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Receptor do peptídeo liberador de gastrina (GRPR) em gliomas: expressão, influência na proliferação celular e mecanismos de sinalização

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Do Ponto de Luz da mente de Deus
Flua Luz às mentes dos homens.
(trecho da Grande Invocação)

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ABREVIATURAS

ADAM-10	Domínio 10 da metalopeptidase (do inglês, metallopeptidase domain 10)
AP-1	Ativador de proteína 1 (do inglês, activator protein-1)
AR	Receptor andrógeno (do inglês, androgen receptor)
ATF-1	Fator ativador de transcrição 1 (do inglês, activating transcription factor 1)
BALB/3T3	Linhagem celular derivada de fibroblasto embrionário de camundongo
BB	Bombesina (do inglês, bombesin)
BB1	Receptor de bombesina 1
BB2	Receptor de bombesina 2
BB4	Receptor de bombesina 4
bFGF	Fator de crescimento de fibroblasto básico (do inglês, basic fibroblast growth factor)
BLP	Peptídeo tipo bombesina (do ingles, bombesin-like peptide)
BRS-3	Receptor de bombesina 3
Caco-2	Linhagem originária de adenocarcinoma intestinal humano
CaMK	Proteína dependente de cálcio calmodulina cinase (do inglês, calcium/calmodulin-dependent protein kinase)
cAMP	Adenosina monofosfato ciclico (do inglês, cyclic adenosine monophosphate)
CID-O	Classificação Internacional de Doenças para Oncologia
CNS	Sistema nervosa central (do inglês, Central nervous system)
COS-7	Linhagem celular de fibroblasto de rim de macaco (do inglês, monkey kidney fibroblast cell line, contain SV-40 viral)
COX-2	Ciclooxigenase 2 (do inglês, Ciclooxygenase-2)
CRE	Elemento responsivo ao AMPc (do inglês, cAMP response element)
CREB	Proteína que se liga à CRE (do inglês, CRE binding protein)
CT (ou SC)	Células-tronco (do inglês, stem cells)

DAB	Diaminobenzidina (do inglês, diaminobenzidine)
DAG	1,2-diacilglicerol
EGF	Fator de crescimento epidérmico (do inglês, epidermal growth factor)
EGFR	Receptor do fator de crescimento epidérmico (do inglês, epidermal growth factor receptor)
ERK	Cinase regulada por sinal extracelular (do inglês, extracellular signal-regulated kinase)
FAK	Cinase de adesão focal (do inglês, focal Adhesion Kinase)
FBS	Soro fetal bovino (do inglês, fetal bovine serum)
FDA	Food and Drug Administration
GFAP	Proteína glial ácida fibrilar (do inglês, glial fibrillary acidic protein)
GPCRs	Receptor acoplado à proteína G (do inglês, G protein-coupled receptor)
GRK2	Cinase 2 de receptor acoplado à proteína G (do inglês, GPCR kinase2)
GRP	Peptídeo liberador de gastrina (do inglês, gastrin releasing peptide)
GRPR	Receptor de GRP (do inglês, gastrin releasing peptide receptor)
HB-EGF	Fator de crescimento epidérmico capaz de ligar heparina (do inglês, heparin-binding-EGF)
HBSS	Hank's Balanced Salt Solution
hGRPR	Receptor do pepitídeo liberador de gastrina humano (do inglês, human Gastrin Releasing Peptide receptor)
HMG-I (Y)	Proteína de grupo de alta mobilidade tipo I (do inglês, high mobility group protein IY)
HNSCC	Carcinoma de células escamosas de cabeça e pescoço (do inglês, head and neck squamous cell carcinomas)
HPAF	Linhagem celular de adenocarcinoma pancreático humano (do inglês, human pancreatic adenocarcinoma cell line)
ICAM-1	Moléculas de adesão intercelular do tipo 1 (do inglês, inter-cellular adhesion molecule 1)

IGF-II	Fator de crescimento semelhante à insulina tipo II (do inglês, Insulin-like growth factor 2)
IGFs	Fatores de crescimento semelhante à insulina (do inglês, insulin-like growth factors)
INCA	Instituto Nacional de Câncer
IP_3	Inositol-1,4,5-trifostato (do inglês, *inositol-1,4,5-Trisphosphate*)
JNK	c-Jun N-terminal kinase
KUZ	Kuzbanian (metaloproteinase)
MAP-2	Proteína associada ao microtúbulo do tipo 2 (do inglês, microtubule-associated protein 2 (marcador neuronal)
MAPK	Proteína cinase ativada por mitógeno (do inglês, mitogen-activated protein kinase)
MMP	Metaloproteinases de membrana (do inglês, Matrix metalloproteinase)
NeuN–p	Proteína nuclear específica de neurônios (do inglês, neuronal nuclear protein)
NMB	Neromedina B (do inglês, neuromedin B)
NMBR	Receptor de NMB
OMS	Organização mundial da saúde
PDGFR	Receptor do fator de crescimento derivado de plaquetas (do inglês, platelet-derived growth factor receptor)
PECAM-1	Molécula-1 de adesão celular endotelial a plaquetas (do inglês, platelet endothelial cell adhesion molecule)
PI3K	Fosfatidilinositol-3-hidroxicinase (do inglês, phosphatidylinositol-3-hydroxikinase)
PIP	Fosfatidilinositol-4-monofosfato (do inglês, phosphatidylinositol-4-phosphate)
PIP_2	Fosfatidilinositol-4,5-bifosfato (do inglês, fosfatidil-inositol 4,5-biofosfato)
PIP_3	Fosfatidilinositol-3-4,5-trifosfato (do inglês, phosphatidylinositol -3,4,5-trisphosphate)
PKA	Protein kinase A
PKC	Protein kinase C

