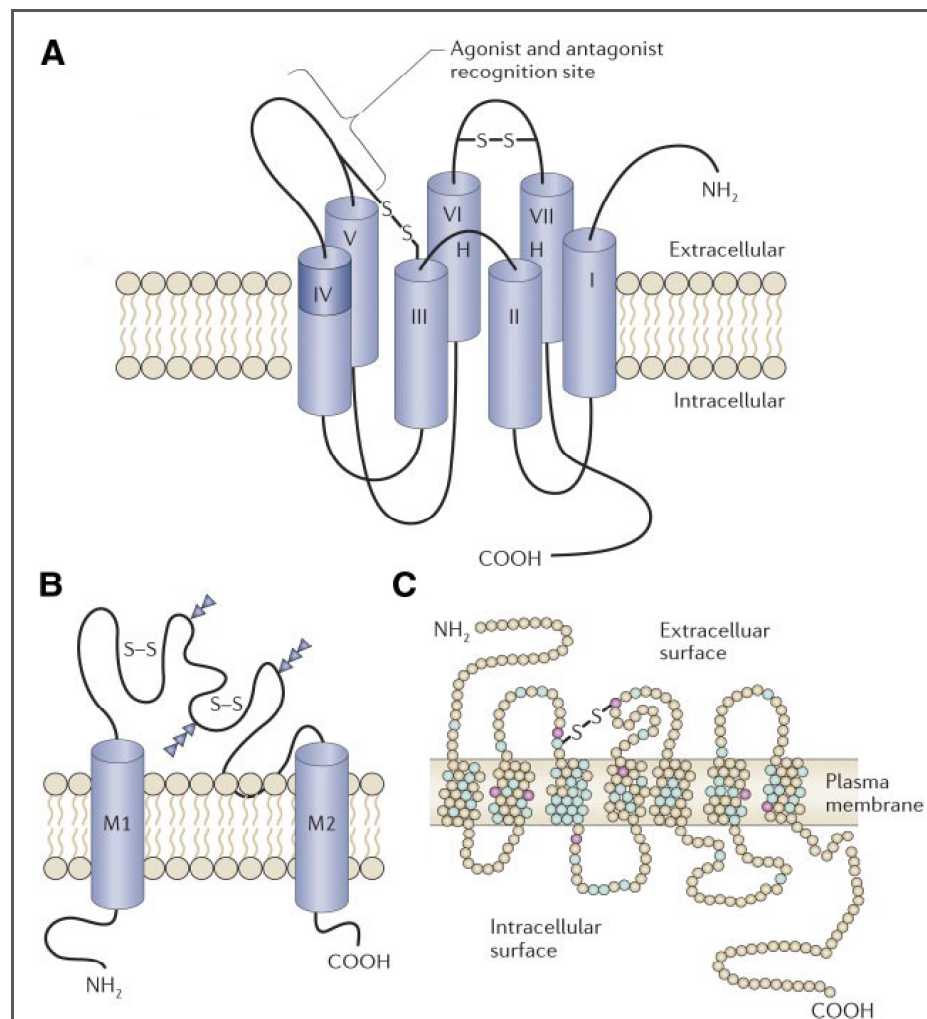


Figura 6. Receptores purinérgicos. (A) receptores P1 para adenosina extracelular são acoplados a proteína G; (B) receptores P2X são acoplados a canais iônicos; (C) receptores P2Y são acoplados a proteína G (adaptado de Burnstock, 2007).

1.2.3 Família das Ectonucleotidases

Os eventos induzidos por nucleotídeos extracelulares são controlados pela ação de ectonucleotidases, as quais representam um importante sistema de modulação da neurotransmissão purinérgica. Assim, após realizada a transdução de sinal, os nucleotídeos extracelulares devem ser rapidamente inativados até adenosina. Diferentes famílias de ectoenzimas trabalham de forma orquestrada nesse processo, entre as quais estão incluídas: as ectonucleosídeo trifosfato-difosfohidrolases (E-NTPDases); as ecto-nucleotídeo pirofosfatase/fosfodiesterases (E-NPP); ecto-5'-nucleotidase/CD73 (ecto-5'-NT/CD73) e as fosfatases alcalinas (PA) (Fig. 7; Zimmermann, 2001; Robson *et al.*, 2006). O foco de estudo da presente tese de doutorado foi as E-NTPDases e a ecto-5'-NT/CD73, as quais atuam de forma integrada, hidrolisando o ATP até adenosina no meio extracelular.

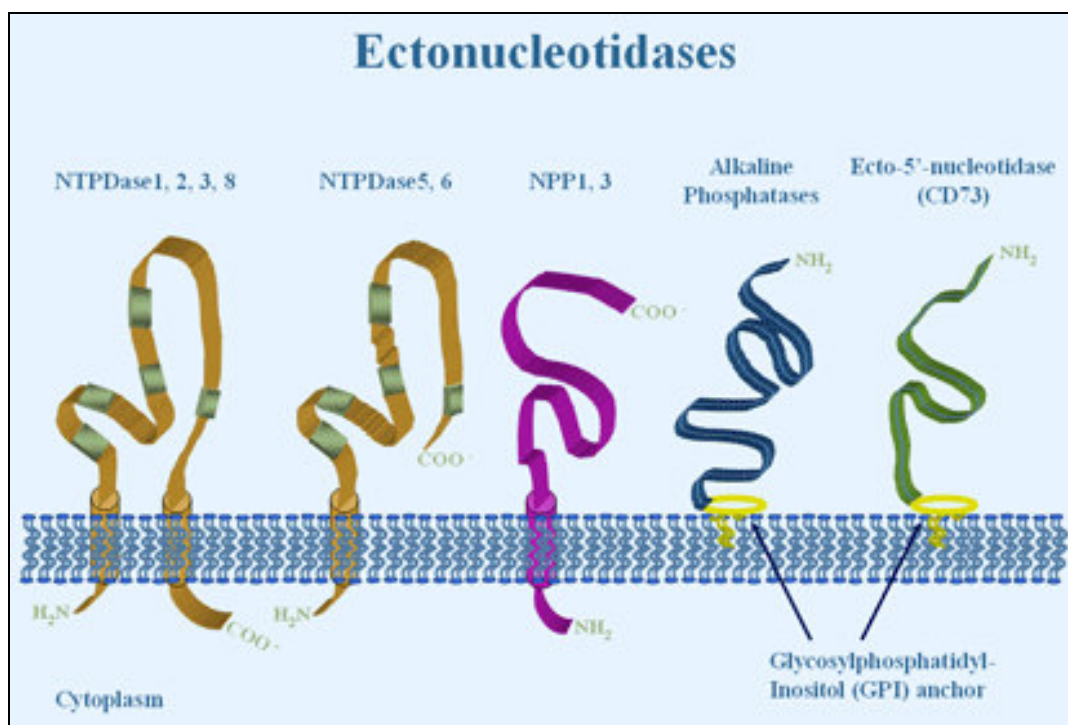


Figura 7. Topografia de membrana das diferentes famílias de enzimas que compõem o grupo das ectonucleotidases (adaptado de www.ccri.ca/sevigny.html).

1.2.3.1 E-NTPDases

Os membros da família E-NTPDase, previamente classificados como E-ATPases, constituem uma classe de ectoenzimas ancoradas à membrana plasmática via domínios hidrofóbicos, com o sítio ativo voltado para o meio extracelular. Essas enzimas são caracterizadas pela sua capacidade em hidrolisar nucleosídeos tri- e difosfatos, pela dependência de cátions divalentes para exercer atividade catalítica, pela insensibilidade a inibidores clássicos de P-, F- e V-ATPases e pela presença de cinco regiões altamente conservadas, denominadas de regiões conservadas da apirase (ACR) (Plesner, 1995). Até o momento foram clonados e caracterizados 8 diferentes membros pertencentes a essa família de enzimas, nomeados de NTPDase1-8: NTPDase1, 2, 3 e 8 são ligadas à membrana da superfície plasmática por dois domínios transmembrana, N e C-terminal citoplasmáticos, sendo as principais responsáveis pela metabolização dos nucleotídeos no meio extracelular; NTPDase5 e 6 exibem localização intracelular, não possuem o domínio transmembrana C-terminal e podem ser clivadas próximo ao domínio N-terminal para formar uma enzima solúvel; NTPDase4 e 7 são localizadas nas membranas de organelas intracelulares com o sítio ativo voltado para o lúmen das mesmas (Zimmermann, 2001; Robson *et al.*, 2006).

A identidade molecular do primeiro membro da família das E-NTPDases (NTPDase1) foi revelada somente na metade da década de 90. Inicialmente, essa enzima havia sido clonada, sequenciada e identificada como o antígeno de ativação celular CD39, um marcador de células B (Maliszewski *et al.*, 1994). Experimentos subsequentes com ATP-difosfohidrolase (apirase) solúvel purificada de batata (Handa and Guidotti, 1996) e com diferentes tecidos de mamíferos (Kaczmarek *et al.*, 1996) confirmaram a homologia entre a enzima metabolizadora de nucleotídeos e o CD39. Estudos posteriores mostraram que a NTPDase1/CD39 é abundantemente expressa nos vasos sanguíneos (células

endoteliais e em células musculares lisas); no pâncreas exócrino; nas células dendríticas; linfócitos e numa variedade de outras células (Robson *et al.*, 2006).

A NTPDase2 está particularmente associada à camada adventícia do endotélio vascular (Robson *et al.*, 2005); a astrócitos em cultura (Wink *et al.*, 2006); a células de Schwann e a outras células gliais do sistema nervoso central e periférico (Langer *et al.*, 2007). Estudos imunohistológicos revelaram que a NTPDase2 em conjunto com a fosfatase alcalina está envolvida no controle da embriogênese posnatal e na neurogênese adulta (Langer *et al.*, 2007). Além disso, a importância dessa enzima no controle da sinalização purinérgica durante o desenvolvimento dos olhos tem sido relatada (Massé *et al.*, 2007).

A expressão da NTPDase3 está principalmente associada aos neurônios e, por meio do controle dos níveis pré-sinápticos de ATP extracelular, pode coordenar múltiplos sistemas, incluindo regulação do apetite e ciclos de descanso e vigília (Belcher *et al.*, 2006). O último membro dessa família de enzimas a ser caracterizado foi a NTPDase8. Essa enzima encontra-se predominantemente expressa no fígado e em menor escala no jejuno e nos rins, estando ausente no cérebro (Bigonnesse *et al.*, 2004). A presença abundante da NTPDase8 em canalículos biliares e em vasos hepáticos de grande calibre sugere o envolvimento dessa ecto-nucleotidase na regulação da secreção da bile e/ou na via de salvação dos nucleosídeos (Fausther *et al.*, 2007).

Todos os membros da família das NTPDases ligados à superfície da membrana plasmática (NTPDase1, 2, 3 e 8), constituem proteínas altamente glicosiladas com peso molecular entre 70-80 kDa; apresentam reatividade imunológica cruzada e podem existir como estruturas monoméricas ou homoligoméricas (diméricas ou tetraméricas) (Stout and Kirley, 1996). Apesar da semelhança estrutural e da habilidade que todas apresentam em hidrolisar nucleotídeos, essas ectonucleotidases podem modular diferentemente a ativação

dos receptores P2 devido a diferenças na preferência por substrato. Enquanto que a NTPDase1 hidrolisa ATP e ADP igualmente bem, as NTPDase3 e NTPDase8 revelam uma preferência maior ao ATP do que sobre o ADP como substrato. A NTPDase2 se destaca pela clara preferência por nucleosídeos trifosfatos, sendo anteriormente classificada como uma ecto-ATPase (Zimmermann, 2001; Kukulski *et al.*, 2005). Assim, a NTPDase1 hidrolisa o ATP quase diretamente até AMP, com uma produção transitória baixa de ADP. Essa propriedade funcional previne de forma efetiva a ativação de receptores P2Y por nucleosídeos difosfato. Ao contrário da NTPDase1, a hidrólise do ATP pela NTPDase2 gera acúmulo de ADP no meio extracelular, o qual é lentamente defosforilado até AMP. Por um lado, ocorre a prevenção da ativação de receptores P2Y sensíveis a ATP, enquanto que por outro ocorre a formação de agonistas para os receptores sensíveis a nucleosídeos difosfato, como os receptores P2Y₁ e P2Y₁₂ presentes nas plaquetas. Dessa forma, o controle dos níveis de nucleotídeos extracelulares exercido pelas NTPDase1 e NTPDase2 tem reflexos importantes na hemostasia e em processos de inflamação e angiogênese em diferentes tecidos. A importância da NTPDase1 nesses processos foi posteriormente confirmada em camundongos mutantes deficientes para NTPDase1 (Enjyoji *et al.*, 1999). Finalmente, as atividades das NTPDase3 e NTPDase8 resultam em padrões intermediários de formação de produtos, levando a um acúmulo transitório de difosfonucleosídeos em presença simultânea de trifosfonucleosídeos. A tabela 1 apresenta os membros da família das E-NTPDases e as respectivas preferências por substrato.

Tabela 1. Nomenclatura e preferência por substratos dos membros da família E-NTPDase em vertebrados.

NOMENCLATURA ATUAL	NOMENCLATURA ANTIGA	PREFERÊNCIA POR SUBSTRATO
NTPDase 1	CD39, ecto-ATP difosfoidrolase, ecto-apirase, ecto-ATPDase	ATP=ADP (1:1)*
NTPDase 2	CD39L1, ecto-ATPase	ATP >>>>ADP (30:1)*
NTPDase 3	CD39L3, HB6	ATP>ADP (3:1)*
NTPDase 4	UDPase (hLALP70v), hLALP70	UDP>GDP,CDP
NTPDase 5	CD39L4, ER-UDPase	UDP>GDP,IDP>>>ADP, CDP
NTPDase 6	CD39L2	GDP>IDP>>UDP,CDP>>ADP
NTPDase 7	LALP1	UTP, GTP, CTP
NTPDase 8	-	ATP>ADP (2:1)*

Adaptado a partir de Zimmermann (2001). * Razão de hidrólise NTP: NDP. As NTPDases de 1 a 3 hidrolisam todos os outros nucleotídeos purínicos e pirimidínicos, similarmente ao ATP e ADP.

1.2.3.2 Ecto-5'-nucleotidase/CD73

Até o momento, sete 5'-nucleotidases foram caracterizadas em humanos, sendo cinco delas localizadas no citosol, uma na matriz mitocondrial e uma ligada à membrana plasmática. A ecto-5'-NT/CD73 associada à membrana plasmática catalisa a hidrólise de nucleosídeos 5'-monofosfatados (como o AMP) até os respectivos nucleosídeos, sendo enzima chave na via de degradação dos nucleotídeos e a principal fonte enzimática de adenosina no meio extracelular (Zimmermann, 1992). Essa enzima consiste em duas subunidades de (glico)proteína com peso molecular de 60-70 kDa e se encontra ligada à membrana plasmática por meio de uma âncora lipídica de GPI, o sítio catalítico é voltado

para o meio extracelular, a atividade hidrolítica é potencializada por cátions divalentes e inibida por ADP, ATP e 5'- α,β - metileno-ADP.

A ecto-5'-NT/CD73 está presente em diferentes tecidos, com expressão abundante no cólon, nos rins, no cérebro, no fígado, no coração e nos pulmões (Zimmermann, 1992; 1996). Nos vasos sanguíneos, a ecto-5'-NT/CD73 é predominantemente associada ao endotélio vascular de vasos de grande calibre, tais como a aorta, a carótida e a artéria coronária (Koszalka *et al.*, 2004). Em linfócitos B e T a expressão da enzima é restrita e está fortemente correlacionada a maturidade celular, enquanto que neutrófilos, eritrócitos e plaquetas apresentam baixa ou nenhuma expressão dessa enzima (Resta *et al.*, 1998; Yamashita *et al.*, 1998; Spychala *et al.*, 1999). Curiosamente, a ecto-5'-NT/CD73 é coexpressa com a NTPDase1/CD39 na superfície das células Treg CD4+/CD25+/Foxp3+, sendo um importante componente da maquinaria imunossupressiva, via geração de adenosina, molécula de ação antiinflamatória que inibe a produção de citocinas e a proliferação das células T (Kobie *et al.*, 2006; Deaglio *et al.*, 2007). Além da importante função de regular a cascata de sinalização purinérgica, a ecto-5'-NT/CD73 também desempenha outras funções não enzimáticas, entre as quais indução da sinalização intracelular e mediadora de processos de adesão célula-célula e célula-matriz e de migração (Fastbom *et al.*, 1987; Schoen *et al.* 1988; Vogel *et al.*, 1991). A figura 8 ilustra como a ação integrada das NTPDases e ecto-5'-NT/CD73 orquestram a ativação dos receptores purinérgicos P1 e P2.

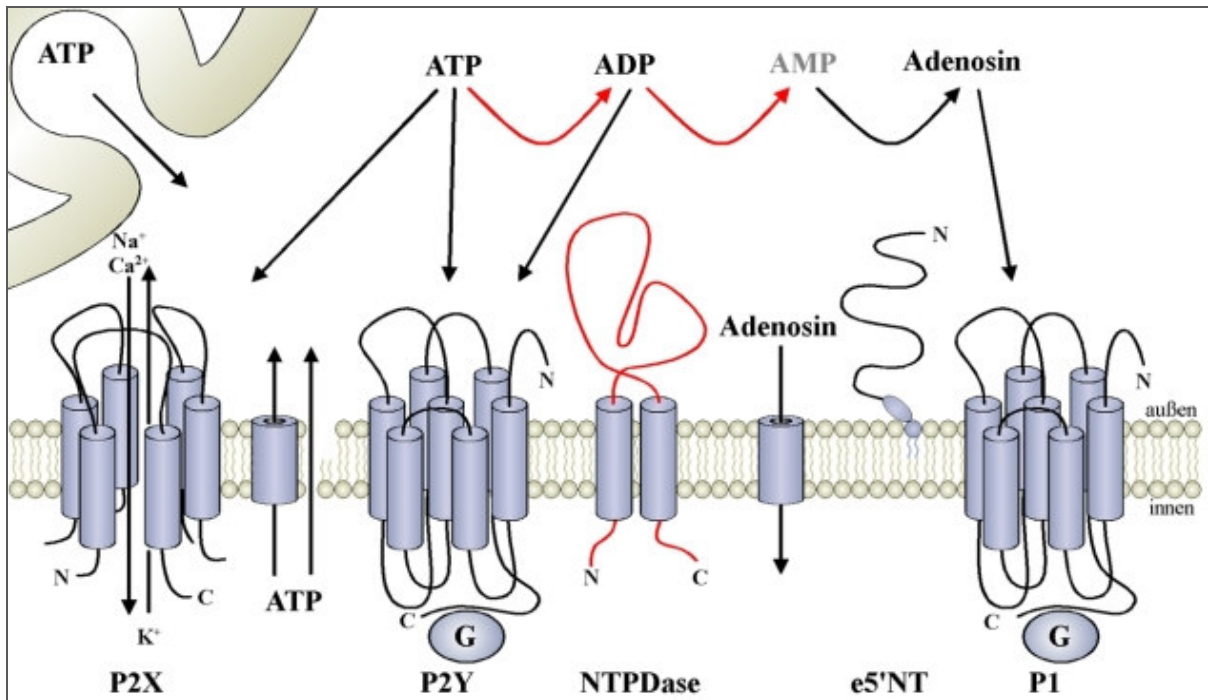


Figura 8. Metabolismo extracelular do ATP até adenosina pela ação conjunta das NTPDases e da ecto-5'-NT/CD73 e o controle da ativação dos receptores purinérgicos (adaptado de www.uni-leipzig.de/research/ntpdase.html).

1.2.4 Sistema Purinérgico e os Tumores

A transformação de uma célula normal em um tumor maligno é um processo multifatorial que, em adição à modulação da expressão de genes que controlam proliferação/diferenciação celular, requer condições específicas que disponibilizem um suporte fisiológico para o desenvolvimento do tumor. Devido à habilidade dos nucleotídeos extracelulares em atuar como fatores tróficos indutores de crescimento e proliferação celular (Rathbone *et al.*, 1999), muitos estudos têm apontado que a sinalização purinérgica pode estar envolvida com a progressão tumoral (White and Burnstock, 2006).

Primariamente, cinco subtipos de receptores purinérgicos do tipo P2 estão relacionados com câncer: P2Y₁, P2Y₂, P2Y₁₁, P2X₅ e P2X₇. As alterações na proliferação de células tumorais podem ser devidas a modulações via P2Y₁ e P2Y₂, enquanto que a

estimulação da diferenciação com subsequente inibição da proliferação envolve P2X₅ e P2Y₁₁ (White and Burnstock, 2006). O receptor P2X₇ é um dos receptores purinérgicos mais estudados em uma série de patologias, incluindo o câncer (Burnstock, 2008). Os efeitos mediados por esse receptor são intrigantes e, dependendo do tipo celular e da forma de ativação, ele pode desencadear efeitos antagônicos de morte ou de proliferação celular (Di Virgilio *et al.*, 2009). Além disso, o receptor P2X₇ é um dos principais mediadores da resposta inflamatória induzida pelo ATP o que pode ter consequências importantes no avanço tumoral. O ATP ativa o fator nuclear de células T ativadas (NFAT), um fator de transcrição chave envolvido na expressão gênica das citocinas e que pode representar um novo mecanismo pelo qual o ATP pode modular a resposta inflamatória inicial (Ferrari *et al.*, 1999). Evidências sugerem que a ativação do P2X₇ também pode modular a função da microglia, estimulando a secreção de citocinas pró-inflamatórias como IL-1 β , IL-6 e TNF- α ou decrescendo a capacidade inflamatória/citotóxica dessas células (Potucek *et al.*, 2006). Provavelmente, a modulação negativa das células microgliais desempenha função importante no controle da citotoxicidade desencadeada pelo ATP em células neuronais. Entretanto, em situações patológicas, como no câncer, essa via de sinalização pode ser utilizada para proteger as células tumorais do ataque imune. De acordo com essa idéia, recentemente foi mostrado em experimentos *in vivo* que o ATP acumula na periferia dos tumores, podendo modular uma série de sinalizações que controlam a resposta inflamatória e que estimulam a proliferação tumoral (Pellegatti *et al.*, 2008).

A adenosina também se encontra aumentada na periferia de tumores cerebrais (Latini and Pedata, 2001) e uma série de evidências apontam a participação desse nucleosídeo na progressão tumoral (Spsychala, 2000). Tumores sólidos freqüentemente

experimentam situações de hipóxia e de necrose que levam à liberação de ATP e de adenosina. Além ser liberada como tal, via transportadores específicos, a adenosina no meio extracelular pode ser resultante da degradação extracelular do ATP por ação das ectonucleotidases, conforme acima descrito. Uma vez no espaço extracelular, a adenosina exerce múltiplos efeitos, que incluem vasodilatação, estímulo à angiogênese e citoproteção. Adicionalmente, as ações anti-inflamatórias e imunossupressivas da adenosina podem inibir a resposta imunológica que controla o crescimento dos tumores (Spsychala, 2000). De uma forma geral, ao redor das células tumorais existe um microambiente imunossupressor hostil que previne a destruição das mesmas pelas células T e a sinalização adenosinérgica desempenha função importante nesse contexto (Lukachev *et al.*, 2007). Um estudo recente mostrou que a deleção dos receptores imunossupressores da adenosina, A_{2A} e A_{2B}, ou o antagonismo farmacológico dos mesmos previniu a inibição das células T anti-tumorais e facilitou a rejeição completa do tumor (Ohta *et al.*, 2006). Além disso, em situação de hipóxia a ativação do receptor A₃ está relacionada com o aumento da expressão de HIF-1 e VEGF e consequente indução de angiogênese (Merighi *et al.*, 2006). Dessa forma, o acúmulo de adenosina extracelular pode estar envolvido na regulação da resposta imune anti-tumoral e, possivelmente, pode ser uma estratégia adaptativa dos tumores para invadir os tecidos e fazer metástases.

1.3 E-NTPDases e Gliomas: a hipótese do estudo

Estudos realizados pelo nosso grupo sugerem que a sinalização purinérgica também pode estar envolvida na progressão dos gliomas: a) gliomas apresentam baixa atividade e expressão das E-NTPDases, particularmente da NTPDase2, quando comparados aos astrócitos; (Wink *et al.*, 2003; Wink *et al.*, 2006); b) ATP extracelular induz estímulo

proliferativo em diferentes culturas de gliomas (Morrone *et al.*, 2003); c) ao contrário do tecido cerebral normal, gliomas apresentam uma clara resistência à morte induzida por concentrações citotóxicas de ATP (Morrone *et al.*, 2005).

Assim, a baixa hidrólise do ATP poderia propiciar o acúmulo desse nucleotídeo na superfície tumoral resultando em proliferação tumoral e citotoxicidade para o tecido normal. Esses dados somados aos dados obtidos por Takano e colaboradores (2001), em que foi proposto um modelo glutamatérgico para a invasão de tumores cerebrais, nos levaram a sugerir uma nova hipótese de trabalho em que ambos, sistema purinérgico e glutamatérgico, estariam envolvidos na proliferação e na invasão dos gliomas. No nosso modelo, propomos que a morte dos neurônios induzida pelo glutamato liberado pelos gliomas levaria ao extravasamento do conteúdo intracelular, onde são encontradas grandes quantidades de glutamato e ATP. Desta forma, o glutamato e o ATP liberados poderiam induzir ainda mais morte neuronal, num estado de retroalimentação positiva. Em acordo com essa idéia, a coinjeção de gliomas com apirase em cérebro de ratos resultou em tumores de menor tamanho e malignidade, indicando que a remoção do ATP do meio extracelular é importante para reduzir a progressão tumoral (Morrone *et al.*, 2006).

Dessa forma, os dados do nosso grupo juntamente com os da literatura sugerem que alterações na ativação dos receptores purinérgicos e na atividade das enzimas envolvidas no metabolismo extracelular de nucleotídeos estejam envolvidas na patologia dos gliomas e que mereçam ser investigadas. Assim, esse estudo visa caracterizar melhor o sistema purinérgico no crescimento de gliomas *in vitro* e *in vivo*, avaliando o papel dos receptores purinérgicos e da restituição da atividade ATPásica aos gliomas, mais especificamente da NTPDase2, em eventos relacionados à progressão tumoral.

2. OBJETIVOS

2.1 Objetivo Geral

Investigar o envolvimento das E-NTPDases e dos receptores purinérgicos na progressão dos gliomas.

2.2 Objetivos Específicos

- Analisar comparativamente a expressão das ectonucleotidases e dos receptores purinérgicos em linhagem celular de glioma C6 e em cultura primária de glioma de rato (modelo de tumor *ex vivo*) (item 3.1);
- Analisar o efeito da restituição da expressão/atividade da NTPDase2 as células de glioma C6 sobre a proliferação dos gliomas *in vitro* e *in vivo* (item 3.2);
- Analisar o efeito da restituição da expressão/atividade da NTPDase2 às células de glioma C6 sobre a resposta do sistema imune e a formação de metástases (item 3.3);
- Analisar o envolvimento dos receptores purinérgicos no controle da secreção de IL-8 e de MCP-1 por células de glioma em cultura (item 3.4);
- Analisar a expressão das E-NTPDase1, 2 e 3 e ecto-5'-NT/CD73 em gliomas implantados em rato e em amostras de tumores cerebrais de pacientes (item 3.5).

3. RESULTADOS

3.1 Artigo 1

**A COMPARATIVE STUDY OF ECTONUCLEOTIDASE AND P2 RECEPTOR
mRNA PROFILES IN C6 CELL LINE CULTURES AND C6 *EX VIVO*
GLIOMA MODEL**

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A comparative study of ectonucleotidase and P2 receptor mRNA profiles in C6 cell line cultures and C6 *ex vivo* glioma model

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Abstract Glioblastoma multiforme is the most common type of primary brain tumour and has the worst clinical outcome. Nucleotides represent an important class of extracellular molecules involved in cell proliferation, differentiation and apoptosis. Alterations in purinergic signalling have been implicated in pathological processes, such as cancer, and glioma cell lines are widely employed as a model to study the biology of brain tumours. Increasing evidence, however, suggests that glioma cell lines may not present all the phenotypic and genetic characteristics of the primary tumours. We have compared the biological characteristics of C6 rat glioma cells in culture and the same cells after their implantation in the rat brain and growth in culture (denominated as the C6 *ex vivo* culture model). Parameters evaluated included cell morphology, differentiation, angiogenic markers, purinergic receptors and ecto-nucleotidase mRNA

profile/enzymatic activity. Analysis of the C6 glioma cell line and C6 *ex vivo* glioma cultures revealed distinct cell morphologies, although cell differentiation and angiogenic marker expressions were similar. Both glioma models co-expressed multiple P2X and P2Y receptor subtypes with some differences. In addition, the C6 glioma cell line and C6 *ex vivo* glioma cultures exhibited similar extracellular ATP metabolism and cell proliferation behaviour when exposed to cytotoxic ATP concentrations. Thus, the disruption of purinergic signalling is a feature shown not only by glioma cell lineages, but also by primary glioma cultures. Our results therefore suggest the participation of the purinergic system in glioma malignancy.

Keywords C6 glioma · ATP · Purinergic receptors · Ectonucleotidases · Cell line culture (Rat) · Glioma *ex vivo* culture (Rat)

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Introduction

Glioblastoma multiforme represents the most common primary brain tumour and, despite recent research efforts in cancer therapy, the prognosis of patients with malignant gliomas has remained dismal (Holland 2001). Malignant gliomas rarely metastasize outside the central nervous system (CNS) but they can diffuse into the host brain, generating tumour microsatellites in the normal brain parenchyma. These microsatellites are not accessible to surgery and are the seed for recurrent tumour growth (Giese et al. 2003). Recent studies have shown that a small subpopulation of the brain tumour cells share key characteristics with neural stem progenitor cells, such as continuous self-renewal, extensive brain parenchymal migration/infiltration and the potential for

full or partial differentiation (Sanai et al. 2005). These findings suggest that brain tumours contain cancer stem cells that are critical for tumour growth. Knowledge of the molecular pathways that govern such stem cell-like behaviour might provide both mechanistic insights and targets for the treatment of glioblastoma multiforme.

Nucleotides represent an important and ubiquitous class of extracellular molecules that interact with specific receptors by activating the signalling pathways that are crucial for the normal function of the nervous system (Ralevic and Burnstock 1998). Extracellular ATP is not only involved in rapid excitatory neurotransmission, but also plays a role in a wide range of other tissues and biological processes including cell proliferation, differentiation and apoptosis (Burnstock 2004). Extracellular nucleotides act via ionotropic receptors (P2X₁₋₇, permeable to Na⁺, K⁺ and Ca²⁺) or G-protein-coupled receptors (eight subtypes, P2Y_{1, 2, 4, 6, 11, 12, 13, 14}; Fields and Burnstock 2006). Nucleotide receptor-mediated cell communication is controlled by ecto-nucleotidases, which hydrolyze ATP into adenosine in the extracellular space. The ENTPD/CD39 family, previously classified as E-type ATPases, constitute a class of ecto-enzymes characterized by their capacity to hydrolyze nucleoside tri- and diphosphates, their strict dependence upon divalent cations and their insensitivity to the classical inhibitors of the P-, F- and V-type ATPases (Plesner 1995). In mammals, at least eight related and homologous enzymes sharing five apyrase-conserved regions (ACRs), named NTDPase1 to NTDPase8, have been cloned and characterized; namely, NTPDase1 (CD39, ATPDase, ecto-apyrase or ecto-ATP diphosphohydrolase), NTPDase2 (CD39L1, ecto-ATPase), NTPDase3 (CD39L3, HB6), NTPDase4 (UDPase, LALP70), NTPDase5 (CD39L4, ER-UDPase, PCPH), NTPDase6 (CD39L2), NTPDase7 (LALP1) and NTPDase8 (Zimmermann 2001). The molecular properties, functional roles and nomenclature of the nucleotidases have been recently reviewed (Zimmermann 2001; Robson et al. 2006).

Disruption of purinergic signalling has been implicated in many pathological processes such as cancer. Investigations suggest that nucleotides exert a synergist effect on cell proliferation together with growth factors, chemokines or cytokines (Lemoli et al. 2004). In astrocytes, extracellular ATP regulates protein kinase (ERK) function by activating P2Y₁, P2Y₂ or P2Y₄ purinoceptors (Lenz et al. 2000; Neary et al. 2003). Additionally, we have previously identified changes in purinergic signalling in various glioma cell lines, when compared with astrocytes, where gliomas have altered extracellular ATP, ADP and AMP catabolism (Wink et al. 2003). Moreover, adenine nucleotides induce cell proliferation in U138MG and U87MG human glioma cell lines (Morrone et al. 2003).

Glioma cell lines have been widely studied as a model for both exploring the biology of brain tumours and as models

for pharmacological studies on P2Y-receptor-mediated effects in the nervous system (Sak and Illes 2005). However, it is becoming clear that, after repeated *in vitro* passages, the cell lines can express phenotypic drift towards cells that can successfully adapt to the tissue culture environment. Consequently, glioma cell lineages might exhibit many biological features that sometimes do not reflect the exact situation *in vivo* (Lee et al. 2006).

In the present study, we have compared some biological differences between the C6 rat glioma cell line in culture and a rat tumour *ex vivo* culture model. The C6 *ex vivo* model was obtained directly from rat biopsy specimens that were previously implanted with the same cell line and then maintained in culture. With this short *in vivo* treatment, we tried to select the cells that could better adapt to the *in vivo* environment. Thus, pre- to post-implantation differences could provide some hint of the molecular pathways that play a role in the survival and growth of the cells in the rat brain. The evaluated parameters included cell morphology, differentiation, angiogenic markers, purinergic receptors and mRNA profile/enzymatic activity of ecto-nucleotidases. The characteristics exhibited by the two distinct glioma models are further discussed and could provide an insight into the way that the nucleotides modulate tumour growth *in vivo*.

Materials and methods

C6 glioma cell line culture

The C6 rat glioma cell line was obtained from the American Type Culture Collection (Rockville, Md., USA). The C6 glioma cell line was grown in culture flasks in culture medium containing 1% DMEM (Gibco BRL), 8.39 mM HEPES (pH 7.4), 23.8 mM NaHCO₃, 0.1% Fungizone (Gibco BRL), 0.032% Garamicin (Gibco BRL) and 5% fetal bovine serum (FBS). Confluent cells were seeded in 24-multiwell plates (TTP plates) at densities of 1×10⁴ cells/well in a final volume of 500 µl culture medium. Cell cultures were maintained in 5% CO₂/95% air at 37°C and allowed to grow to confluence.

C6 glioma cell implantation in rat brain

The rat C6 glioma cell line was cultured to approximately 70% confluence and then cells were trypsinized (0.25% trypsin/EDTA solution), washed once in DMEM/5% FBS, spun down and suspended in DMEM/5% FBS. A total of one million cells in a 3 µL volume were injected at a depth of 6.0 mm into the right striatum (coordinates with regard to bregma: 0.5 mm posterior and 3.0 mm lateral) of male Wistar rats (250–270 g, 8 weeks old) anesthetized by intraperitoneal administration of ketamine/xilazine (Takano et al. 2001). The

negative control group was treated similarly, except 3 μL DMEM/5% FBS was injected. The procedures for glioma implantation were approved by the Ethical Committee of the Hospital de Clinicas de Porto Alegre.

Rat brain fixation and histopathology

Rats were decapitated and their brains were immediately removed and fixed in 4% paraformaldehyde for 72 h, followed by embedding in paraffin. Brains were sectioned at 5 μm thickness on a MicromHM200 microtome (Eryostar) and stained with haematoxylin-eosin as a standard histopathological technique.

Primary culture from rat glioma biopsy (C6 *ex vivo* glioma model)

At 20 days after C6 glioma cell implantation, the rats were decapitated, the whole brain was removed and the tumor area was isolated to prepare the glioma primary culture. Briefly, rat glioma samples were removed and dissociated mechanically in a Ca^{2+} - and Mg^{2+} -free balanced salt solution (CMF-BSS, pH 7.4; containing 137 mM NaCl, 5.36 mM KCl, 0.27 mM Na_2HPO_4 , 1.1 mM KH_2PO_4 , 6.1 mM glucose). Following centrifugation at 1,000 g for 10 min, the pellet was suspended in culture medium consisting of 1% DMEM (Gibco BRL), 8.39 mM HEPES (pH 7.4), 23.8 mM NaHCO_3 , 0.1% Fungizone (Gibco BRL), 0.032% Garamicin (Gibco BRL) and 5% FBS. Glioma cells were then plated at a density of 5.0×10^5 cells on 24-multiwell plates or flasks pre-coated with poly-L-lysine. The cultures were maintained in 5% CO_2 /95% air at 37°C, allowed to grow to confluence (8–10 days in culture) and then used for *in vitro* assays. The medium was changed every 2 days.

Ectonucleotidase enzymatic assay

To determine ATP, ADP and AMP hydrolysis, 24-multiwell plates containing C6 glioma cell line or rat glioma primary culture (C6 *ex vivo* glioma) were washed three times with phosphate-free incubation medium in the absence of the nucleotides. The reaction was started by the addition of 200 μl of the incubation medium containing 2 mM CaCl_2 , 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES (pH 7.4) and 1 mM of ATP or ADP at 37°C. For AMP hydrolysis, the same incubation medium was used with the exception that 2 mM MgCl_2 was used instead of CaCl_2 and the final nucleotide concentration was 2 mM. Following incubation (20 min for ATP/ADP or 10 min for AMP hydrolysis), the reaction was stopped by removing an aliquot of the incubation medium and transferring it to a pre-chilled tube containing trichloro-acetic acid (5% w/v). The release of inorganic phosphate (Pi) was measured by

the malachite green method (Chan et al. 1986) with KH_2PO_4 as a Pi standard. Controls to determine non-enzymatic Pi release were performed by incubating the cells in the absence of the substrate or the substrate in the absence of the cells. To determine the protein concentration, the cell cultures were dried, solubilized with 100 μL 1 N NaOH and frozen overnight. The protein concentration was measured by the Coomassie Blue method (Bradford 1976) with bovine serum albumin as the standard. All samples were run in triplicate. Specific activity was expressed as nanomol Pi released per minute per milligram of protein.

Analysis by reverse transcription with polymerase chain reaction

Total RNA from C6 glioma cell line or rat glioma primary cultures (C6 *ex vivo* glioma) was isolated with Trizol LS reagent (Invitrogen, Carlsbad, Calif., USA) in accordance with the manufacturer's instructions. The cDNA species were synthesised with M-MLV Reverse Transcriptase (Promega, Madison, Wis., USA) from 5 μg total RNA in a final volume of 25 μL with a random hexamer primer in accordance with the manufacturer's instructions. One microliter of cDNA was used as a template for the polymerase chain reaction (PCR) in a total volume of 20 μL with 0.5 μM of each specific primer (Table 1), 50 μM dNTP and 1 U Taq polymerase (Phonotria Biotecnologia, Belo Horizonte, MG, Brazil) in the supplied reaction buffer. As a control for cDNA synthesis, reverse transcription with PCR (RT-PCR) for β -actin was performed (data not shown). The PCR cycling conditions were as follows: 1 min at 95°C, 1 min at 94°C, 1 min at the annealing temperature, 1 min at 72°C for 35 cycles, with a final extension at 72°C for 10 min. Ten microliters was analysed on a 1.5% agarose gel containing ethidium bromide and visualized under ultraviolet light. Negative controls were performed with templates substituted by DNase/RNase-free distilled water for each PCR.

Nucleotide glioma cell treatment

The C6 glioma cell line or rat glioma primary cultures (C6 *ex vivo* glioma) were seeded in 24-multiwell plates and treated with ATP (0.1, 0.5 or 5.0 mM) for 24 h. The medium was then removed and the cells were washed and detached by phosphate-buffered saline and 200 μL 0.25% trypsin/EDTA solution (Gibco BRL). The cells were counted in a hemocytometer.