PKD	Protein kinase D
PLC	Fosfolipase C (do inglês, phospholipase C)
PTEN	Phosphatase and tensin homolog
Pyk2	Tirosina cinase rica em prolina (do inglês, proline-rich tyrosine kinase)
Rat-1 cells	Linhagem de fibroblasto de rato
ROCK	Proteína cinase associada à Rho (do inglês, Rho-associated coiled-coil forming protein kinase)
SCLC	Carcinoma de pequenas células de pulmão (do inglês, Human small cell lung carcinoma)
SFKs	Src family kinases
SNC	Sistema nervoso central
Swiss 3T3	Linhagem celular de fibroblasto de camundongo
TACE	Enzima que converte o TNF-α (do inglês, TNF converting enzyme)
TCGA	The Cancer Genome Atlas Research Network
TNF	Fator de necrose tumoral (do inglês, tumor necrosis factor)
VEGF	Fator de crescimento endothelial vascular (do inglês, vascular endothelial growth factor)
WHO	World Health Organization

RESUMO

Gliomas correspondem à grande maioria de tumores do sistema nervoso central (SNC), sendo que os glioblastomas multiformes são os mais comuns e mais malignos. O tratamento destes tumores ainda é ineficiente, pois a sobrevida média de pacientes com glioblastomas é de um ano após o diagnóstico. A ressecção cirúrgica, quando possível, acompanhada de radioterapia e quimioterapia são o tratamento padrão. A tendência é que a quimioterapia assuma um papel cada vez maior, devido ao desenvolvimento de quimioterápicos mais seletivos e com menores efeitos prejudiciais. Um peptídeo que vem sendo bastante estudado como fator de crescimento em vários tipos de câncer é o peptídeo liberador de gastrina (GRP), que atua no seu receptor (GRPR). O GRP pertence à família de peptídeos semelhantes à bombesina, e tem sido mostrado que o GRPR é super-expresso em muitos tipos tumorais. Os agonistas de GRPR, GRP e bombesina, estimulam o crescimento de diferentes tipos de células tumorais. Os objetivos para este estudo foram avaliar a expressão de GRPR em gliomas, avaliar a influência da ativação e inibição do GRPR sobre o crescimento de células de glioma e avaliar o envolvimento de efetores atuantes na sinalização celular desencadeada por GRPR, como fosfatidilinositol-3-hidroxicinase, PI3K.

Foi encontrada a expressão de RNA mensageiro de GRPR, pela reação da transcriptase reversa seguida da reação em cadeia da polimerase (RT-PCR, r*everse transcription polymerase chain reaction*) e a expressão ao nível protéico, por imuno-histoquímica, em linhagens de gliomas de rato (C6) e de humanos (U-87 e U373). O efeito sobre a proliferação celular do RC-3095, um

antagonista de GRPR, foi analisado nestas linhagens. RC-3095 inibiu a proliferação celular 48 horas após o tratamento, nas linhagens U-87 e C6, mas não na linhagem U-373. A inibição sobre a proliferação foi de 45% em C6, na dose 0,1 μM, e de 24% e 26% em U-87 nas doses de 0,1 μM e 1,0 μM, respectivamente. Células quiescentes tratadas com bombesina proliferaram 50% a mais em relação ao controle. Esta indução na proliferação celular foi prevenida pelo antagonista de GRPR. Quando as células quiescentes foram pré-tratadas com inibidor de PI3K, LY294002, a bombesina não foi capaz de induzir proliferação, sendo, portanto, esta indução na proliferação dependente de PI3K.

Depois da análise nas linhagens, foi avaliada a expressão de GRPR em material derivado de biópsias de 34 pacientes. GRPR esteve amplamente expresso em 100% destes tumores. Não foi encontrada expressão de GRPR em células gliais em amostras de necrópsias de cérebro de pacientes sem neoplasia, enquanto que a expressão de GRPR em neurônios humanos foi observada. A forte expressão de GRPR nestes 34 tumores não teve relação significativa com o grau tumoral ou com a sobrevida do paciente.

Considerando a hipótese de que gliomas, assim como outros tumores, podem se originar de uma pequena população de células-tronco (CT) dentro do tumor, avaliamos o efeito da bombesina sobre esta população de células que em tumores do SNC, formam neuro-esferas. Bombesina aumentou em 100% o numero de neuro-esferas após sete dias ao tratamento em cultura de glioma (U-87). Adicionalmente, foi observado que os tumores de pacientes que

expressam GRPR também apresentam uma sub-população de células expressando o marcador de CT, CD-113.

Esses achados sugerem que o GRPR tenha função no desenvolvimento e manutenção de gliomas induzindo proliferação de células tronco e diferenciadas. O bloqueio de GRPR, assim como a combinação dele com a inibição de outros agentes associados ao GRPR na cascata de sinalização celular, podem servir como estratégia potencial no tratamento destes tumores.

ABSTRACT

Gliomas represent the vast majority of tumors of the central nervous system (CNS), and the glioblastoma multiform constitutes the most common and most malign one among them. The treatment of these tumors is still inefficient, the median survival of patients of only one year after the diagnosis. Surgical resection, when possible, radiotherapy and chemotherapy are the standard treatment. The trend is that chemotherapy will assume a more important role, due to the development of targeted agents with less side effects. The gastrin releasing peptide (GRP) is a peptide implicated in cancer progression. Belonging to the family of bombesin-like peptides, GRP and its receptor, GRPR, have been shown to be over-expressed in many tumor types, and GRP and bombesin stimulate growth of different cell types. The aims of this study were to evaluate the expression of GRPR in gliomas, the effects of GRPR activation and inhibition on cell growth, and the involvement of active signaling effectors triggered by GRPR such as phosphatidylinositol-3-hydroxikinase (PI3K).

GRPR expression was found in rat (C6) and human (U87, U373) glioma cell lines, both as mRNA examined by reverse transcription polymerase chain reaction (RT-PCR) as well as protein examined by immunohistochemistry. The effect of RC-3095, a GRPR antagonist, on cell proliferation was analyzed in these cell lines. RC-3095 inhibited cell proliferation in U-87 and C6, but not U-373 cells, 48 hours after treatment. Proliferation was inhibited by 45% in C6 cells , at 0.1μM of RC-3095 and 24% and 26% in U-87 at doses of 0.1 μM and 1.0 μM, respectively. Quiescent cells treated with bombesin proliferated 50% more than untreated cells. This induction in cell proliferation was reversed by

the antagonist of GRPR. When these quiescent cells were pre-treated with the PI3K inhibitor, LY294002, bombesin was not able to induce proliferation, indicating that that this proliferation is PI3K-dependent.

The expression of GRPR was also evaluated in biopsy-derived cells from 34 patients of the Hospital of Clinics of Porto Alegre. GRPR was widely expressed in 100% of these tumors. This high expression was not found in samples from autopsies of brains of patients without cancer. In these samples, GRPR was expressed only in neurons, but not in glial cells. The strong expression of GRPR in 34 tumors showed have neither relationship with the tumor grade nor with the patient survival.

Considering the growing hypothesis that gliomas and other tumors can be originated from a small population of stem cells within the tumor, was important to assess the effect of bombesin on this population of cells that, in tumors of the CNS, are neurospheres. Bombesin increased 100% the number of neuros peers seven days after the treatment. Additionally, was observed that the same tumors of patients that express GRPR also have a subpopulation of cells expressing stem cell marker, CD-113.

These findings suggest that GRPR has a function in development and maintaince of gliomas inducing proliferation of stem and differentiated cells. The blockade of GRPR, as well as its combination it with the inhibition of other agent associated to the GRPR in the waterfall of cellular pathway signaling can serve like potential strategy in the treatment of these tumors.

INTRODUÇÃO

1. Gliomas

A classificação dos tumores do sistema nervoso central (SNC) é bastante complexa. Na mais nova edição da Classificação Internacional de Doenças para Oncologia (CID-O), publicada em 2007 pela Organização Mundial da Saúde (OMS) novos itens e variantes de cada subtipo tumoral foram incluídos. De forma mais genérica, os tumores do SNC são classificados em: tumores dos tecidos neuro-epiteliais, que incluem os gliomas e os tumores do plexo coróide; os neurais e mistos; os tumores da região pineal; os tumores embrionários, nos quais se enquadram os meduloblastomas e os neuroblastomas; os tumores de crânio e nervo para-espinhal; os tumores das meninges entre outros (Louis et al., 2007). Glioma é o termo utilizado para designar um grupo heterogêneo de tumores originários das células da glia encefálica e são classificados de forma mais genérica em astrocitomas, oligodendrogliomas, ependimomas e os gliomas mistos, oligoastrocitomas (Figarella-Branger e Bouvier, 2005; Louis et al., 2007).

Os gliomas correspondem à aproximadamente 70% de todos os tumores cerebrais. Dentre os gliomas, os astrocitomas são os mais comuns e, entre estes, os glioblastomas multiformes são os que acometem a maioria de pacientes. Os tumores primários do SNC correspondem a menos de 2% do total de cânceres em adultos (Sathornsumetee e Rich, 2008; Wen e Kesari, 2008; Ohgaki, 2009).

Nos Estados Unidos, a cada ano, são diagnosticados cerca de 22.500 novos casos de tumores primários do SNC em adultos, sendo que, entre esses, os gliomas correspondem a cerca de 14.000 casos. Na população em geral a

incidência é de aproximadamente 5 casos para cada 100.000 pessoas (Wen e Kesari, 2008).

No Brasil, a incidência de gliomas não é muito bem conhecida. Segundo o Instituto Nacional de Câncer (INCA), em um período de quatro anos (de 1994 a 1998), os tumores encefálicos encaminhados aos hospitais do Instituto foram um total de 90, correspondendo a 1,4% do total de casos deste período.