Statistical analysis

Results are presented as means \pm SD. Data were analysed by ANOVA (analysis of variance), followed by Tukey's

Table 1 Primer sequences, annealing temperatures (*T_a*) and fragment sizes (*F* forward, *R* reverse, *GFAP* glial fibrillary acidic protein, *VEGF* vascular endothelial growth factor)

Gene	Primer sequence	T _a (°C)	Fragment size
GFAP F	5'CGAATGCCCCCTCCACTCC3'	61	732
GFAP R	5'GTCGTTGGCTTCGTGCTTG3'		
Vimentin F	5'GGTCCGTGTCCTCGTCCTC3'	61	456
Vimentin R	5'CCCGCATCTCCTCCTCGTA3'		
Nestin F	5'GGCAGCGTTGGAACAGAGGTTGGA3'	60	299
Nestin R	5'CTCTAAACTGGAGTGGTCAGGGCT3'		
CD31 F	5'GCTGTCTACTCAGTCATGG3'	58	226
CD31 R	5'CTTCCTTCTGGATGGTGAAG3'		
Flt1 F	5'ATGCCAGCAAGTGGGAGTT3'	58	277
Flt1 R	5'ATCACCATCAGAGGCCCTC3'		
VEGF F	5'GCACATAGGAGAGATGAGC3'	58	347
VEGF R	5'TAGTTCCCGAAACCCTGAG3'		
ETA F	5'GGCTTCGTCATGGTACCCT3'	60	536
ETA R	5'TTCATGGGGACCGAGGTCA3'		
ETB F	5'TGACGCCACCCACTAAGAC3'	60	286
ETB R	5'GCGATCAAGATATTGGGACC3'		
P2Y1 R	5'AGATGAAATAACTTCGCAGG3'	58	338
P2Y1 F	5'TGTTCAATTTGGCTCTGGC3'		
P2Y2 F	5'CTTCGCCCTCTGCTTCTCTG3'	58	222
P2Y2 R	5'TTGGCATCTCGGGCAAAGC3'		
P2Y4 F	5'GGCATTGTCAGACACCTTG3'	58	530
P2Y4 R	5'AAGACAGTCAGCACACACAG3'		
P2Y6 F	5'CGCTTCCTCTTCTATGCCA3'	58	478
P2Y6 R	5'AGGCTGTCTTGGTGATGTG3'		
P2Y12 F	5'GACTACAAGATCACCCAGG3'	58	245
P2Y12 R	5'CCTCCTGTTGGTGAGAATC3'		
P2Y13 F	5'GCCGACTTGATAATGACAC3'	58	176
P2Y13 R	5'ATGATCTTGAGGAATCTGTGC3'		
P2Y14 F	5'TCTTTTACGTGCCAGCTC3'	58	217
P2Y14 R	5'CTGTCAAAGCTGATGAGCC3'		
P2X1 F	5'AAGGTCAACAGGCGCAACC3'	60	338
P2X1 R	5'AACACCTTGAAGAGGTGACG3'		
P2X2 F	5'GTGCAGAAAAGCTACCAGG3'	60	406
P2X2 R	5'GGATGGTGAAATTTGGGGC3'		
P2X3 F	5'GCTGCGTGAACACTACAGCTC3'	60	360
P2X3 R	5'ACTGGTCCCAGGCCCTGTGC3'		
P2X4 F	5'CTTGGATTCCGGATCTGGG3'	60	457
P2X4 R	5'GGAATATGGGGCAGAAGGG3'		
P2X5 F	5'GCACCTGTGAGATCTTTGC3'	60	218
P2X5 R	5'TCGGAAGATGGGGCAGTAG3'		
P2X6 F	5'CAGGACCTGTGAGATCTGG3'	60	369
P2X6 R	5'TCCTGCAGCTGGAAGGAGT3'		
P2X7 F	5'TCCCTTTCAGGGGAACT C3'	60	465
P2X7 R	5'GTACGGTGAAGTTTTCGGC3'		
NTPDase1 F	5'GATCATCACTGGGCAGGAGGAAGG3'	60	543
NTPDase1 R	5'AAGACACCGTTGAAGGCACACTGG3'		
NTPDase2 F	5'GCTGGGTGGGCCGGTGGATACG3'	60	331
NTPDase2 R	5'ATTGAAGGCCCGGGGACGCTGAC3'		
NTPDase3 F	5'CGGGATCCTTGCTGTGCGTGGCATTCTT3'	60	267
NTPDase3 R	5'TCTAGAGGTGCTCTGGCAGGAATCAGT3'		
NTPDase5 F	5'GGGATCCTTTGAGATGTTTAAACAGCACT3'	60	156
NTPDase5 R	5'GAATTCTTGGTTACCACCATACTGGTA3'		
NTPDase6 F	5'GAATTCCTTGTCCGGGATGACTGTGTT3'	60	156
NTPDase6 R	5'ATCTGAGTGGATCCTCCGCCCAA3'		
Ecto-5'NT/CD73 F	5'CCCGGGGGCCACTAGCACCTCA3'	60	473
Ecto-5'NT/CD73 R	5'GCCTGGACCACGGGAACCTT3'		

test. *P*-values of <0.05 were taken to indicate statistical significance.

Results

Tumour tissues from rat glioma

A histopathological evaluation of the implanted rat glioma is given in Fig. 1. As previously demonstrated by our group, the implanted tumour showed all the histological characteristics of a glioblastoma multiforme (Morrone et al. 2006). Briefly, the tumours consisted of atypical cells with mitotic features, plus degenerative changes with necrosis, the presence of haemorrhagic areas, oedema, lymphocyte infiltration and endothelial cell proliferation.

In vitro characterization of C6 cell line culture and the C6 *ex vivo* glioma model

The C6 glioma cell line and the C6 *ex vivo* glioma model cultured under the same conditions displayed profound morphological differences *in vitro*. Whereas the C6 glioma cell line cultures readily proliferated as a cell monolayer exhibiting a characteristic homogeneous morphology (Fig. 2a), the cells obtained from the implanted glioma (C6 *ex vivo* glioma) had a highly heterogeneous morphology and proliferated as multicellular spheres (Fig. 2b). Such sphere formation persisted until the 5th passage *in vitro*.

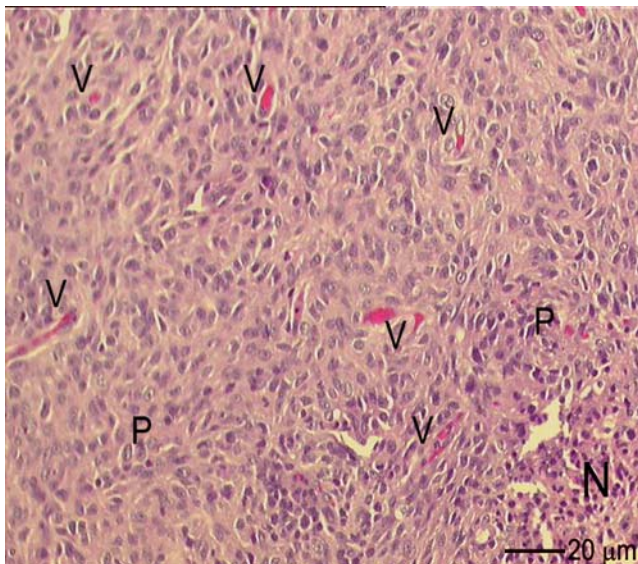


Fig. 1 Histopathological features of the implanted gliomas. Sections of implanted rat glioma were stained with haematoxylin and eosin. Histological characteristics that define glioblastoma multiforme, as seen in the implanted gliomas: necrosis (N), microvascular proliferation (V), peripheral pseudopalisading (P), giant cell formation and nuclear pleomorphism (magnification $\times 200$)

However, following the 5th passage, the cells returned to growth as a cell monolayer, with a morphology similar to that of the C6 glioma cell line (Fig. 2c).

As shown in Fig. 3, the expression of intermediary filaments as markers of the differentiation state was evaluated. Glial fibrillary acidic protein (GFAP) and vimentin were employed as markers of glial cell lineage, whereas nestin was used as a marker of neural stem cells. Astrocyte culture cDNA was taken as a positive control for GFAP and vimentin expression. The C6 glioma cell line and the C6 *ex vivo* glioma model exhibited a similar pattern of cell differentiation markers, presenting vimentin and nestin mRNA expression, whereas GFAP mRNA expression was absent.

Angiogenic markers of C6 cell line culture and C6 *ex vivo* glioma model

Angiogenesis is a key event in the natural progression of gliomas. In order to evaluate the angiogenic profile exhibited by the C6 cell line and the C6 *ex vivo* cultures, the expression of angiogenic markers was analysed by RT-PCR. Both the C6 culture models expressed all angiogenesis markers evaluated (CD31, flt1, VEGF, ETA and ETB), in accordance with the highly angiogenic characteristic exhibited by malignant gliomas (Jain et al. 2007; data not shown).

Co-expression of multiple P2X and P2Y receptor subtypes in C6 cell line culture and C6 *ex vivo* glioma model

Extracellular nucleotides exert a variety of biological actions such as cell proliferation or cell death through purinergic receptors. To determine which mRNA P2X and P2Y receptors were expressed by the C6 cell line culture and the C6 *ex vivo* glioma model, mRNA was isolated from both cell cultures and was analysed by RT-PCR. Amplicons of the expected sizes were obtained for P2X₂, P2X₄ and P2X₅ in the C6 glioma cell culture, whereas the C6 *ex vivo* glioma culture expressed only P2X₄ mRNA (Fig. 4).

Similarly, amplicons of the expected sizes were obtained for the P2Y receptors (Fig. 5). The mRNA expression of P2Y receptors was the same in both glioma models except that P2Y₆ was only expressed by the C6 *ex vivo* glioma culture. The purinoceptor mRNA profile expression of the C6 and C6 *ex vivo* glioma cultures is summarized in Table 2.

Ectonucleotidase mRNA expression and enzymatic activities in C6 cell line culture and C6 *ex vivo* glioma model

Since the C6 cell line culture and the C6 *ex vivo* glioma model demonstrated the co-expression of multiple P2X and P2Y

receptor subtypes and since the signalling events induced by the extracellular adenine nucleotides is believed to be controlled by the conjugated action of NTPDases and ecto-5'-nucleotidase/CD73 (ecto-5'-NT/CD73; Zimmermann 2001; Robson et al. 2006), we investigated the mRNA expression of NTPDases 1–3, 5 and 6 and ecto-5'-NT/CD73 in the C6 cell line culture and the C6 *ex vivo* glioma model. Both glioma cell culture models showed a similar mRNA expression pattern for all ectonucleotidases evaluated. Whereas amplicons were obtained for NTPDase2, NTPDase3, NTPDase5, NTPDase6 and ecto-5'-NT/CD73, NTPDase1 transcript was absent in both cell types (Fig. 6a). In addition, the C6 glioma cell line and the C6 *ex vivo* cell cultures exhibited a similar pattern of nucleotide metabolism, such that the extracellular ATP and ADP were found to be poorly hydrolyzed by both glioma cultures when compared with AMP. Statistical analysis revealed that AMP hydrolysis was higher in both glioma cultures when compared with ATP and ADP hydrolysis (Fig. 6b).

Effect of extracellular ATP on C6 and C6 *ex vivo* cell proliferation

High extracellular ATP has been shown to induce cell death in various cell types by activating the P2X₇R (Khakh and Kennedy 1998; Rathbone et al. 1999) or by acting as a chemotactic signal for microglial activation in CNS (Haynes et al. 2006). Previous studies published by our laboratory have shown that U138MG and C6 glioma cell lines exhibit a clear resistance to cell death induced by cytotoxic concentrations of ATP when compared with normal brain cells (Morrone et al. 2005). Therefore, we evaluated whether the C6 *ex vivo* glioma cells maintained this similar response to high extracellular ATP concentrations (Fig. 7). The C6 cell line and the C6 *ex vivo* glioma cells were treated with ATP (0.1, 0.5 and 5.0 mM) for 24 h and the cells were counted in a haemocytometer. Figure 7 shows that, even in the presence of cytotoxic ATP concentrations, no significant differences were found in cell proliferation in the C6 cell line and C6 *ex vivo* glioma cells, when compared with controls. These results are in agreement with previously published data (Morrone et al. 2005) and suggest that glioma cell cultures are resistant to cell death induced by ATP.

Discussion

Glioblastoma multiforme is a highly aggressive primary brain tumour giving a mean survival of less than 12 months after diagnosis, despite maximal treatment (Stupp et al. 2005). One traditional and essential approach to studying the molecular features and pathogenesis of glioblastoma

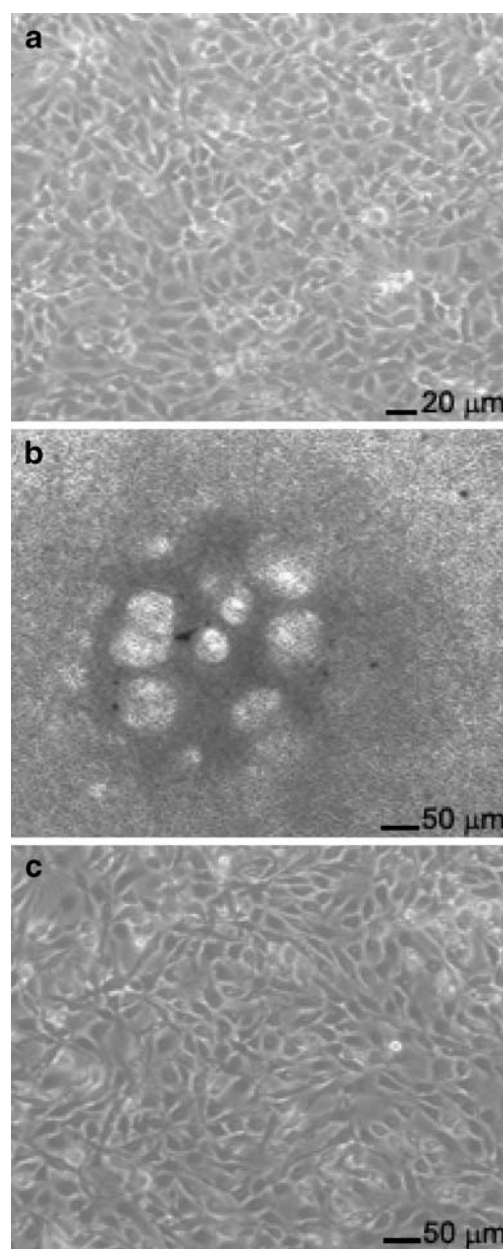


Fig. 2 Characterization of C6 glioma cell line and primary culture from rat glioma biopsy (C6 *ex vivo* glioma model). Microphotographs of the C6 glioma cell line (a), C6 *ex vivo* glioma cell culture with spheres or tumour-spheres from the glioblastoma at the first passage (b), C6 *ex vivo* glioma cell culture at the fifth passage (c). Images were captured by using a digital camera coupled to an inverted microscope (Nikon Eclipse TE300)

multiforme is the use of cell lines derived from malignant gliomas (Ponten and Macintyre 1968; Giard et al. 1973). However, increasing evidence suggests that glioma cell lines often lose the phenotypic and the genetic characteristics of the primary tumours because of repeated *in vitro* passages (Lee et al. 2006). In light of these new findings, we have asked whether the C6 glioma cell line, a nitrosurea-induced tumour widely used as a glioma model

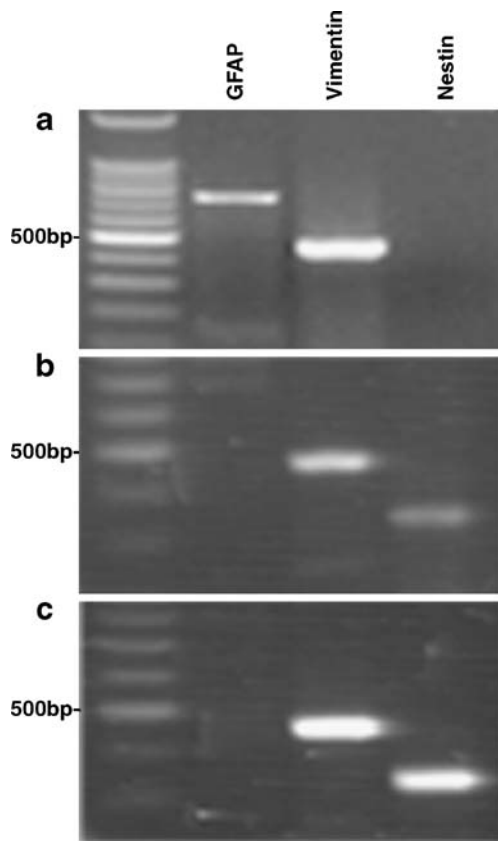


Fig. 3 Evaluation of glial fibrillary acidic protein (GFAP), vimentin and nestin mRNA expression in the C6 glioma cell line and C6 *ex vivo* glioma model. Cells were submitted to RNA extraction and processed for expression analysis of target genes. RT-PCR analysis generated fragments of the expected size. The length (bp) of the PCR products obtained with each pair of primers is given in Table 1. **a** Astrocyte cells were used as a positive control for GFAP and vimentin expression. **b** C6 glioma cell line culture. **c** C6 *ex vivo* glioma cell culture. The data are representative of five different glioma cultures analysed in independent experiments

for *in vitro* studies, can maintain the characteristics of primary tumours in relation to purinergic signalling. To answer this question, we have compared the expression of cell differentiation and angiogenesis markers, purinergic receptors and ectonucleotidases in rat C6 glioma cell line cultures with those of primary cultures from rat glioma biopsies (C6 *ex vivo* glioma).

Tumorigenicity, resistance to therapy and biological features presented by tumoural cells have been suggested to be related to a selected population of cells with a stem-like phenotype (Galli et al. 2004; Lee et al. 2006). Therefore, we have compared the morphological characteristics of the C6 cell line and the C6 *ex vivo* glioma in culture. Whereas the C6 cells proliferate as a cell monolayer, the C6 *ex vivo* glioma cells grow as spheres, a characteristic feature of tumour stem cells that are responsible for tumour expansion (Sanai et al. 2005; Lee et al. 2006; Ghods et al. 2007). Our *ex vivo* glioma model is able to form spheres until the 5th passage, even in the presence of fetal bovine serum,

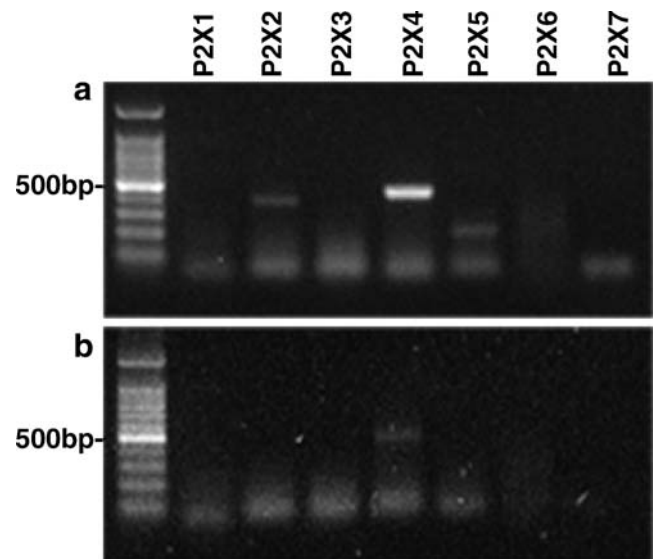


Fig. 4 Analysis of mRNA expression for P2X receptor subtypes in the C6 glioma cell line and C6 *ex vivo* glioma model. Cells were submitted to RNA extraction and processed for expression analysis of target genes (P2X₁₋₇). RT-PCR analysis generated fragments of the expected size. The length (bp) of the PCR products obtained with each pair of primers is given in Table 1. **a** C6 glioma cell line culture. **b** C6 *ex vivo* glioma cell culture. The data are representative of five different glioma cultures analysed in independent experiments

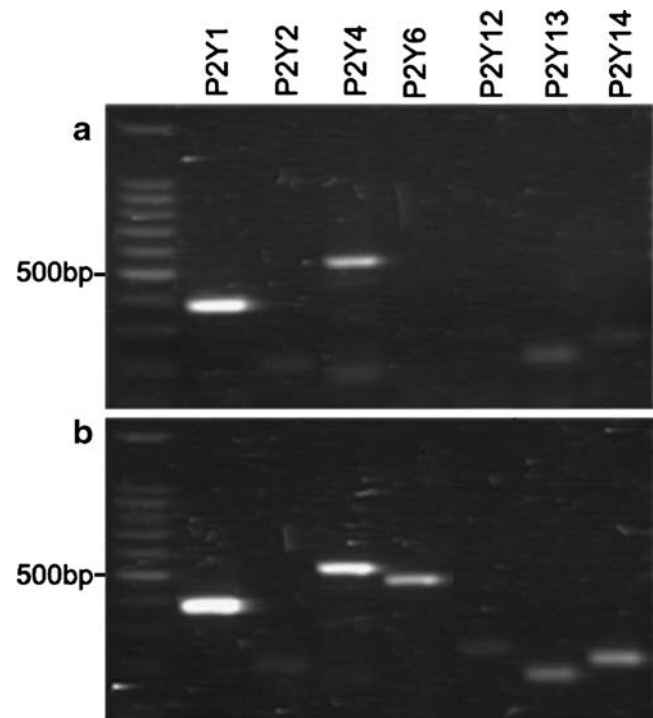


Fig. 5 Analysis of mRNA expression for P2Y receptor subtypes in the C6 glioma cell line and C6 *ex vivo* glioma model. Cells were submitted to RNA extraction and processed for expression analysis of target genes (P2Y_{1, 2, 4, 6, 12, 13, 14}). RT-PCR generated fragments of the expected size. The length (bp) of the PCR products obtained with each pair of primers is given in Table 1. **a** C6 glioma cell line culture. **b** C6 *ex vivo* glioma cell culture. The data are representative of five different glioma cultures analysed in independent experiments

Table 2 Summary of mRNA P2X and P2Y receptor subtypes identified in the C6 glioma cell line and C6 *ex vivo* glioma model by RT-PCR (+ present, ± weakly present, - absent). The PCR data are representative of five different glioma cultures analysed in independent experiments

	P2X ₁	P2X ₂	P2X ₃	P2X ₄	P2X ₅	P2X ₆	P2X ₇	P2Y ₁	P2Y ₂	P2Y ₄	P2Y ₆	P2Y ₁₂	P2Y ₁₃	P2Y ₁₄
C6 cell line	-	+	-	+	+	-	-	+	±	+	-	±	+	±
C6 <i>ex vivo</i>	-	-	-	+	-	-	-	+	±	+	+	±	+	+

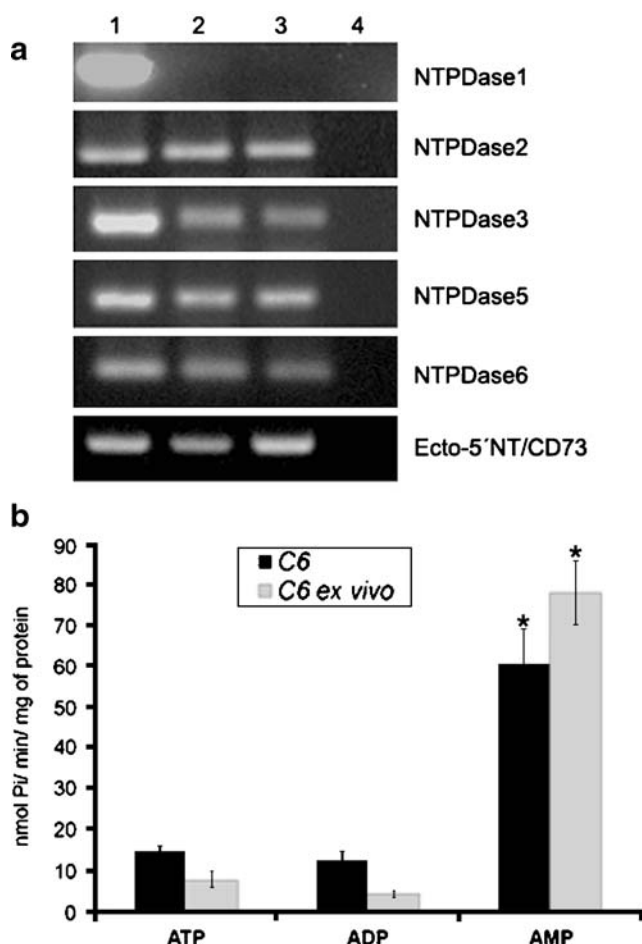


Fig. 6 Analysis of NTPDase and ecto-5'-NT/CD73 expression in C6 glioma cell line and C6 *ex vivo* glioma model. **a** RT-PCR analysis of NTPDase and ecto-5'-NT/CD73 expression. Total RNA was isolated from C6 glioma and C6 *ex vivo* glioma cultures and the cDNA was analysed by PCR with specific primers. Plasmids containing the sequences of all NTPDases and ecto-5'-NT/CD73 were used as positive controls (*lane 1* positive control, *lane 2* C6 glioma cell line, *lane 3* C6 *ex vivo* glioma model, *lane 4* negative control). The length (bp) of the PCR products obtained with each pair of primers is given in Table 1. The data are representative of five different glioma cultures analysed in independent experiments. **b** Enzymatic activity for ATP, ADP and AMP hydrolysis by C6 glioma and C6 *ex vivo* glioma cells. Glioma cells were cultured in 24-multiwell plates and submitted to enzymatic assay. Specific activity was expressed as *nmol Pi/min/mg of protein*. Data represent the means \pm SD of five independent experiments performed in triplicate. Data were analysed by ANOVA, followed by Tukey's test. *Statistical difference between AMP hydrolysis when compared with ATP and ADP hydrolysis

demonstrating the behaviour of tumoural stem cells in culture (Yuan et al. 2004). Although the two glioma models studied here present differences in cell morphology, the patterns of cell maturation markers are similar in both cultures, providing evidence for the presence of vimentin and nestin and the absence of GFAP expression. Nestin is expressed abundantly in neuroepithelial stem cells during early neurogenesis but is absent in the most mature cells of the CNS (Lendahl et al. 1990). The nestin expression in the C6 cell line and in the C6 *ex vivo* glioma cultures might be correlated to the presence of the immature cell type, which can continually divide without undergoing terminal differentiation.

ATP regulates cell proliferation, motility, survival and differentiation in a variety of cell types through purinergic receptors (Fields and Burnstock 2006). The disruption of purinergic signalling is also involved in the tumour process (White and Burnstock 2006). With regard to purinergic receptor expression, we have found that both the C6 cells and the C6 *ex vivo* glioma cultures express specific purinergic receptors in common, namely, P2X₄, P2Y₁, P2Y₂, P2Y₄, P2Y₁₂, P2Y₁₃ and P2Y₁₄. The expression of P2X₄ in the C6 glioma cells has been previously reported (Guo et al. 2004) and is related to a subset of tumour cells

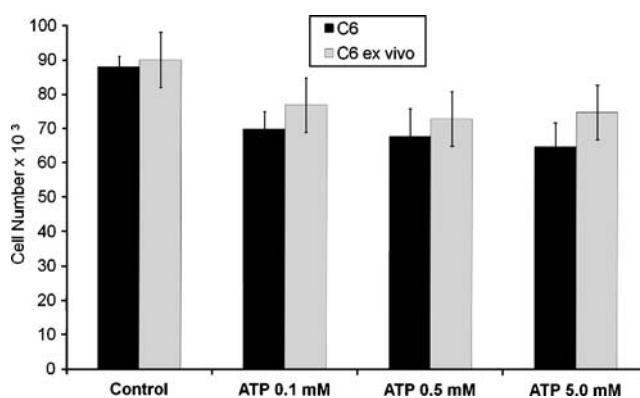


Fig. 7 Effect of extracellular ATP on C6 glioma and C6 *ex vivo* glioma in culture. Glioma cells were cultured in 24-multiwell plates and were exposed to 0.1, 0.5 or 5 mM ATP for 24 h. Following ATP treatment, cells were detached and counted in a haemocytometer. Results are expressed as cell number. Data represent the means \pm SD of three independent experiments performed in triplicate. Data were analysed by ANOVA, followed by Tukey's test. No significant differences were observed between groups

associated with activated microglia, which, by releasing cytokines, can favour angiogenesis and invasion process in gliomas (Guo et al. 2004; Deininger et al. 2000). P2Y₁ has a dual role; it has been implicated in decreased cell proliferation in many cancers such as melanoma (White et al. 2005) but is also involved in cell proliferation stimuli in glioma cells (Czajkowski et al. 2004). P2Y₂ is usually related to increased cell proliferation by activation of the phospholipase C pathway (White and Burnstock 2006). Finally, P2Y₁₂ has been reported to mediate cell proliferation in C6 glioma cells by activating the ERK1/ERK2 pathway (Czajkowski et al. 2004).

Interestingly, the presence of P2Y₆ and the absence of P2X₅ mRNA expression in the C6 *ex vivo* glioma cultures possibly indicate that they have important roles in cell proliferation in several manners. For instance, P2Y₆ is related to increased cell survival and the inhibition of apoptosis in osteoclasts and 1321N1 astrocytoma cells, respectively (Korkov et al. 2005; Kim et al. 2003), whereas P2X₅ inhibits cell proliferation and stimulates markers of cell differentiation (White and Burnstock 2006). In addition, P2X₇, which mediates cell apoptosis, is notably absent in both the C6 cell line and the C6 *ex vivo* glioma cultures. The differences in P2 receptor expression observed between the two glioma cultures might be attributable, at least in part, to the *in vivo* environment passage when the C6 cells are exposed to vascular blood and a variety of signalling factors able to modulate purinergic receptor expression.

Important mechanisms are involved in the control of the extracellular nucleotide levels and, hence, regulate P2-mediated effects. Several ectonucleotidases are involved in ATP extracellular hydrolysis to adenosine including the NTPDases and the ecto-5'-NT/CD73. The C6 cells and the C6 *ex vivo* glioma cultures poorly metabolize the ATP/ADP outside the cells, whereas the AMP is efficiently hydrolyzed. The nucleotide hydrolysis pattern exhibited by these two different types of glioma cell cultures is in agreement with previous studies published by our group (Wink et al. 2003). Consistent with the high AMP hydrolysis, the mRNA for ecto-5'-NT/CD73 is clearly detectable in both cell cultures. Such results emphasize the inversion of extracellular ATP metabolism in glioma cells when compared with normal astrocytes, as previously reported by our group (Wink et al. 2003; Morrone et al. 2006).

Finally, as in C6 glioma cells (Morrone et al. 2005), cytotoxic ATP concentrations do not alter C6 *ex vivo* glioma cell proliferation, which is in agreement with the absence of the P2X₇ receptor (Di Virgilio 2000). Notably, the extracellular nucleotide metabolism, the pattern of mRNA expression of ectonucleotidases and the normal cell proliferation rate, even in the presence of high ATP concentrations, exhibited by the C6 *ex vivo* glioma cells indicate that the disruption of purinergic signalling is a

characteristic that is expressed not only by the glioma cell lineages, but also by a primary glioma culture.

Overall, our study has found that the C6 glioma cell line and the C6 *ex vivo* glioma model, which represents a closer model of original tumours, exhibit similar alterations in ATP extracellular metabolism and normal proliferation responses when exposed to high ATP concentrations. The results presented herein and the data previously published by our group suggest that changes in extracellular ATP metabolism are a characteristic of this kind of tumour. Further studies are necessary to evaluate whether the disruption of purinergic signalling contributes to glioma malignancy.

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3.2 ARTIGO 2

SELECTIVE NTPDASE2 EXPRESSION MODULATES *IN VIVO* RAT GLIOMA GROWTH

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Selective NTPDase2 expression modulates *in vivo* rat glioma growth

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The ectonucleoside triphosphate diphosphohydrolases (E-NTPDases) are a family of ectoenzymes that hydrolyze extracellular nucleotides, thereby modulating purinergic signaling. Gliomas have low expression of all E-NTPDases, particularly NTPDase2, when compared to astrocytes in culture. Nucleotides induce glioma proliferation and ATP, although potentially neurotoxic, does not evoke cytotoxic action on the majority of glioma cultures. We have previously shown that the co-injection of apyrase with gliomas decreases glioma progression. Here, we tested whether selective re-establishment of NTPDase2 expression would affect glioma growth. NTPDase2 overexpression in C6 glioma cells had no effect on *in vitro* proliferation but dramatically increased tumor growth and malignant characteristics *in vivo*. Additionally, a sizable platelet sequestration in the tumor area and an increase in CD31 or platelet/endothelial cell adhesion molecule-1 (PECAM-1), vascular endothelial growth factor and OX-42 immunostaining were observed in C6-Enhanced Yellow Fluorescent Protein (EYFP)/NTPDase2-derived gliomas when compared to controls. Treatment with clopidogrel, a P2Y₁₂ antagonist with anti-platelet properties, decreased these parameters to control levels. These data suggest that the ADP derived from NTPDase2 activity stimulates platelet migration to the tumor area and that NTPDase2, by regulating angiogenesis and inflammation, seems to play an important role in tumor progression. In conclusion, our results point to the involvement of purinergic signaling in glioma progression. (*Cancer Sci* 2009; 100: 1434–1442)

Glioblastoma multiforme, the most common form of malignant brain tumor, is relatively resistant to therapeutic strategies and has a median survival after first diagnosis of only around 12 months. This median survival has remained unchanged for decades despite multiple clinical trials designed to optimize radiation and/or chemotherapy regimens.^(1,2) Although systemic metastases of malignant gliomas are relatively rare, the highly infiltrative nature exhibited by these tumors is the main cause of treatment failure and high recurrence rates. Recent works suggest that malignant gliomas have a stem cell population, which is fundamental for tumor maintenance and growth. Such cellular subpopulations seem to be more resistant to radiotherapy, exhibit enhanced proliferative and migratory potential, and can overcome diverse paths of differentiation.⁽³⁾ In addition to neoplastic cells, a number of non-malignant cells such as lymphocytes, endothelial cells, microglial cells, and macrophages comprise the tumor microenvironment and appear to be associated with tumor progression by influencing cell proliferation and angiogenesis.⁽⁴⁾

Glioma invasion is a multifactorial process consisting of numerous genetic and physiological alterations, which affect glioma cell interactions with neurons, glia, and vascular cells in the central nervous system.⁽³⁾ Among the pathological alterations that give tumor cells invasive potential, purinergic signaling is emerging as an important component. By activating specific

purinergic receptors (P2X and P2Y), extracellular ATP has been shown to mediate a variety of biological functions,⁽⁵⁾ including events related to cell proliferation, cell differentiation, and cell death.⁽⁶⁾ The effects of nucleotides and nucleosides on purinergic receptors are regulated by the action of ectonucleotidases, such as ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) and ecto-5'-nucleotidase or CD73 (ecto-5'-NT/CD73). These ectoenzymes can efficiently hydrolyze ATP, ADP, and AMP to adenosine in the extracellular space, with the latter being taken up by specialized transporters. The molecular properties, functional roles, and nomenclature of ectonucleotidases have been extensively reviewed in Zimmermann and Robson *et al.*^(7,8)

Accumulating evidence published by our group and others suggests that purinergic signaling is involved in the growth and progression of gliomas. In previous studies, we demonstrated that glioma cell lines have altered extracellular ATP, ADP, and AMP catabolism, showing low rates of extracellular ATP hydrolysis and high rates of extracellular AMP hydrolysis when compared to astrocytes in culture.⁽⁹⁾ Adenine nucleotides induce cell proliferation in diverse human glioma cell lines⁽¹⁰⁾ and the majority of glioma cell lines are resistant to cell death induced by cytotoxic ATP concentrations.⁽¹¹⁾ As ATP is poorly hydrolyzed by glioma cells, it could potentially accumulate in the tumor periphery resulting in glioma cell proliferation and neuronal toxicity. It has been suggested that the purinergic^(9–12) and glutamatergic systems⁽¹³⁾ are involved in this process. In this model, we hypothesize that neuronal death induced by glutamate released from gliomas results in the liberation of the intracellular milieu, which normally has high ATP and glutamate concentrations, leading to more neuronal cell death and glioma proliferation in a positive feedback cycle. Because gliomas, contrary to astrocytes in culture, exhibit low NTPDase expression and activity,⁽¹²⁾ this feedback is not blocked. In agreement with this hypothesis, we have previously shown that the co-injection of apyrase, an ATP scavenger enzyme, with gliomas significantly decreases the growth and malignancy of gliomas implanted in rats, indicating that ATP plays an important role in glioma proliferation *in vivo*.⁽¹²⁾

Considering that NTPDase2 is the dominant E-NTPDase member expressed by astrocytes in culture,⁽¹⁴⁾ the present study aimed to better characterize the participation of nucleotides and the role of E-NTPDases in glioma progression by restoring NTPDase2 expression and activity in rat C6 glioma cells. The *in vitro* and *in vivo* impact of NTPDase2 expression on glioma growth was evaluated. The results shown here could give new insights into how nucleotides modulate glioma progression *in vivo*.

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Materials and Methods

Cell culture. The C6 rat glioma and COS-7 cell lines (American Type Culture Collection, Rockville, Maryland, USA) were grown in culture flasks and maintained in DMEM containing 1% DMEM, 8.39 mM HEPES (pH 7.4), 23.8 mM NaHCO₃, 0.1% fungizone, and 0.032% gentamicin (Gibco BRL, Invitrogen Co., Carlsbad, CA, USA) and supplemented with 5 or 10% (v/v) FBS (Cultilab, Campinas, SP, Brazil) for rat C6 glioma or COS-7 cells respectively. Cells were kept at a temperature of 37°C, humidity of 95% and 5% CO₂ in air.

DNA construction. Enhanced Yellow Fluorescent Protein (EYFP)-NTPDase2 was constructed by inserting NTPDase2 from pGW1-hCD39L1⁽¹⁵⁾ using *EcoRI* into the PBS-SKII plasmid (Stratagene, La Jolla, CA, USA), sequencing it, and then inserting it into pEYFP-C1 (Clontech Laboratories Inc., Palo Alto, CA, USA) using *BamHI* and *SallI* restriction sites.

Transient transfection. COS-7 cells were seeded in 24-multiwell plates and grown in DMEM with 10% FBS. Cells at 80% confluence were transfected with Lipofectamine 2000 (Life Technologies, Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions with 1 µg/well of PBS-SKII-hNTPDase2 (COS-7-NTPDase2), pEYFP-hNTPDase2 (COS-7-EYFP/NTPDase2), or pEYFP empty vector (COS-7-EYFP).

C6 glioma cells were grown in DMEM with 5% FBS. Cells were seeded in 25 cm² culture flasks and transfected with Lipofectamine 2000, using 1 µg of pEYFP (C6-EYFP) or pEYFP/NTPDase2 (C6-EYFP/NTPDase2). Twenty-four hours after transfection, the transfected cells were selected with 2.0 mg/mL G418 (Sigma Chemical Co., St. Louis, MO, USA).

The functionality of the plasmid encoding the EYFP or EYFP/NTPDase2 sequences was confirmed by evaluating the ATPase and ADPase activities in COS-7 and C6 glioma-transfected cells, as described below.

E-NTPDase activity assay. Three days after transfection, the ATP/ADPase activities were determined in COS-7 and C6 glioma cells, as described previously,⁽⁹⁾ in incubation medium (2 mM CaCl₂, 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES [pH 7.4], and 3 mM of nucleotides ATP or ADP) incubated at 37°C. The release of inorganic phosphate (Pi) and the protein concentration were measured by the Malachite Green⁽¹⁶⁾ and Coomassie Blue methods⁽¹⁷⁾ respectively. Specific activity was expressed as nmol Pi released/min/mg protein.

In vitro assessment of glioma cell proliferation. C6, C6-EYFP, or C6-EYFP/NTPDase2 glioma cells were seeded at 2 × 10³ cells/well in DMEM with 5% FBS in 96-multiwell plates and allowed to grow for increasing amounts of time (3, 4, 5, or 6 days). Then, the cell number was assessed by cell counting with a hemocytometer and cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma Chemical Co., St. Louis, MO, USA).

Glioma implantation. C6, C6-EYFP, or C6-EYFP/NTPDase2 glioma cells were cultured to approximately 70% confluence and a total of 1 × 10⁶ cells in 3 µL DMEM with 5% FBS was injected in the right striatum at a depth of 6.0 mm (coordinates with regard to bregma: 0.5 mm posterior and 3.0 mm lateral) of male Wistar rats (250–270 g, 8 weeks old) anesthetized by i.p. administration of ketamine and xilazine.⁽¹³⁾ The same procedure was carried out for the negative control group, except that 3 µL DMEM with 5% FBS (no cells) was injected. All procedures used in the present study followed the Principles of Laboratory Animal Care from NIH and were approved by the Ethical Committee of the Hospital de Clínicas de Porto Alegre.

In vivo clopidogrel treatment. One day after C6-EYFP/NTPDase2 glioma implantation, clopidogrel (10 mg/kg/day; Sanofi Winthrop, Toulouse, France) was administered by gavage to rats over a period of 19 days. Clopidogrel was prepared in PBS (pH 7.4)

at a final concentration of 8.4 mg/mL. Control animals were treated with the equivalent volume of PBS.

Pathological analysis and tumor volume quantification. Twenty days after glioma implantation, rats were decapitated and the entire brain was removed, sectioned and fixed in 10% paraformaldehyde (PFA) in PBS (pH 7.4). The fixed tissue sections were then stained with hematoxylin–eosin (HE) and pathological analysis of the slides was carried out by two pathologists in a blinded manner. Glioma cell proliferation was assessed by counting the number of mitotic glioma cell nuclei in ten randomly chosen fields (×200) per tumor (Olympus America Inc., Center Valley, PA, USA). Tumor size was quantified by analyzing images captured with a digital camera connected to a microscope with Image Tool Software (Department of Dental Diagnostic Science, The University of Texas Health Science Center, San Antonio, TX, USA). The total volume (mm³) of the tumor was computed by summing the segmented areas and by multiplication of the slice resolution.

Immunohistochemical staining. The anesthetized rats were perfused with 4% PFA in PBS (pH 7.4). Following post-fixation incubation in 4% PFA for 24 h, the brains were cryoprotected in 30% sucrose solution, embedded in OCT freezing medium (Tissue-Tek; Sakura Finetek, Torrance, CA, USA) and snap frozen in isopentane in dry ice and stored at –80°C until use. To carry out the immunohistochemical (IHC) analysis, cryostat sections (5 µm) were fixed in acetone, blocked in 1% albumin solution, and incubated overnight (4°C) with the following specific antibodies: mouse anti-rat mAb CD31 or platelet/endothelial cell adhesion molecule-1 (PECAM-1) (1:30) and rabbit anti-rat pAb P-selectin (1:500; BD Pharmingen, BD Biosciences, Mountain View, CA, USA); rabbit anti-rat pAb Ki67 (1:300) and mouse anti-rat mAb OX-42 (1:200; Abcam Inc., Cambridge, MA, USA); mouse anti-human mAb vascular endothelial growth factor (VEGF) (1:30; Dako, CA, USA). Next, tissue sections were incubated with biotinylated secondary antibody and streptavidin–avidin–biotin (kit Lsab; Dako, USA). The peroxidase reaction was carried out using 3,3'-diaminobenzidina tetrahydrochloride, according to the manufacturer's specifications. Finally, sections were counterstained with Harris haematoxylin. The IHC slides were analyzed by two pathologists in a blinded manner. The microvessel density measurements for CD31/PECAM-1 staining were carried out using Weidner's method.⁽¹⁸⁾ In addition to the morphologically identifiable vessels with a lumen, each positive endothelial cell cluster of immunoreactivity in contact with the selected field was counted as an individual vessel. The VEGF analysis was carried out according to methods of Cascinu and colleagues.⁽¹⁹⁾ VEGF expression showed cytoplasmic localization and only clearly immunoreactive cells were considered positive. The VEGF quantitative analysis was scored as the percentage of immunoreactive cells. Additionally, the P-selectin and OX-42 immunostaining were also scored as the percentage of immunoreactive cells. All immunohistological evaluations were made in ten randomly chosen fields (×200) per tumor (Olympus BH-2 microscope). In cases of disagreement, a final score was determined by consensus after independent re-examination.