Quanto à malignidade, os astrocitomas são divididos em: astrocitomas de baixo grau, que incluem graus I e II e de alto grau, que incluem graus III e IV, Veja a classificação de cada grau na Tabela 1.

Tumores astrocíticos/ grau tumoral	I	II	III	IV
astrocitoma subependimal de células gigantes	*			
astrocitoma pilocítico	*			
astrocitoma pilomixoide		*		
astrocitoma difuso		*		
xantoastrocitoma pleomórfico		*		
astrocitoma anaplásico			*	
glioblastoma				*
glioblastoma de células gigantes				*
gliosarcoma				*

Tabela 1- Classificação de astrocitomas quanto à malignidade (adaptado de Louis et al., 2007).

Existe uma tendência à maior incidência destes tumores em países industrializados e desenvolvidos. Alguns trabalhos indicam que a população caucasiana tem uma maior incidência que a africana ou a asiática (revisado por Ohgaki, 2009). Gliomas malignos são 40% mais comuns em homens do que em mulheres e a idade média de pacientes diagnosticados com glioblastomas

é de 64 anos e de 45 anos para os casos de gliomas anaplásicos (Fisher et al., 2007).

Aproximadamente 5% dos pacientes com gliomas malignos têm histórico de glioma familiar. Alguns destes casos familiares são associados com raras síndromes genéticas como neurofibromatose dos tipos 1 e 2, a síndrome de Li-Fraumeni e Síndorme de Turcot. No entanto, para a maior parte dos casos familiares não é identificada uma causa genética (Wen e Kesari, 2008).

A terapia padrão para um novo glioma diagnosticado envolve ressecção cirúrgica, radioterapia e quimioterapia. A quimioterapia utilizada atualmente, aprovada pela *Food and Drug Administration* (FDA) em 2005, é a temozolomida que é uma agente alquilante e pode ser administrada durante ou depois da radioterapia (Sathornsumetee e Rich, 2008).

Os gliomas malignos não podem ser totalmente eliminados cirurgicamente por causa de sua natureza infiltrativa, mas os pacientes são submetidos à ressecção cirúrgica máxima sempre que possível. O procedimento cirúrgico *debulking* (quando o tumor não pode ser removido completamente e é retirado parcialmente) reduz os sintomas de massa crítica e fornece tecido para o diagnóstico histológico e estudos moleculares. Avanços nos métodos cirúrgicos têm melhorado a segurança da cirurgia e aumentado o alcance de maior parte da ressecção (Asthagiri et al., 2007).

A radioterapia é a terapia mais importante no tratamento de gliomas malignos. A adição de radioterapia no tratamento aumenta a sobrevida dos

pacientes de um intervalo de 3 a 4 meses a um intervalo de 7 a 12 meses. Após a radioterapia padrão, 90% dos tumores reaparecem na área original. Então, estratégias que aumentam a dose de radiação com o uso de braquiterapia e radiocirurgia estereotáxica são utilizadas, mas não conseguiram melhorar a sobrevida (Tsao et al., 2005; Wen et al., 2008).

A quimioterapia está assumindo um papel cada vez mais importante no tratamento dos gliomas malignos. Duas meta-análises sugerem que a quimioterapia adjuvante resulta em um aumento modesto na sobrevida (de 6 a 10% de aumento na sobrevida de 1 ano) (Fine et al., 1993; Stewart et al., 2002; Wen et al., 2008).

Segundo Stupp et al. (2005), a combinação de radioterapia e temozolomida gerou um aumento na sobrevida mediana (de 12,1 meses para 14,6 meses s, P <0,001) comparada à radioterapia sozinha. Além disto, a taxa de sobrevida em dois anos entre os pacientes que receberam radioterapia e temozolomida foi significativamente maior do que a taxa entre os pacientes que receberam radioterapia isoladamente (de 10,4% para 26,5%).

Porém, mesmo usando os recursos cirúrgicos, de radioterapia e temozolomida, a sobrevida dos pacientes continua baixa. Cerca de 74% dos pacientes morrem com menos de dois anos do diagnóstico. Pouco mais de 3% dos pacientes com gliomblastoma multiforme permanecem vivos após cinco anos do diagnóstico (Ohgaki, 2009).

1.1 Biologia de gliomas e busca de novos alvos terapeuticos

A oncogênese de gliomas, particularmente de glioblastomas, é dirigida muitas vezes por processos biológicos que incluem a ativação de receptores de fatores de crescimento. Muitos destes receptores como o receptor do fator de crescimento epidérmico (EGFR, *epidermal growth factor receptor*, também conhecido como HERB1-*human epidermal growth factor receptor*), receptor do fator de crescimento derivado de plaquetas (PDGFR, *platelet-derived growth factor receptor*) e o receptor do fator de crescimento endotelial vascular (VEGFR, *vascular endotelial growth factor receptor*) estão superexpressos, amplificados e ou mutados em gliomas. A transformação de células normais em glioblastomas primários parece ser causada principalmente pela: amplificação de EGFR; diminuição da fosfatase PTEN (*phosphatase and tensin homolog*) e deleção do gene p16. Os glioblastomas secundários estão mais relacionados a mutações do gene p53 e à superexpressão de PDGFR (Zhu e Parada, 2002; Wen e Kesari, 2008).

Outro aspecto da oncogênese de gliomas que vem sendo fortemente estudado é a hipótese das células-tronco (CT) tumorais. Esta basea-se que mutações oncogênicas ocorridas em uma pequena fração de células, como CT no tecido normal levariam à formação de CT do câncer e células diferenciadas dirigindo o processo tumorigênico (Marx, 2007). Fatores de crescimento como EGF e FGF mantem as CT tumorais em cultura. Dois recentes trabalhos demostraram a indução de alterações em EGFR, PTEN e p53 em CT sadias e permitiram observar características muito semelhantes às encontradas nos tumores. Zheng et al. (2008) mostraram que a concomitante deleção de p53 e

PTEN em CT neurais aumenta significativamente a proliferação e a capacidade dessas células de se renovarem. Adicionalmente, Li et al. (2009) deletaram PTEN de CT cerebrais normais e infectaram essas com EGFR e essas células apresentaram muitas características observadas nas CT tumorais e, consequentemente, nos tumores como gliomas (CT em gliomas será melhor abordado no item 4).

O bloqueio dos receptores de fatores de crescimento e as vias de sinalização desencadeadas por eles têm sido mais recentemente estudados como promissores alvos na terapia de tumores. Muitas destas estratégias de terapia já foram aprovadas e estão sendo usadas na clínica. Alguns exemplos são: Cetuximab, anticorpo IgG1 monoclonal contra EGFR, já foi aprovado para o tratamento de carcinoma pavimentocelular de cabeça e pescoço e câncer colorretal. Bevacizumab, anticorpo IgG monoclonal contra fator de crescimento endotelial vascular (VEGF, *vascular endotelial growth factor*), é a primeira linha de tratamento para câncer de pequenas células de pulmão e colorretal metastático. Trastuzumab, anticorpo monoclonal contra o fator de crescimento epitelial humano tipo 2 (HER2, *human epidermal growth factor receptor 2*), está sendo utilizado no tratamento de cânceres de mama que superexpressam este receptor. Imatinib, inibidor de tirosina cinases, é usado no tratamento de leucemias e tumores gastrointestinais. Em gliomas, o quimiterápico utilizado é a temozolamida, um agente alquilante. Portanto, pertencente a uma geração mais antiga de quimioterápicos. O entendimento da biologia de gliomas, com maior estudo nas vias de sinalização ativadas no processo tumorigênico, faz-se necessário para o encontro de terapias mais seletivas e principalmente mais eficazes. Peptídeos da família da bombesina têm sido estudados em muitos

tumores e são um potente mitógeno que induz a proliferação e o crescimento celular. Além disso, são altamente expressos em neoplasias (Patel et al., 2006; Cornélio et al., 2007; Jensen et al., 2008).

2. Caracterização da bombesina e seus análogos

A bombesina é um peptídeo composto por 14 aminoácidos (Figura 1, a) isolado da pele da rã de origem europeia *Bombina bombina,* popularmente conhecida como *Sapo-de-barriga-de-fogo,* (Figura 1, b) (Erspamer et al., 1970; Anastasi et al., 1971).

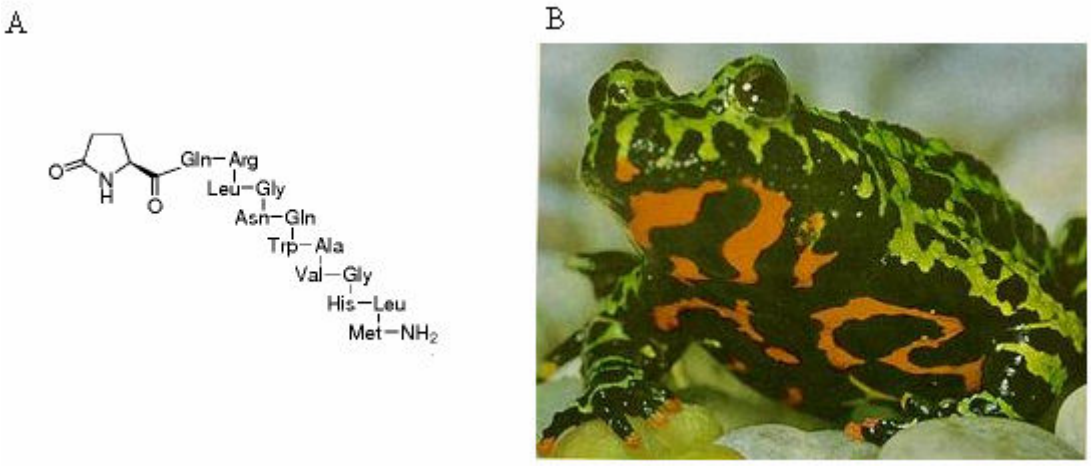

Figura 1. Estrutura química da bombesina (a) e anfíbio *Bombina bombina* (b).

Erspamer et al. (1984) isolaram muitos outros peptídeos da pele de anfíbios. Devido à similaridade estrutural, eles dividiram estes peptídeos em três grupos: família bombesina, a qual inclui a bombesina e a alitensina; família ranatensina, que inclui ranatensina, litorina e seus derivados; e a família filolitorina (Figura 2).