Statistical analysis. Data were expressed as mean ± SD and were subjected to one-way analysis of variance (ANOVA) followed by Tukey–Kramer post-hoc test (for multiple comparisons). Differences between mean values were considered significant when $P < 0.05$.

Results

Effect of NTPDase2 expression on in vitro growth of C6 rat glioma cells. A plasmid encoding the EYFP/NTPDase2 sequence was constructed and its functionality tested by transfecting COS-7 cells and evaluating ATP/ADPase activities. Three days after transfection, COS-7 exhibited ATP/ADPase activities (170.0 ± 24.4

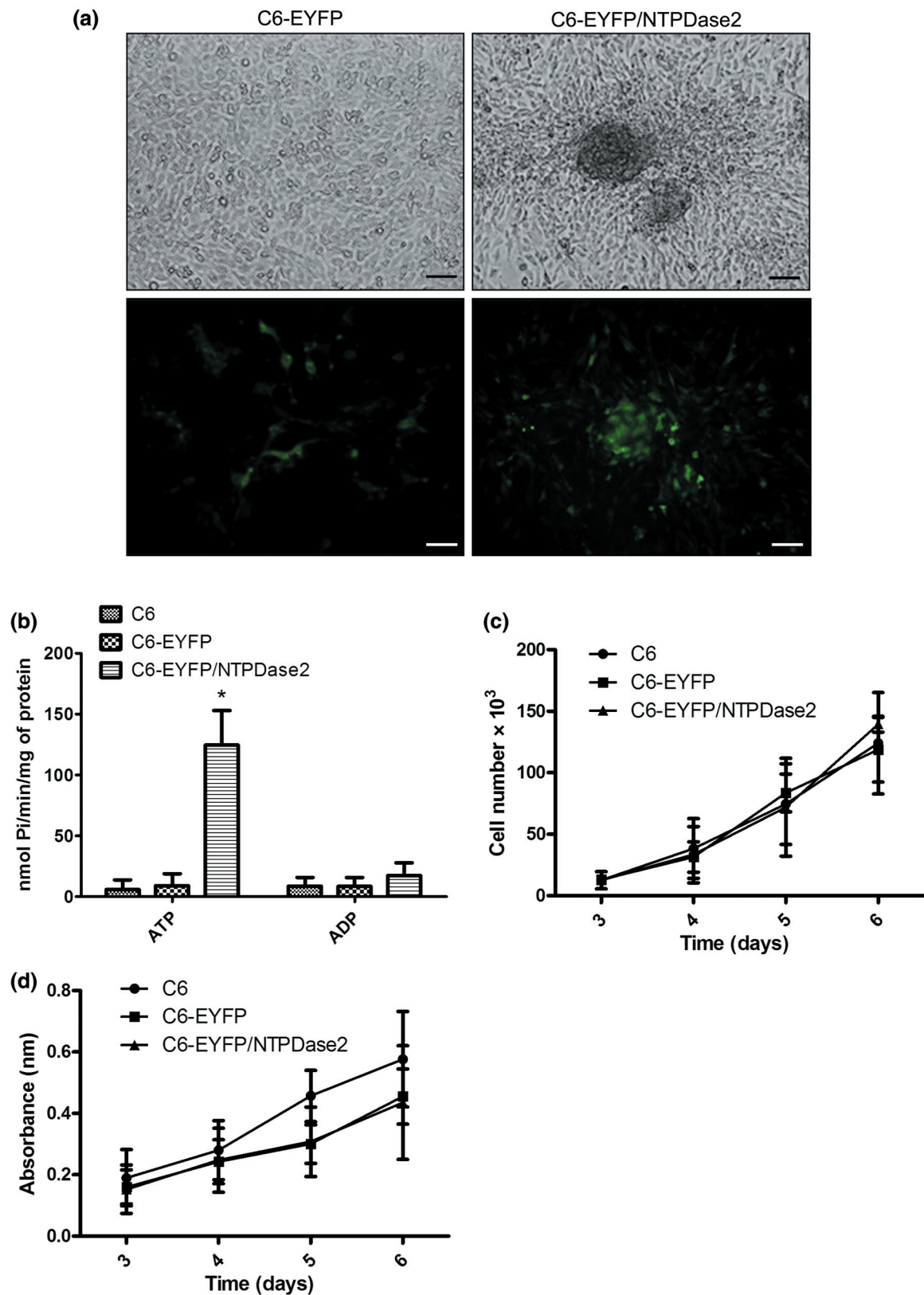


Fig. 1. NTPDase2 overexpression in C6 glioma cells. (a) Representative pictures of C6-EYFP and C6-EYFP/NTPDase2 cells in culture 72 h after transfection. C6 glioma cells were transfected with a plasmid encoding EYFP (C6-EYFP) or EYFP/NTPDase2 (C6-EYFP/NTPDase2). Scale bars = 20 μ m. (b) ATP/ADPase activities of C6 cells transfected as in (a). (c,d) The C6, C6-EYFP, and C6-EYFP/NTPDase2 cells (2×10^3 cells/per well) were grown in DMEM with 5% FBS and the cell number or cell viability was assessed by (c) cell counting or (d) MTT assay. The values represent the mean \pm SD of at least three independent experiments carried out in triplicate. Data were analyzed by ANOVA followed by post-hoc comparisons (Tukey–Kramer test). *Significantly different from the ATPase activity of non-transfected cells or cells transfected with EYFP empty vector ($P < 0.05$).

Table 1. Histological characteristics of implanted gliomas

Histology	C6 (n = 12)	C6-EYFP (n = 6)	C6-EYFP/NTPDase2 (n = 8)	C6-EYFP/NTPDase2 Clopidogrel (n = 5)
Coagulative necrosis	6/12	4/6	8/8	2/5
Intratumoral hemorrhage	4/12	2/6	8/8	2/5
Lymphocytic infiltration	10/12	6/6	8/8	4/5
Peritumoral edema	5/12	2/6	8/8	2/5
Peripheral pseudopalisading	5/12	3/6	6/8	1/5
Mitotic index: mitosis/ high power fields	14.5 ± 5.0	16.6 ± 4.7	24.4 ± 3.0*	8.0 ± 2.0*†

*Significant difference between C6-YN2 and control groups (C6 and C6-Y), †significant difference between C6-YN2 and C6-YN2 clopidogrel-treated group. $P < 0.05$ as determined by ANOVA, followed by Tukey–Kramer test.

and 8.8 ± 0.68 nmol Pi/min/mg protein for ATP and ADP, respectively), indicating an active NTPDase2. The activity of this chimeric NTPDase was similar to the non-chimeric form and cells transfected with EYFP empty vector did not exhibit ATP/ADPase activities (data not shown).

Next, NTPDase2 expression was restored in C6 glioma cells and the *in vitro* glioma growth was evaluated. Expression of NTPDase2 resulted in green fluorescence at the cell surface 72 h post-transfection (Fig. 1a), and these cells exhibited a high ATPase activity with an ATP/ADPase ratio of around 12, as expected for this enzyme (Fig. 1b). The *in vitro* growth of C6, C6-EYFP, and C6-EYFP/NTPDase2 glioma cells was accessed by cell count (Fig. 1c) and MTT assay (Fig. 1d). No significant differences in the growth of these cells were observed.

Effect of NTPDase2 expression on *in vivo* glioma growth. To determine the involvement of NTPDase2 on *in vivo* glioma growth, C6, C6-EYFP, or C6-EYFP/NTPDase2 glioma cells were implanted by intracranial injection into adult Wistar rats. Twenty days after glioma implantation, rat brains were fixed and the tissue blocks were processed for HE staining. Despite a similar growth rate *in vitro*, a dramatic increase (approximately 200% compared to both C6 parental and C6-EYFP) in the *in vivo* growth of C6-EYFP/NTPDase2-derived gliomas was observed (Fig. 2a–c). The average volume of C6-EYFP/NTPDase2-derived gliomas was 160 ± 22 mm³, compared to 100 ± 15 mm³ and 80 ± 18 mm³ for C6- or C6-EYFP-derived gliomas respectively.

Pathological analysis revealed that C6-EYFP/NTPDase2-derived gliomas not only exhibited higher tumor volume when compared to control groups, but also show pathological characteristics related to increased glioma malignancy (Fig. 3; Table 1). All of the C6-EYFP/NTPDase2-derived gliomas exhibited extensive coagulative necrosis, intratumoral hemorrhage, lymphocytic infiltration, and peritumoral edema (Table 1). In addition, a significant increase in the mitotic index and Ki67 immunostaining in the C6-EYFP/NTPDase2 group was observed (Fig. 4; Table 1), reflecting the high proliferation rate exhibited by these gliomas *in vivo*. These results suggest that something other than the proliferative rate of the C6-EYFP versus C6-EYFP/NTPDase2-derived gliomas accounts for the difference in growth potential. It has previously been shown that NTPDase2, by preferentially removing ATP, can favor extracellular ADP accumulation and consequent P2Y₁ and P2Y₁₂ receptor modulation.^(20,21) In our experimental conditions, ADP could potentially promote platelet recruitment to the tumor area, favoring the malignant phenotype exhibited by C6-EYFP/NTPDase2 gliomas *in vivo*. To test this hypothesis, we verified the presence of activated platelets, angiogenesis, and the inflammatory process in C6-EYFP/NTPDase2-derived gliomas.

NTPDase2 expression in C6 glioma promotes platelet activation, angiogenesis, and induction of the inflammatory response. To examine the effect of NTPDase2 expression on platelet activation, cryostat sections from implanted gliomas were immunostained with a P-selectin antibody (Fig. 5; Table 2). P-selectin (CD62P) is present on the membrane of endothelial cells and platelets following

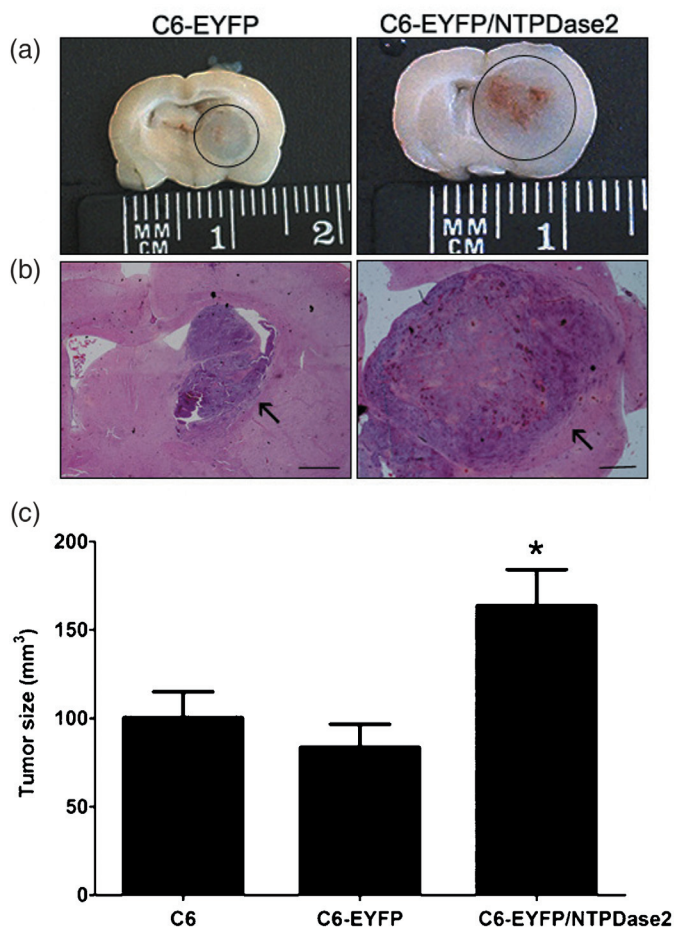


Fig. 2. NTPDase2 expression stimulates C6 glioma growth *in vivo*. To determine the glioma growth *in vivo*, equal amounts of C6, C6-EYFP, or C6-EYFP/NTPDase2 cells (1×10^6 cells) were implanted in the right striatum of Wistar rat brains by stereotaxical surgery. The animals were killed 20 days later and glioma sections were dissected and analyzed for tumor growth. (a) Photographs of rat brain slices of C6-EYFP- and C6-EYFP/NTPDase2-implanted gliomas. The gliomas are marked with a circle. (b) Representative sections of C6-EYFP- and C6-EYFP/NTPDase2-implanted gliomas stained with HE. Scale bars = 0.5 mm. (c) Tumor size quantification of implanted gliomas. Tumor size was evaluated 20 days following glioma implantation. Data represent the mean \pm SD of at least six animals per group. Data were analyzed by ANOVA, followed by Tukey–Kramer post-hoc test. *Significantly different from C6 and C6-EYFP gliomas ($P < 0.05$).

cellular activation.^(22,23) C6-EYFP/NTPDase2-derived gliomas exhibited a higher platelet activation ($70\% \pm 10$) when compared to C6-EYFP-derived gliomas ($10 \pm 3\%$). Additionally, in C6-EYFP/NTPDase2 gliomas, the activated platelets were associated with blood vessels and proliferating areas in the tumor periphery.

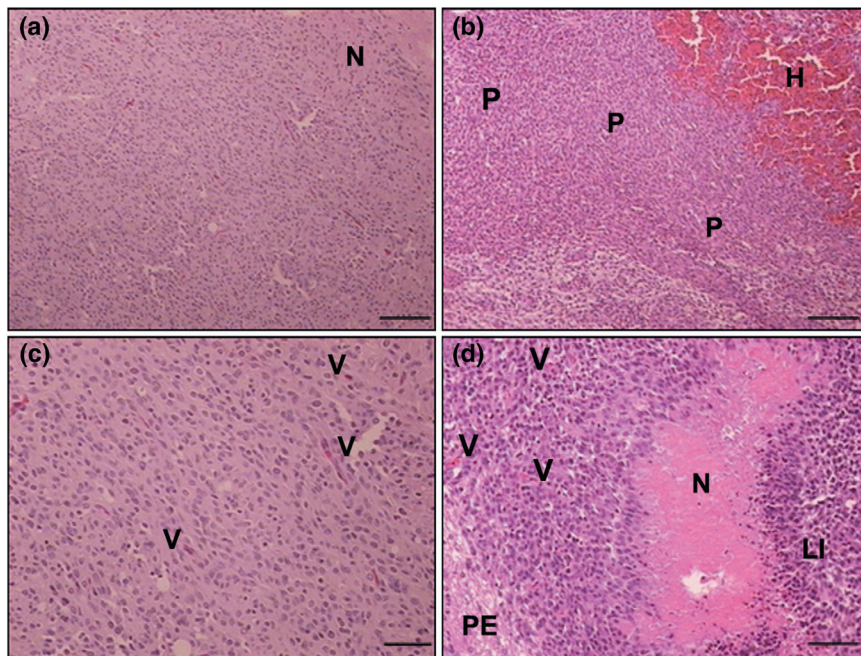


Fig. 3. NTPDase2 expression increases the malignancy of C6 gliomas *in vivo*. Representative HE sections of implanted (a,c) C6-EYFP and (b,d) C6-EYFP/NTPDase2 gliomas denote histological characteristics that define glioblastoma multiforme. Histological characteristics exhibited by C6-EYFP/NTPDase2 gliomas indicate a dramatic increase in the malignant potential: extensive intratumoral hemorrhage (H), peripheric pseudopalisade (P), necrosis (N), peritumoral edema (PE), lymphocytic infiltration (LI), and vascular proliferation (V). The complete analysis is presented in Table 1. Scale bars = 100 μ m (a,b); 50 μ m (c,d).

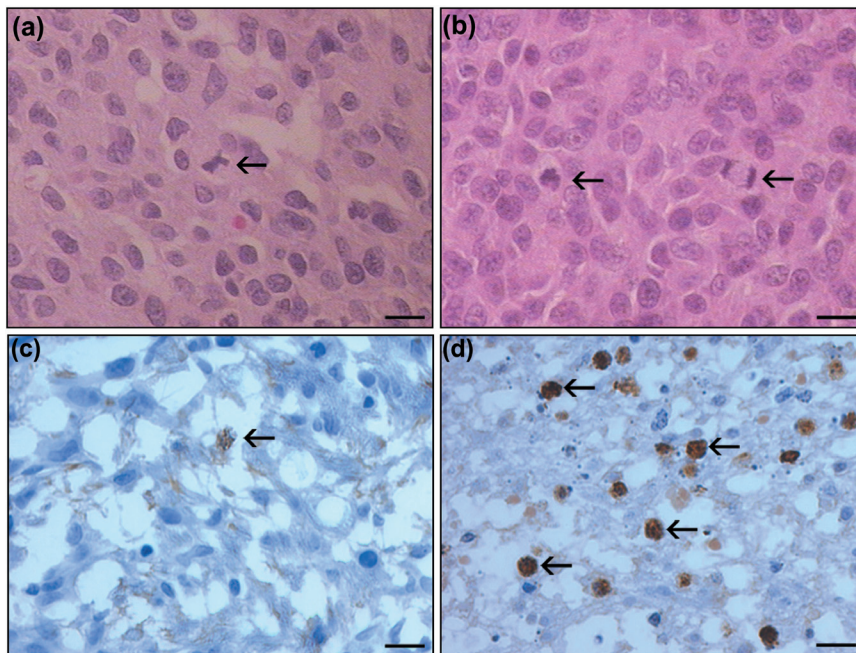


Fig. 4. NTPDase2 expression increases C6 glioma proliferation *in vivo*. *In vivo* glioma proliferation was assessed by histological analysis of (a,b) HE staining and (c,d) immunohistochemical analysis of Ki67-positive glioma cell nuclei. (a,c) C6-EYFP glioma; (b,d) C6-EYFP/NTPDase2 glioma. Arrows indicate mitotic cells. (d) C6-EYFP-NTPDase2 gliomas exhibit an increased number of Ki67-positive cells when compared to (c) C6-EYFP-derived gliomas. The mitotic index analysis is presented in Table 1. Scale bars = 10 μ m.

Next, angiogenesis was evaluated by IHC for CD31/PECAM-1 and VEGF (Fig. 5; Table 2). The re-establishment of NTPDase2 in C6 glioma resulted in a 3.5-fold increase in CD31-positive microvessels in C6-EYFP/NTPDase2-derived gliomas when compared to C6-EYFP gliomas (14.0 ± 2.0 and 4.0 ± 1.1 blood vessels respectively). Moreover, VEGF immunostaining was also increased in C6-EYFP/NTPDase2 gliomas when compared to C6-EYFP gliomas (90 ± 5 and $70 \pm 10\%$ respectively). The marked increase in these two markers corresponds to increased angiogenesis in C6-EYFP/NTPDase2-derived tumors *in vivo*.

To evaluate the involvement of the inflammatory response in the increased C6-EYFP/NTPDase2 glioma malignance, immunostaining for OX-42, a macrophages and microglial marker, was carried out (Fig. 5; Table 2). The results show that C6-EYFP/NTPDase2-derived gliomas exhibit increased macrophage/microglial

activation when compared to C6-EYFP gliomas (70 ± 10 and $40 \pm 10\%$ respectively). The activated macrophage/microglial cells were mainly associated with blood vessels.

Taken together, these data suggest that the ADP generated from NTPDase2-mediated ATP hydrolysis, among other effects, may potentially be involved in platelet activation, angiogenic stimulation, and recruitment of inflammatory cells in an interrelated manner. Importantly, these events all play a crucial role in tumorigenesis.^(4,22-24)

Involvement of ADP-activated platelets in the malignancy of gliomas expressing NTPDase2. Considering ADP is an important platelet activator, we tested whether platelet recruitment may contribute to the increased malignancy of C6-EYFP/NTPDase2-derived gliomas. To test this hypothesis, Wistar rats implanted with C6-EYFP/NTPDase2 glioma cells were treated with clopidogrel,

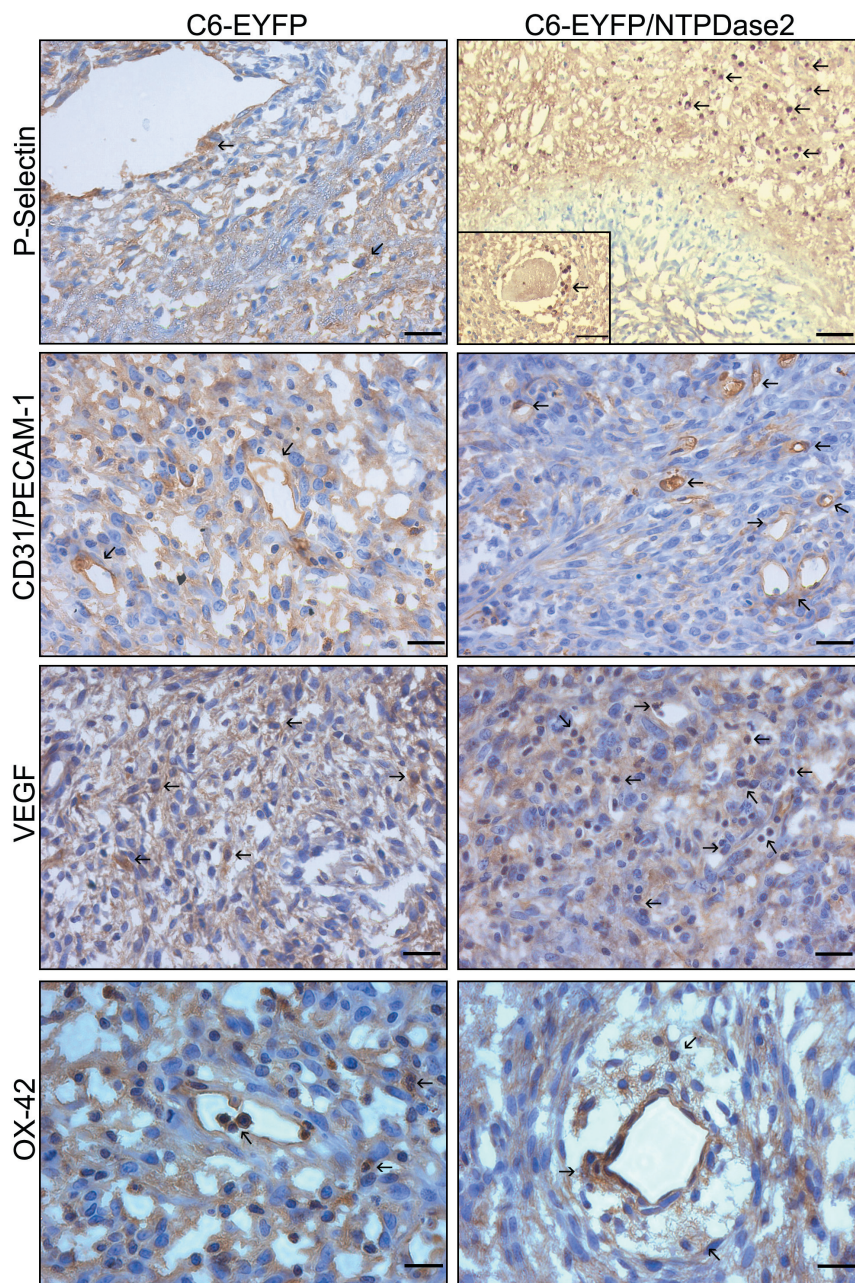


Fig. 5. NTPDase2 expression promotes platelet activation, angiogenesis and inflammation on C6 gliomas *in vivo*. Immunohistochemistry analysis of C6-EYFP- and C6-EYFP/NTPDase2-implanted gliomas. The immunopositivity is indicated by arrows for: P-selectin (platelet activation; insert shows platelet aggregation around a blood vessel), CD31/PECAM-1, vascular endothelial growth factor (VEGF) (vascular proliferation), and OX-42 (microglial/macrophage cells). The C6-EYFP/NTPDase2 gliomas exhibit an increased number of immunopositive cells for all markers when compared with C6-EYFP gliomas. These differences were quantified and are presented in Table 2. Scale bars = 20 μ m (P-selectin; CD31/PECAM-1; VEGF), 10 μ m (P-selectin insert; OX-42).

Table 2. Immunohistochemical analysis of P-selectin, CD31/PECAM-1, VEGF and OX42 in implanted gliomas

Markers	C6-EYFP (n = 4)	C6-EYFP/NTPDase2 (n = 4)	C6-EYFP/NTPDase2 Clopidogrel (n = 5)
P-selectin (%)	10 \pm 3	70 \pm 10*	20 \pm 6 [†]
CD31 (blood vessels)	4 \pm 1	14 \pm 2*	3 \pm 1 [†]
VEGF (%)	70 \pm 10	90 \pm 5*	70 \pm 8 [†]
OX-42 (%)	40 \pm 10	70 \pm 10*	50 \pm 15 [†]

Twenty days following glioma implantation, rats implanted with gliomas (C6, C6-EYFP, C6-EYFP/NTPDase2 or C6-EYFP/NTPDase2 clopidogrel-treated) were processed for immunohistochemical analysis. For CD31 quantification, each positive endothelial cell was counted as an individual vessel. The VEGF quantitative analysis was scored as percentage of immunoreactive cells. The P-selectin and OX42 immunostaining were scored as the percentage of immunoreactive cells. All immunohistological evaluations were made in 10 independent high-magnification (\times 200) fields, per glioma. Mean \pm SD were performed on at least 4 animals per group. *Indicates significant difference between C6-EYFP and C6-EYFP/NTPDase2 implanted gliomas, [†]indicates significant difference between C6-EYFP/NTPDase2 and C6-EYFP/NTPDase2 clopidogrel-treated group. $P < 0.05$ as determined by ANOVA, followed by Tukey–Kramer test. VEGF, vascular endothelial growth factor.

which was administered daily over a period of 19 days. Clopidogrel is a P2Y₁₂ receptor antagonist with anti-platelet and anti-aggregating properties.⁽²⁵⁾ Treatment with clopidogrel markedly decreased the presence of malignant pathological characteristics

and reduced the mitotic index and tumor size by 66% and 25%, respectively, when compared to C6-EYFP/NTPDase2-untreated animals (Fig. 6; Table 1). In addition, clopidogrel treatment decreased P-selectin, CD31/PECAM-1, VEGF, and OX-42

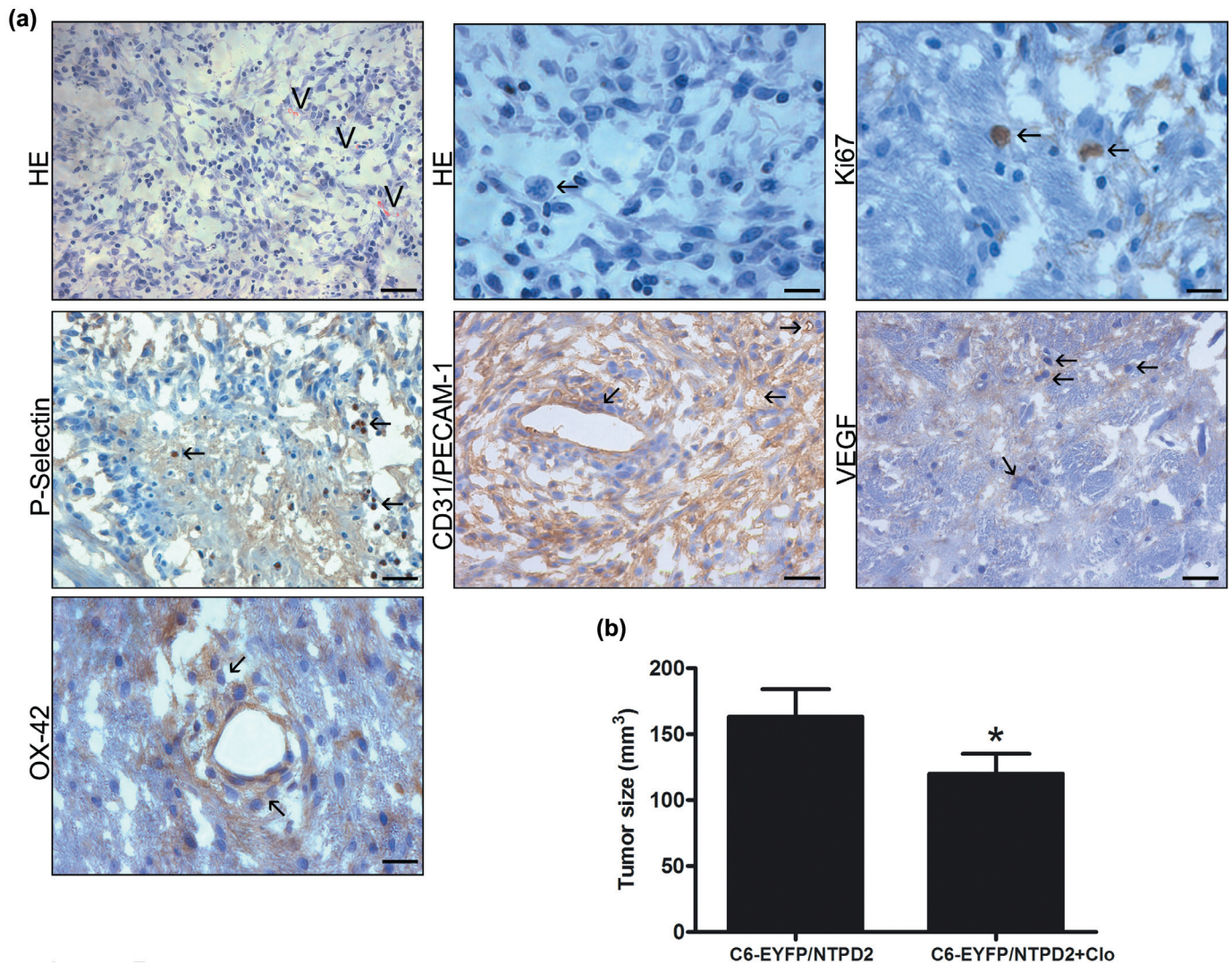


Fig. 6. Clopidogrel treatment decreases the malignancy of C6-EYFP/NTPDase2 gliomas. One day after C6-EYFP/NTPDase2 glioma implantation, clopidogrel was administered daily to rats for a period of 19 days. The animals were killed, glioma sections excised, and tumor malignancy was analyzed by (a) histology and immunohistochemistry and (b) tumor size. (a) Representative HE sections of C6-EYFP/NTPDase2 of clopidogrel-treated rats show a decrease in the glioma malignancy when compared to C6-EYFP/NTPDase2 tumors of untreated-rats (Fig. 3b,d). Note that histological characteristics related to high malignancy, such as intratumoral hemorrhage, necrosis, and peritumoral edema, were absent from clopidogrel-treated rats. Vascular proliferation (V) and number of mitotic cells (arrows) were also decreased by clopidogrel treatment. The differences in histological characteristics were analyzed and are presented in Table 1. Arrows indicate the immunopositivity for: Ki67 (mitotic cells), P-selectin (platelet activation), CD31/PECAM-1, vascular endothelial growth factor (VEGF) (vascular proliferation) and OX-42 (microglial/macrophage cells). These differences were quantified and are presented in Table 2. Scale bars = 20 μ m (HE, CD31/PECAM-1 and VEGF), 10 μ m (mitotic cells, P-selectin). (b) Tumor size quantification of rats with C6-EYFP/NTPDase2-derived gliomas untreated (C6-EYFP/NTPD2) or treated with clopidogrel (C6-EYFP/NTPD2+Clo). Data represent the mean \pm SD of five animals per group. Data were analyzed by ANOVA, followed by Tukey-Kramer post-hoc test. *Significantly different from untreated C6-EYFP/NTPDase2-implanted rats ($P < 0.05$).

expression to the levels observed in C6-EYFP-derived gliomas (Table 2). These data suggest that ADP-mediated platelet activation plays an important role in the increased malignancy of C6-EYFP/NTPDase2-derived gliomas.

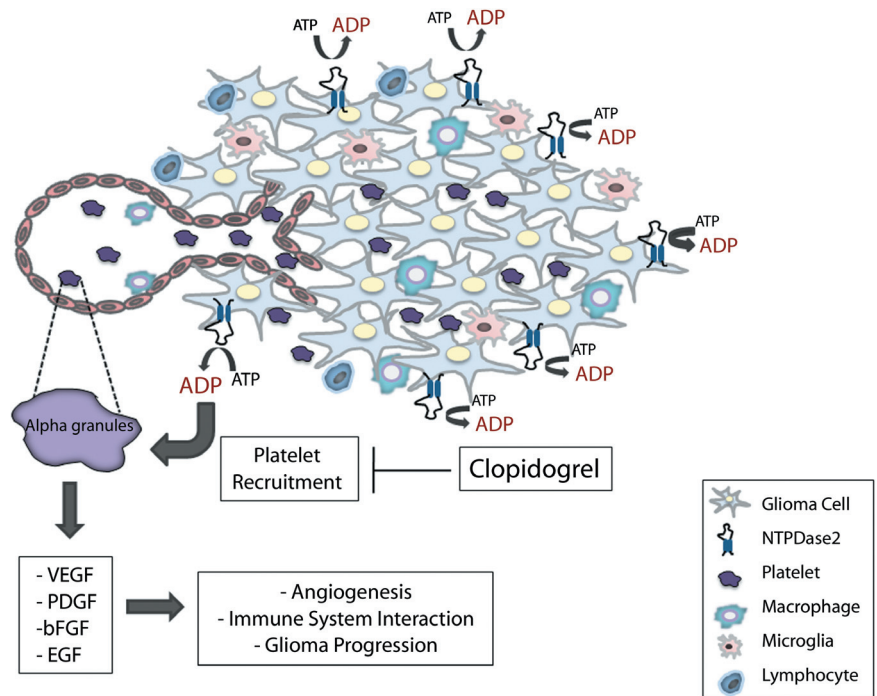
Discussion

The present work demonstrates a novel function of purinergic signaling in glioma growth and malignancy *in vivo*. First, we evaluated the effect of NTPDase2 overexpression on *in vitro* cell proliferation. We observed that ectopic NTPDase2 expression did not affect cell proliferation or cell viability of C6 cells in culture. However, in an *in vivo* rat glioma model, NTPDase2 overexpression resulted in a dramatic increase of glioma cell

growth and malignancy when compared to controls. We further demonstrated that the presence of NTPDase2 at the surface of C6-EYFP/NTPDase2 glioma cells increases the capacity of these tumors to recruit platelets and macrophages/microglia, most probably via activation of ADP-dependent P2 receptors. Indeed, clopidogrel, a P2Y₁₂ antagonist, strongly reduced the growth of tumors expressing NTPDase2.

Interestingly, we have previously shown that the co-injection of apyrase (low ATP/ADPase ratio) in a C6 rat glioma experimental model markedly reduces glioma development,⁽¹²⁾ whereas the results of the present work indicate that NTPDase2 (high ATP/ADPase ratio) has the opposite effect. This inverse effect could be due to the fact that whereas NTPDase1 hydrolyses ATP and ADP approximately equally well, NTPDase2 has an increased

Fig. 7. Possible pathways connecting NTPDase2 overexpression to ADP generation, platelet recruitment, and glioma progression. Overexpression of NTPDase2, due to its preference for ATP over ADP, would strongly favor ADP accumulation in the tumor periphery and vascular interface and consequently induce endothelial cell migration and platelet activation via ADP-dependent P2Y receptors. Upon activation, platelets release a variety of substances from alpha-granules, which stimulate angiogenesis, recruitment of immune cells, and tumor proliferation. In our experimental model, clopidogrel, a P2Y₁₂ antagonist and inhibitor of ADP-mediated platelet activation, markedly reduced glioma growth and angiogenesis. We hypothesize that the overexpression of NTPDase2 in glioma cells produces accumulating amounts of ADP around the tumor and this activates ADP-dependent receptors on platelets, which may play an important role in the increased malignancy of C6-EYFP/NTPDase2 gliomas. bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor.



preference for ATP over ADP as a substrate. Therefore, the enzymatic activity of NTPDase2 could favor extracellular ADP accumulation.^(7,8) Data from the literature show that extracellular ADP plays an important role in cell growth and inflammation in the central nervous system. For example, ADP has been implicated in eye development,⁽²⁶⁾ endothelial cell migration,⁽²⁷⁾ stem cell proliferation,⁽²⁸⁾ and platelet activation.⁽²⁹⁾ Additionally, there is strong evidence indicating that platelets play an important role in tumor progression and metastasis and regulate tumor cell invasion and angiogenesis.^(22,30-32) Considering that platelets express P2 receptors, which are activated by ADP (P2Y₁ and P2Y₁₂), we hypothesized that ADP produced by the NTPDase2 overexpressed in the implanted glioma cells could activate these receptors, leading to increased platelet recruitment and activation (Fig. 7). The latter processes promote angiogenesis as well as the recruitment of other inflammatory cells.⁽²²⁾ In agreement with our hypothesis, C6-EYFP/NTPDase2 gliomas exhibited a massive accumulation of platelets in the tumor area when compared to the control tumors. This effect was associated with increased expression of CD31/PECAM-1 and VEGF, indicating an enhanced angiogenic response, as these proteins have been shown to stimulate endothelial cell proliferation and migration, and *in vivo* angiogenesis.^(33,34) It is important to note that angiogenesis can not only be stimulated by ADP generated by NTPDase2 present on C6-EYFP/NTPDase2 gliomas but also by ADP secreted from activated platelets.⁽²⁹⁾

In addition, we also demonstrated that the implanted C6-EYFP/NTPDase2 gliomas contain more macrophage/microglial cells compared to the control tumors. We speculate that these cells are recruited to tumors by activated platelets. Indeed, recent reports have demonstrated that platelets are essential in the initiation of an inflammatory response⁽³⁵⁾ and that the adhesive interactions between platelets, leukocytes, and cancer cells promote inflammation and metastasis.⁽²³⁾ Additionally, macrophages/microglia present in large numbers in the implanted C6-EYFP/NTPDase2 gliomas could further increase the capacity of these tumors to stimulate an inflammatory response, thus increasing the malignant characteristics of these gliomas. Similar to what was seen in this study, microglial cells have been repeatedly identified in brain tumors,⁽⁴⁾ and although the exact role of these

cells in cancer progression remains unclear, studies suggest that they can promote or facilitate the inflammatory response by secreting cytokines and growth factors.⁽⁴⁾ Interestingly, some of these cytokines and growth factors can be secreted as a result of P2 receptor stimulation by extracellular nucleotides. For example, ATP stimulates microglia to release interleukin-6 and interleukin-10^(36,37) and ADP regulates the extension of microglial processes.⁽³⁸⁾

In accordance with the role of ADP-activated platelets in glioma progression, we observed that treatment with clopidogrel, a P2Y₁₂ antagonist, markedly reduced tumor size, malignancy characteristics, angiogenesis, and the inflammatory process in C6-EYFP/NTPDase2-derived gliomas to the levels observed for control gliomas. These results are in agreement with previous works defending the use of anticoagulants in cancer therapy to prevent platelet interaction with tumor vasculature^(39,40) and show that ADP-activated platelets might be important players contributing to the increased malignancy of C6-EYFP/NTPDase2 gliomas (Fig. 7).

In conclusion, our results confirm the hypothesis that purinergic signaling is involved in the progression of gliomas *in vivo*. Additionally, our findings reveal a previously underestimated role for ADP in tumor promotion and reinforce the important roles carried out by the different E-NTPDase members, which, by working in a highly coordinated enzymatic chain, maintain the extracellular nucleotide equilibrium and control the effects mediated by purinergic receptors. The data reported here point to the pharmacological modulation of P2 receptors as a novel therapeutic modality for the treatment of brain tumors in the future.

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3.3 Artigo 3

SELECTIVE NTPDase2 OVEREXPRESSION IN GLIOMAS DRIVES INFLAMMATION, PLATELET ACTIVATION AND METASTASIS

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INFLAMMATION, PLATELET ACTIVATION AND METASTASIS**

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Abstract

Gliomas are the most common and devastating type of primary brain tumor. Many non-neoplastic cells, such as immune cells comprise the tumor microenvironment and create an inflammatory milieu fundamental for cancer development. Disruption of purinergic signalling has been implicated in cancer. ATP and its breakdown products ADP and adenosine display cytokine-like properties and may participate in the interactions among cancer and immune cells. The nucleotide receptor-mediated cell communication is controlled by ectonucleotidases, such as ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), which hydrolyze extracellular nucleotides. We have shown that, in opposite to astrocytes, gliomas, exhibit a low E-NTPDase activity; ATP induces glioma cell proliferation and the co-injection of apyrase/NTPDase1 with gliomas decreases glioma progression *in vivo*. However, recently we have shown that the NTPDase2 restoration to gliomas dramatically increased tumor growth *in vivo*. The contradictory results obtained using different ATP scavengers suggest those interactions between tumor, immune cells and purines as key elements in the anti- or pro-tumor responses. Here we evaluated whether the NTPDase2 restoration to gliomas could modulate the systemic inflammatory response and the distant metastasis. NTPDase2 overexpression increased the *in vitro* C6 glioma cell adhesion properties, modulated the pro-inflammatory cytokine production and the platelet reactivity *in vivo*. Alterations on lung tissue suggestive of glioma metastasis were also observed. These results suggest that disruption of purinergic signaling creates an inflammatory microenvironment that modulates tumor cell migration and malignity *in vivo*.