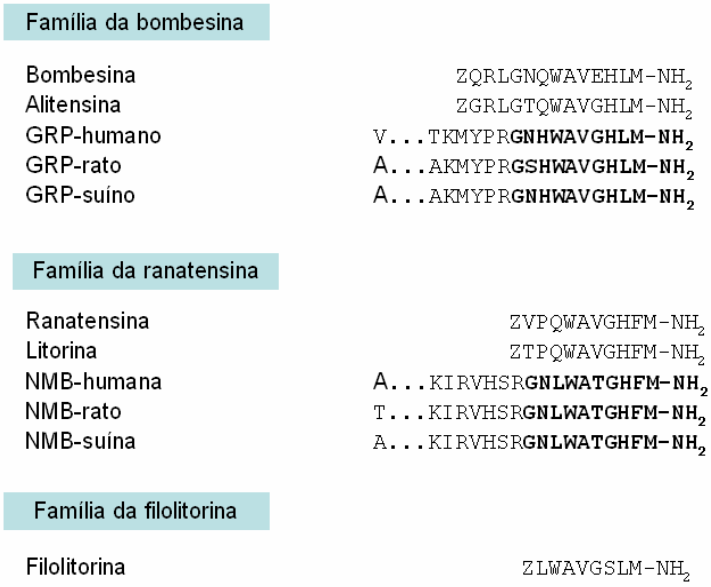

Figura 2. Estrutura da bombesina e famílias análogas (adaptado de Ohki-Hamazaki et al., 2005).

Estudos subseqüentes mostraram o isolamento de dois outros peptídeos análogos à bombesina em mamíferos: o peptídeo liberador de gastrina ou GRP (*gastrin releasing peptide*) e a neuromedina B ou NMB *(neuromedin B*) (McDonald et al., 1979; Minamino et al., 1983). O GRP foi isolado de tecidos gástricos de suínos e recebeu este nome devido à sua potente indução da liberação de gastrina. Ele é um peptídeo composto por 27 aminoácidos e possui os sete últimos aminoácidos da região carboxi-terminal semelhantes aos da bombesina, no que se refere à atividade biológica destes peptídeos. NMB foi isolada do cordão espinhal de suínos e mostra idêntica similaridade com ranatensina nos sete últimos aminoácidos da porção caboxi-terminal, exceto a substituição de uma treonina em NMB por uma valina na ranatesina (Miamino et al., 1985).

Existem três RNAs mensageiros (mRNA) maduros de GRP em humanos que codificam proteínas com 148, 141 e 138 aminoácidos. Todos codificam

identicamente o peptídeo GRP_{1-27}, mas diferem na seqüência que codifica a extensão C-terminal. Os três mRNAs de GRP são originados de uma simples fita de mRNA nascente, por *splicing* alternativo (Spindel et al., 1986; Sausville et al., 1986).

O GRP é processado a partir de um precursor bem maior do que em sua forma análoga à bombesina, preproGRP, com 148 aminoácidos. Dois produtos ativos podem ser originados do processamento do preproGRP; um GRP_{1-27} e outro GRP_{18-27}, este também conhecido como GRP-10 ou NMC (Erspamer et al.,1988; Patel et al., 2006). Durante o processamento, ocorre uma amidação no aminoácido 27. A NMB é codificada com 76 aminoácidos e processada para uma forma madura com 32 aminoácidos. GRP e NMB possuem 48% de similaridade. O gene codificador de GRP está localizado no cromossomo 18, enquanto o gene para NMB no cromossomo 15.

3. Receptores ativados por bombesina

A bombesina tem afinidade por quatro subtipos de receptores. Destes, três estão presentes em mamíferos: BB1 ou NMBR, BB2 ou GRPR e BRS-3. O BB4-R está presente somente em cérebro de anfíbios. O BB4-R tem maior afinidade por bombesina do que por GRP, e apresenta 56%, 61% e 70% de aminoácidos idênticos com GRPR humano, NMBR humana e BRS-3 humano, respectivamente (Nagalla et al., 1995). Dentre os três subtipos de receptores presentes em mamíferos, a bombesina apresenta maior afinidade por BB2 (revisado por Jensen et al., 2008).

O BB1 também é conhecido como NMBR por apresentar uma maior afinidade por NMB do que por GRP, (>100X). O BB2, por sua vez, é conhecido

como GRPR por apresentar maior afinidade por GRP como ligante endógeno. Ele tem alta afinidade por bombesina e GRP e baixa afinidade por NMB (Tabela 2). O terceiro tipo de receptor pelo qual a bombesina tem afinidade é o BRS-3. Neste caso, ainda não é conhecido o ligante endógeno em mamíferos, mas tanto GRP como neuromedina C e NMB apresentam baixa afinidade por este receptor (Fathi et al., 1993b; Liu et al., 2002; Patel et al., 2006).

Peptídeo	Afinidade pelo receptor (nM)			
	NMBR (BB1)	GRPR (BB2)	BRS-3	BB4R
Bombesina	32 ± 2	2.1± 0.1	>1000	14 ± 1
GRP	4800	6.6 ± 1.3	>1000	79 ± 12
NMB	1.8 ± 0.3	700± 200	>1000	11 ± 1
Agonista de BRS-3	9 ± 0.8	0.2 ± 0.07	0.32 ± 0.8	0.41± 0.06

Tabela 2. Propriedade de ligação de receptores da família da bombesina (adaptado de Patel, 2006).