Key words: Gliomas, lung, inflammation, ATP, E-NTPDases

Introduction

Glioblastoma multiforme (GBM) is the most common and devastating type of primary brain tumor. These tumors are characterized by diffuse infiltration of the brain parenchyma, recurrent growth and poor prognosis for survival (1). In addition to neoplastic and cancer stem cells, the progression of malignant gliomas involves the participation of an inflammatory microenvironment (2). Recent studies have shown that an inflammatory milieu consisting of immune cells and their secretory products create a growth factor-rich environment fundamental for tumor maintenance and growth (3).

Cytokines and chemokines and their receptors have been accepted as regulators of cross-talk among the cancer cells, the immune cells and the vascular endothelium (4). Purinergic signalling, involving ATP released from neural and immune cells and its breakdown products ADP and adenosine also display cytokine-like properties (5). ATP modulates the cytokine gene expression within the nervous and immune system (6) and it controls the secretion of pro-inflammatory cytokines, such as IL-1 β , IL-6 and TNF- α (7; 8). The platelet activation by ADP has also been implicated in inflammation. Platelets are essential in the initiation of an inflammatory response (9) and the adhesive interactions between platelets, leukocytes, and cancer cells play an important role in tumor progression and metastasis (10; 11). Adenosine, the final product of the nucleotide hydrolysis, accumulates in the tumor interstitium (12) and modulates cell proliferation, angiogenesis and may suppress the anticancer immune response (13; 14). The nucleotide receptor-mediated cell communication is controlled by ecto-nucleotidases, such as ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) and ecto-5'-nucleotidase/CD73 (ecto-5'-NT/CD73), which can efficiently hydrolyze ATP, ADP, and AMP to adenosine in the extracellular space. The molecular properties,

functional roles, and nomenclature of ectonucleotidases have been extensively reviewed in Robson et al (2006) (15).

Disruption of purinergic signalling has been implicated in pathological processes such as cancer (16). For example, in gliomas we have previously demonstrated that the low E-NTPDase activity might favor ATP accumulation in the tumor interstitium and the consequent tumor progression (17; 18; 19). Accordingly, the co-injection of apyrase/NTPDase1 (low ATPase/ADPase ratio) with glioma cells decreased the growth of tumors implanted in rats (20). To better characterize the ectonucleotidase participation on *in vivo* glioma growth, we have restored to glioma cells the NTPDase2, the major ectonucleotidase expressed by astrocytes. Surprisingly, NTPDase2 overexpression dramatically increased glioma growth and its malignancy in an *in vivo* glioma model (21). These contradictory results obtained by using NTPDase2 (high ATPase/ADPase ratio) as an ATP scavenger revealed additional components in the interactions between tumor, immune cells and purines and point to the ADP signal and platelets recruitment as key elements in the anti- or pro-tumor responses.

Considering that platelet recruitment and inflammatory process are the hallmark of NTPDase2-derived gliomas (21), and that both processes are related to poor prognosis and metastasis in a variety of tumors (22; 23), in the present study we evaluated whether the NTPDase2 restoration to gliomas could modulate the systemic inflammatory response and the distant metastasis. Our results reveal that NTPDase2 overexpression increases the *in vitro* C6 glioma cell adhesion properties, and modulates the pro-inflammatory cytokine production and the platelet function *in vivo*. Additionally, alterations on lung tissue suggestive of glioma metastasis were also observed and support the notion that disruption of purinergic signaling creates an inflammatory microenvironment that may modulate tumor cell migration.

Material and methods

Cell culture and transient transfection. The C6 rat glioma cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA), grown in culture flasks and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 1% DMEM, 8.39 mM HEPES (pH 7.4), 23.8 mM NaHCO₃, 0.1% fungizone, 0.5 U/ml penicillin/streptomycin (Gibco BRL) and supplemented with 5% (v/v) fetal bovine serum (FBS; Cultilab, Brazil). Cells were kept at a temperature of 37°C, humidity of 95% / 5% CO₂ in air. Cell transfection with pEYFP empty vector (Enhanced Yellow-green Fluorescent Protein) (C6-EYFP) or pEYFP-NTPDase2 (C6-EYFP/NTPDase2) was performed with Lipofectamine 2000 (Life Technologies, Inc.) as previously described (21).

Cell adhesion assay. C6-EYFP or C6-EYFP/NTPDase2 glioma cells were seeded at density of 3×10^4 cells/well in 96 multiwell plates and incubated for 1 h at 37°C in absence or presence of extracellular matrix components (ECM) collagen type I, laminin and fibronectin (15 µg/mL) or NTPDase inhibitors suramin and Evans blue (100 µM). The non-adherent cells were removed by washing with PBS. Adherent cells were fixed with 4% paraformaldehyde (PFA) for 10 min; stained for 10 min with 100 µL 0.5% crystal violet diluted in 20% methanol which was eluted in 100 µL 10% acetic acid (v/v). The optical density of each well was measured at 570 nm (24).

Glioma implantation. The glioma implantation was made as described by Braganhol et al. (2009) (21). Briefly, C6, C6-EYFP or C6-EYFP/NTPDase2 glioma cells (1×10^6 cells) were injected in the right striatum of male *Wistar* rats (250-270 g, 8 weeks-old) anesthetized by intraperitoneal administration of ketamine/xilazine. The

negative control group (naive) was carried out via the same procedure and received an injection of 3 μ L of DMEM/5% FBS in the right striatum. All procedures used in the present study followed the “Principles of Laboratory Animal Care” from National Institutes of Health (NIH) and were approved by the Ethical Committee of the Hospital de Clínicas de Porto Alegre.

Pathological analysis. Twenty days after glioma implantation, rats were decapitated and the liver, kidney and lungs were removed, sectioned and fixed in 4% PFA in PBS (pH 7.4). The fixed tissue sections were then stained with hematoxylin and eosin (H&E) and pathological analysis of the slides was performed by a pathologist blinded to the groups.

Immunohistochemical staining. Twenty days after glioma implantation, rats were decapitated and the lungs embedded in O.C.T. freezing medium (Tissue-Tek[®], Sakura Finetek, USA) and snap-frozen in isopentane in dry ice and stored at -80 °C until used. To perform the immunohistochemical analysis (IHC), cryostat sections (5 μ m) were fixed in acetone, blocked in 1% albumin solution and incubated overnight (4°C) with the following specific antibodies: rabbit anti-rat pAb P-selectin (1:500; BD Pharmingen, USA) and rabbit anti-rat mAb nestin (1:200; Chemicon, USA & Canada). Next, tissue sections were incubated with biotinylated secondary antibody and Streptavidin-Avidin-Biotin (kit Lsab, Dako, USA). The peroxidase reaction was performed using 3, 3'-diaminobenzidine tetrahydrochloride (DAB), according to the manufacturer's specifications. Finally, sections were counterstained with Harris haematoxylin. The IHC slides were analyzed by a pathologist in a blinded manner.

Determination of cytokines levels in blood serum. Twenty days after glioma implantation, the blood samples of animals of the naive, C6, C6-EYFP and C6-EYFP/NTPDase2 groups were collected by cardiac puncture. The blood samples were centrifuged at $1,300 \times g$ at 4°C for 10 min. The supernatant was rapidly frozen and stored at -70°C for the later measurement of TNF- α , IL-1 β , IL-6 and IL-10 levels using specific enzyme-linked immunosorbent assay (ELISA) kits, according to the recommendations of the supplier (R&D Systems).

Platelet count. The blood for platelets count was collected by cardiac puncture from anesthetized ketamin/xilazin control and glioma implanted rats into vacutainer plastic tube containing 120 mM sodium citrate. Blood samples were analyzed in a Coulter JT counter. The data are expressed as the number of platelets $\times 10^3/\mu\text{L}$.

Isolation of platelet. The platelets were prepared in accordance with the method of Lunkes and colleagues (2004) (25) with minor modifications. Total blood was collected by cardiac puncture into a flask containing 120 mM sodium citrate as anticoagulant. The total blood citrate system was centrifuged at $160 \times g$ during 15 min in order to remove the residual blood cells and to obtain the platelet-rich plasma (PRP). The PRP was centrifuged at $1,400 \times g$ for 20 min and washed twice by centrifugation at $1,400 \times g$ for 10 min with 3.5 mM HEPES isosmolar buffer pH 7.5 containing 142 mM NaCl, 2.5 mM KCl and 5.5 mM glucose. The washed-platelets were suspended in HEPES buffer for enzyme assays.

Enzyme assay. The analysis of platelet nucleotide hydrolysis was performed as described in Zanin and colleagues (2009) (26). Briefly, the enzyme reaction was started

by the addition of ATP, ADP or AMP as substrate to a final concentration of 1.0 mM in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 5 mM KCl, 6 mM glucose, 5 mM CaCl₂, for ATP and ADP, or 5 mM MgCl₂ for AMP, pH 7.5, in a final volume of 200 μ L. The incubation was stopped by adding trichloroacetic acid (TCA) (5%, final concentration). The release of inorganic phosphate (Pi) was measured by the malachite green method (27) using KH₂PO₄ as a Pi standard. The protein concentration was measured by the Coomassie Blue method (28) using bovine serum albumin as standard. All samples were run in triplicate. Specific activity was expressed as nmol Pi released/min/mg of protein.

Platelet aggregation. The blood samples for platelet aggregation assays were collected by cardiac puncture, and centrifuged at $160 \times g$ for 15 min at room temperature to achieve a PRP suspension. Platelet aggregation assays were performed on a SpectraMax microplate reader (Molecular Devices, USA). Briefly, platelet agonists (10 μ M ADP), 2.0 mM CaCl₂ and Tyrode/BSA were mixed in 96-well-flat-bottom plates. Aggregation was triggered by the addition of 100 μ L of platelet suspension in a final reaction volume of 150 μ L. The plate was incubated for 2 min at 37 °C before the beginning of stirring and readings were followed at 650 nm every 20 s for 20 min. Changes in turbidity were measured in absorbance units and the results were obtained as area under the aggregation curves.

Statistical analysis. Data were expressed as mean \pm S.D. of at least three independent experiments and were subjected to one-way analysis of variance (ANOVA) followed by Tukey-Kramer post-hoc test (for multiple comparisons) or *t*-student test,

when necessary. Differences between mean values were considered significant when $P < 0.05$.

Results

NTPDase2 overexpression increased the C6 glioma cell adhesion *in vitro*.

The overexpression of NTPDase2 on C6 glioma promoted clear morphological differences in the cells in culture. While C6-EYFP glioma cells proliferate as a cell monolayer, the C6-expressing NTPDase2 cell proliferates as multicellular spheres, suggesting an increased cell-cell adhesion potential (Fig. 1A). To determine whether the NTPDase2 overexpression modulates the cell adhesion process, we assessed the interaction of glioma cells with extracellular matrix components (ECM) in a cell adhesion assay. NTPDase2 overexpression caused a 2-fold increase in glioma adhesion when compared to C6-EYFP cells. The presence of laminin and fibronectin promoted an additional increase of 1.5 times in the cell adhesion (Fig. 1B). Moreover, the treatment with suramin and Evans blue, two NTPDase inhibitors, reduced the increase of cell adhesion promoted by the NTPDase2 overexpression to control levels (Fig. 1C).

NTPDase2 expression in implanted gliomas promoted histological changes in the lung tissue. Considering that NTPDase2 restoration to C6 glioma modulated some important mechanisms related to tumor invasion, such as angiogenesis and inflammation on central nervous system (CNS) (21) and cell-cell and cell-ECM interaction *in vitro* (Fig. 1), we asked whether the NTPDase2 overexpression could promote histological alterations in distant organs. Following 20 days of glioma implantation, the main sites for cancer metastasis, including lung, kidney and liver, were removed and processed for histological analysis (Fig. 2). While no histological

changes were observed in the liver and kidney, important alterations characteristic of an initial malignant process, such as focal hemorrhage, extensive lymphocytic infiltration and hyper-cellularity were observed in the lungs from NTPDase2 glioma-implanted rats (Fig. 2 and Table 1). In addition, to examine the possible presence of glioma cells in the lung, the expression of nestin, a glioma cell marker, was evaluated by IHC (Fig. 3). Surprisingly, lung tissues from NTPDase2 glioma implanted rats exhibited the presence of nestin positive cells, which is suggestive of glioma metastasis (Fig. 3). Considering that gliomas rarely metastasize, this result suggests that NTPDase2 restoration to gliomas promotes changes in the tumor microenvironment that sustain the spread of cells to circulation and establishment in a secondary organ.

NTPDase2 overexpression in implanted gliomas induced an increase in pro-inflammatory cytokine levels. Previous studies have shown that an inflammatory milieu composed by immune cells and their secretory cytokines contribute to tumor progression and metastasis (4). Taking in consideration that nestin positive cells in lung tissues were localized within the inflammatory infiltrate and around the blood vessels, and that the purinergic signaling controls the inflammation, we thought that cytokines could mediate the communication between tumor cells and the lung tissue. Accordingly, C6-EYFP/NTPDase2 glioma-implanted rats exhibited an expressive increase of pro-inflammatory cytokine serum levels by 2.4, 2.2 and 12 times for IL-1 β , TNF- α and IL-6, respectively. On the other hand, the serum levels of the anti-inflammatory cytokine IL-10 remained unchanged (Fig. 4). This is consistent with our previous study that reports the increased malignity of NTPDase2-derived gliomas (21), and sustains the idea that disruption of inflammatory process by purinergic signalization could be involved in the peripheral effects caused by NTPDase2 overexpression in gliomas.

NTPDase2 overexpression in implanted gliomas modulated the platelets activation. Considering that ADP is a platelet activator, we hypothesized that the ADP produced by NTPDase2 activity could promote platelet recruitment and mediate glioma metastasis. The ectonucleotidase activities, important modulators of P2-mediated platelet activation, were evaluated. Figure 5 shows that while ATP hydrolysis was increased in platelets from both groups of glioma implanted rats, ADP hydrolysis was reduced in about 50% only in platelets from C6-EYFP/NTPDase2 glioma-implanted rats. In addition, a significant increase on AMP hydrolysis was observed. The ADP and AMP hydrolysis in platelets from C6-EYFP-implanted glioma rats remained unchanged (Fig. 5A). Platelet number and platelet aggregation have shown a significant increase in C6-EYFP/NTPDase2 glioma-implanted rats (35% and 25%, respectively) when compared to control group (Fig. 5B and C). Finally, lung cryostat sections from rat-implanted gliomas were immunostained with a P-selectin antibody and showed a sizable platelet sequestration in the lung tissue from NTPDase2 glioma-implanted rats (Fig. 5D). Taken together, these results suggest a relationship between purinergic signaling disruption, platelet activation and glioma invasiveness.

Discussion

The present work demonstrates a novel function for purinergic signaling in glioma progression and metastasis. First, we evaluated the role of NTPDase2 on *in vitro* cell adhesion. We observed that ectopic NTPDase2 overexpression increased the cell-cell and cell-ECM interaction in cell culture. Moreover, in an *in vivo* rat glioma model, NTPDase2 overexpression resulted in important pathological alterations in lung tissue suggestive of glioma metastasis. We further investigated the involvement of

inflammatory process and platelet activation on lung alterations. Indeed, the presence of NTPDase2 at surface of glioma cells promoted an increase in IL-1 β , TNF- α and IL-6 pro-inflammatory cytokine serum levels and in the platelet activation and recruitment to lung tissue.

The data present herein, allied to previous works showing that NTPDase2 overexpression is involved in hepatic cancer progression, eye formation disruption and increased glioma growth *in vivo* (29; 30; 31; 21) suggest that this enzyme may play a general role on process related to cell proliferation, adhesion and migration. The importance of the NTPDase2 in glioma cell-cell and cell-ECM interactions has been confirmed in *in vitro* experiments. The NTPDase2 overexpression promoted an increase in glioma cell adhesion, which was favored by laminin and fibronectin, ECM synthesized by gliomas, and prevented by suramin and Evans blue, two known NTPDase inhibitors. These data reveal a possible role of NTPDase2 overexpression *per se*, and also as extracellular ADP source in processes related to tumor implantation, motility and invasion within brain parenchyma and blood vessels. Accordingly, it was previously demonstrated that increased ADP receptor P2Y₁₂ expression in C6 glioma cells has been associated with changes in cell morphology and adhesion and increased survival (32).

Further experiments performed on *in vivo* rat glioma model indicated that NTPDase2 overexpression orchestrates the inflammatory response and the glioma invasiveness to peripheral organs probably through its hydrolysis product, ADP. Surprisingly, the NTPDase2 restoration to gliomas markedly induced histological alterations in the pulmonary tissue, which was followed by the presence of nestin positive cells, characteristics suggestive of glioma metastasis. Data from literature have shown that gliomas rarely metastasize from brain tissue (33). Although the mechanisms

involved on this glioma feature are not completely understood, studies suggest that the cerebral environment does not favor the glioma metastasis (34). Then, we hypothesized that the NTPDase2 overexpression and consequent ADP accumulation around the implanted gliomas could induce changes in the glioblastoma-environment interaction, leading to increased immune cell recruitment, cytokine production and platelet activation. The latter processes are thought to drive the tumor cell epithelial-mesenchymal transition, a process that modulates cell adhesion and motility and constitutes the most important mechanism of tumor metastasis (4; 10; 35). In agreement with our main hypothesis, NTPDase2 overexpression in gliomas triggered an important increase of IL-1 β , TNF- α and IL-6 pro-inflammatory cytokine serum levels when compared to control. The relevance of these cytokines on growth, motility and invasiveness of tumor cells has been previously proposed. For example, IL-1 β affects the pattern of tumor-host interactions (36), and its genetic ablation in mice results in absence of metastatic tumors *in vivo* (37). Additionally, TNF- α up-regulates the selectin overexpression on endothelial cells, which promotes tumor cell adhesion and migration (38; 39). Finally, IL-6 is known to accelerate tumorigenesis and its administration during tumor initiation resulted in increased malignancy and multiplicity of tumor (40). These data are in accordance to the theory of “inflaming metastasis” (3), and suggest a relevant role for purinergic signaling in this process.

In addition, we also demonstrate that the NTPDase2 overexpression in gliomas modulated the nucleotide metabolism and recruitment of platelets to lung tissue. While the increase in the ATP hydrolysis was similar in both groups of glioma implanted rats, the platelets from NTPDase2 glioma-implanted rats exhibited a decrease in ADP hydrolysis. Considering that platelets express P2 receptors, which are activated by ADP (P2Y₁ and P2Y₁₂), we speculated that the possible ADP increase on platelet periphery

would modulate its aggregation and recruitment properties. Accordingly, NTPDase2 overexpression promoted an increase in platelet aggregation. It is important to note that the compensatory increase of AMP hydrolysis to adenosine, a potent inhibitor of platelet aggregation, did not prevent this effect. In addition, the NTPDase2 overexpression induced an increase in the platelet count, which could be a result of the inflammatory response developed by these animals. Taken together, the increased platelet reactivity to ADP-mediated activation, the platelet recruitment to pulmonary tissue and the increase in the pro-inflammatory cytokine levels may be important components of actions mediated by NTPDase2 overexpression and support the idea that the formation of platelet-tumor aggregates and inflammation facilitate immune evasion and implantation of tumor cells at distant sites (Fig. 6) (23).

In summary, the data presented in this work suggest that disruption of purinergic signaling is involved in the glioma invasiveness. Furthermore, our findings reveal an important function of extracellular nucleotides, mostly ADP, in modulating cancer-related inflammation events, and point out NTPDase2 overexpression as a marker of metastatic tumors. Altogether, these results contribute to a better understating of nucleotide implication in cancer malignance and metastasis.

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Figure legends

Figure 1. NTPDase2 overexpression increases the C6 glioma cell adhesion *in vitro*. **(A)** Representative pictures of C6-EYFP and C6-EYFP/NTPDase2 (C6NT2) cells in culture. Arrow indicates the sphere formation in C6NT2 glioma cells (magnification x 20). **(B and C)** The C6-EYFP and C6-NT2 cell adhesion assay was performed in presence of **(B)** 15 µg/mL ECM components collagen type I (Col I), laminin (Lam) or fibronectin (Fib) or **(C)** NTPDase inhibitors Evans blue (EB) or suramin. Controls in absence of treatment were performed in parallel. The results are expressed as the optical density measured at 570 nm. The values represent the mean ± SD of at least three independent experiments performed in triplicate. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey-Kramer test).

* Significantly different from C6-EYFP glioma cells; # significantly different from C6NT2 glioma cells ($P < 0.05$).

Figure 2. NTPDase2 overexpression in gliomas promoted histological changes in the pulmonary tissue. To perform the histological analysis in peripheral organs, equal amounts of C6-EYFP or C6-EYFP/NTPDase2 (C6NT2) cells (1×10^6 cells) were implanted in the right striatum of *Wistar* rat brains by stereotaxical surgery. The animals were killed 20 days later and the liver, kidney and lung were dissected, processed for hematoxylin/eosin (H&E) standard stain and the histological analysis was performed by a pathologist in a blinded manner. Representative sections of liver, kidney and lung are shown. Histological characteristics exhibited by lung tissue from C6NT2 glioma implanted rats indicate the presence of pathological alterations: extensive immune cell infiltrate (IC) and hemorrhage (H) (magnification x 20). The analyses were performed in five animals per group. The complete histological evaluation is presented in Table 1.

Figure 3. NTPDase2 overexpression promoted the presence of nestin positive cells in lung tissue. The presence of glioma cells on lung tissue was assessed by immunohistochemical analysis of nestin positive cells, a glioblastoma multiform marker. Arrows indicate nestin positive cells around blood vessels and inflammatory infiltrate in the lung tissue from C6-EYFP/NTPDase2 (C6NT2) glioma implanted rats (magnification x 40). The insert represent a higher magnification (x 100) of nestin positive cells. Experiments were performed in five animals per group.

Figure 4. NTPDase2 overexpression in gliomas promoted an increase in the cytokine serum levels of glioma implanted rats. Following 20 days of C6-EYFP or C6-EYFP/NTPDase2 (C6NT2) glioma implantation, the rats were anesthetized with ketamin/xilazin and the blood was obtained by cardiac puncture. The (A) IL-1 β , (B) TNF- α , (C) IL-6 and (D) IL-10 serum levels (pg/mL) were evaluated by ELISA following the costumer's instructions. Animals not submitted to the surgery (Control) or animals injected with DMEM/5% FBS in absence of tumor cells (Naive) were taken as controls. The values represent the means \pm S.D. of five animals per group. Data were analyzed by ANOVA followed by post-hoc comparisons (Tukey's test).

*, **, *** Significantly different from all groups and $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

Figure 5. NTPDase2 overexpression induced alterations in platelet reactivity. (A) The ATP/ADP and AMPase activities in platelets from control (animals not submitted to surgery), C6-EYFP or C6-EYFP/NTPDase2 (C6NT2) glioma implanted rats. (B) Platelet count from C6-EYFP or C6NT2 glioma implanted rats; C6-EYFP was taken as

control. (C) Platelet aggregation from C6-EYFP or C6NT2 glioma implanted rats. The platelet aggregation results are expressed as the percentage in relation to control (animals not submitted to surgery). (D) The presence of platelet activation and recruitment to lung tissue was assessed by immunohistochemical analysis of P-selectin positive cells, a platelet activation marker. Arrows indicate P-selectin positive platelets (magnification x 40). The values represent the mean \pm SD of at least three independent experiments performed in triplicate. Data were analyzed by ANOVA followed by *post-hoc* comparisons (Tukey-Kramer test). *, *** Significantly different from controls and $P < 0.05$, $P < 0.001$, respectively.

Figure 6. Possible pathways connecting NTPDase2 overexpression, platelet recruitment, cytokine release and glioma metastasis. The NTPDase2 overexpression, which hydrolyze preferentially ATP, induces ADP accumulation in the tumor interstitium and consequent ADP-dependent platelet activation. Upon activation, platelets express cell adhesion molecules and release a variety of substances from alpha-granules, which are essential in the initiation of an inflammatory response and metastasis. We hypothesized that C6-NTPDase2 tumor microenvironment composed by pro-inflammatory cytokines and activated platelets would modulate the tumor cell adhesion and motility and induce the formation of tumor-platelet aggregates. Studies suggest that platelet-coated tumor cells are protected from immunological assault in the circulation and exhibit a tendency to embolize the microvasculature and extravasate within metastatic site. Accordingly, activated platelets and nestin positive cells were found in the lung tissue from NTPDase2 glioma implanted rats and support the idea that disruption in purinergic signaling is involved in glioma invasiveness.

Figure 1

A

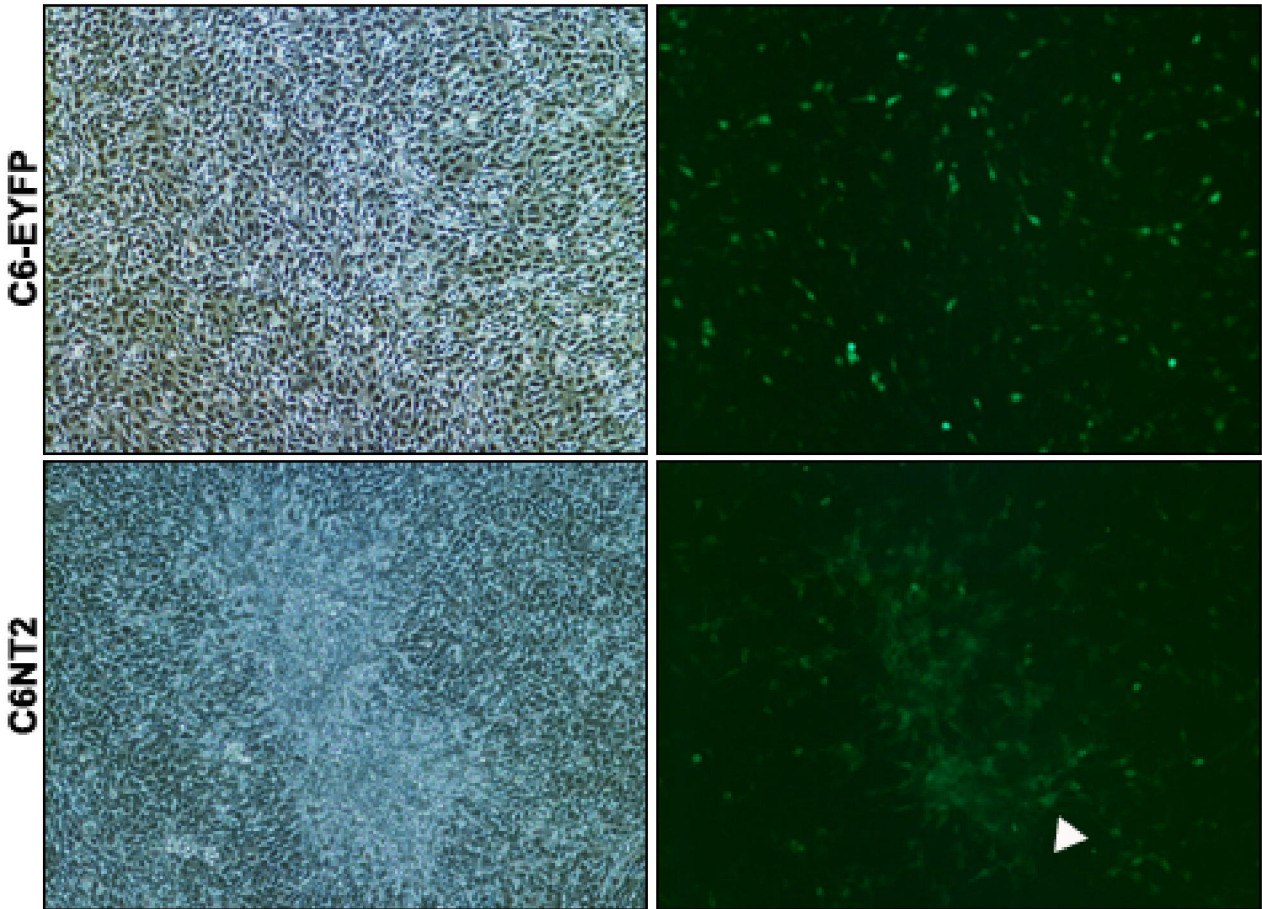
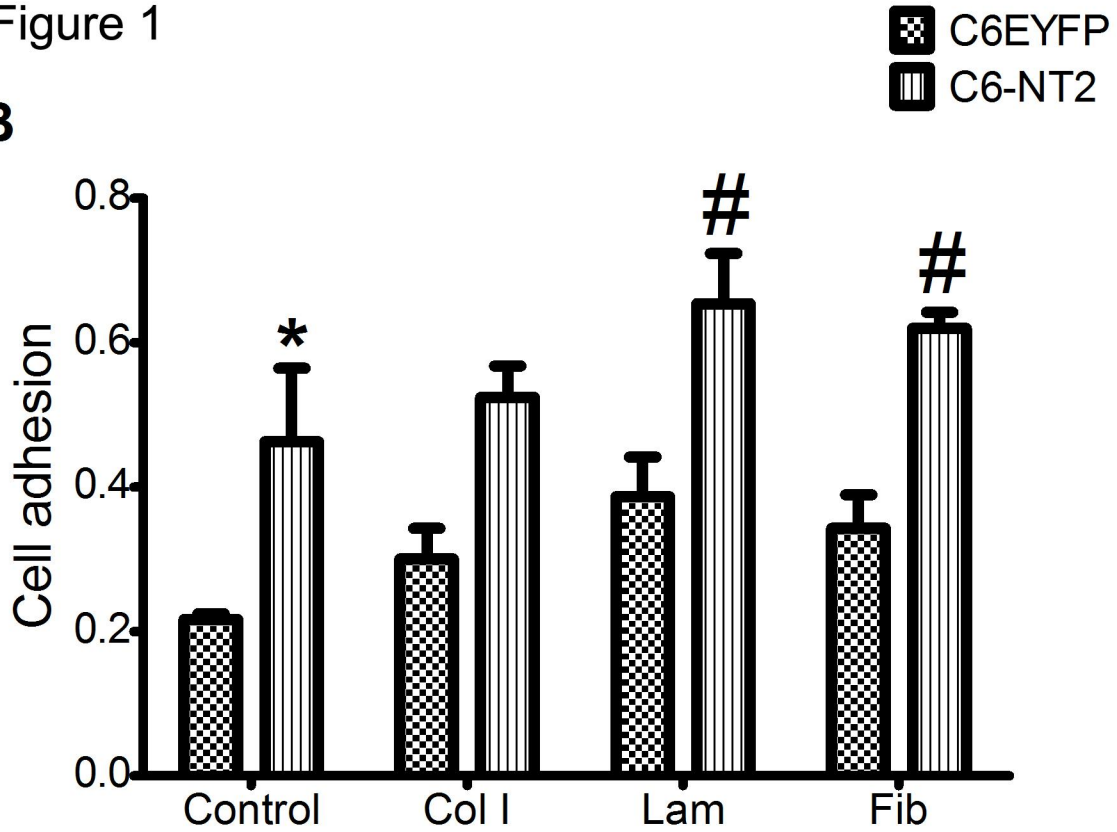


Figure 1

B



C

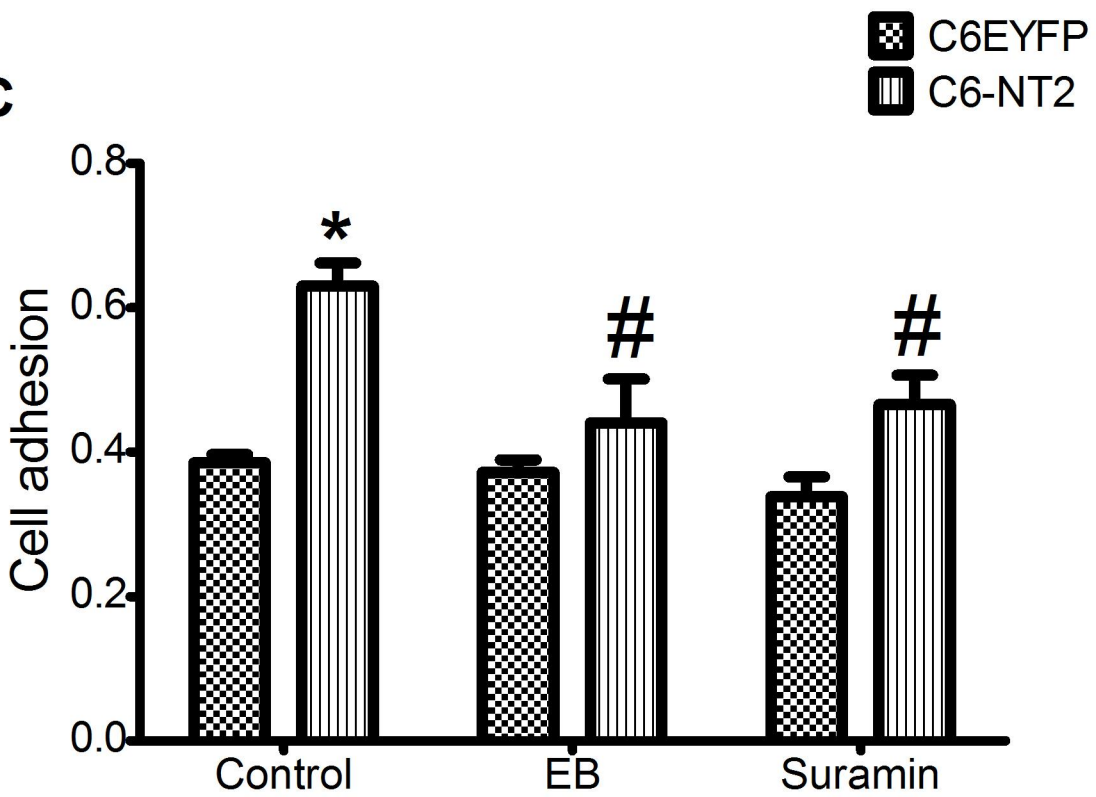


Figure 2

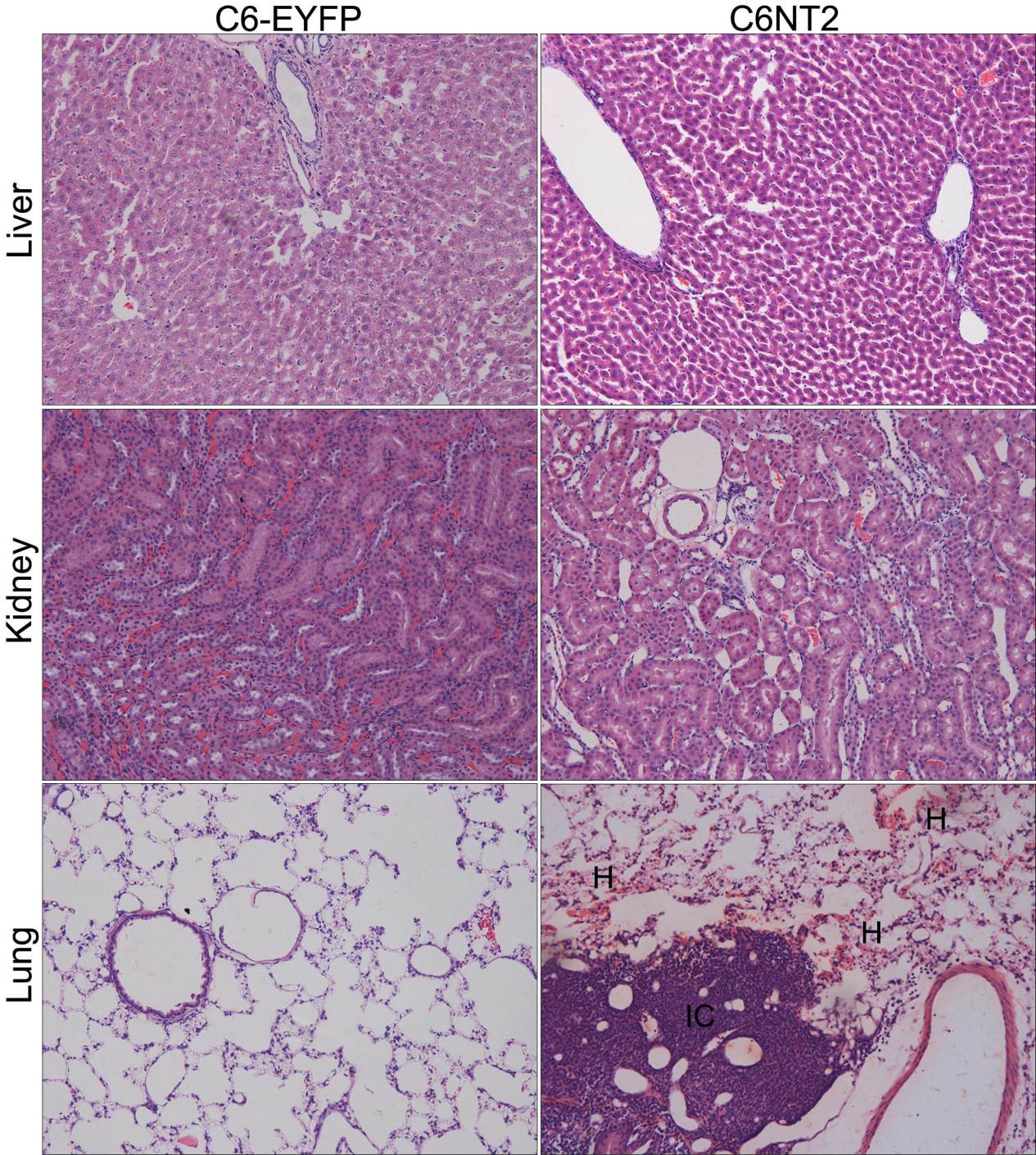


Figure 3

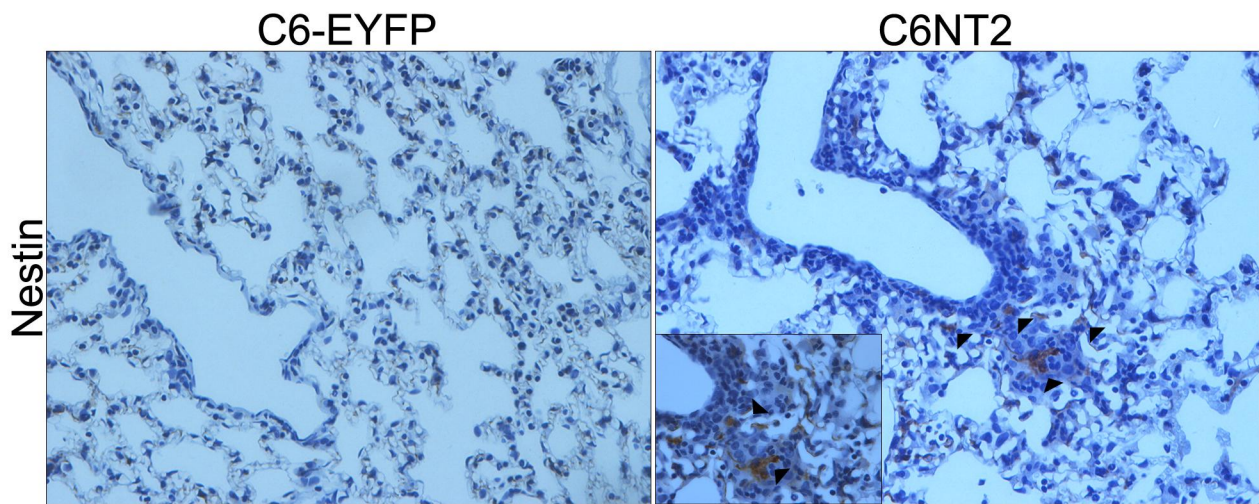


Figure 4

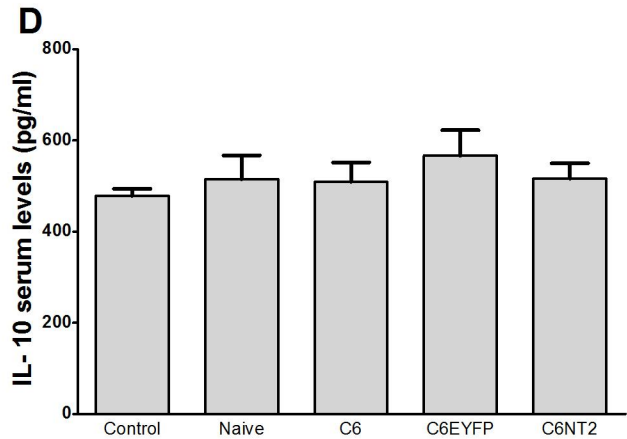
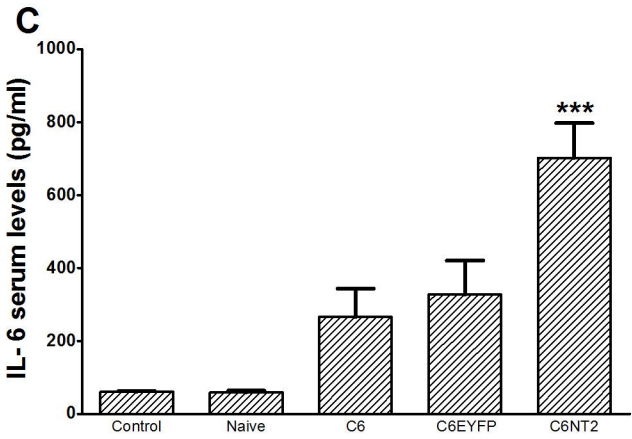
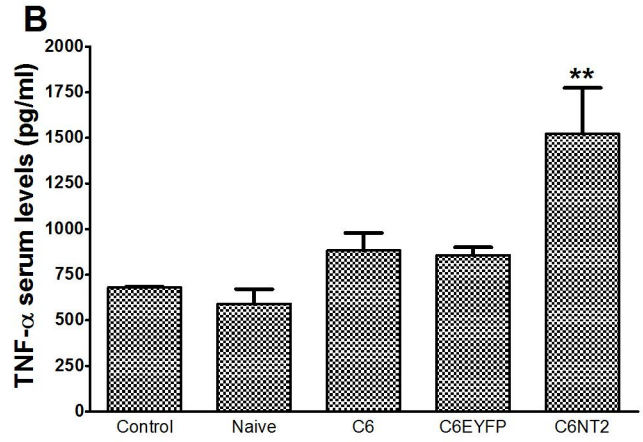
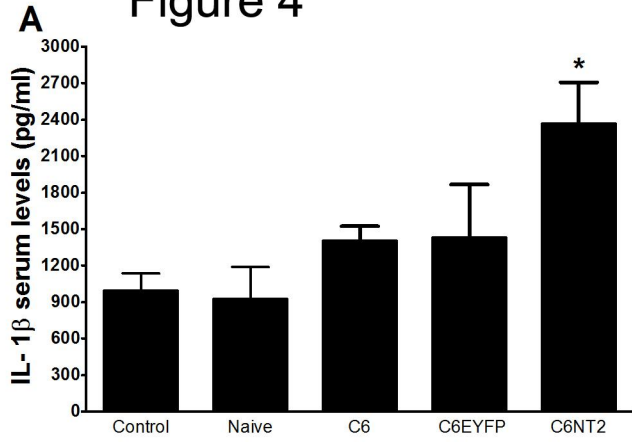
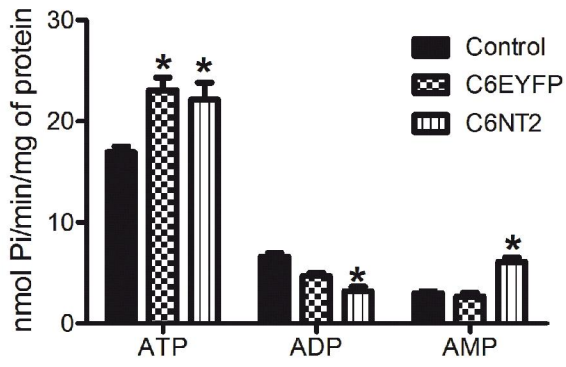
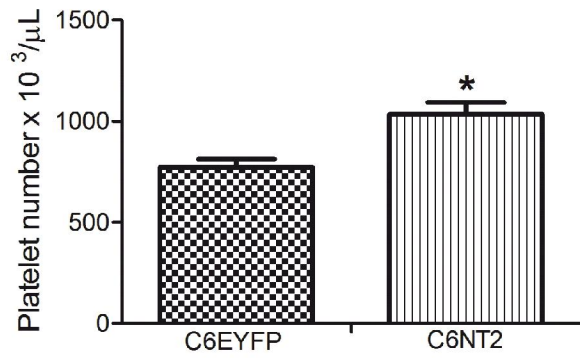


Figure 5

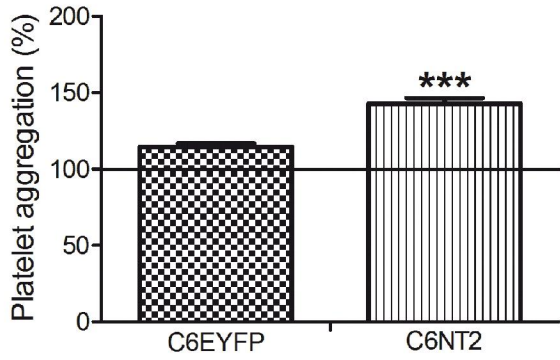
A



B



C



D

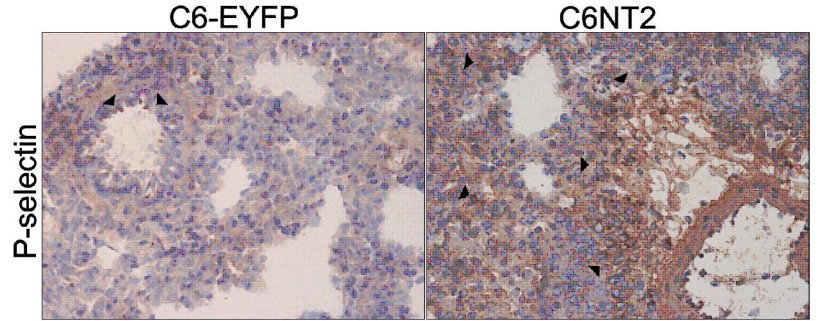


Figure 6

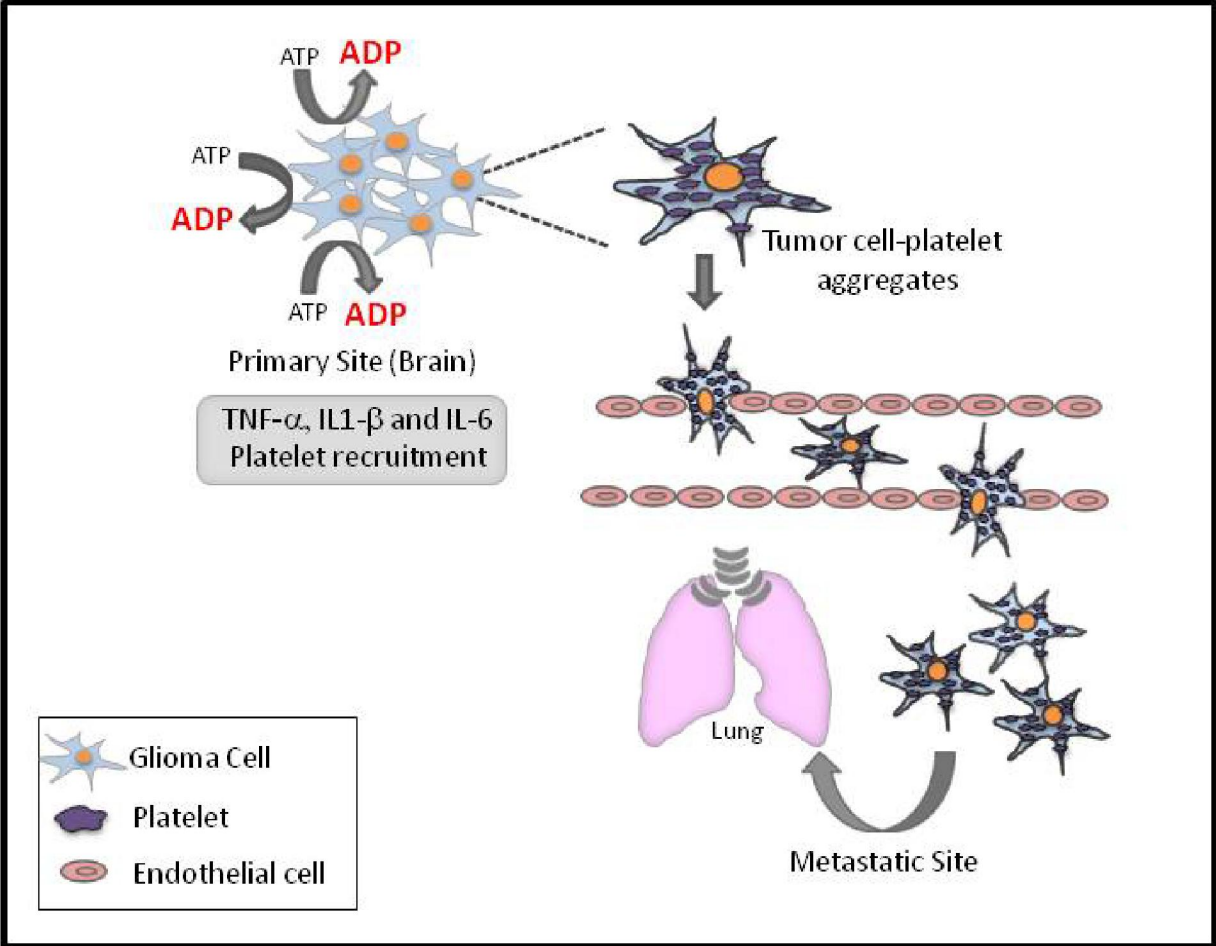


Table 1. Histological characteristics of lung from rats implanted with gliomas.

Histology	C6 (n=5)	C6-EYFP (n=5)	C6NT2 (n=5)
Focal hemorrhage	1/5	2/5	5/5
Lymphocytic infiltration	1/5	0/5	4/5
High Cellularity	0/5	0/5	5/5

Twenty days following glioma implantation, lungs from rats implanted with C6, C6-EYFP or C6-EYFP/NTPDase2 (C6NT2) were processed for hematoxylin and eosin (H&E) differential staining. The histological analysis was performed by a pathologist in a blinded manner. The histological variables (focal hemorrhage, lymphocytic infiltration and high cellularity) were scored as present or absent. The results are expressed as the number of animals positive for the pathological characteristic in relation to the total number of animals per group.

3.4 Artigo 4

EXTRACELLULAR NUCLEOTIDES CONTROL IL-8 AND MCP-1 SECRETION IN U251MG GLIOMA CELL LINE

Elizandra Braganhol, Filip Kukulski, Sébastien A. Lévesque, Michel Fausther, Elise G. Lavoie, Fariborz Bahrami, Fethia Ben Yebdri, Ana Maria O. Battastini and Jean Sévigny.

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Abstract

Gliomas are the most common and devastating primary brain tumors. The progression of malignant tumors involves the participation of an inflammatory microenvironment, consisting of immune cells and their secretory products. Cancer cells also exhibit properties characteristic of immune cells, such as TLR expression, which are subsequently used to promote tumor growth. Upon activation, TLR induces the production of a variety of cytokines, including IL-8 and MCP-1. These cytokines promote the recruitment of immune cells neutrophils and monocytes, respectively, and they are also involved in tumor cell proliferation and angiogenesis. Purinergic signalling, involving ATP released from neural and immune cells and its breakdown products display cytokine-like properties. By activating purinergic receptors, ATP and ADP modulate the immune cell recruitment to injured areas and the secretion of pro-inflammatory cytokines by immune cells. Previously, we have shown that gliomas hydrolyze poorly extracellular nucleotides when compared to astrocytes in culture. Moreover, astrocytes have been shown to release nucleotides. The preservation of nucleotide release during astrocyte to glioma transformation may favor a robust purinergic signaling, which may maintain an inflammatory environment favorable to developing tumors. In agreement with this hypothesis, in the present work we show that extracellular nucleotides control basal and TLR-induced glioma IL-8 and MCP-1 secretion via P2 receptor activation and thus glioma cell proliferation.

Key words: glioma, ATP, P2 receptors, IL-8, MCP-1.

Introduction

The glioblastoma multiform (GBM) remains one of the most aggressive and devastating brain tumors despite decades of efforts to find effective treatments [1-3]. Accumulating evidence indicates that chronic inflammatory process significantly contributes to the progression of malignant gliomas [4-6]. To trigger inflammatory response, gliomas secrete a number of factors such as cytokines and chemokines [7; 8]. Some studies also suggest that during cancerogenesis, cancer cells acquire many properties characteristic of immune cells which are subsequently used to promote tumor growth [9; 10]. For example, tumor cells abundantly express Toll-like receptors (TLRs) that seem to be involved in tumor progression and chemoresistance. TLRs play a key role in the innate immunity where they serve to recognize invading microbes and initiate appropriate immune responses [11]. These receptors can also be activated by various endogenous ligands originating from dying cells or extracellular matrix that are produced during tumor expansion in the affected tissue [12]. Upon stimulation, the majority of TLRs induce the activation of NF- κ B pathway, which orchestrates the production of a variety of inflammatory cytokines including IL-8 and MCP-1 [13]. These two cytokines promote the recruitment of immune cells neutrophils and monocytes, respectively, which play a major role in tumorigenesis [14; 15]. In addition to immune cell recruitment, IL-8 is also involved in glioma cell proliferation and angiogenesis [16], while MCP-1 controls the migration of neural progenitor cells toward the tumor bulk [17]. Moreover, both cytokines serve as mediators of immune cells differentiation and tumor supportive functions, including tissue invasion and metastasis, resistance to chemotherapy and to apoptosis [18].

Purinergic signaling is exerted by extracellular nucleotides such as ATP, ADP, UTP and UDP via P2 receptors [19]. P2 receptors are represented by two subfamilies: the G-protein-coupled receptors (P2Y_{1, 2, 4, 6, 11-14}) and the ligand-gated ion channels (P2X₁₋₇). The P2Y subtypes differ in their selectivity toward adenine (ATP, ADP) and uracil nucleotides (UTP, UDP) while all P2X receptors are activated by ATP [20; 21]. Interestingly, P2 receptor activation has been implicated in cytokine/chemokine release from various cells types. For example, ATP and ADP stimulate cytokine production of microglial cells [22; 23]. The autocrine stimulation of P2Y₆ is required for LPS-induced IL-8 secretion from monocytes [24; 25] while the stimulation of these cells with Pam₃CSK₄ requires the activation of both P2Y₆ and P2Y₂ receptors [26].

We have previously demonstrated that glioma cells exhibit a dramatically reduced capacity to hydrolyze extracellular nucleotides compared to astrocytes from which they originate [27; 28]. Moreover, astrocytes have been shown to release nucleotides [29; 30]. The preservation of nucleotide release during astrocyte to glioma transformation may favor a robust purinergic signaling in developing tumors. In agreement, in the present work we show that extracellular nucleotides control basal and TLR-induced glioma IL-8 and MCP-1 secretion via P2 receptor activation and thus glioma cell proliferation.

Results

Extracellular nucleotides are involved in basal and TLR-induced IL-8 and MCP-1 secretion by U251MG glioma cells

It has been demonstrated by us and others that extracellular nucleotides control IL-8 and MCP-1 production in monocytes/macrophages [24; 26; 33; 34]. As these

chemokines play an important role in glioma growth, we hypothesized that IL-8 and MCP-1 release by human U251MG glioma cells may also be extracellular-nucleotide dependent. In agreement with this hypothesis, we observed that either the medium of unstimulated U251MG glioma cultures or cells treated with LPS, a TLR4 ligand, contained significant amounts of extracellular ATP, as determined by luciferin/luciferase assay (Fig. 1). Interestingly, we also found that U251MG cells express relatively low ecto-ATPase activity (12.1 ± 2.1 and 5.6 ± 3.3 nmol Pi/min/mg of protein for ATP and ADP hydrolysis, respectively) when compared to astrocytes in culture [35], which may favor P2 receptor activation by nucleotides accumulating within a tumor. Noteworthy, the nucleotide metabolism rate in glioma cells was not affected in the presence of LPS (data not shown).

Next, we investigated whether nucleotides are involved in chemokine production by U251MG glioma cells. We first observed that these cells have a high basal IL-8 and MCP-1 secretion (Fig. 2A and 2B, respectively) which was in agreement with previous reports [36; 37]. To determine whether extracellular nucleotides play a role in this spontaneous cytokine secretion, we incubated U251MG glioma cells in the presence of apyrase, a nucleotide scavenger, to deplete the medium from extracellular nucleotides. Apyrase markedly diminished (~30% decrease) spontaneous IL-8 and MCP-1 secretion. Importantly, heat-inactivated apyrase had no effect on chemokine level in U251MG medium, indicating that extracellular nucleotides are indeed involved in basal IL-8 and MCP-1 secretion by these cells. We next aimed to determine the P2 receptor(s) subtype responsible for the spontaneous cytokine secretion. RT-PCR showed that U251MG glioma cells express multiple P2 receptors (P2X₄, P2X₇, P2Y₁, P2Y₄, P2Y₆, P2Y₁₁₋₁₄;

Table 1). To define P2 receptors involved in IL-8 and MCP-1 secretion, glioma cells were treated for 18 h with the following P2 antagonists: suramin and PPADS (general P2 receptor antagonists), MRS2500 (selective for P2Y₁), MRS2578 (P2Y₆), NF157 (P2Y₁₁ and P2X₁), and KN62 (P2X₇). The spontaneous secretion of IL-8 and MCP-1 was decreased by suramin (45% and 55% decrease for IL-8 and MCP-1, respectively; Fig. 2A and 2B) and MRS2578 (~35% decrease for IL-8 and MCP-1, respectively; Fig. 2A and 2B). Taken together, these results indicate that extracellular nucleotides via P2Y₆, and possibly also other P2 receptors, are responsible for at least in part for the basal IL-8 and MCP-1 production by U251MG cells.

U251MG glioma cells express TLRs that have been shown to promote tumor growth and malignancy via induction of cytokine production [18]. Therefore, we tested whether extracellular nucleotides are also involved in TLR-induced IL-8 and MCP-1 secretion. To find out which TLR induce chemokine secretion by U251MG glioma, these cells were incubated with the agonists for the TLRs that are thought to be involved in tumor progression [18; 38]. This stimulation was carried out in reduced-serum conditions (0.5% FBS) to limit the basal IL-8 and MCP-1 secretion which was already high in the presence of 10% FBS (Fig. 2A and B). The potency of TLR ligands on IL-8 and MCP-1 release was in the following rank order: LPS (TLR4) >> Flagelin (TLR5) > Poly(I:C) (TLR3) > PAM₃CSK₄ (TLR1/2) (data not shown). Hence, LPS was used to trigger chemokine release in the subsequent experiments. To determine the role of extracellular nucleotides in LPS-induced chemokine production by glioma cells, we incubated these cells with LPS in the presence or absence of a range of P2 receptors antagonists, as above for basal chemokine secretion. Fig. 3 shows that as for the basal chemokine release, LPS-

induced IL-8 and MCP-1 secretion was decreased significantly by suramin and MRS2578. Note that, in contrast to IL-8, the MCP-1 secretion by glioma cells was still high in serum-reduced conditions, and suramin and MRS2578 decreased this release (Fig. 3B).

The strong inhibition of LPS-induced IL-8 and MCP-1 secretion to MRS2578 suggests that this response is largely mediated by P2Y₆ receptor. To further confirm the role of P2Y₆, we knockdown this receptor with predesigned shRNAs. As shown in Fig. 4A, three shRNA molecules used, namely 14075, 14076 and 14077 decreased the relative expression of mRNA for P2Y₆ gene by 40–70% compared to the control cells expressing scrambled shRNA. Due to the lack of reliable antibodies against P2Y₆, we were unable to confirm the knockdown of this receptor at the protein level. In agreement with a role of P2Y₆ in IL-8 and MCP-1 release, the spontaneous secretion of these chemokines by the cells with a reduced P2Y₆ expression were decreased by ~50% and ~30% compared to the control cells, respectively (Fig. 4B and 4C). P2Y₆ knockdown also diminished LPS-induced IL-8 secretion (Fig. 4D), however, it had only modest effect on LPS-induced MCP-1 release which was not significantly different from the control cells (Fig. 4E). Note that the two shRNA molecules that did not reduce P2Y₆ mRNA, namely 14073 and 14074, were also ineffective in blocking IL-8 and MCP-1 secretion, which further confirmed the requirement of P2Y₆ for chemokine release by glioma cells tested. Taken together, these data suggest that extracellular nucleotides control basal and LPS-induced chemokine secretion by U251MG glioma cells via P2Y₆ receptor.

Extracellular milimolar ATP concentrations markedly enhances LPS-induced IL-8 and MCP-1 secretion by U251MG glioma cells.

The above experiments suggest that U251MG cells possess a low ecto-ATPase activity which favor the accumulation of significant amounts of ATP due to basal or induced (with LPS) release of this nucleotide. They also showed that the basal and LPS-induced chemokine secretion involve the activation of P2Y₆ receptors which is specific for UDP. Since these glioma cells (Table 1) as well as their precursors astrocytes [33] express P2X₇ receptor we hypothesized that high milimolar ATP concentrations may have an effect on IL-8 and MCP-1 release from U251MG cells. The above experiments showed that P2X₇ antagonist did not affect chemokine release by LPS-treated glioma (Fig. 3), suggesting that glioma cells alone are unable to produce high ATP levels required for P2X₇ activation that, however, could be easily generated *in vivo* together with adjacent cells or migrating immune cells. To test this hypothesis, U251MG cells were stimulated with exogenous ATP alone (0.5, 1.0 and 3.0 mM) or in combination with a suboptimal concentration of LPS (10 ng/mL for IL-8 release and 1 ng/mL for MCP-1 release; these doses were determined by preliminary dose-response experiments which are not shown). Importantly, none of these ATP concentrations caused glioma cell-death (data not shown). As demonstrated in Fig. 5, ATP alone did not induce a significant chemokine release. However, the combination of 3 mM ATP with LPS increased IL-8 and MCP-1 release by glioma cells by ~6 and 2 fold compared to LPS alone, respectively (Fig.5A and 5B).

The effect of ATP and LPS on IL-8 and MCP-1 release was inhibited by suramin (~80% decrease) and the specific P2X₇ antagonists A438079 and KN62 (~50%

inhibition; Fig. 6A and 6B). The effect of the latter molecules confirmed that millimolar ATP used in these assays most probably activated P2X₇ receptor. However, suramin which is not an antagonist of P2X₇ was even stronger inhibitor of IL-8 and MCP-1 release suggesting that in addition to P2X₇, other(s) P2 receptor(s) was required for LPS/ATP-induced chemokine production. In agreement, the blockage of P2Y₆ with MRS2578 also very efficiently prevented both IL-8 and MCP-1 secretion in response to ATP and LPS (~90% inhibition of IL-8 and MCP-1 secretion). These results suggest that P2X₇ receptor activation boosts LPS/P2Y₆-induced IL-8 and MCP-1 secretion by U251MG cells. The strong inhibitory effect of MRS2578 on this response together with the fact that the activation of P2X₇ alone was insufficient to activate chemokine release indicate that P2Y₆ receptor is instrumental for glioma IL-8 and MCP-1 release.

Extracellular nucleotide-induced cytokine secretion triggers glioma cell proliferation

As the proinflammatory cytokines promote tumor growth [5], we examined whether LPS-induced and nucleotide-mediated IL-8 and MCP-1 release are important for U251MG glioma cell proliferation. To this end, sub-confluent glioma cultures were treated for 48 h with LPS (100 ng/mL) in the presence or absence of suramin and MRS2578 that were shown to inhibit chemokine release. As shown in Fig. 7A, the addition of LPS increased cell proliferation by 60% compared to untreated cells. This effect was prevented by both P2 antagonists tested (Fig. 7A) and correlated with a decrease in IL-8 and MCP-1 secretion (Fig. 7B and 7C). In agreement with the results from the preceding sections, A438079, a P2X₇ antagonist, did not affect LPS-induced

chemokine release nor cell proliferation. The role of IL-8 on glioma growth was further demonstrated by using IL-8 receptor antagonist (SB225002) and the hIL-8 neutralizing antibody. Both treatments reduced cell counts by 80% and 70% compared to control cells grown in the absence of these inhibitors, respectively. These results show that extracellular nucleotides via P2Y₆ receptor modulate glioma cell proliferation *in vitro* probably by controlling cytokine secretion.

Discussion

The present work demonstrates a novel function of purinergic signaling in proinflammatory chemokine production by glioma cells which may have very important implication in tumor growth. Specifically, we found that human U251MG glioma cells have a basal ATP release which could be markedly increased upon stimulation with LPS. Accordingly, a previous study has reported that high quantities of ATP accumulate in the interstitium of human ovarian carcinoma [39]. This fact in conjunction with a low ATP/ADPase activity at the surface of U251MG glioma cells may favor the sustained activation of various P2 receptors expressed in these cells. In keeping with this, we further showed that extracellular nucleotides trigger basal and TLR-induced IL-8 and MCP-1 secretion. Indeed, the administration of apyrase, suramin, the general P2 receptor antagonist, and MRS2578, a specific P2Y₆ antagonist, significantly reduced the cytokine secretion either by unstimulated or LPS-treated U251MG cells. The involvement of P2Y₆ in the release of IL-8 (basal and LPS-induced) and MCP-1 (basal only) was further confirmed with specific shRNAs, as the cells with knocked down P2Y₆ expression produced markedly less IL-8 compared to the control cells. P2Y₆ knockdown had no a

significant effect on LPS-induced MCP-1 secretion but there was a clear tendency indicating a decrease in this chemokine release in cells treated with specific P2Y₆ siRNA. Note that LPS stimulation increases glioma IL-8 secretion by 4-fold whereas MCP-1 not even by 2-fold (Fig.4D and 4E, respectively) so it was expected to see only a modest effect of P2Y₆ knockdown on LPS-induced MCP-1 secretion. We also found that high millimolar concentrations of exogenous ATP can markedly increase IL-8 and MCP-1 release by glioma cells stimulated with LPS. The effect of ATP was probably mediated by P2X₇ receptor as the antagonists of this receptor significantly inhibited this response. Finally, in line with the role of TLRs in tumor progression [18], we showed that LPS treatment induced glioma cell proliferation which correlated with P2 receptor-dependent cytokine secretion. In agreement with these results, previous studies from our group showed that the co-injection of apyrase (to deplete extracellular nucleotides and thus prevent P2 activation) with gliomas in rat brain decreased the presence of inflammatory infiltrate in the implanted tumors and their pathological characteristics [40].

Previous studies have already suggested an important role of purinergic signaling in cancer progression [41; 42]. Interestingly, glioma cells often exhibit alterations in purinergic signaling compared to the cells from which they develop such as an impaired extracellular ATP metabolism, the disruption of P2 receptor expression and function, and an increased resistance to ATP-induced cell death [42; 28]. These characteristics are common to different glioma cells lines where they seem to increase their malignance potential [27; 40; 43]. The novel observation made here is that the extracellular nucleotides control glioma IL-8 and MCP-1 secretion. A robust production of these chemokines may represent an important strategy adopted by tumor cells to progress and

invade normal tissues [10; 18]. In addition, the results presented here demonstrate that two P2 receptors, namely P2Y₆ and P2X₇, play an important role in glioma IL-8 and MCP-1 secretion. Of these receptors, P2Y₆ appears instrumental for these responses while P2X₇ is required for their potentiation. Our results also showed that the basal or LPS-induced autocrine stimulation of glioma cells with endogenous nucleotides causes P2Y₆ activation. This is consistent with already reported role of P2Y₆ in TLR1/2 and TLR4-induced IL-8 secretion by human monocytes [24-26]. In contrast, the activation of P2X₇ appears to require high millimolar ATP concentrations that could not be produced by glioma cells in the experimental conditions used however they can be generated by tumor cells together with adjacent cells, often damaged by growing tumors or due to ongoing inflammation, as well as from immune cells infiltrating tumors. To our knowledge, this is the first demonstration of the role of P2X₇ in chemokine production which may represent a peculiar property of P2X₇ in cancer cells and an important adaptation of these cells. Indeed, in normal cells expressing P2X₇, its activation often leads to cell death [44] while in glioma cells it promotes inflammatory response required for tumor growth and progression [45].

In summary, the data presented in this work suggest that extracellular nucleotides are necessary for IL-8 and MCP-1 secretion by U251MG glioma cells. As IL-8 and MCP-1 secreted by tumor cells and a variety of immune cells can trigger angiogenesis and tumor cell proliferation, the extracellular nucleotide receptors (P2Y₆ and/or P2X₇) may reveal as novel anti-cancer therapy target.

Material and methods

Materials

LPS from *Escherichia coli* O111:B4, nucleotides (ATP and UDP), pyridoxal-phosphate-6-azophenyl-2, 4-disulfonate (PPADS), suramin and semi-purified potato apyrase grade VII were purchased from Sigma Chemical (St. Louis, MO, USA). Pam3CSK4 and flagellin from *Salmonella typhimurium* were purchased from InvivoGen (San Diego, CA, USA). MRS2500, MRS2578, NF157, KN62 and A438079 were purchased from Tocris Bioscience (Bristol, UK). Human IL-8 neutralizing Ab MAB208 were provided by R&D Systems (Minneapolis, MN, USA) and a matching isotype mouse IgG1 Ab (used as a control in cell proliferation assays) by Sigma. Fetal bovine serum (FBS), HEPES and glutamate were obtained from Wisent (St-Bruno, Canada) and DMEM medium from Gibco (NJ, USA).

Treatment preparation

FBS used for cell culture and cellular assays was heat-inactivated by 30 min incubation at 56°C. TLR agonists (Pam₃CSK₄, LPS, flagellin and poly(I:C), nucleotides and P2 receptor antagonists PPADS, suramin, MRS2500, NF157 and A438079) were reconstituted in endotoxin-free water (Sigma). Prior to cell stimulation, LPS was sonicated for 10 min in a water bath sonicator. MRS2578 and KN62 were dissolved in sterile DMSO (Sigma) at the concentration of 10 and 1.5 mM, respectively, and further diluted with Dulbecco's modified Eagle's medium (DMEM) with 0.5% or 10% FBS to obtain 1 and 0.3 mM, respectively. Appropriate controls containing 0.01 and 0.2% DMSO were performed.

U251MG glioma cell culture and stimulation

The U251MG glioma cell line (a kind gift from Dr. L. Valliere, CRRI, CRCHUL, Québec) was grown in culture flasks in the presence of DMEM supplemented with 10% FBS. Cells from passages 10 to 20 were used. Due to the increased chemokine release in the presence of 10% FBS in the culture medium, U251MG glioma cells were stimulated with TLR agonists or exogenous nucleotides in DMEM containing 0.5% FBS. Confluent U251MG glioma cells in a 24 well-plate were stimulated with LPS (1, 10 or 100 ng/mL; TLR4 agonist) in the presence or absence of P2 receptor antagonists (100 μ M suramin or PPADS, 10 μ M MRS2578, 1 μ M MRS2500 or NF157, 3 μ M KN62 and 25 μ M A438079) or ATP (0.1, 0.5, 1.0 or 3.0 mM). P2 antagonist or nucleotides were added to the cells 30 min before the addition of LPS. In addition to LPS, the glioma cells were also stimulated with Pam₃CSK₄ (1 μ g/mL; TLR1/2 agonist), poly(I:C) (1 μ g/mL; TLR3 agonist) and flagellin (100 ng/mL; TLR5 agonist). The stimulation of U251MG glioma cells was carried out for 18 h at 37°C in a humid atmosphere containing 5% CO₂. The resulted conditioned media were centrifuged (1000g, 10 min, 4°C) to remove the detached cells, the supernatants collected and frozen at -80°C until used for ELISA, as described below.

IL-8 and MCP-1 ELISA

IL-8 and MCP-1 secreted by U251MG glioma cell line were quantified in the supernatants of these cells by Human IL-8 CytoSet™ (Biosource) or human CCL2/MCP-1 DuoSet (R&D Systems), following the manufacturers' instructions. Human recombinant (hr) IL-8 or MCP-1 were used as standards.

RT-PCR

Total RNA was isolated from the cells using Trizol as recommended by the manufacturer (Invitrogen) and quantified spectrophotometrically at 260 nm. One microgram of RNA was reverse transcribed to cDNA in 20 μ L of the reaction mixture containing 50 μ M oligo(dt)20, 10 mM dNTPs, 40 U/mL RNase Out, 200 U/ μ L Superscript III reverse transcriptase dissolved in the supplied buffer (all from Invitrogen) and 0.1mM dithiothreitol (Roche, Ontario, Canada). The reactions were performed for 60 min at 50°C and stopped by heating at 70°C for 15 min followed by the addition of 2U E. coli RNase H (Invitrogen). The PCR reactions were performed in 25 μ L of the reaction mixture containing 1 μ L cDNA, 10 pmol of the primer, 10 mM dNTP, 5 U/ μ L of Taq DNA polymerase (New England BioLabs). After initial denaturation for 4 min at 94°C, the amplifications were carried out for 35 cycles of denaturation at 95°C for 45 s, annealing at primer specific temperature for 45 s and extension at 74°C for 45 s. The PCR was ended by 7 min incubation at 74°C. The same program was used for the amplification of the gene of reference, which was GAPDH. Sequences of primers and expected PCR fragment sizes are listed in Ben Yebdri *et al*, 2009 [26]. As a control for contaminations of the RNA preparation with genomic DNA, the crude product of the RNA extraction procedure without any reverse transcription reaction was used as template for a PCR reaction. No signal was detected in these samples without cDNA synthesis indicating that they were free of genomic DNA. RT-PCR products were separated on 1.5% agarose gels containing ethidium bromide and photographed under UV illumination.

P2Y₆ knockdown

A partial P2Y₆ depletion was achieved by the lentiviral infection of U251MG glioma cells according to the protocol adapted from the Invitrogen's ViraPower™ Lentiviral Expression System by the Gene Expression Lab. Lentiviral vectors containing scrambled shRNA (control) or shRNAs targeting human P2Y₆ (clones 14073, 14074, 14075 and 14076; Sigma) were produced in 293FT cells. On day 1, confluent glioma cultures in a 75 cm² cell culture flask containing 10 mL of DMEM with 6 µg/mL Polybrene[®] were incubated with the indicated viral vectors overnight at 37°C in a humid atmosphere containing 5% CO₂. Starting from day 2 up to day 15 after infection, the medium was replaced every three days with a fresh one containing an antibiotic puromycin (1.5 µg/mL) to select for stably infected cells. Depletion of P2Y₆ mRNA in U251MG glioma cells growing in the presence of puromycin was confirmed by quantitative qRT-PCR using FastStart SYBR Green Master (Roche) following the manufacturer's instructions. Standard curves for plasmids encoding the P2Y₆ or GAPDH sequences were performed simultaneously and were used to quantify the P2Y₆ absolute mRNA expression in the samples. The cells treated with specific P2Y₆ shRNAs or scrambled shRNA were used for IL-8 and MCP-1 secretion assays.

Ecto-ATPase activity assay

The ATP/ADPase activities were determined in U251MG glioma cells, as described previously [27]. Briefly, confluent glioma cultures in the incubation medium containing 2 mM CaCl₂, 120 mM NaCl, 5 mM KCl, 10 mM glucose and 20 mM HEPES (pH 7.4) were incubated with 1 mM nucleotides (ATP or ADP) for 10 min at 37°C. The release of

inorganic phosphate (Pi) and the protein concentration of cell homogenate were measured by the Malachite Green [31] and Coomassie Blue methods [32], respectively. Specific activity was expressed as nmol Pi released/min/mg protein.

Glioma ATP release measurement

The supernatants of unstimulated U251MG cells (2.0×10^4 cells) in DMEM/10% FBS (basal ATP release) or cells stimulated with LPS (100 ng/mL; induced ATP release) for 5 min in DMEM/0.5% FBS were collected and centrifuged (1000g, 10 min, 4°C). ATP was quantified using a firefly luciferase bioluminescence kit (Sigma), according to the manufacturer's instructions. Sample readings were done using Luminoskan Ascent microplate luminometer (Thermo Electron Corporation, Miliford, MA).

Glioma cell proliferation *in vitro*

U251MG glioma cells were seeded at 1.0×10^3 cells/well in DMEM with 10% FBS in 24-well plates, which corresponded to ~20% confluence. On day 2, the medium was replaced with a fresh one containing 0.5% FBS. The LPS stimulation was performed on day 3 in DMEM/0.5% FBS. The P2 antagonists (100 μ M suramin, 10 μ M MRS2578 or 25 μ M A438079), the IL-8 receptor antagonist (1 μ M SB225002) or the hIL-8 neutralizing Ab (1 μ g/mL) were added to the cells 30 min before LPS. Cell number and viability were assessed 48 h later using hemocytometer and trypan blue exclusion.

Statistical analysis

Data were expressed as mean \pm S.D. of at least three independent experiments and were subjected to one-way analysis of variance (ANOVA) followed by Tukey-Kramer post-hoc test (for multiple comparisons) or T-student test when necessary. Differences between mean values were considered significant when $P < 0.05$.

Acknowledgments

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Figure legends

Figure 1. U251MG glioma cells release ATP. Glioma cells were incubated in DMEM/10% FBS for 30 min or stimulated with LPS (100 ng/mL) in DMEM/0.5% FBS for 5 min and the conditioned medium was collected to evaluate the basal or stimulated ATP release, respectively. Data show means \pm S.D. of at least three independent experiments, each performed in triplicate. **Significantly different from control ($^{**}P < 0.01$).

Figure 2. Extracellular nucleotides control basal IL-8 and MCP-1 secretion by U251MG glioma cells. Glioma cells were incubated for 18 h in DMEM/10% FBS in presence or absence of a nucleotide scavenger 2 U/mL apyrase (Apy); apyrase boiled (Apy boil); or with the indicated P2 receptor antagonists (suramin, Sur). Secreted IL-8 (panel A) or MCP-1 (panel B) was measured by ELISA. Data show means \pm S.D. of at least three independent experiments, performed in duplicate ($^{*}P < 0.05$). * Significantly different from the control.

Figure 3. Extracellular nucleotides modulate LPS-induced IL-8 and MCP-1 secretion by U251MG glioma cells. Glioma cells were stimulated in DMEM/0.5% FBS for 18 h with lipopolysaccharide (LPS, 100 ng/mL) or vehicle (Control) in the presence or absence of the indicated P2 receptor antagonists added to the cells 30 min before LPS. Secreted IL-8 (panel A) or MCP-1 (panel B) was measured by ELISA. Data show means \pm S.D. of at least three independent experiments, performed in duplicate. *Significantly different from control; #significantly different from LPS stimulated glioma cells ($^{*}\#P < 0.05$).

Figure 4. P2Y₆ receptor knockdown decreases IL-8 and MCP-1 release by U251MG glioma cells. Glioma cells were infected with lentivirus carrying scrambled shRNA (Control) or shRNA targeting human P2Y₆ mRNA as described in the Materials and methods. (A) qRT-PCR for P2Y₆ mRNA expression levels; (B) IL-8 and (C) MCP-1, shRNA treated cells were incubated for 18 h in DMEM/10% FBS and the cytokine basal secretion was evaluated. (D) IL-8 and (E) MCP-1, the shRNA cells were incubated in DMEM/0.5% FBS (control, filled bars) or they were stimulated with LPS (empty bars) and IL-8 or MCP-1 secretion were measured. Data show means ± S.D. of four independent experiments, performed in triplicate. * and ***Significantly different from control; #significantly different from LPS stimulated glioma cells (*# $P < 0.05$; *** $P < 0.001$).

Figure 5. Extracellular ATP increases LPS-induced IL-8 and MCP-1 secretion by U251MG glioma cells Glioma cells were stimulated with suboptimal concentration of LPS (10 ng/mL for IL-8 or 1.0 ng/mL for MCP-1) for 18 h in the presence or absence of ATP (0.5; 1.0 or 3.0 mM) as indicated. ATP was added to the cells 30 min before LPS. Secreted IL-8 (panel A) or MCP-1 (panel B) was measured by ELISA. Data show means ± S.D. of at least three independent experiments, performed in triplicate. *Significantly different from control (* $P < 0.05$).

Figure 6. P2 receptor antagonists inhibit IL-8 and MCP-1 secretion by U251MG glioma cells stimulated with ATP and LPS. Glioma cells were stimulated with suboptimal

concentration of LPS and 3.0 mM ATP as described in material and methods, in the presence or absence of P2 receptor antagonists pre-incubated with the cells for 30 min. Secreted IL-8 (panel A) or MCP-1 (panel B) was measured by ELISA. Data show means \pm S.D. of at least three independent experiments, performed in triplicate. *Significantly different from control; #significantly different from LPS plus ATP stimulated glioma cells (*,# $P < 0.05$).

Figure 7. Extracellular nucleotides are required for LPS-induced U251MG glioma cell proliferation. (A) Glioma cells were treated with LPS (100 ng/mL) for 48 h in the presence or absence of the indicated P2 antagonists (Sur, suramin; MRS2578; A438079), IL-8 receptor antagonist (SB225002) or the hIL-8 neutralizing antibodies added to the cells 30 min before LPS. After 48 h of treatment, the cell number was assessed with a hemocytometer in the presence of trypan blue. In parallel, secreted IL-8 (B) or MCP-1 (C) was measured by ELISA. Data show means \pm S.D. of at least three independent experiments, performed in triplicate. * Significantly different from control; #significantly different from LPS treated glioma cells (*,# $P < 0.05$).