3.1 NMBR

O gene de NMBR está localizado no cromossomo 6p21 em humanos e em ratos no cromossomo 10. Tanto o NMBR humano quanto o de rato e camundongo possuem três éxons e dois íntrons (Corjay et al., 1991; Wada et al., 1991; Ohki-Hamazaki et al., 2000). O receptor NMBR é composto por 390 aminoácidos e possui 55% de similaridade com GRPR e 47% de similaridade com BRS-3 (Fathi et al., 1993a).

A expressão de mRNA de NMBR foi descrita em humanos, ratos, camundongos e primatas (Ohki-Hamazaki et al., 2000; Sano et al., 2004). Este receptor é expresso no SNC, no trato gastrointestinal e em células de músculo

liso (Von Schrenck et al., 1989; Severi et al., 1991; Bitar e Coy, 1992). Além disto, este receptor está presente em diferentes tumores, incluindo os de pequenas células de pulmão, os de células epiteliais de brônquio, carcinoma intestinal, câncer de epitélio de ovário, linhagens de glioblastomas e linhagem de células pancreáticas (DeMichele et al., 1994; Sun et al., 2000b; Jensen et al., 2008).

O NMBR é um receptor acoplado à proteína G e sua estimulação com NMB induz a proliferação celular em glioma C6 de ratos e em células de câncer de pulmão, enquanto que inibidores seletivos revertem este efeito (Moody et al., 1992; Moody et al., 2000).

Outros peptídeos que pertencem à família de NMB, como ranatensina e litorina, têm afinidade por NMBR (Wang et al., 1992; Katsuno et al., 1999). A NMB atinge o máximo de ligação ao receptor, a 22º C, entre 5 e 15 minutos (Benya et al., 1992; Ohki-Hamazaki et al., 2000).

O receptor de NMB é internalizado rapidamente e o agonista degradado. Mais de 70% da NMB é internalizada em até 60 minutos em glioma C6 de rato e em fibroblastos transfectados com NMBR (Benya et al., 1992; 1994; Wang et al., 1993). O receptor é reciclado e volta para a superfície celular, independentemente de síntese protéica (Benya et al., 1994a). O NMBR é acoplado à $G\alpha_q$, que ativa a isoforma β de fosfolipase C, catalisando a hidrólise de fosfatidilinositol-4,5-bifosfato (PIP_2) na membrana, resultando nos produtos inositol-1,4,5-trifostato (IP_3) e 1,2-diacilgliocerol (DAG) (Figura 3). O IP_3 liga-se ao receptor do retículo endoplasmático e promove a liberação de Ca^{++} para o citosol, enquanto DAG ativa a proteína cinase C (PKC). A NMB ativa as proteínas cinases ativadas por mitógeno p42 e p44 (p42/p44MAPK, *mitogen-*

activated protein kinase) e induz a fosforilação das proteínas p1256^{FAK}, paxilina e a molécula de adesão focal. A NMB aumenta os níveis de expressão do fator de transcrição c-fos em linhagens de glioma de rato e em pequenas células de pulmão (Moody et al., 1992; 1995; Lach et al., 1995; Ohki-Hamazaki et al., 2000). A NMB aumenta os níveis de adenosina-3,5-monofosfato cíclico (cAMP, *cyclic adenosine monophosphate*) em glioma de rato (Moody et al., 1995).

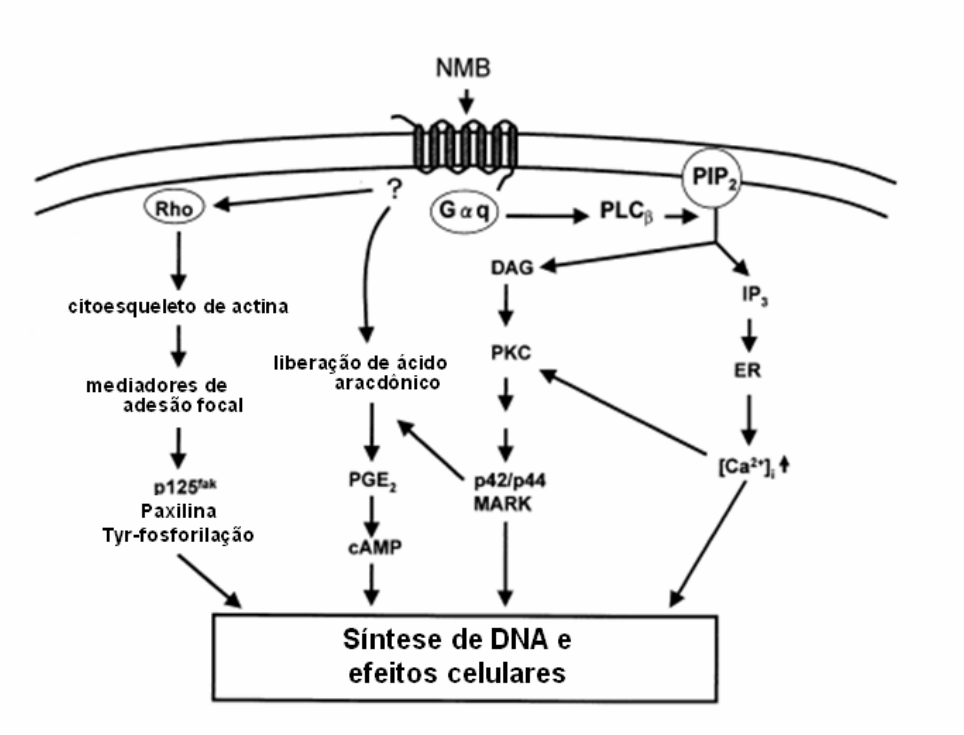

Figura 3. Possível cascata de sinalização ativada por NMBR (adaptado de Ohki-Hamazaki et al., 2000).