Figure 1

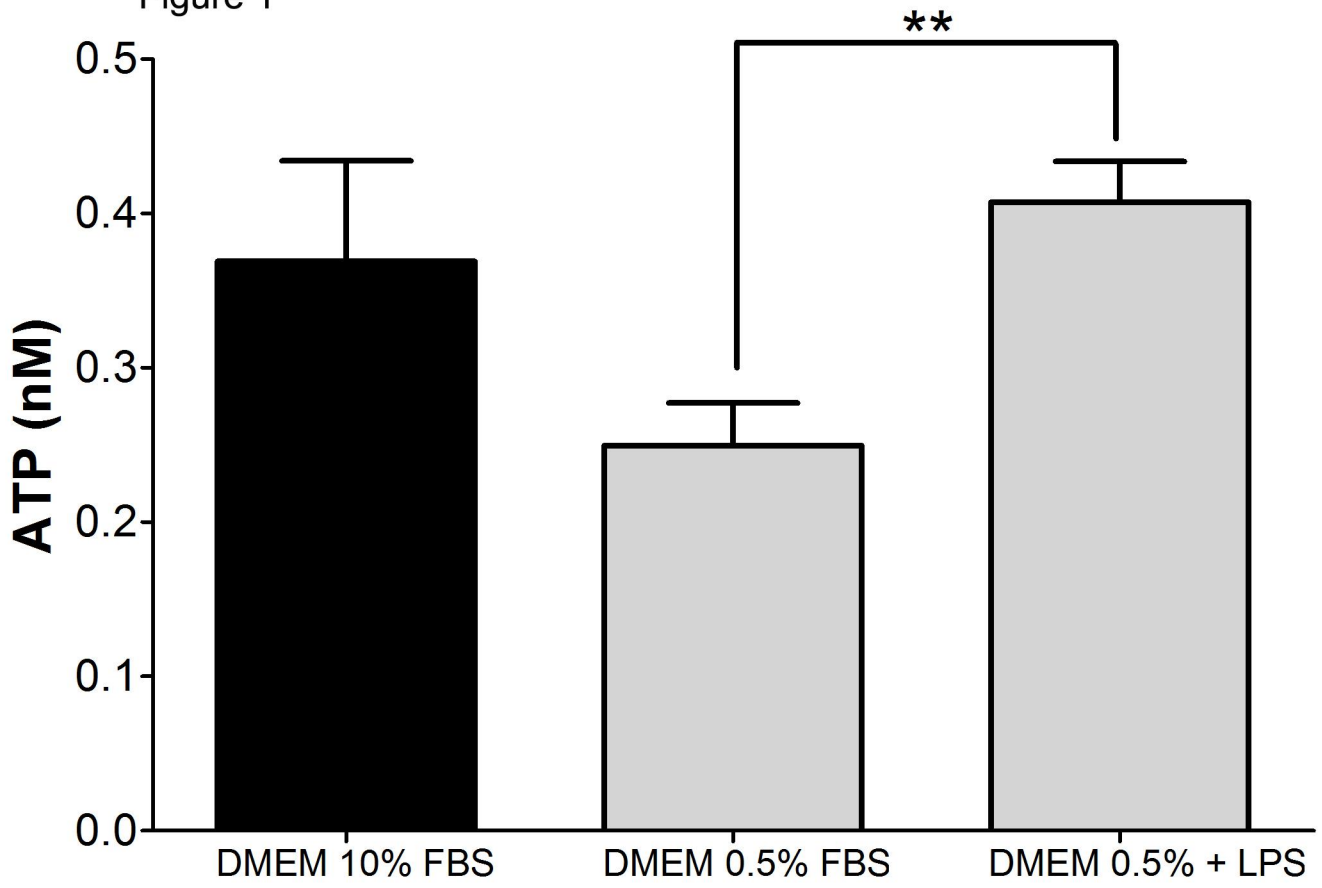


Figure 2

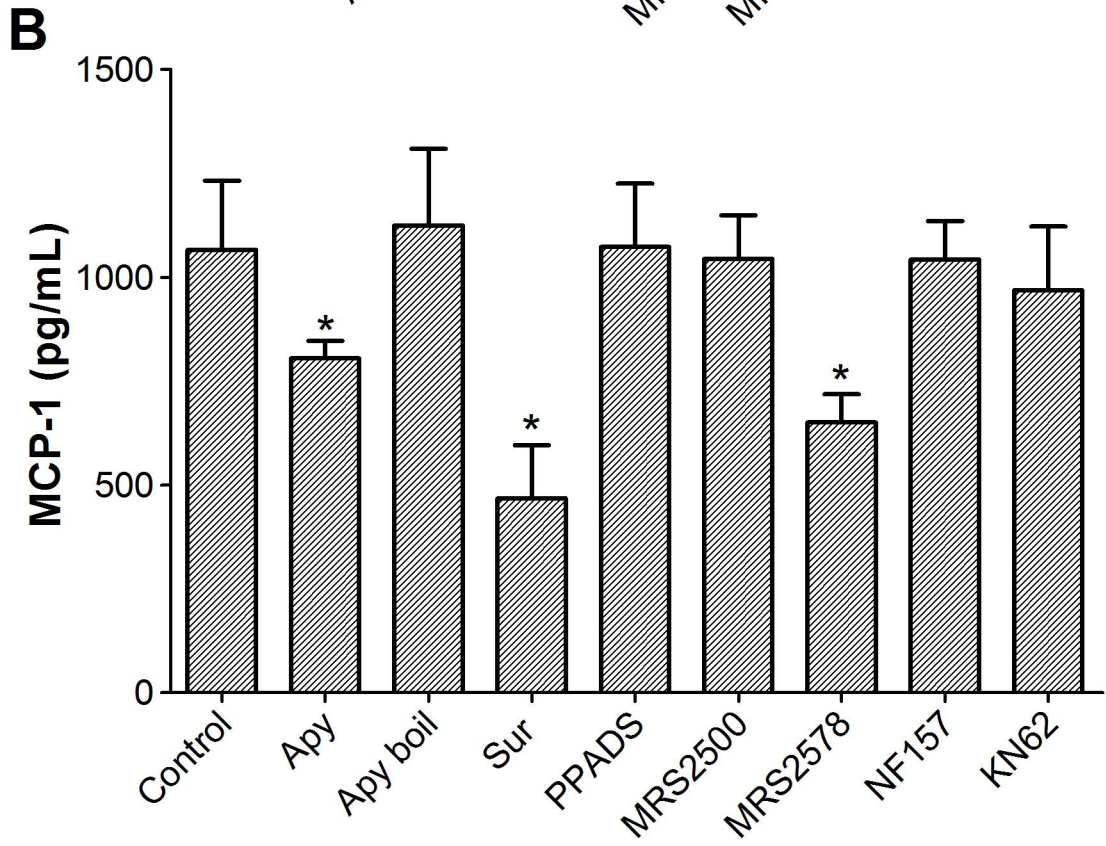
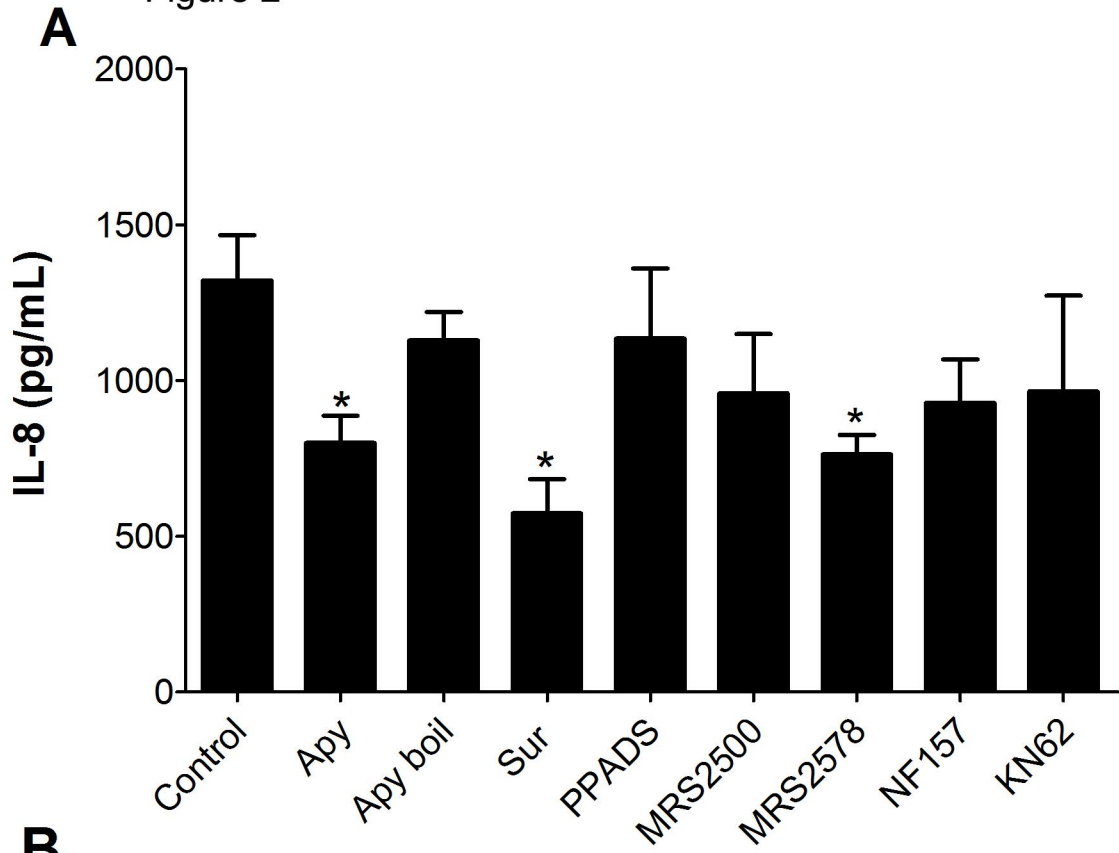
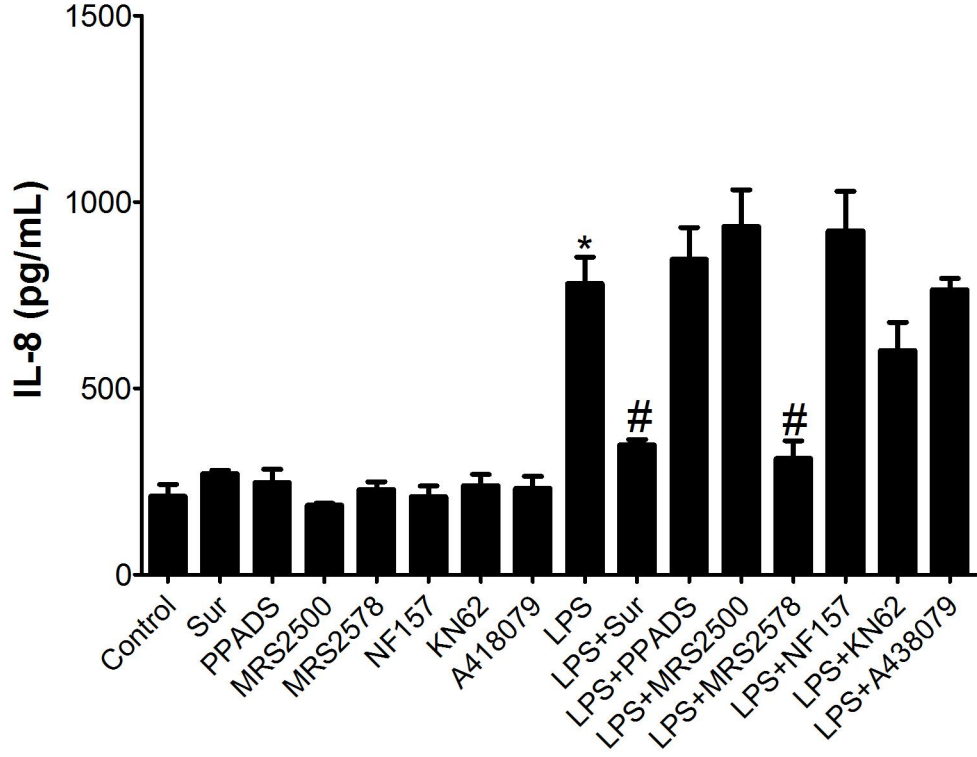


Figure 3

A



B

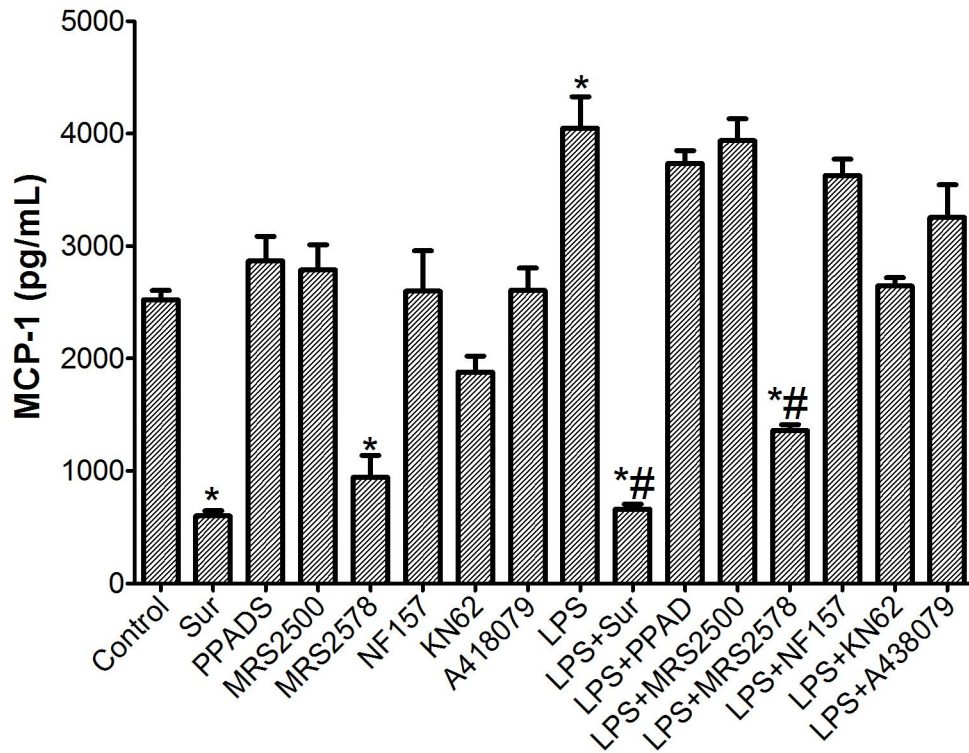


Figure 4

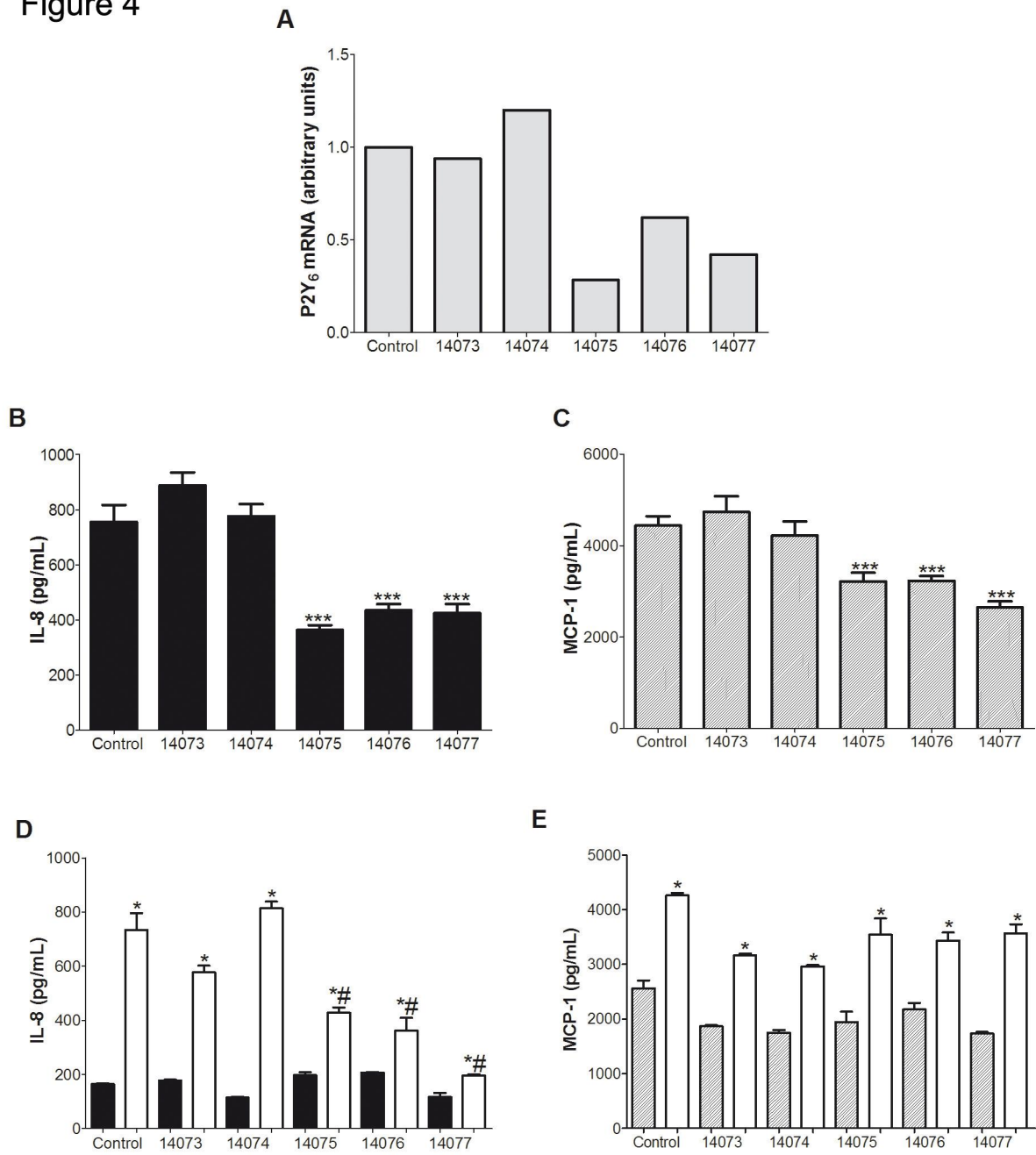


Figure 5

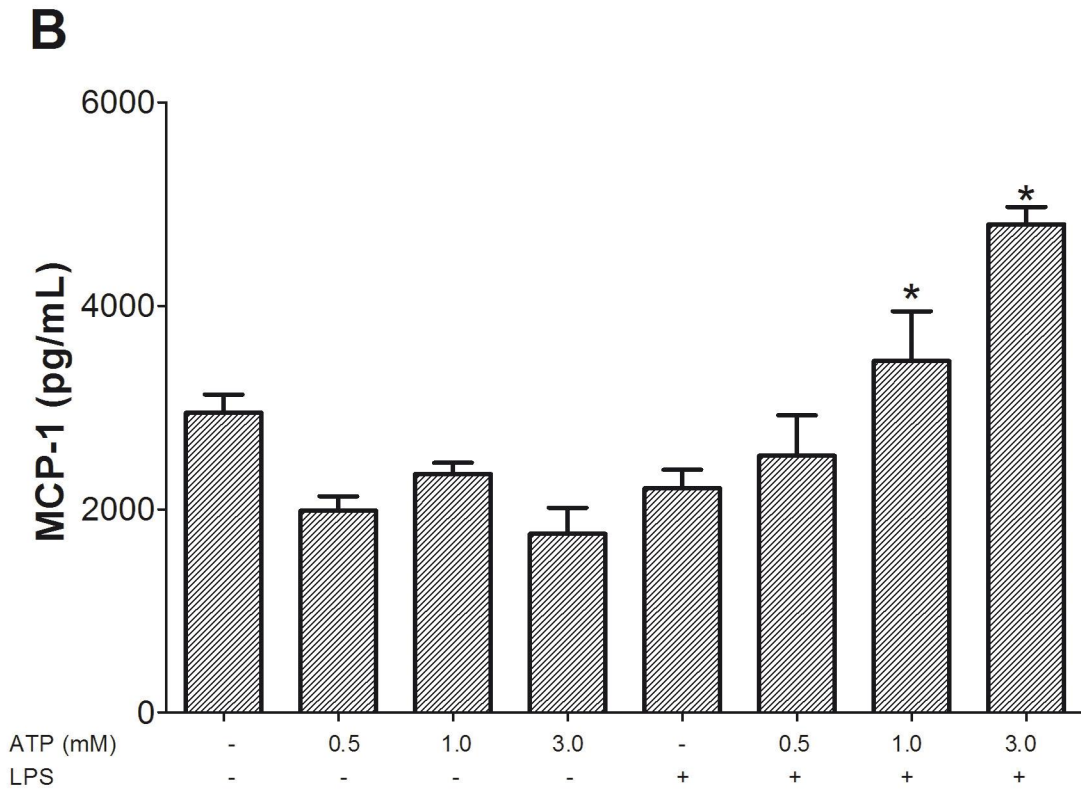
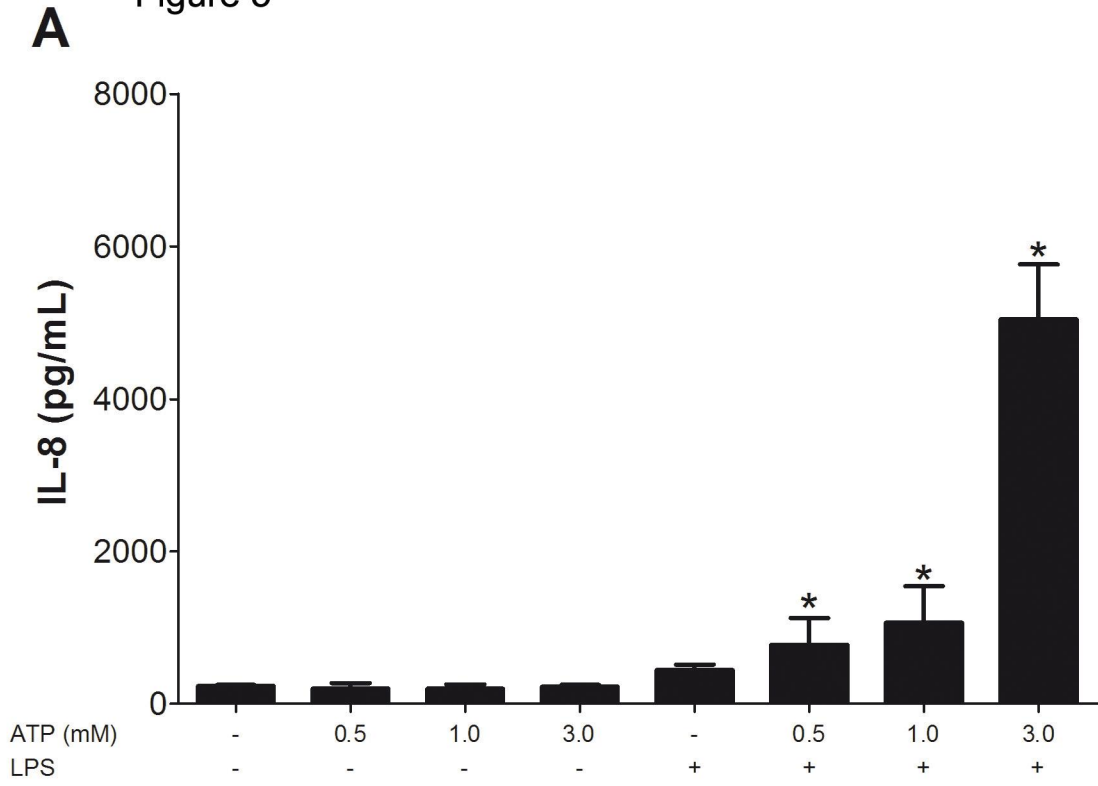
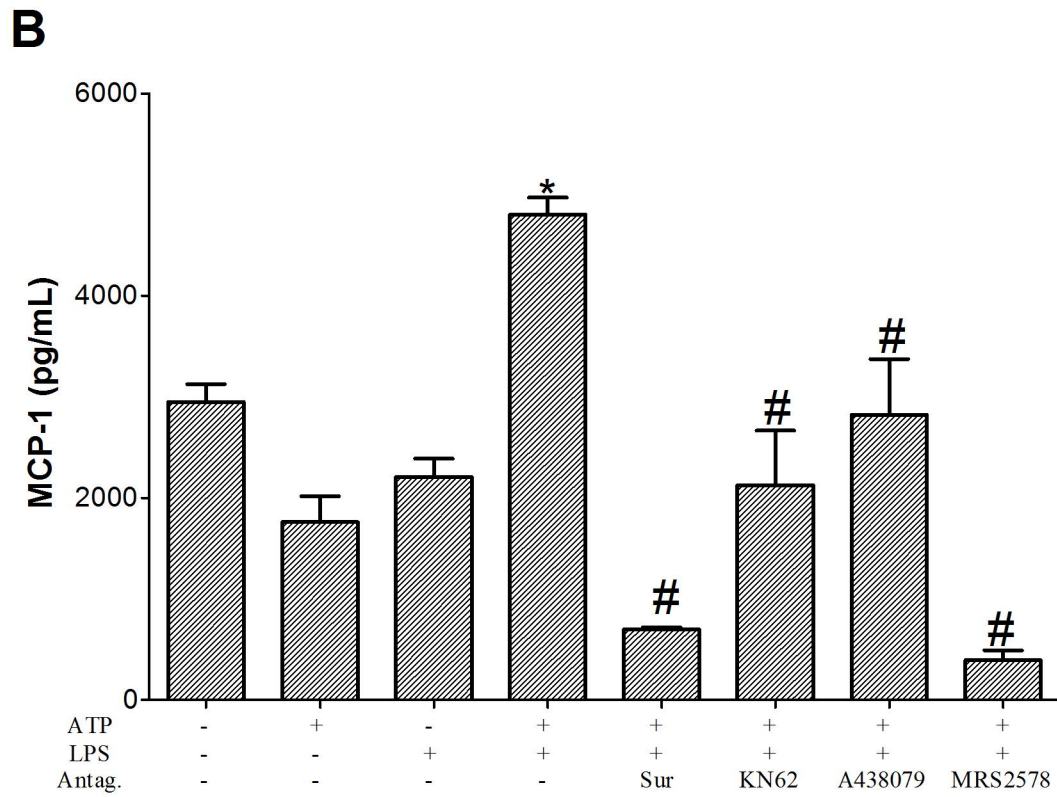
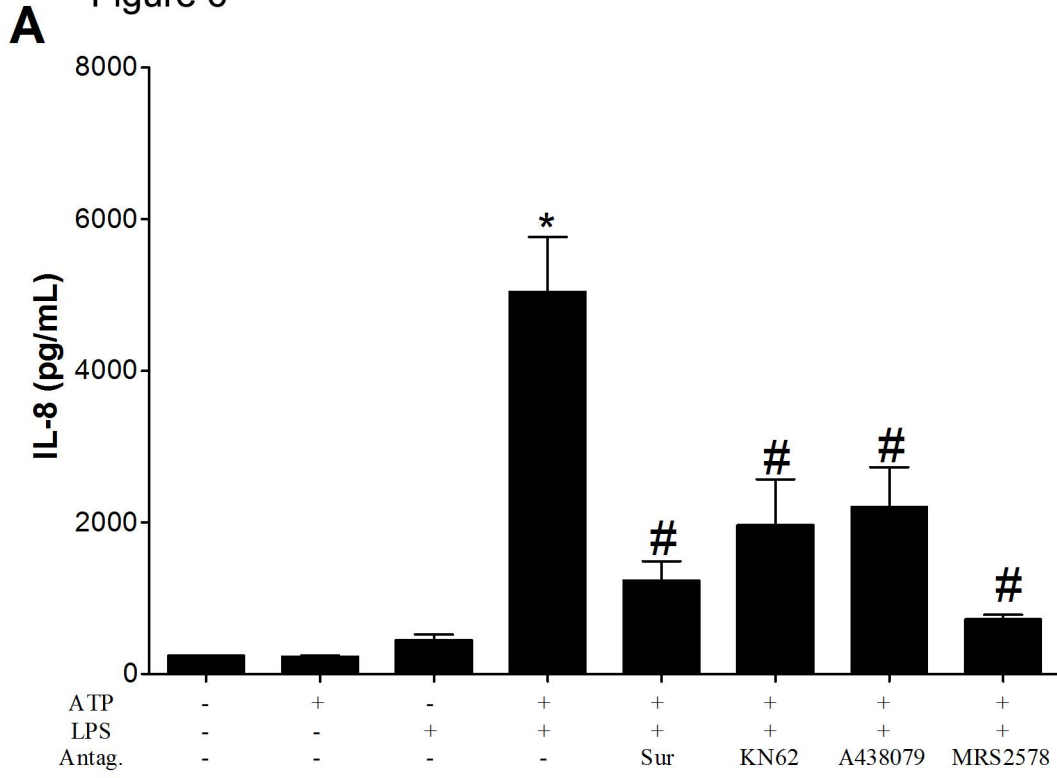
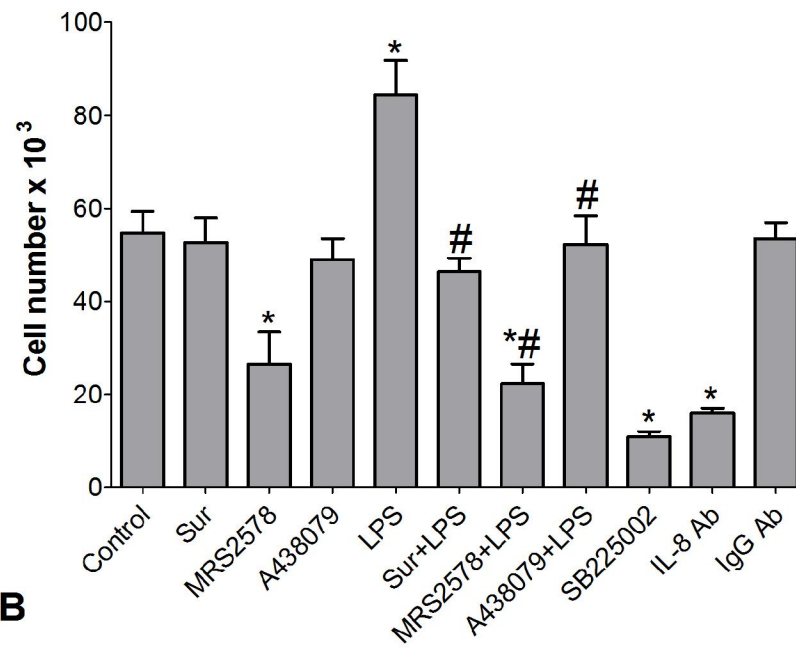


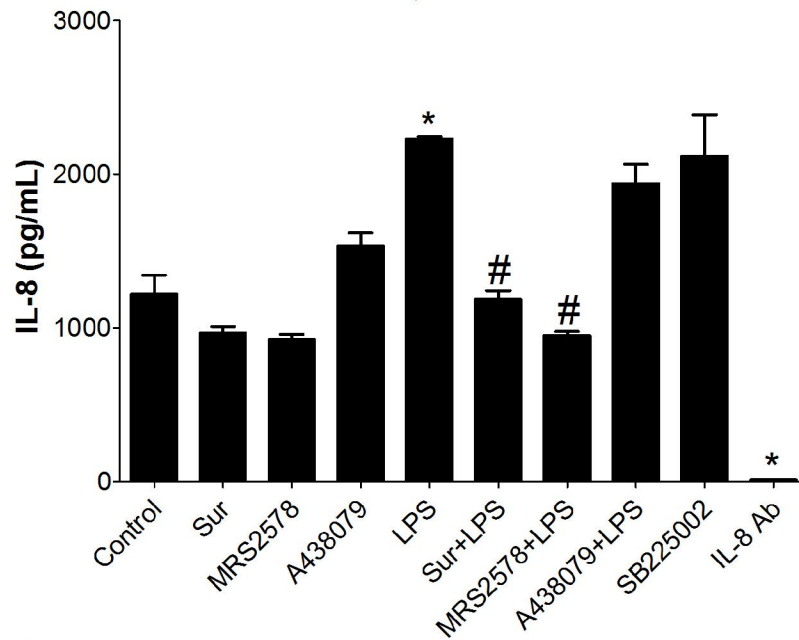
Figure 6



A Figure 7



B



C

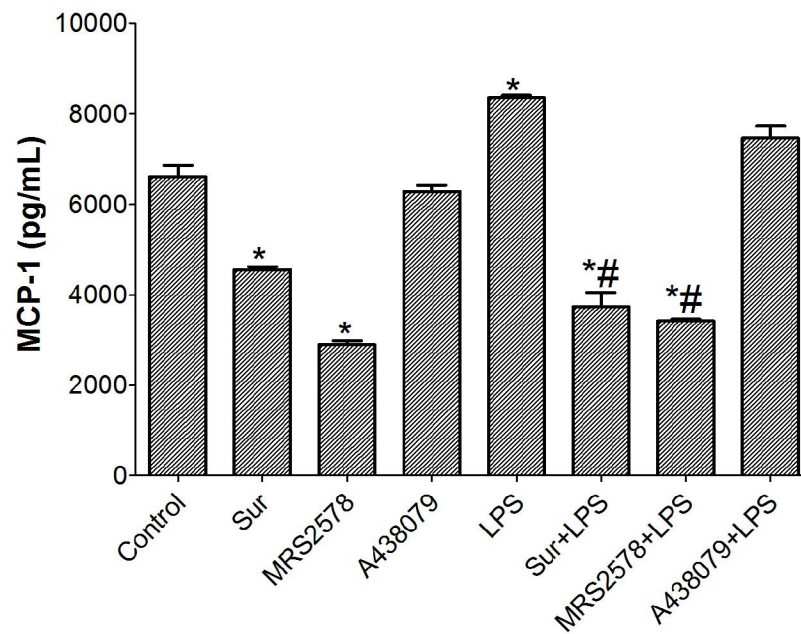


Table 1. Summary of mRNA P2X and P2Y receptor subtypes identified in the U251MG glioma cell line by RT-PCR (+ present; - absent). The PCR data are representative of three different glioma cultures analyzed in independent experiments.

Cell line	P2X ₁	P2X ₂	P2X ₃	P2X ₄	P2X ₅	P2X ₆	P2X ₇	P2Y ₁	P2Y ₂	P2Y ₄	P2Y ₆	P2Y ₁₁	P2Y ₁₂	P2Y ₁₃	P2Y ₁₄
U251MG	-	-	-	+	-	-	+	+	-	+	+	+	+	+	+

3.5 Artigo 5

STUDY OF PLASMA MEMBRANE BOUND NTPDASES AND ECTO-5'- NUCLEOTIDASE/CD73 IN RAT AND HUMAN BRAIN TUMORS

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Abstract

Background: Extracellular nucleotides and nucleosides might influence aspects of cancer biology. In that, ATP and adenosine regulate events related to cell death and proliferation, participate in angiogenesis and regulate tumor immunosuppression. Nucleotide receptor-mediated cell communication in CNS is controlled by ectonucleotidases, such as ectonucleoside triphosphodiphosphohydrolases (E-NTPDases) and ecto-5'-nucleotidase/CD73 (ecto-5'-NT/CD73) that can efficiently hydrolyze ATP to adenosine in the extracellular space.

Methods: The purpose of this study was to characterize the expression profile of the membrane-bound NTPDases1, 2 and 3 and ecto-5'-NT/CD73 in rat and human brain tumor tissues by immunohistochemistry and RT-PCR techniques.

Results: The expression of NTPDase 1 and 2 were not detected; the NTPDase3 was poorly expressed, while the ecto-5'-NT/CD73 was prominently expressed in all tumors analyzed. This study suggests that disruption in the ectonucleotidase expression seems to favor the ATP and the adenosine in the tumor periphery, which could mediate the tumor advance via purinergic receptor activation.

Conclusion: Our results support the notion that purinergic signaling is involved in glioma progression.

Background

Malignant gliomas are the most common type of primary brain tumors in adults. This class of glial tumors is composed of anaplastic astrocytoma (AA), anaplastic oligodendroglioma, and glioblastoma multiforme (GBM). They can also exist as mixed lineage tumors, most commonly as anaplastic oligoastrocytoma [1]. These tumors show a high proliferation rate, variability in tumor histopathology and diffusely infiltrate adjacent brain tissue [1]. Although recent research efforts in cancer therapy, the prognosis of patients with malignant gliomas has remained dismal [2].

Nucleotides represent an important class of extracellular molecules that control signalling pathways crucial for the normal function of the nervous system [3]. Extracellular ATP has been shown to mediate a variety of events related to cell proliferation, differentiation and death [4]. Extracellular nucleotides act via ionotropic receptors (P2X₁₋₇) or G-protein-coupled receptors (P2Y_{1, 2, 4, 6, 11-14}) [5]. Nucleotide receptor-mediated cell communication is controlled by ectonucleotidases, such as ectonucleoside triphosphodiphosphohydrolases (E-NTPDases) and ecto-5'-nucleotidase/CD73 (ecto-5'-NT/CD73). The E-NTPDase family comprises eight members. Four of these, NTPDases 1-3 and 8, hydrolyze extracellular nucleotides. As NTPDases 4–7 are mainly intracellular enzymes and NTPDase8 is not expressed in SNC, they will not be considered here. The NTPDase1–3 are anchored to the cell membrane by two transmembrane domains. These enzymes hydrolyze adenine-based nucleotides, as well as other nucleotides such as UTP, GTP, ITP, CTP and their respective nucleoside diphosphates, leading ultimately to the generation of the corresponding nucleoside monophosphates as final products. Ecto-5'-NT/CD73 catalyses the hydrolysis of

nucleoside monophosphates generated from the NTPDase activity to nucleosides (e.g., AMP→adenosine). The molecular properties, functional roles, and nomenclature of ectonucleotidases have been extensively reviewed in Zimmermann and Robson *et al* [6; 7].

There is increasing evidence that purinergic signaling may contribute to cancer progression [4]. We have previously showed that glioma cell lines have altered extracellular ATP, ADP, and AMP catabolism when compared to astrocytes in culture, exhibiting low rates of extracellular ATP hydrolysis and high rates of extracellular AMP hydrolysis [8]. Extracellular ATP and adenosine induce glioma cell proliferation and, in opposite to neuronal tissue, the majority of glioma cell lines are resistant to cell death induced by cytotoxic ATP concentrations [9]. Further studies showed that the co-injection of apyrase, an ATP scavenger, with gliomas decreased the growth of tumors implanted in rats, indicating that ATP plays an important role in glioma proliferation *in vivo* [10]. Finally, glioma cells exhibit high expression and activity of ecto-5'-NT/CD73 [11] an enzyme that has been reported in a variety of tumors and it is correlated with tumor angiogenesis, invasiveness and poor prognosis [12].

In the present study we have characterized the expression of NTPDase1-3 and ecto-5'-NT/CD73 in glioma implanted in rat brain and in human brain tumor tissues obtained from surgical resection. Similar to findings obtained in glioma cell lines, our results reveal that while the NTPDase members were absent or poorly expressed, the ecto-5'-NT/CD73 expression was positive in all biopsies analyzed. These data sustain the idea that altered ectonucleotidase expression may contribute to glioma progression and point the purinergic signaling as target of therapeutic investigation.

Materials and Methods

Cell culture

The C6 rat glioma cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA), grown in culture flasks and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 1% DMEM, 8.39 mM HEPES (pH 7.4), 23.8 mM NaHCO₃, 0.1% fungizone, 0.5U/ml penicillin/streptomycin (Gibco BRL) and supplemented with 5% (v/v) fetal bovine serum (FBS; Cultilab, Brazil). Cells were kept at a temperature of 37°C, humidity of 95% / 5% CO₂ in air.

Glioma implantation

The glioma implantation was made as previously described (10). Briefly, C6 glioma cells (1x10⁶ cells) were injected in the right striatum of male *Wistar* rats (250-270g, 8 weeks old) anesthetized by intraperitoneal administration of ketamine/xilazine. The negative control group was performed via the same procedure, and received an injection of 3 µL of DMEM/5% FBS in the right striatum. All procedures used in the present study followed the "Principles of Laboratory Animal Care" from National Institutes of Health (NIH) and were approved by the Ethical Committee of the Hospital de Clínicas de Porto Alegre.

Immunohistochemistry Techniques

a) DAB revelation: Frozen sections from rat glioma tissues were processed as follow. Sections of 6 µm were obtained, fixed in 10% phosphate-buffered formalin mixed with cold acetone, and stained using rabbit polyclonal antibodies raised against rat NTPDase1 (rNu-6_LI₄; 1:250), NTPDase2 (BZ3-4F; 1:250), NTPDase3 (rN3-1_LI₅; 1:250) and ecto-5'-

nucleotidase (rNu-9_LI₅; 1:2000). Briefly, sections were incubated overnight at 4°C with primary antibodies, washed in phosphate-buffered saline, and blocked with 7% normal goat serum. Primary antibody binding sites were detected using biotin-labeled goat anti-rabbit secondary antibodies. Endogenous peroxidase activity was previously blocked by incubating with 0.4% hydrogen peroxide in phosphate-buffered saline for 10 min. After several washes with phosphate-buffered saline, the sections were stained with horseradish peroxidase complex (Vector Laboratories, Burlington, Ontario, Canada) as specified by manufacturer's recommendations. Peroxidase activity was revealed using DAB (Sigma, St. Louis, MO) as a substrate. Sections were counterstained with aqueous hematoxylin (Biomedex, Foster City, CA) for 30 s. Negative controls for immunohistochemistry were generated by substituting the primary antibody with pre-immune or non-immune rabbit IgG. The antibodies for ectonucleotidases were homemade prepared (Sévigny's laboratory) and its use and specificity have been extensively described in previous studies [13-17].

b) Immunofluorescence: Frozen cryostat sections (6 µm) from rat glioma tissues were fixed in 10% phosphate-buffered formalin mixed with cold acetone and washed three times for 5 min in TBS. Tissue sections were then incubated in 5% normal goat serum prepared in TBS containing 0.25% Triton X-100 for 30 min at room temperature. These sections were incubated overnight at 4°C with the following primary antibodies: rabbit anti-rat NTPDase1 (rNu-6_LI₄; 1:250), mouse anti-rat mAb nestin (1:200; Chemicon, USA), mouse anti-rat mAb CD31/PECAM-1 (1:30; BD Pharmingen, BD Biosciences, USA) each diluted in 1% normal goat serum prepared in TBS containing 0.25% Triton X-100. They were then incubated with Alexa488-conjugated goat anti-rabbit or

Alexa633-conjugated goat anti-mouse (1:1000) for 60 min at room temperature. Sections were counterstained with DAPI blue (1:10000) for 5 min at room temperature.

Human Subjects

In this study, we evaluated specimens resected from patients with primary brain tumors who underwent operations at Hospital de Clínicas de Porto Alegre (HCPA) from 2008 to 2009. This study was conducted under protocol # 07/588, which was approved by the institutional review board of The Federal University of Rio Grande do Sul, HCPA and National Commission of Ethic in Research (CONEP). Informed written consent was obtained from all patients. Two pathologists provided the histological diagnoses according to the revised World Health Organization classification.

RT-PCR

Human tumor biopsies obtained from surgical resection were conserved in RNALater (Invitrogen) and stored at -80°C until be processed. Total RNA was isolated from the biopsies using the RNeasy kit purification (Qiagen®) following the manufacturer's instructions. One microgram of RNA was reverse transcribed to cDNA in 20 µL of the reaction mixture containing 50 mM oligo(dt)20, 10 mM dNTPs, 40 U/µL RNase Out, 200 U/µL Superscript III reverse transcriptase dissolved in the supplied buffer (all from Invitrogen). The PCR reactions were performed in 25 µL of the reaction mixture containing 1 µL cDNA, 10 pmol of the primer in the supplied PCR mix buffer (Platinum PCR Supermix, Invitrogen). After initial denaturation for 5 min at 95°C, the amplifications were carried out for 35 cycles of denaturation at 94°C for 45 s, annealing

at primer specific temperature for 45 s and extension at 72°C for 45 s. The PCR was ended by 7 min incubation at 72°C. The same program was used for the amplification of the gene of reference, which was GAPDH. Sequences of primers and expected PCR fragment sizes are listed in Table 1. RT-PCR for plasmids containing the sequence of each gene analyzed was run simultaneously as positive controls. Negative controls were performed with templates substituted by DNase/RNase-free distilled water for each PCR reaction. RT-PCR products were separated on 1.5% agarose gels containing a fluorescent nucleic acid stain (SyBR[®] Gold, Molecular Probes, Invitrogen) and photographed under UV illumination.

Results

NTPDase and ecto-5'-NT/CD73 expression in rat glioma tissue

C6 rat glioma cells were injected in the rat brain and following 20 days of implantation, the brain tissues were processed for immunohistochemical analysis. The histological evaluation characterized the implanted rat tumors as glioblastoma multiforme (Fig. 1). Briefly, the tumors consisted of atypical cells with mitotic features, plus degenerative changes with necrosis, the presence of hemorrhagic areas, edema, lymphocyte infiltration and endothelial cell proliferation. Figure 2 shows the immunohistochemical evaluation of NTPDase1-3 and ecto-5'-NT/CD73 expression at protein level in rat glioma tissues. NTPDase1 was expressed by blood vessels from both tumor bulk and tumor periphery and probably by the inflammatory infiltrate mainly constituted by macrophage/microglial cells. The NTPDase2 expression was limited to specific cells localized in the tumor periphery, which could be identified as NTPDase2

positive neural progenitors [18]. The NTPDase3 was weakly expressed in the glioma and in the tissue around the tumor, while ecto-5'-NT/CD73 expression was prominent in the tumor area. In addition, the identity of NTPDase1 positive cells was better evaluated by immunofluorescence experiments. As shown in figures 3 and 4, the NTPDase1 expression co-localized with the endothelial cell marker CD31/PECAM-1, but not with nestin, a glioblastoma multiforme marker. These results indicate that NTPDase1 expression is predominant in the blood vessels, while it is not present in glioma cells.

NTPDase and ecto-5'-NT/CD73 expression in human brain tumors

To determine the distribution of ectonucleotidases in human brain tumor biopsies, the total RNA was extracted from tissue samples and the expression of NTPDase1-3 and ecto-5'-NT/CD73 mRNA was evaluated by RT-PCR. The analysis of 14 samples from grade II, III and IV tumors (Table 2) showed that the NTPDase1 and 2 expression was absent in all tumor biopsies, while the NTPDase3 were expressed in low levels. Furthermore, ecto-5'-NT/CD73 was highly expressed in all samples (Table 3; Fig. 5). These results are in accordance to the pattern of ectonucleotidase expression from rat implanted gliomas (Fig. 2) and they are consistent with the idea that altered ectonucleotidase expression could be involved in brain tumor progression.

Discussion

The existence of purinoceptors (P1 and P2) in the CNS is well established [19] and the activation of these receptors elicits a range of physiological and pathological events that control neurotransmission, cell death and proliferation and immune response. It seems probable that the hydrolysis of nucleotides by ectonucleotidases expressed in CNS and in malignant brain tumors will result in a differential P1 and P2 activation, thereby modifying the final response. Hence, the knowledge of the pattern of ectonucleotidase distribution in brain tumors is essential to comprehension of purinergic receptor function in malignant processes in CNS. This study evaluates for the first time the ectonucleotidase expression in rat and human brain tumors. The expression of NTPDase members were low or absent in the rat glioma and in the human brain tumor biopsies, while the ecto-5'-NT/CD73 was highly expressed. The results suggest that this ectonucleotidase distribution could be a characteristic of brain tumors.

All members of the NTPDase family hydrolyze ATP and ADP to AMP, but they differ in their preferences for these substrates. In the present study, we showed that NTPDase1, which hydrolyzes ATP and ADP with almost equal preference [7; 20] was expressed in blood vessels, but it was absent in rat and human tumor cells. The absence of NTPDase1 expression in tumor cells could be related to poor cancer prognosis [21]. In addition, our findings are consistent with data from previous studies that identify the NTPDase1 as the major ectonucleotidase expressed by endothelial cells [22; 23] and suggest a possible role to this enzyme in tumor blood flow and thrombogenesis.

In contrast to NTPDase1, NTPDase2 has a significantly higher preference for the hydrolysis of ATP over ADP [7; 20]. Staining of NTPDase2 in rat glioma was confined

to some specific cells around the tumor periphery and NTPDase2 mRNA expression was absent in the human tumor biopsies. Although the identity of the NTPDase2 positive cells remains unclear, we suggest that these cells are neural precursors, which are NTPDase2 positive, and could be attracted to glioma bulk and contribute to increase the tumor malignity [24]. NTPDase3 reveals a preference for ATP over ADP as substrate [7; 20]. Weak NTPDase3 expression was found in rat and in human tumor analyzed. Although the NTPDase3 expression has been reported in bladder cancer and in hepatoma cell lines [25; 26] its functional significance in cancer progression remains to be further evaluated. Interestingly, we found prominent expression of ecto-5'-NT/CD73 in the rat glioma sections and in all human brain tumors analyzed. Our results are in agreement with the potential role of ecto-5'-NT/CD73 in control the cell growth and invasiveness in a variety of tumors [27; 28; 29; 30]. Ecto-5'-NT/CD73 is the best-characterized enzymatic source of extracellular adenosine [31]. This nucleoside is involved in events of cell proliferation, angiogenesis and may suppress the anticancer immune response [12]. Furthermore, ecto-5'-NT/CD73 has functional properties in the control of cell growth, maturation, differentiation, cell-cell and cell-matrix interactions and in drug resistance [12; 27; 28].

The pattern of NTPDases and ecto-5'-NT/CD73 expression observed in the rat glioma tissue and in human tumor biopsies, together with previous works showing that: ATP accumulates preferentially in the tumor interstitium [32]; glioma cell lines exhibit altered extracellular ATP metabolism when compared to astrocytes in culture [8] and ATP and adenosine induce glioma proliferation [9] suggest that disruption in the ectonucleotidase expression seems to favor the ATP and the adenosine in the tumor periphery, which may favor immunosuppression, tumor associated macrophages (TAM)

recruitment and angiogenesis. Accordingly, we have previously shown that the co-injection of apyrase/NTPDase1 (low ATP/ADPase ratio) in a C6 rat glioma experimental model markedly reduces glioma growth [10], whereas the results of a recent work indicate that NTPDase2 (high ATP/ADPase ratio) has the opposite effect [33]. Thus, these findings support the hypothesis that purinergic signaling regulate key pathways necessary to control the tumor advance and that an adequate pharmacological manipulation of this system could reduce the glioma malignity.

Conclusion

In conclusion, the main characteristic exhibited by brain tumors is the absence of NTPDase1 and 2 expression and a prominent presence of ecto-5'-NT/CD73. Reasons for this pattern of distribution remain to be better evaluated, but it suggest that these enzymes are strategically placed to influence the activity of P1 and P2 receptors through the generation, or hydrolysis, of agonists such as ATP, ADP, or adenosine.

Competing interest

The authors declare that they have no competing interest.

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Figure legends

Figure 1. Histological features of the implanted gliomas. Sections of implanted rat glioma were stained with haematoxylin and eosin. Histological characteristics that define glioblastoma multiforme, as seen in the implanted gliomas: necrosis (N), microvascular proliferation (V), peripheral pseudopalisading (P), lymphocytic infiltration (LI). Magnification x10, insert x20.

Figure 2. Immunohistochemistry of rat glioma tissue for NTPDase1, 2 and 3 and ecto-5'-NT/CD73. Glioma sections were incubated with ectonucleotidase antibodies, as indicated. The tumor area was limited and it was indicated as glioma; glioma periphery is indicated as GP. Note the presence of NTPDase1 positivity in blood vessels (close head arrows) and inflammatory infiltrate (open head arrows); NTPDase2 positive cells were indicated in the tumor area (open arrows). No staining was seen in negative controls (insert). Magnification x20. Data are representative of the analysis of five animals.

Figure 3. Immunofluorescence staining of rat glioma tissue for CD31/PECAM-1, an endothelial cell marker, and NTPDase1, as indicated. Merged image of CD31/PECAM-1 (red) and NTPDase1 (green). Colocalized staining of CD31/PECAM-1 and NTPDase1 in blood vessels is yellow (open head arrows). Magnification x20. Data are representative of the analysis of five animals.

Figure 4. Immunofluorescence staining of rat glioma tissue for nestin, a glioblastoma multiforme marker, and NTPDase1, as indicated. Merged image of nestin (red) and

NTPDase1 (green). No colocalized staining of nestin and NTPDase1 was observed. Glioma cell nestin positive and blood vessel NTPDase1 positive were indicated (open head arrows). Magnification x20. Data are representative of the analysis of five animals.

Figure 5. Ectonucleotidase expression in human brain tumor biopsies. Human brain tumor samples were resected from patients with primary gliomas and the ectonucleotidase expression was analyzed by RT-PCR. The PCR products were separated on a 1.5% agarose gel. As a control for cDNA synthesis, GAPDH-PCR was performed. Lane identification: (C+) positive control; (1, 2) glioma samples; (C-) Negative control. Representative picture of 2 samples of glioma grade IV. The complete analyzes is shown in the Table 3.

Figure 1

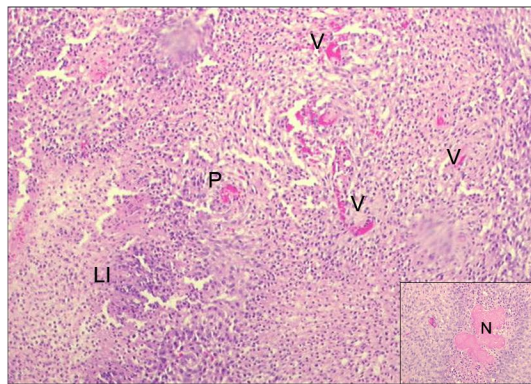


Figure 2

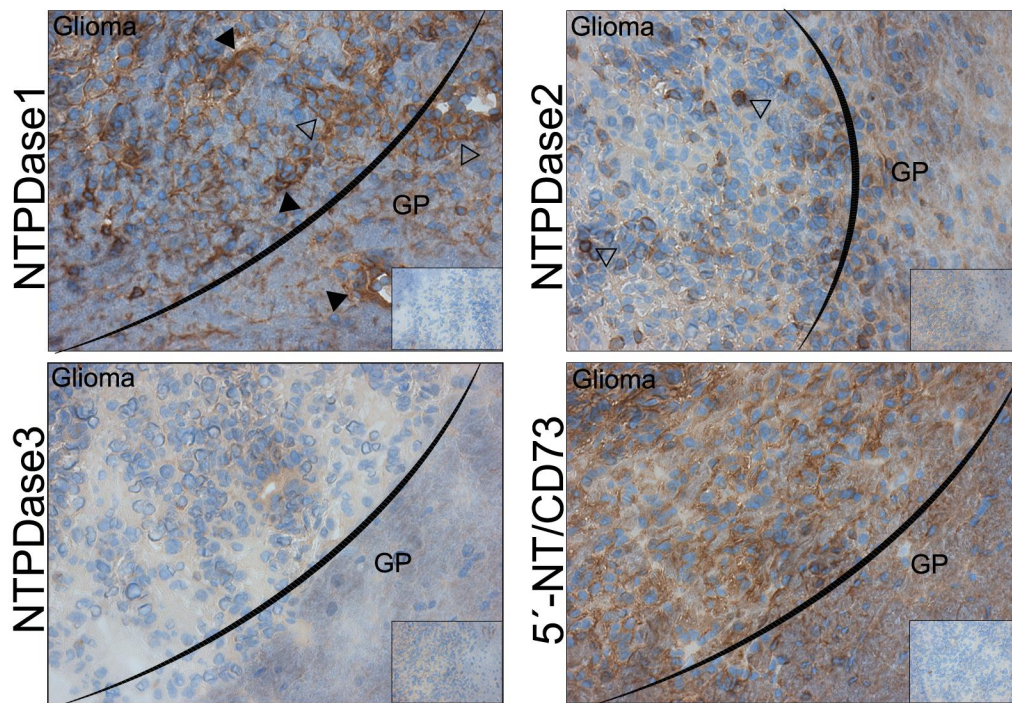


Figure 3

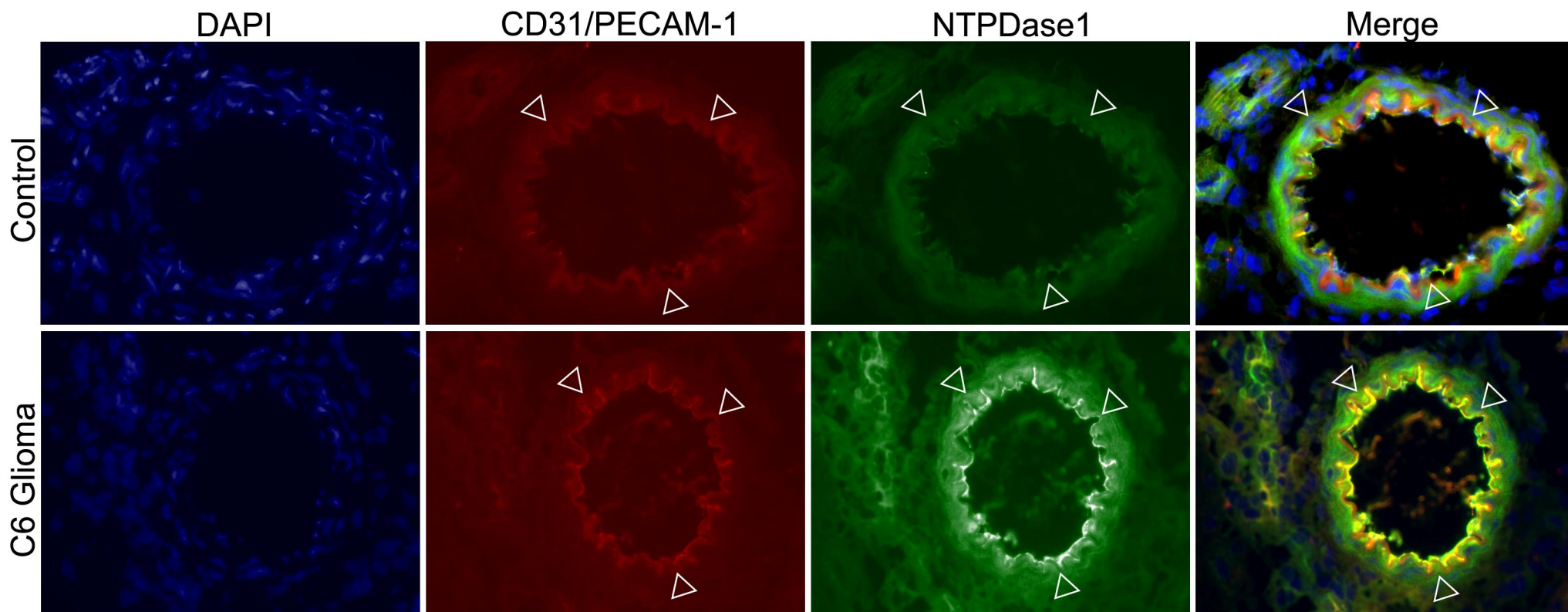


Figure 4

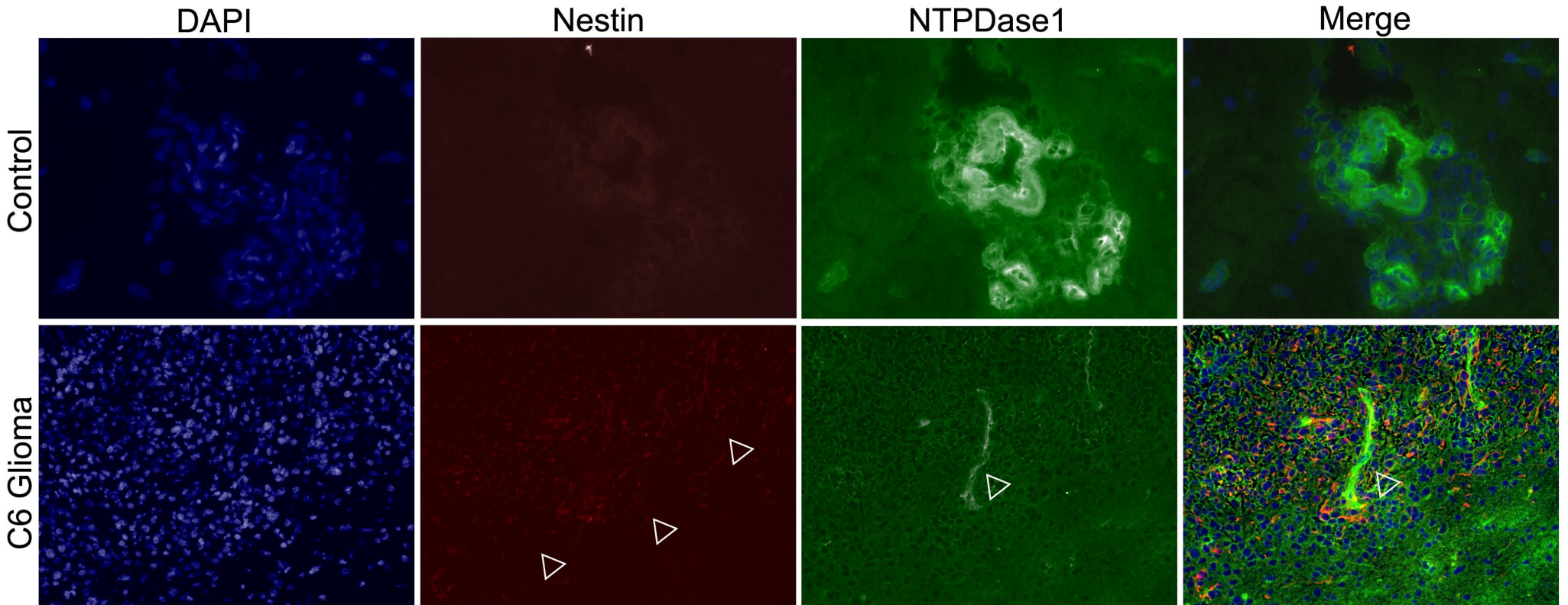


Figure 5

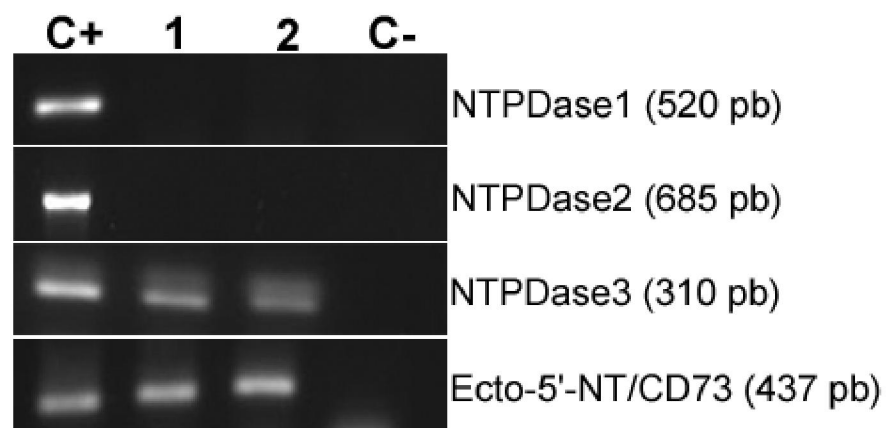


Table 1. Primer sequences, annealing temperatures (Ta) and fragment sizes (F forward, R reverse).

<i>Gene</i>	<i>Primer Sequence</i>	<i>Ta (°C)</i>	<i>Fragment (bp)</i>
NTPD1 F	5'CCACATCCAGAACCCTGTCT3'	60	520
NTPD1 R	5'CAGAACAAAGCATTGCCAGA3'		
NTPD2 F	5'GCTGGGTGGGCCCGGTGGATACG3''	59	685
NTPD2 R	5'ATTGAAGGCCCGGGGACGCTGAC3'		
NTPD3 F	5'TTGACCTCAGGGCTCAGTTT3'	58	310
NTPD3 R	5'TGAGGGGGTTCACTGCTTAC3'		
5'-NT/CD73 F	5'TGGAACCACGTATCCATGTG3'	60	437
5'-NT/CD73 R	5'ATGCTCAAAGGCCTTCTTCA3'		
GAPDH F	5'CAAAGTTGTCATGGATGACC3'	52	195
GAPDH R	5'CCATGGAGAAGGCTGGGG3'		

Table 2. Characteristics of the human brain tumors used in this study.

	<i>Grade I</i>	<i>Grade II</i>	<i>Grade IV</i>
<i>No of Cases</i>	7	3	4
<i>Gender</i>			
Male	2	0	2
Female	5	3	2
<i>Age (years)</i>			
Median (range)	39 (6-69)	30 (29-32)	48 (30-65)
<i>Histological diagnosis</i>			
Pilocytic astrocytoma	2		
Neuroepithelial tumor	1		
Meningioma	3		
Schwanoma	1		
Difuse astrocytoma		1	
Atypical meningioma		1	
Ependymoma		1	
Glioblastoma			4

Table 3. Summary of NTPDase1-3 and ecto-5'-NT/CD73 mRNA expression in the human brain tumors analyzed by RT-PCR (+ present; - absent).

<i>Tumor Grade</i>	<i>No of Cases</i>	<i>NTPDase1</i>	<i>NTPDase2</i>	<i>NTPDase3</i>	<i>Ecto-5'-NT/CD73</i>
I	7	-	-	+	+
II	3	-	-	+	+
IV	4	-	-	+	+

4. DISCUSSÃO

Os gliomas são os tumores primários mais comuns e devastadores do SNC. Apesar de intensos esforços em desenvolver terapias efetivas, esses tumores continuam sendo letais, constituindo um dos grandes desafios da oncologia (Konopka and Bonni, 2003). Além das alterações moleculares e genéticas previamente descritas, tais como a amplificação do receptor para EGF (EGFR) e deleção ou mutação das vias da CDK, p53 e PTEN (Jemal *et al.*, 2006), o nosso grupo de pesquisa tem proposto que alterações da sinalização purinérgica também podem ser um componente da progressão dos gliomas.

De fato, estudos prévios mostram que, ao contrário dos astrócitos, gliomas apresentam baixa expressão e atividade das NTPDases, o que parece favorecer o acúmulo de ATP no meio extracelular (Wink *et al.*, 2003). Além disso, esse nucleotídeo induz estímulo proliferativo nos gliomas, enquanto é citotóxico para as células neuronais normais (Morrone *et al.*, 2005). Com base nesses dados, nós propusemos que a restauração da atividade ATPásica nos gliomas poderia ser uma boa estratégia para abolir a estimulação dos receptores purinérgicos e assim prevenir as ações protumorais mediadas pelo ATP. De acordo com essa idéia, experimentos realizados usando o modelo de implante de tumor *in vivo* mostraram que a co-injeção de apirase/NTPDase1 com gliomas em cérebro de ratos foi suficiente para diminuir o tamanho e a malignidade dos tumores implantados (Morrone *et al.*, 2006). Assim, o objetivo central dessa Tese de Doutorado foi melhor caracterizar a hipótese purinérgica de invasão dos gliomas, avaliando o envolvimento dos receptores purinérgicos e das E-NTPDases, mais especificamente da NTPDase2, na proliferação *in vitro* e *in vivo* dessas células tumorais. Outros parâmetros relacionados com malignidade tumoral, incluindo o processo

inflamatório e a formação de metástases também foram analisados e podem dar uma idéia de como o sistema purinérgico pode estar envolvido nesses eventos.

Uma das formas mais tradicionais de avaliar as características moleculares e a patogênese dos gliomas e de outros tipos de tumores é o uso de linhagens celulares derivadas de tumores malignos (Ponten and Macintyre 1968; Giard *et al.*, 1973). Entretanto, estudos sugerem que as linhagens celulares frequentemente perdem as características genéticas e fenotípicas presentes nos tumores originais devido as repetidas passagens *in vitro* (Lee *et al.*, 2006). Assim, nós nos questionamos o quanto a linhagem de glioma de rato C6, a qual é amplamente utilizada em nossos estudos *in vitro* e *in vivo*, poderia manter as características de tumores primários com relação ao sistema purinérgico (item 3.1). Para elucidar essa questão, nós comparamos algumas diferenças biológicas entre a linhagem de glioma de rato C6 em cultura e um modelo de cultura de tumor de rato C6 *ex vivo*. O modelo de glioma C6 *ex vivo* foi obtido a partir de uma cultura primária de gliomas de ratos previamente implantados via cirurgia estereotáxica com a mesma linhagem celular. Por meio dessa curta exposição ao microambiente *in vivo*, nós tentamos selecionar as células tumorais que pudessem melhor representar os tumores primários de humanos.. Entre os parâmetros avaliados foram incluídos morfologia celular, marcadores de diferenciação e de angiogênese, expressão dos receptores purinérgicos e das NTPDases e citotoxicidade frente ao ATP.

Primeiramente, a análise histológica dos tumores implantados em cérebro de ratos indicou a presença de todas as alterações patológicas características de um glioblastoma multiforme, entre as quais presença de figuras mitóticas, necrose, edema, hemorragia, infiltrado inflamatório e proliferação vascular (item 3.1, Figura 1). Posteriormente, foi

realizada a caracterização das culturas de linhagem de glioma C6 e das culturas de glioma C6 *ex vivo*. Ao contrário da linhagem C6, as culturas de glioma C6 *ex vivo* apresentaram profundas diferenças morfológicas *in vitro*, proliferando até a 5ª passagem como esferas multicelulares, morfologia característica de células tronco. Provavelmente as esferas deram lugar a monocamada celular devido à suplementação do meio de cultura com soro fetal bovino um indutor de diferenciação celular hábil em reduzir a população de células tronco tumorais presentes na cultura (Lee *et al.*, 2006). Com relação aos marcadores de diferenciação celular, ambos modelos de cultura de glioma C6 apresentaram expressão de vimentina e nestina, marcador de células tronco neurais e tumorais, enquanto que a expressão de GFAP, marcador de astrócitos maduros, foi ausente. Similar aos nossos dados, um estudo publicado recentemente mostra que a linhagem de glioma C6 apresenta uma pequena população de células imaturas CD133 e nestina positivas, caracterizadas como células tronco tumorais, as quais são as responsáveis pela propagação do tumor *in vivo* (Zhou *et al.*, 2009).

No mesmo estudo, nós avaliamos o perfil de expressão dos receptores purinérgicos P2X e P2Y nas culturas de linhagem de glioma C6 e de glioma C6 *ex vivo*. Os distintos modelos de cultura apresentaram a expressão de vários receptores em comum, entre eles: P2X₄, P2Y₁, P2Y₂, P2Y₄, P2Y₁₂, P2Y₁₃ e P2Y₁₄, os quais estão envolvidos em processos tumorais. Por exemplo, a expressão de P2X₄ está relacionada com a interação glioma-microglia e a consenquente produção de citocinas angiogênicas (Deininger *et al.*, 2000; Guo *et al.*, 2004), enquanto que os receptores P2Y₁, P2Y₂ e P2Y₁₂ estão envolvidos com proliferação de células tumorais por meio da ativação das vias da PLC e ERK1/ERK2 (Czajkowski *et al.*, 2004; White and Burnstock, 2006). Outra

semelhança encontrada entre as culturas foi a ausência da expressão do receptor P2X₇. Interessantemente, em células normais a ativação sustentada do receptor P2X₇ desencadeia apoptose. Entretanto, em células tumorais esse receptor se encontra ausente ou, quando expresso, apresenta alteração de função, induzindo proliferação e resistência a morte celular induzida por ATP (Di Virgilio *et al.*, 2009). A principal diferença encontrada entre as células C6 em cultura e as culturas dos gliomas implantados, foi a presença do receptor P2Y₆ somente na cultura de glioma C6 *ex vivo*. Esse receptor é ativado por UDP e está relacionado à proliferação celular e resistência a apoptose em células de astrocitoma (Kim *et al.*, 2003). Além disso, esse receptor medeia importantes ações do sistema imune, como recrutamento de leucócitos e produção de citocinas (Warny *et al.*, 2001). Provavelmente, a presença de mediadores inflamatórios que compõem o microambiente dos gliomas modulou positivamente a expressão desse receptor durante a passagem *in vivo* das células C6.

Um componente essencial do controle da ativação dos receptores purinérgicos é a presença de NTPDases e da ecto-5'-NT/CD73, as quais hidrolisam o ATP até adenosina no meio extracelular (Robson *et al.*, 2006). A expressão dessas ectoenzimas foi avaliada nas culturas de linhagem de glioma C6 e de glioma C6 *ex vivo*. Com exceção da NTPDase1, os demais membros estão expressos em ambos modelos de cultura. Um estudo sugere que a ausência de expressão da NTPDase1 está associada com mau prognóstico de pacientes com tumor de pâncreas (Künzli *et al.*, 2007). Além disso, a homologia entre a NTPDase5 e o protooncogene *PCPH* foi descrita (Páez *et al.*, 2001) e, por meio do controle da disponibilidade do ATP intracelular para as reações de fosforilação, essa ectonucleotidase intracelular também está relacionada com a regulação

de vias de proliferação celular como as MAPK (Villar *et al.*, 2009). Apesar de 4 dos 5 membros da família das NTPDases estarem expressos nas culturas de gliomas, as atividades ATP/ADPásicas foram modestas quando comparadas aos astrócitos. Esse resultado sugere duas possibilidades: o mRNA para essas enzimas não estar sendo traduzido para proteína ou, a proteína não estar sendo traduzida corretamente, estando presente na membrana, porém sem atividade enzimática. De acordo com a elevada atividade AMPásica, a expressão da ecto-5'-NT/CD73 foi positiva em ambas culturas de glioma. A presença dessa enzima tem sido relatada em uma variedade de tumores e o aumento de expressão está correlacionado com o aumento de malignidade. Estudos *in vivo* em modelo de tumor de mama mostram que o silenciamento dessa enzima diminuiu drasticamente a malignidade e o poder de invasão dos tumores implantados. Além disso, em nosso laboratório mostramos que a expressão da ecto-5'-NT/CD73 aumenta com a confluência das culturas de células de gliomas, o que é coerente com a sua função de proteína de adesão e migração celular (Bavaresco *et al.*, 2008). Em resumo, o padrão de hidrólise extracelular de nucleotídeos apresentado pelas culturas de linhagem de glioma C6 e de glioma C6 *ex vivo* estão em acordo com dados previamente publicados pelo nosso grupo (Wink *et al.*, 2003). Tais resultados indicam que a inversão do metabolismo extracelular de nucleotídeos quando comparados com astrócitos em cultura parece ser uma característica importante dos gliomas o que pode ter consequências importantes sobre a estimulação dos receptores P2 presentes nessas células.

O último parâmetro analisado no item 3.1 foi o efeito de altas concentrações de ATP (0,1; 0,5 e 5,0 mM) sobre a proliferação das culturas de glioma C6 *ex vivo*. Os efeitos tóxicos do ATP em células neuronais já foram amplamente descritos e estão

relacionados com diversas patologias do SNC, como por exemplo isquemia cerebral, doenças neurodegenerativas e desordens neuroinflamatórias (Burnstock, 2008). A distribuição estratégica das NTPDases no SNC (NTPDase1 em células microgliais; NTPDase2 em astrócitos; NTPDase3 em neurônios) parece estar relacionada com as funções de controle da resposta neuroimune, neuroproteção e neurotransmissão que esses tipos celulares desempenham (Burnstock, 2008). Morrone e colaboradores (2005) previamente mostraram que a linhagem de glioma C6 é resistente a morte celular induzida por ATP extracelular. Similarmente, o ATP também não alterou a proliferação celular de culturas de glioma C6 *ex vivo*, o que pode estar relacionado a ausência da expressão do receptor P2X₇, principal mediador dos efeitos tóxicos do ATP (Di Vigilio *et al.*, 2009).

De uma forma geral, os resultados do item 3.1 mostram que culturas de linhagem de glioma C6 e de glioma C6 *ex vivo*, exibem padrões similares de expressão de receptores P2, metabolismo extracelular de nucleotídeos e proliferação celular frente a elevadas concentrações de ATP. Esses dados sugerem que alterações no sistema de sinalização purinérgica são características presentes não somente em linhagens celulares de glioma, mas também em culturas de glioma *ex vivo*, as quais representam um modelo de cultura mais próximo aos tumores originários dos pacientes.

Tendo em conta os dados obtidos no item 3.1, e dados anteriores do nosso grupo (Morrone *et al.*, 2006) o próximo passo desse trabalho foi avaliar a proliferação dos gliomas *in vitro* e *in vivo*, após restaurar a atividade ecto-ATPásica na linhagem de glioma C6 por meio da superexpressão da NTPDase2, enzima majoritária nos astrócitos (item 3.2). Para tanto, utilizamos a técnica de transfecção utilizando um plasmídeo

constituído pelas sequências da NTPDase2 e da EYFP (proteína fluorescente amarela/verde). Como controle da transfecção, foi utilizado um plasmídeo contendo somente a sequência da EYFP (controle vetor vazio). A transfecção celular foi feita seguindo o protocolo da Lipofectamina[®] e as células C6 transfectadas (C6-EYFP; C6-EYFP/NTPDase2) foram selecionadas com o antibiótico G418. A eficiência da transfecção foi avaliada pela expressão da proteína fluorescente verde e pela habilidade das células C6 em hidrolisar o ATP extracelular. Conforme resultados mostrados na Figura 1 (item 3.2), as células de glioma C6 passaram a apresentar fluorescência, porém somente as células que receberam o plasmídeo contendo a sequência da NTPDase2 passaram a expressar atividade ATPásica e, em menor extensão, ADPásica. A razão de hidrólise ATP/ADP ficou em torno de 12, o que é esperado para essa enzima que previamente foi caracterizada como um ecto-ATPase, devido a sua preferência por ATP como substrato. Em experimentos *in vitro*, a superexpressão da NTPDase2 não alterou a taxa de proliferação e a viabilidade das células C6.

Com o objetivo de avaliar o impacto da superexpressão da NTPDase2 no crescimento dos gliomas *in vivo*, células C6-EYFP (controle) e C6-EYFP/NTPDase2 foram implantadas no *striatum* do cérebro de ratos via cirurgia esterotáxica conforme previamente descrito (Takano *et al.*, 2001). A avaliação do tamanho do tumor e das características de malignidade foi realizada 20 dias após o implante do tumor utilizando lâminas histológicas coradas com HE. Apesar da taxa similar de proliferação celular *in vitro*, a superexpressão da NTPDase2 causou um dramático e inesperado aumento do crescimento dos gliomas *in vivo*. Esse efeito foi acompanhado pelo aumento das características de malignidade dos tumores implantados. Todos os gliomas C6-

EYFP/NTPDase2 apresentaram extensivas áreas de necrose coagulativa, hemorragia intratumoral, infiltrado linfocítico, edema peritumoral e aumento do índice mitótico (Tabela 1, item 3.2). Os resultados da superexpressão da NTPDase2 na proliferação *in vitro* e *in vivo* do glioma C6, sugerem que a interação das purinas com outros sistemas presentes no microambiente *in vivo*, como por exemplo o sistema angiogênico e inflamatório são fundamentais para o aumento tumoral observado.

Em uma etapa subsequente desse trabalho, nós tentamos elucidar como duas ectonucleotidases pertencentes a mesma família de enzimas, apirase/NTPDase1 (Morrone *et al.*, 2006) e a NTPDase2 utilizada nesse trabalho, resultaram em efeitos opostos quando utilizadas como “scavengers” de ATP no modelo de implante de glioma *in vivo*. Apesar de ambas enzimas hidrolisarem nucleosídeos tri- e difosfatos, a preferência pelos substratos é diferenciada (Zimmermann, 2001). Enquanto que a NTPDase1 hidrolisa o ATP e o ADP igualmente bem, a NTPDase2 hidrolisa preferencialmente o ATP, favorecendo o acúmulo de ADP no meio extracelular (Kukulski *et al.*, 2005; Robson *et al.*, 2006). Dados da literatura mostram que o ADP desempenha funções importantes na proliferação celular e em processos inflamatórios no SNC. Por exemplo, ADP está envolvido no desenvolvimento dos olhos (Massé *et al.*, 2007), migração de células endoteliais (Shen and Dicorleto, 2008), proliferação de células progenitoras neurais (Mishra *et al.*, 2006) e ativação plaquetária (Gachet, 2008). Além disso, existem fortes evidências indicando que as plaquetas desempenham funções muito importantes na progressão tumoral, formação de metástases e na regulação da angiogênese (Folkman, 1995; Pinedo *et al.*, 1998; Mohle *et al.*, 2007; Sierko and Wojtukiewicz, 2007). Assim, considerando que as plaquetas expressam receptores P2, os quais são ativados por ADP

(P2Y₁ e P2Y₁₂), nós hipotetizamos que o ADP produzido pela atividade da NTPDase2 superexpressa nos gliomas implantados poderia ativar esses receptores, resultando em recrutamento e ativação das plaquetas para o sítio tumoral. Esse processo, por sua vez, estimularia angiogênese, bem como o recrutamento de outras células inflamatórias (Sierko and Wojtukiewicz, 2007). De acordo com a nossa hipótese, os gliomas C6-EYFP/NTPDase2 exibiram um acúmulo elevado de plaquetas na área tumoral quando comparados aos tumores controle. Esse efeito foi associado com o aumento da expressão de CD31/PECAM-1 e VEGF, marcadores de angiogênese (Pipili-Synetos *et al.*, 1998; Kisucka *et al.*, 2006). É importante observar que o processo angiogênico pode ser estimulado não somente pelo ADP gerado pela atividade da NTPDase2, mas também pelo ADP secretado pelas plaquetas ativadas (Gachet, 2008).

Além disso, também foi observado um aumento de macrófagos/células microgлияis associados ao glioma C6-EYFP/NTPDase2. Nós especulamos que essas células imunes poderiam ser recrutadas para o tumor pelas plaquetas ativadas. Estudos recentes têm mostrado que as plaquetas são componentes essenciais da iniciação da resposta inflamatória (Sprague *et al.*, 2007) e que as interações adesivas entre plaquetas, leucócitos e células tumorais estimulam a inflamação (Chen and Geng, 2006). A presença de macrófagos/células microgлияis nos tumores C6-EYFP/NTPDase2 também poderia contribuir para aumentar a malignidade dos tumores implantados. A presença de células microgлияis tem sido repetidamente indentificada em tumores cerebrais e, apesar da sua função não estar completamente elucidada, estudos sugerem que elas podem promover uma resposta inflamatória protumoral, via secreção de citocinas e fatores de crescimento (Watters *et al.*, 2005). Notavelmente, muitas dessas citocinas, como a IL-6 e a IL-10,

podem ser secretadas pelas células microgлияis em resposta à estimulação dos receptores P2 pelos nucleotídeos extracelulares. Além disso, o ATP e o ADP regulam os processos de quimiotaxia e fagocitose nessas células (Haynes *et al.*, 2006).

Finalmente, de acordo com a idéia que a ativação das plaquetas pelo ADP contribui para a maior malignidade dos gliomas C6-EYFP/NTPDase2, o tratamento dos animais com clopidogrel, um antagonista de receptor P2Y₁₂ que previne a ativação das plaquetas pelo ADP, reduziu o tamanho, as características de malignidade e os processos de angiogênese e de inflamação aos níveis dos tumores controle. Similar aos resultados aqui reportados, muitos estudos mostram que processos hipercoagulativos estão associados à patologia do câncer e defendem o uso de anticoagulantes como terapia adjuvante no tratamento de tumores (Chiodoni *et al.*, 2006; Gerotziafas *et al.*, 2008). A Figura 7 (item 3.2) apresenta de forma esquemática os resultados obtidos no item 3.2.

Considerando os dados do item 3.2 que mostram que o recrutamento de plaquetas e a resposta inflamatória constituem características marcantes do aumento de malignidade dos gliomas superexpressando a NTPDase2 e, ainda, que esses processos estão relacionados com mau prognóstico e formação de metástases (Borsig, 2008; Tsuruo and Fujita, 2008), no item 3.3 nós investigamos os efeitos periféricos da superexpressão da NTPDase2 sobre o sistema inflamatório e sobre metástases em órgãos distantes.

A partir da observação que as células de glioma C6 superexpressando a NTPDase2 passaram a proliferar como esferas multicelulares (Figura 1, item 3.3), sugerindo um aumento das interações adesivas célula-célula, nós avaliamos a função da NTPDase2 como proteína de adesão. De fato, a superexpressão da NTPDase2 aumentou em 2 vezes a adesão das células C6 quando comparado com as células controle. A

possível interação da NTPDase2 com componentes da matriz extracelular (ECM) produzidos pelos gliomas também foram avaliadas (Bellail *et al.*, 2004). Os resultados mostraram que a presença de laminina e de fibronectina promoveram um aumento adicional de 1,5 vezes na adesão das células superexpressando a NTPDase2. Além disso, o tratamento das células C6 com suramina e Evans blue, inibidores da atividade das NTPDases (Munkonda *et al.*, 2007), preveniram o aumento da adesão celular causado pela superexpressão da NTPDase2. Esses dados sugerem que a NTPDase2 *per se* ou como fonte geradora de ADP extracelular pode desempenhar funções como proteína de adesão, interagindo com componentes da ECM. Essas interações terão consequências importantes em processos de implantação do tumor, motilidade e invasão do parênquima cerebral e dos vasos sanguíneos. Além disso, esses resultados estão de acordo com o aumento da malignidade apresentado pelos gliomas C6-NTPDase2 *in vivo* (item 3.2).

Nesse estudo mostramos também que a superexpressão da NTPDase2 em gliomas modula a resposta inflamatória sistêmica e a invasão dos gliomas para os órgãos periféricos (item 3.3). Surpreendentemente, a restauração da NTPDase2 aos gliomas induziu alterações histológicas no tecido pulmonar características de um processo maligno inicial, com presença de hemorragia focal, infiltrado linfocítico e aumento de celularidade em torno dos vasos sanguíneos (Tabela 1, item 3.3). Experimentos de imunohistoquímica demonstraram a presença de células nestina-positivas no tecido pulmonar, indicando a presença de células metastáticas. Dados da literatura mostram que gliomas raramente fazem metástases a partir do SNC (Smith *et al.*, 1969). Apesar dos mecanismos envolvidos nessa característica peculiar apresentada pelos gliomas não serem completamente elucidados, existem muitas especulações a respeito desse assunto.

Alguns estudos sugerem que os gliomas são hábeis em invadir a corrente circulatória, porém são destruídos pela resposta imune, enquanto que outros defendem a idéia de que o microambiente cerebral não favorece a metástase dos gliomas (Hoelzinger *et al.*, 2007).

Assim, baseados nos dados da literatura, nós propomos que a superexpressão da NTPDase2 e a geração de ADP no meio extracelular poderia induzir modificações na interação entre os gliomas e o microambiente cerebral, levando a um aumento nos processos de adesão celular, no recrutamento de células imunes e de plaquetas e na produção de citocinas. De acordo com a nossa hipótese, a superexpressão da NTPDase2 em gliomas promoveu um aumento muito significativo nos níveis séricos das citocinas pró-inflamatórias IL-1 β , TNF- α e IL-6, enquanto que o nível de IL-10, citocina anti-inflamatória, não foi alterado. A relevância dessas citocinas na transição das células tumorais do fenótipo epitelial para o mesenquimal, processo que modula a adesão e a motilidade celular e que constitui um dos mecanismos mais importantes da metástase tumoral tem sido amplamente discutida (Sierko and Wojtukiewicz, 2007; Mantovani *et al.*, 2008; Wu and Zhou, 2009). A IL-1 β afeta o padrão de interação entre tumor e hospedeiro (Apte *et al.*, 2006) e em modelo experimental de tumor de mama, o bloqueio da produção dessa citocina previne a formação de metástases (Voronov *et al.*, 2003). O TNF- α regula positivamente a expressão de selectinas nas células endoteliais, o que promove aumento de adesão entre plaquetas e células tumorais ao endotélio, facilitando a migração celular (Mannel *et al.*, 1994; Stoelcker *et al.*, 1995). Finalmente, a IL-6 acelera a tumorigênese, e a administração dessa citocina durante os períodos iniciais do desenvolvimento tumoral acarreta em aumento da malignidade e multiplicidade do tumor (Grivennikov *et al.*, 2009). Esses dados estão em acordo com a teoria da “metástase

inflamatória” (Mantovani, 2009) e sugere o envolvimento do sistema purinérgico nesse processo.

Novamente, a participação das plaquetas foi evidenciada nos efeitos periféricos promovidos pela superexpressão da NTPDase2. Foi observado alteração no metabolismo extracelular de nucleotídeos e recrutamento de plaquetas para o tecido pulmonar. Plaquetas originárias de ambos grupos de animais com glioma apresentaram aumento da atividade ATPásica, o que pode ser uma estratégia de defesa contra os efeitos citotóxicos do ATP que tem seus níveis aumentados em quadros inflamatórios (Bodin and Burnstock, 1998). Porém, somente as plaquetas dos animais implantados com os gliomas superexpressando a NTPDase2 apresentaram diminuição significativa na hidrólise do ADP. Assim, nós especulamos que um possível aumento de ADP na superfície das plaquetas poderia modular as suas propriedades de agregação e de recrutamento via ativação dos receptores P2Y₁ e P2Y₁₂. De acordo com essa idéia, a superexpressão da NTPDase2 promoveu um aumento na reatividade plaquetária e na presença de aglomerados de plaquetas no tecido pulmonar. Uma importante observação a ser feita é que mesmo o aumento compensatório da hidrólise do AMP até adenosina, uma potente inibidora da agregação plaquetária, não preveniu o aumento de reatividade das plaquetas. Além disso, um aumento da contagem de plaquetas foi observado nesses animais, o que pode ser reflexo do processo inflamatório, conforme reportado para pacientes que sofrem de artrite reumatóide (Yazici *et al.*, 2010). Em conjunto, esses dados sugerem que a superexpressão da NTPDase2 causa mudanças importantes na resposta inflamatória, as quais promovem a formação de um microambiente favorável para o desenvolvimento do glioma e para a formação de metástases. O aumento da reatividade e o recrutamento de

plaquetas para o tecido pulmonar, juntamente com o aumento de citocinas proinflamatórias são coerentes com a idéia que a formação de agregados de células tumorais e plaquetas e a resposta inflamatória facilitam a evasão do sistema imune e a implantação das células tumorais em sítios distantes (Borsig, 2008). A Figura 6 presente no item 3.3 integra essas idéias.

Assim, apesar da restauração da atividade e expressão da NTPDase2 ao glioma C6 ter resultado ações opostas as esperadas inicialmente, aumentando a malignidade dos gliomas implantados e tornando-os hábeis em fazer metástases (itens 3.2 e 3.3), esses resultados confirmam que a sinalização purinérgica está envolvida na progressão dos gliomas. Entretanto, como todos os produtos de degradação do ATP apresentam atividade biológica e podem ativar diferentes receptores purinérgicos, a manipulação desse sistema deve ser feita de forma cuidadosa a fim de não criar um novo desequilíbrio pelo acúmulo de outro agonista, como o ADP. Esses resultados reforçam a importância da sobreposição natural da expressão de diferentes E-NTPDases em células normais. Por exemplo, nos astrócitos a NTPDase2 é a enzima predominantemente expressa, porém as NTPDase1, 3, 5 e 6 também estão presentes. A combinação das atividades hidrolíticas delas gera uma razão de hidrólise ATP/ADP em torno de 3:1, o que provavelmente é a melhor condição para manter o controle da ativação dos receptores P1 e P2 (Wink *et al.*, 2006).

Os resultados obtidos nos itens 3.2 e 3.3 utilizando o modelo de implante de glioma *in vivo* sustentam a idéia de que a resposta imune desempenha função essencial na malignidade dos gliomas e que a sinalização purinérgica exerce a modulação dessa resposta. Sabe-se que durante a carcinogênese as células tumorais adquirem muitas propriedades características de células imunes, como a capacidade de secretar citocinas e

de expressar TLRs, os quais são subsequentemente utilizados para promover o crescimento tumoral (Kelly *et al.*, 2006; Chen *et al.*, 2008). Uma variedade de citocinas podem ser produzidas pelas células tumorais espontaneamente ou em resposta a estimulação dos TLR. Entre elas estão incluídas a IL-8 e a MCP-1, as quais estão envolvidas com o recrutamento de neutrófilos e de monócitos, respectivamente, e com processos de angiogênese e migração celular (de la Iglesia *et al.*, 2008; Maggé *et al.*, 2009).

Assim, a capacidade reduzida dos gliomas em metabolizar os nucleotídeos extracelulares somada aos nucleotídeos liberados pelos astrócitos e pelas células imunes que compõem o microambiente tumoral *in vivo*, podem favorecer uma sinalização purinérgica robusta nos gliomas. Efetivamente, no item 3.4 dessa Tese nós mostramos que os nucleotídeos extracelulares controlam a secreção basal e induzida por TLRs das citocinas IL-8 e MCP-1 em linhagem de glioma humano U251MG via ativação de receptores P2 e que essa modulação estimula a proliferação celular.

Os experimentos mostraram que a linhagem de glioma humano U251MG apresenta liberação basal de ATP, a qual pode ser aumentada pela estimulação das células com LPS, um agonista de TLR4. Esses resultados estão em concordância com um estudo prévio que relatou o acúmulo de ATP no interstício de carcinoma de ovário (Pellegati *et al.*, 2008). Assim, a liberação de ATP em conjunto com a baixa atividade ATP/ADPásica dessas células poderia favorecer a ativação dos receptores P2 expressos pela linhagem de glioma U251MG, entre os quais estão incluídos P2X₄, P2X₇, P2Y₁, P2Y₄, P2Y₆, P2Y₁₁₋₁₄. A administração de apirase, do antagonista inespecífico de receptores P2 (suramina) e do antagonista específico de P2Y₆ (MRS2578), significativamente reduziram tanto a

secreção basal como a estimulada por LPS das citocinas no glioma U251MG. O envolvimento do receptor P2Y₆ na secreção de IL-8 (basal e estimulada por LPS) e de MCP-1 (basal) foi posteriormente confirmado em experimentos de silenciamento desse receptor.

Considerando que o glioma U251MG expressa o receptor P2X₇, o qual está envolvido com regulação da resposta inflamatória e tumorigênese, nós especulamos que altas concentrações de ATP poderiam ter efeito sobre a secreção de IL-8 e de MCP-1 nessas células. Provavelmente, as células do glioma são incapazes de liberar níveis elevados de ATP. Entretanto, no microambiente *in vivo* essas concentrações podem ser alcançadas com a contribuição dos astrócitos e das células imunes recrutadas ao sítio tumoral. Para avaliar essa hipótese, as células U251MG foram tratadas com ATP sozinho ou em combinação com concentrações subótimas de LPS. Os resultados mostraram um efeito sinérgico do ATP e LPS na liberação de IL-8 e MCP-1 pelas células do glioma. O efeito de concentrações milimolares de ATP provavelmente foi mediado pelo receptor P2X₇, primeiramente porque altos níveis de ATP não desensibilizam esse receptor e dois antagonistas específicos de P2X₇ (KN62 e A438079) significativamente inibiram o efeito sinérgico sobre a liberação de IL-8 e de MCP-1.

Finalmente, a função dos TLRs na progressão tumoral foi reforçada (Chen *et al.*, 2008) pois o tratamento com LPS induziu proliferação celular da linhagem U251MG, a qual foi correlacionada com a ativação dos receptores P2 e com a secreção de citocinas. Esses resultados somados ao estudo publicado previamente, mostrando que a coinjeção de apirase diminui a malignidade e a presença de infiltrado inflamatório nos gliomas

(Morrone *et al.*, 2006), reforçam a participação dos receptores P2 no processo inflamatório nos tumores *in vivo*.

Os resultados apresentados no item 3.4 demonstram que dois receptores purinérgicos, P2Y₆ e P2X₇, modulam a secreção das citocinas IL-8 e MCP-1 pelas células de glioma U251MG. O receptor P2Y₆ parece ser instrumental para essas respostas, enquanto que a ativação do receptor P2X₇ é requerida para a potencialização dos seus efeitos. Além disso, a liberação endógena de nucleotídeos pelos gliomas parece estimular de forma autócrina os receptores P2Y₆. Essa observação é consistente com estudos prévios que reportam o envolvimento do receptor P2Y₆ na secreção de IL-8 estimulada por TLR1/2 e TLR4 em monócitos de humanos (Warny *et al.*, 2001; Kukulski *et al.*, 2007; Ben Yebdri *et al.*, 2009). Por outro lado, a ativação dos receptores P2X₇ requer elevadas concentrações de ATP (na faixa mM) que podem ser geradas pelas próprias células tumorais em colaboração com as células adjacentes, como por exemplo células danificadas pela invasão tumoral, pelo processo inflamatório ou mesmo a partir das células imunes recrutadas para a área do tumor. Essa é a primeira demonstração do envolvimento do receptor P2X₇ em produção de citocinas em células tumorais. Esse achado pode representar uma propriedade peculiar do P2X₇ e uma adaptação importante das células tumorais, uma vez que a ativação sustentada desse receptor em células normais leva à morte, enquanto que em gliomas resulta em resposta inflamatória a qual é requerida para a progressão tumoral (Di Virgilio *et al.*, 2009).

De uma forma geral, os resultados mostrados nos itens 3.1 a 3.4 dessa Tese demonstram que o desequilíbrio das concentrações extracelulares de nucleotídeos foi decisivo nos eventos de estímulo à progressão tumoral observados, entre os quais: a

resistência a morte induzida por ATP; o aumento da progressão tumoral *in vivo*, formação de metástases; a modulação da resposta inflamatória, incluindo a ativação/recrutamento de plaquetas e a secreção de citocinas.

Considerando a importância das ectonucleotidases no controle da ativação dos receptores purinérgicos (Robson *et al.*, 2006), no item 3.5 nós comparamos a expressão das NTPDases e da ecto-5'-NT/CD73 em gliomas implantados em cérebro de ratos com biópsias de tumores cerebrais humanos obtidas por ressecção cirúrgica.

Experimentos de avaliação da expressão das NTPDases e da ecto-5'-NT/CD73 por imunistoquímica em gliomas implantados em ratos e por RT-PCR em biópsias de tumores cerebrais revelaram ausência da expressão das NTPDase1 e 2. A NTPDase3 foi pobremente expressa, enquanto que a ecto-5'-NT/CD73 foi expressa abundantemente em todos os tecidos analisados. O padrão de expressão das enzimas avaliadas foi similar entre tumores derivados de rato e de humanos, o que sugere que essas alterações são características de tumores cerebrais e fazem parte do seu potencial de malignidade.

Experimentos de colocalização de expressão entre a NTPDase1 e a proteína CD31/PECAM-1, marcadora de endotélio vascular, e a nestina, uma marcadora de glioblastoma multiforme, também foram realizados nos gliomas implantados em cérebro de ratos. O resultado obtido nesses experimentos identificou os pequenos pontos de imunopositividade para NTPDase1 presentes no tumor com os vasos sanguíneos, uma vez que a marcação da NTPDase1 colocalizou perfeitamente com CD31/PECAM-1. Esse resultado está de acordo com estudos prévios que mostram que a NTPDase1 é predominante expressa no SNC em vasos sanguíneos (Braun *et al.*, 2000).

A expressão da NTPDase2 foi restrita a poucas células presentes na periferia dos gliomas, enquanto que a sua expressão em nível de mRNA foi ausente nos tumores humanos analisados. Apesar da identidade das células NTPDase2-positivas permanecer indefinida, nós sugerimos que essas células podem ser precursores neurais, os quais sabidamente expressam essa enzima (Shukla *et al.*, 2005) e são atraídos para a massa tumoral por fatores secretados pelos gliomas contribuindo para aumentar a malignidade desses tumores (Sanai *et al.*, 2005). Além disso, foi verificada uma baixa expressão da NTPDase3 nos tecidos tumorais analisados. A presença dessa enzima tem sido relatada em diferentes tipos de linhagens celulares tumorais, como por exemplo células de câncer de bexiga e de fígado (Wood *et al.*, 2002; Stella *et al.*, 2009), entretanto estudos adicionais são necessários para determinar a sua significância na progressão tumoral.

Finalmente, foi observada uma imunomarcção proeminente da ecto-5'-NT/CD73 nos gliomas de rato e elevados níveis de mRNA para essa enzima em todas as biópsias de tumores de humanos analisadas. Esses resultados estão em acordo com a potencial função da ecto-5'-NT/CD73 no crescimento e invasividade de diversos tipos de tumores (Turnay *et al.*, 1989; Navarro *et al.*, 1998; Sadej *et al.*, 2006; Wang *et al.*, 2007). A ecto-5'-NT/CD73 é a melhor e mais bem caracterizada fonte enzimática de adenosina extracelular (Zimmermann, 2001). Esse nucleosídeo está envolvido em eventos de proliferação celular, angiogênese e imunossupressão tumoral (Spsychala, 2000). Além disso, a ecto-5'-NT/CD73 apresenta propriedades funcionais no controle do crescimento, da maturação e da diferenciação celular e também nas interações célula-célula, célula-matriz e na resistência a drogas (Spsychala, 2000; Turnay *et al.*, 1989; Navarro *et al.*, 1998).

O padrão de expressão das NTPDases e da ecto-5'-NT/CD73 observado nos gliomas implantados em ratos e em tumores cerebrais de humanos em conjunto com dados prévios do nosso grupo (Wink *et al.*, 2003; Morrone *et al.*, 2005; Morrone *et al.*, 2006), fortemente sugerem que alterações na expressão das ectonucleotidases favorecem o acúmulo de ATP e de adenosina no interstício tumoral. Muitas funções protumorais têm sido atribuídas ao ATP e à adenosina extracelulares, incluindo imunossupressão, recrutamento de células imunes, angiogênese e proliferação celular (Inoue, 2006; Feoktistov *et al.*, 2009; Fishman *et al.*, 2009; Neary and Zimmermann, 2009). Além disso, nós especulamos que alterações gênicas como superexpressão de genes ligados à proliferação celular e deleção de genes supressores tumorais também podem afetar o padrão de expressão das ectonucleotidases. Por exemplo, os genes da NTPDase1 e 2 estão localizados na mesma região cromossomal dos genes supressores tumorais PTEN e DAPK1 (Koul, 2008; Cohen and Kimchi, 2001). Enquanto que a NTPDase3 e a ecto-5'-NT/CD73 estão localizadas em regiões próximas as que codificam genes envolvidos com proliferação e sobrevivência celular (Dallol *et al.*, 2002; Huanq *et al.*, 2005; Gozgit *et al.*, 2008; Park *et al.*, 2009). Assim, alterações na expressão das NTPDases e da ecto-5'-NT/CD73 podem acontecer paralelamente a genes marcadores tumorais. A Tabela 2 faz um apanhado dessas informações.

Em conclusão, o principal achado do item 3.5 é que biópsias de tumores cerebrais humanos seguem o mesmo padrão de expressão das linhagens celulares de gliomas e dos gliomas implantados em animais. Esse resultado, além de validar os nossos modelos *in vitro* e *in vivo* de gliomas, também reforça a hipótese purinérgica de progressão desses tumores. Assim, a manipulação farmacológica dos receptores purinérgicos ou das

enzimas que compõem esse sistema constitui uma boa estratégia para o combate dos tumores cerebrais humanos.

Tabela 2. Localização cromossomal dos genes que codificam a expressão das NTPDase1-3 e da ecto-5'-NT/CD73 e a possível correlação com a expressão de genes envolvidos com o câncer.

Nome da Proteína	Nome do Gene	Número de Identificação	Localização Cromossomal	Função
NTPDase1	<i>ENTPD1</i>	ID 953	10q24	
PTEN (phosphatase and tensin homolog)	<i>PTEN</i>	ID 5728	10q23.3	Supressor tumoral (Koul, 2008)
NTPDase2	<i>ENTPD2</i>	ID 954	9q34	
DAPK1 (death-associated protein kinase 1)	<i>DAPK1</i>	ID 1612	9q34.1	Supressor tumoral (Cohen and Kimchi, 2001)
NTPDase3	<i>ENTPD3</i>	ID 956	3p21.3	
ROBO1 (roundabout, axon guidance receptor, homolog 1)	<i>ROBO1</i>	ID 6091	3p12	Migração celular (Dallol <i>et al.</i> , 2002)
Ecto-5'-NT/CD73	<i>NT5E</i>	ID 4907	6q14-q21	
HDAC-2 (histone deacetylase 2)	<i>HDAC2</i>	ID 3066	6q21	Sobrevivência e proliferação celular (Huang <i>et al.</i> , 2005)
PIM-1 (pim-1 oncogene)	<i>PIMI</i>	ID 5292	6q21.2	Sobrevivência e proliferação celular (Gozgit <i>et al.</i> , 2008)
SOD-2 (superoxide dismutase 2, mitochondrial)	<i>SOD2</i>	ID 6648	6q25.3	Marcador prognóstico para o câncer (Park <i>et al.</i> , 2009)

5. CONCLUSÕES

5.1 Gerais

- ✓ O sistema purinérgico está envolvido com a progressão dos gliomas.
- ✓ Alterações no padrão de expressão das ectonucleotidases e dos receptores purinérgicos parecem ser uma característica de malignidade desses tumores.
- ✓ O desequilíbrio dos níveis extracelulares de nucleotídeos orquestra múltiplos eventos relacionados ao aumento da malignidade tumoral, principalmente via modulação do sistema inflamatório.

5.2 Específicas

- ✓ A linhagem de glioma C6 e o modelo de glioma C6 *ex vivo* exibiram padrões similares de marcadores purinérgicos, sugerindo que alterações nesse sistema são uma característica presente não somente em linhagens celulares de glioma, mas também em culturas de glioma *ex vivo*, as quais representam um modelo mais próximo aos tumores primários.
- ✓ A similaridade de expressão de marcadores de diferenciação celular e de angiogênese entre a linhagem de glioma C6 e o modelo de glioma C6 *ex vivo* valida o uso da linhagem C6 como um bom modelo de glioma em nossos estudos;
- ✓ A superexpressão da NTPDase2 nos gliomas não alterou a proliferação da linhagem C6 *in vitro*, porém aumentou dramaticamente a proliferação dos gliomas *in vivo*.
- ✓ O aumento da malignidade dos tumores *in vivo* está relacionada ao possível acúmulo dos níveis de ADP extracelular e consequente recrutamento de plaquetas, aumento de angiogênese e da resposta inflamatória.
- ✓ A superexpressão da NTPDase2 nos gliomas aumentou o potencial de adesão célula-célula e célula-ECM em glioma C6, o que pode contribuir para a malignidade desses tumores.

- ✓ A superexpressão da NTPDase2 no glioma C6 promoveu o surgimento de metástase no tecido pulmonar, a qual foi acompanhada de aumento da resposta inflamatória e da reatividade das plaquetas.
- ✓ Os receptores purinérgicos, mais especificamente P2Y₆ e P2X₇, modulam a secreção espontânea e induzida por TLR das citocinas IL-8 e MCP-1 em linhagem de glioma humano U251MG.
- ✓ O ATP e LPS, agonista de TLR4, promoveram efeito sinérgico sobre a secreção de IL-8 e de MCP-1.
- ✓ O tratamento com LPS induziu a proliferação das células do glioma U251MG, o qual foi dependente da secreção de citocinas e da ativação de receptores purinérgicos P2.
- ✓ A análise de gliomas implantados em cérebro de ratos e de tumores cerebrais de humanos revelou ausência de expressão das NTPDase1 e 2, baixa expressão da NTPDase3 e elevados níveis de expressão da ecto-5'-NT/CD73.
- ✓ Os dados do padrão de expressão das ectonucleotidases em tumores cerebrais humanos sugerem que alterações no sistema de sinalização purinérgica constituem uma característica de malignidade nesses tumores e podem ser alvo de tratamento.

6. PERSPECTIVAS

Como continuação dessa Tese, pretende-se focalizar os seguintes objetivos:

- Estudo do impacto da superexpressão da NTPDase2 sobre o crescimento dos gliomas *in vivo* sobre os momentos iniciais da implantação do tumor (3-5 dias pós cirurgia);
- Avaliação do envolvimento da adenosina e dos receptores P1 na malignidade dos gliomas superexpressando a NTPDase2;
- Determinação do potencial do meio condicionado de cultura de gliomas em induzir a reatividade das plaquetas, tentando elucidar os possíveis receptores purinérgicos envolvidos;
- Estudo do envolvimento do sistema purinérgico na produção das citocinas IL-8 e MCP-1 em modelo de glioma *in vivo*.
- Avaliação da participação do sistema purinérgico em eventos de imunossupressão verificada em diversos tipos tumorais, incluindo os gliomas;
- Determinação do envolvimento do sistema purinérgico na modulação da função dos macrófagos e dos linfócitos, componentes importantes da malignidade tumoral;
- Quantificação da expressão das E-NTPDases em biópsias de tumores cerebrais humanos por PCR em tempo real, tentando correlacionar a expressão das enzimas com o prognóstico dos pacientes;
- Estudo da expressão dos receptores purinérgicos P1 e P2 em biópsias de tumores cerebrais humanos;

- Realização de ensaios de atividade de hidrólise de nucleotídeos *in situ* em biópsias de tumores cerebrais humanos.

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8. ANEXOS

8.1 OUTROS ARTIGOS CIENTÍFICOS REALIZADOS EM CO-AUTORIA DURANTE O PERÍODO DO DOUTORADO

1. Zanotto-Filho A, **Braganhhol E**, Schroder R, Pasquali M, Battastini AMO and Moreira JC. Targeting NFkappaB as strategy to induce glioma cells death. *Manuscrito em preparação.*
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8.2 INSTRUÇÕES DAS REVISTAS AOS AUTORES PARA SUBMISSÃO DE ARTIGO CIENTÍFICO

8.2.1 Artigo 3: Cancer Science

Cancer Science

[The Official Journal of the Japanese Cancer Association](#)

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Yusuke Nakamura

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TopAuthor Guidelines

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- New subject categories (effective from 1 November 2009)
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2 Okumura H, Ishii H, Pichiorri F et al. Fragile gene product, Fhit, in oxidative and replicative stress responses. *Cancer Sci* 2009 doi: 10.1111/j.1349-7006.2009.01168.x

Benz PJ, Soll J, Bölter B. Protein transport in organelles: The composition, function and regulation of the Tic complex in chloroplast protein import. *FEBS Journal*, 2009. doi: 10.1111/j.1742-4658.2009.06874.x

Book

3 Ringsven MK, Bond D. *Gerontology and Leadership Skills for Nurses*, 2nd edn. Albany, NY: Delmar Publishers, 1996.

Chapter in a Book

4 Phillips SJ, Whisnant JP. Hypertension and stroke. In: Laragh JH, Brenner BM, eds. *Hypertension: Pathophysiology, Diagnosis, and Management*, 2nd edn. New York: Raven Press, 1995; 465-78.

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5 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* (Serial online) 1995 Jan-Mar; 1(1): (24 screens). [Cited 5 Jun 1996.] Available from URL: <http://www.cdc.gov/ncidod/eid/index.htm>

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8.2.2 Artigo 4 : European Journal of Immunology

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Stadler, M. B., Arnold, D., Frieden, S., Luginbühl, S. and Stadler, B. M., Single nucleotide polymorphisms as a prerequisite for autoantigens. *Eur. J. Immunol.* 2005 DOI 10.1002/eji.200425481.

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Revised October 2009

Standard Abbreviations

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Å	angstrom	DMSO	dimethylsulfoxide	HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
aa	amino acid	DNA	deoxyribonucleic acid	HGF	hepatocyte growth factor
Ab	antibody	DNase	deoxyribonuclease	HIV	human immunodeficiency virus
Ag	antigen	DNP	dinitrophenyl	HLA	human histocompatibility leukocyte Ag
AIDS	acquired immunodeficiency syndrome	dNTP	2'-deoxynucleoside 5'-triphosphate (dATP, dCTP, dGTP, dTTP)	HMG	3-hydroxy-3-methyl-glutaryl
ANOVA	analysis of variance			HPLC	high performance liquid chromatography
AP-1	activator protein 1	ds	double-stranded	HRP	horseradish peroxidase
APC	antigen-presenting cell	DTT	dithiothreitol	HSC	hematopoietic stem cell
AZT	3'-azido-3-deoxythymidine	E:T ratio	effector to target ratio	HSP	heat shock protein
		EAE	experimental autoimmune encephalomyelitis	HSV	herpes simplex virus
BALF	bronchoalveolar lavage fluid	EBV	Epstein-Barr virus	HTLV	human T lymphocyte virus
BALT	bronchus-associated lymphoid tissue	EC50	50% effective concentration	HUVEC	human umbilical vein endothelial cells
BCG	Bacillus Calmette-Guérin	ECL	enhanced chemiluminescence		
BCR	B cell receptor	ECM	extracellular matrix		
β2m	β2 microglobulin	ED50	50% effective dose	i.m.	intramuscular(ly)
bFGF	basic fibroblast growth factor	EDTA	ethylenediamine tetraacetic acid	i.n.	intranasal(ly)
β-gal	β-galactosidase	EGF	epidermal growth factor	i.p.	intraperitoneal(ly)
BM	bone marrow	EGTA	ethylene glycol-bis(b-aminoethyl ester)-N,N',N'-tetraacetic acid	i.v.	intravenous(ly)
bp	base pair			IC50	inhibitory concentration of 50%
Bq	Becquerel	ELISA	enzyme-linked immunosorbent assay	ICAM	intercellular adhesion molecule
BrdU	5-bromo-2'-deoxyuridine	ELISPOT	enzyme-linked immunospot	ICOS	inducible costimulator
BSA	bovine serum albumin	EM	electron microscopy	Id	idiotype
BSE	bovine spongiform encephalopathy	EMSA	electrophoretic mobility shift assay	ID50	50% infective or inhibiting dose
		ER	endoplasmic reticulum	IDDM	insulin-dependent diabetes mellitus
C region	constant region of Ig	ERK	extracellular signal-regulated kinase	IDO	indoleamine 2,3-dioxygenase
C/EBP	CCAAT/enhancer-binding protein	ES	embryonic stem (cell)	IFN	interferon
CC	CC chemokine	Fab	Ag-binding fragment	Ig	immunoglobulin
cDNA	complementary deoxyribonucleic acid (cDNA)	FACS	fluorescence-activated cell sorter	IGF	insulin-like growth factor
CDR	complementarity determining region	Fc	crystallizable fragment (of immunoglobulin)	IκB	inhibitory NF-κB
CFA	complete Freund's adjuvant	FC(B)S	fetal calf (bovine) serum	IL	interleukin
CFSE	carboxyfluorescein diacetate succinimidyl ester	FGF	fibroblast growth factor	IMDM	Iscove's modified Dulbecco's medium
CFU	colony-forming unit	FISH	fluorescent <i>in situ</i> hybridization	IMEM	Iscove's minimal essential medium
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate	FITC	fluorescein isothiocyanate	iNOS	inducible nitric oxide synthase
ChIP	Chromatin immunoprecipitation	FLICE	Fas-associated death domain-like IL-1β-converting enzyme	IP	immunoprecipitation
CHO	Chinese hamster ovary	FLIP	FLICE inhibitory protein	ITAM	immunoreceptor tyrosine-based activation motif
Ci	curie	FMLP	formyl-methionyl-leucyl-phenylalanine	ITIM	immunoreceptor tyrosine-based inhibitory motif
CIITA	class II transactivator	FOXP3/ Foxp3	forkhead box p3 (human/mouse)	IU	international unit
CLIP	class II-associated invariant-chain peptide	Fura 2-AM	fura 2-acetoxymethyl ester	J region	joining region of Ig or TCR
cM	centimorgan	g	gram	JAK	Janus kinase
CMV	cytomegalovirus	g	unit of gravity	JNK	c-Jun NH ₂ -terminal kinase
cNMP	cyclic NMP (cAMP, cCMP, cGMP, cIMP, cUMP)	GABA	γ-aminobutyric acid	k	kilo
CNS	central nervous system	GALT	gut-associated lymphoid tissue	K _a	association constant
CoA	coenzyme A	GAPDH	glyceraldehyde 3-phosphate dehydrogenase	K _D	affinity constant
Con A	concanavalin A	GC	germinal center	KO	knockout
COX	cyclooxygenase	G-CSF	granulocyte CSF	L	ligand
CpG	cytosine guanine dinucleotide	GFP	green fluorescent protein	L chain	light chain of Ig
cpm	counts per minute	GM-CFU	granulocyte-macrophage colony forming unit	LAK	lymphokine-activated killer
CREB	cAMP response element binding protein	GM-CSF	granulocyte-macrophage CSF	LD50	50% lethal dose
cRNA	complementary RNA	gp	glycoprotein	LN	lymph node
CSF	colony-stimulating factor	GPI	glycosylphosphatidylinositol	LPS	lipopolysaccharide
CTL	cytotoxic T lymphocyte	GST	glutathione S-transferase	LTR	long terminal repeat
CTLA	cytolytic T lymphocyte-associate Ag	GVH	Graft-vs.host (reaction)	LUC	luciferase
CXC	CXC chemokine	h	hour	m	meter
		H chain	heavy chain of Ig	M	molar
3D	three-dimensional	H&E	hematoxylin and eosin	mAb	monoclonal Ab
D region	diversity region of Ig or TCR	HA	hemagglutinin	MACS	magnetic-activated cell sorting
Da	dalton(s)	HBSS	Hanks' balanced salt solution	MALT	mucosa-associated lymphoid tissue
DAPI	4',6-diamidino-2-phenylindole dihydrochloride	HCV	hepatitis C virus	MAPK	mitogen-activated protein kinase
DC	dendritic cell			MCP-1	monocyte chemoattractant protein-1
Δ (delta)	change			M-CSF	macrophage CSF
DMEM	Dulbecco's modified Eagle's medium			2-ME	2-mercaptoethanol
				MEK	mitogen-activated protein kinase kinase

MEM	minimum essential medium	PAMP	pathogen-associated molecular pattern	SHIP	Src homology 2 domain-containing inositol 5' phosphatase
MFI	mean fluorescence intensity	PBL	peripheral blood lymphocyte	SHP	Src homology 2 domain-containing tyrosine phosphatase
mg	milligram	PBMC	peripheral blood mononuclear cell	siRNA	small interfering RNA
µg	microgram	PBS	phosphate-buffered saline	SIV	simian immunodeficiency virus
MHC	major histocompatibility complex	PCR	polymerase chain reaction	SOCS	suppressor of cytokine signalling
MIF	macrophage migration inhibitory factor	PDGF	platelet derived growth factor	SRBC	sheep red blood cell
min	minute	PE	phycoerythrin	ss	single-stranded
MIP	macrophage-inflammatory protein	PECAM-1	platelet endothelial cell adhesion molecule-1	STAT	signal transducer and activator of transcription
mL	milliliter	PerCP	peridinin chlorophyll protein	SV-40	simian virus 40
µL	microliter	PFU	plaque-forming unit		
MLC	mixed lymphocyte culture	PHA	phytohemagglutinin	$t_{1/2}$	half-life
MLN	mesenteric lymph node	PI	propidium iodide	TAP	transporter associated with Ag processing
MLR	mixed leukocyte reaction	PI3K	phosphatidylinositol 3-kinase	Tat	terminal deoxynucleotidyl-transferase
MMP	matrix metalloproteinase	PIPES	piperazine-N,N'-bis(2-ethane sulfonic acid)	TBS	Tris-buffered saline
MOI	multiplicity of infection	PKC	protein kinase C	TBST	TBS with Tween 20
MOPS	4-morpholinepropanesulfonic acid	PMA	phorbol myristate acetate	TCR	T cell receptor for antigen
MΦ	macrophage	PMN	polymorphonuclear leukocyte	TdT	terminal deoxynucleotidyl-transferase
M_r	relative molecular mass	PMSF	phenylmethylsulfonyl fluoride	Tg	transgene/transgenic
mRNA	messenger RNA	polyI:C	polyinosinic-polycytidylic acid	TGF	transforming growth factor
MS	multiple sclerosis	PRR	pattern-recognition receptor	Th	T helper (cell)
MyD88	myeloid differentiating factor 88			TLR	Toll-like receptor
n	number in study or group	r	recombinant	TNF	tumor necrosis factor
NAD	nicotinamide adenine dinucleotide	R	receptor	TNP	trinitrophenyl
NADH	reduced NAD	RACE	rapid amplification of cDNA end	TRAIL	TNF-related apoptosis-inducing ligand
NADP	NAD phosphate	RAG	recombination-activating gene	Treg	regulatory T cell
NADPH	reduced NAD phosphate	RANTES	regulated upon activation normal T-cell expressed and secreted	Tris	tris(hydroxymethyl)aminomethane
NBT	nitroblue tetrazolium	RBC	red blood cell	tRNA	transfer RNA
NDP	nucleoside 5'-diphosphate (ADP, CTP, GDP, IDP, UDP)	RFLP	restriction fragment length polymorphism	TUNEL	Tdt-mediated dUTP nick end labeling
NF	nuclear factor	RNA	ribonucleic acid	U	unit
NFAT	nuclear factor of activated T cells	RNase	ribonuclease	UV	ultraviolet
NF-κB	nuclear factor κB	rpm	revolutions per minute	V region	variable region of Ig
NK cell	natural killer cell	rRNA	ribosomal RNA	V(D)J	variable (diversity) joining
NKT cell	natural killer T cell	ROS	reactive oxygen species	v/v	volume to volume ratio (%)
NMP	nucleoside 5'-monophosphate (AMP, CMP, GMP, IMP, UMP)	RSV	respiratory syncytial virus	VCAM	vascular cell adhesion molecule
NO	nitric oxide	RT	reverse transcription	wk	week
NOD	nonobese diabetic	RT-PCR	reverse transcriptase polymerase chain reaction	WT	wild-type
NP-40	Nonidet P-40	s	seconds	XID	X-linked immunodeficiency
nt	nucleotide	S	Svedberg unit of sedimentation coefficient	Zap70	ζ-associated protein 70
NTP	nucleoside 5'-triphosphate (ATP, CTP, GTP, ITP, UTP)	s.c.	subcutaneous(ly)		
NTPase	nucleoside 5'-triphosphatase (ATPase, CTPase, GTPase, ITPase, UTPase)	SCF	stem cell factor		
OD	optical density	SCID	severe combined immunodeficiency		
OVA	ovalbumin	SD	standard deviation		
p	probability	SDS	sodium dodecyl sulfate		
PAGE	polyacrylamide gel electrophoresis	SE	standard error		
		SEM	standard error of the mean		

8.2.3 Artigo 5: BMC Cancer

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In press article

3. Kharitonov SA, Barnes PJ: **Clinical aspects of exhaled nitric oxide.** *Eur Respir J*, in press.

Published abstract

4. Zvaifler NJ, Burger JA, Marinova-Mutafchieva L, Taylor P, Maini RN: **Mesenchymal cells, stromal derived factor-1 and rheumatoid arthritis [abstract].** *Arthritis Rheum* 1999, **42**:s250.

Article within conference proceedings

5. Jones X: **Zeolites and synthetic mechanisms.** In *Proceedings of the First National Conference on Porous Sieves: 27-30 June 1996; Baltimore.* Edited by Smith Y. Stoneham: Butterworth-Heinemann; 1996:16-27.

Book chapter, or article within a book

6. Schnepf E: **From prey via endosymbiont to plastids: comparative studies in dinoflagellates.** In *Origins of Plastids. Volume 2.* 2nd edition. Edited by Lewin RA. New York: Chapman and Hall; 1993:53-76.

Whole issue of journal

7. Ponder B, Johnston S, Chodosh L (Eds): **Innovative oncology.** In *Breast Cancer Res* 1998, **10**:1-72.

Whole conference proceedings

8. Smith Y (Ed): *Proceedings of the First National Conference on Porous Sieves: 27-30 June 1996; Baltimore*. Stoneham: Butterworth-Heinemann; 1996.

Complete book

9. Margulis L: *Origin of Eukaryotic Cells*. New Haven: Yale University Press; 1970.

Monograph or book in a series

10. Hunninghake GW, Gadek JE: **The alveolar macrophage**. In *Cultured Human Cells and Tissues*. Edited by Harris TJR. New York: Academic Press; 1995:54-56. [Stoner G (Series Editor): *Methods and Perspectives in Cell Biology*, vol 1.]

Book with institutional author

11. Advisory Committee on Genetic Modification: *Annual Report*. London; 1999.

PhD thesis

12. Kohavi R: **Wrappers for performance enhancement and oblivious decision graphs**. *PhD thesis*. Stanford University, Computer Science Department; 1995.

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