3.1.1 Camundongos deficientes em NMBR

Camundongos deficientes em NMBR expostos à baixa temperatura apresentaram 58% de redução da temperatura corporal comparados ao tipo selvagem, mesmo quando tratados com NMB. Em relação à contração muscular e ao controle da saciedade, é sugerido que GRPR, e não NMBR,

tenha maior parte na mediação destes efeitos, pois os animais *knockouts* para NMBR não apresentam diferenças em relação ao tipo selvagem (Ladenheim et al., 1994; revisado por: Ohki-Hamazaki et al., 2000). Em relação ao comportamento social e à agresividade, estes animais não mostram diferenças quando comparados ao tipo selvagem (Ohki-Hamazaki et al., 1999).

3.2 BRS3

BRS-3 em humanos é composto por 399 aminoácidos (Fathi et al., 1993b). Ele já foi clonado a partir de ratos, camundongos, porcos, carneiros e macacos Rhesus. Em frangos, foi encontrado um receptor com similaridade a BRS-3 e BB4, presentes em anfíbios, e que foi chamado de chBRS-3 (Iwabuchi et al., 2003). O gene codificador do BRS-3 humano está localizado no cromossomo Xq25, e contém dois íntrons e três éxons (Gorbulev et al., 1994; Fathi et al., 1993b; Weber et al., 1998).

Entre os três subtipos de receptores de bombesina presentes em mamíferos, o BRS-3 é o que apresenta a distribuição da expressão mais diferenciada. O BRS-3 é expresso no SNC e em testículos de macacos e ratos, mas não em testículos de camundongos e carneiros (Ohki-Hamazaki et al., 1997; Weber et al., 2003; Sano et al., 2004). Foi detectada a sua expressão em pâncreas, tireóide e tecido periférico de ovários de macacas (Sano et al., 2004), e também em células epiteliais bronquiais humanas, rim de ratos e pulmão de fetos de camundongos (DeMichele et al., 1994; Emanuel et al., 1999; Fleishchmann et al., 2000; Dumesny et al., 2004; Tan et al. 2006).

No SNC, a expressão de BRS-3 não é tão intensa como as de NMBR e GRPR, sendo sua maior expressão localizada na formação hipocampal e na

área hipotalâmica (Jennings et al., 2003; Jensen et al., 2008). O BRS-3 é expresso em alguns tipos de tumores como: tumores de pequenas células de pulmão, carcinomas de pulmão, tumores renais, pancreáticos, sarcomas de Ewing, tumores pituitários, de ovários e de próstata (Sun et al., 2000; Reubi et al., 2002; Schulz et al., 2006). Não há estudos mostrando sua expressão em gliomas.

Alguns agonistas sintéticos para BRS-3 foram projetados na tentativa de serem seletivos para este receptor. Dois destes foram bem mais seletivos para BRS-3 do que os demais receptores de bombesina e que a própria bombesina. Usando estes agonistas, viu-se que o BRS-3 ativa a fosfolipase C e mobiliza cálcio (Weber et al., 2002; Jensen et al., 2008). Não é conhecido qualquer antagonista que seja seletivo para BRS-3 (Jensen et al., 2008).

3.2.1. Camundongos deficientes em BRS-3

Camundongos *knockouts* em BRS-3 desenvolveram obesidade moderada associada com hipertensão e prejuízo no metabolismo da gliocose. Estas mudanças ainda foram associadas com o aumento de lecitina no sangue e hiper-fadiga. Os autores deste estudo sugerem que o BRS-3 possa ter uma importante função no mecanismo responsável pelo balaço de energia e controle do peso corporal (Ohki-Hamazaki et al., 1997). Em outros estudos com camundongos *knockouts* para esse receptor, os autores sugerem que o BRS-3 tenha efeito sobre funções comportamentais como: regulação de isolamento social, modulação da emoção incluindo formas de ansiedade (Yamada et al., 2000a; 2002).

3.3 GRPR

O GRPR humano é composto por 384 aminoácidos (Figura 4) e apresenta alta homologia com GRPR de rato, com 90% de identidade (Corjay et al., 1991; Ohki-Hamazaki et al., 2005; Jensen et al., 2008). A similaridade do GRPR humano com o NMBR humano e o BRS-3 humano é de 52% e 51% respectivamente (Jensen et al., 2008). O GRPR foi completamente ou parcialmente clonado em 21 espécies e as regiões mais conservadas são domínios transmembranas e o terceiro domínio intracelular (Baldwin et al., 2007).

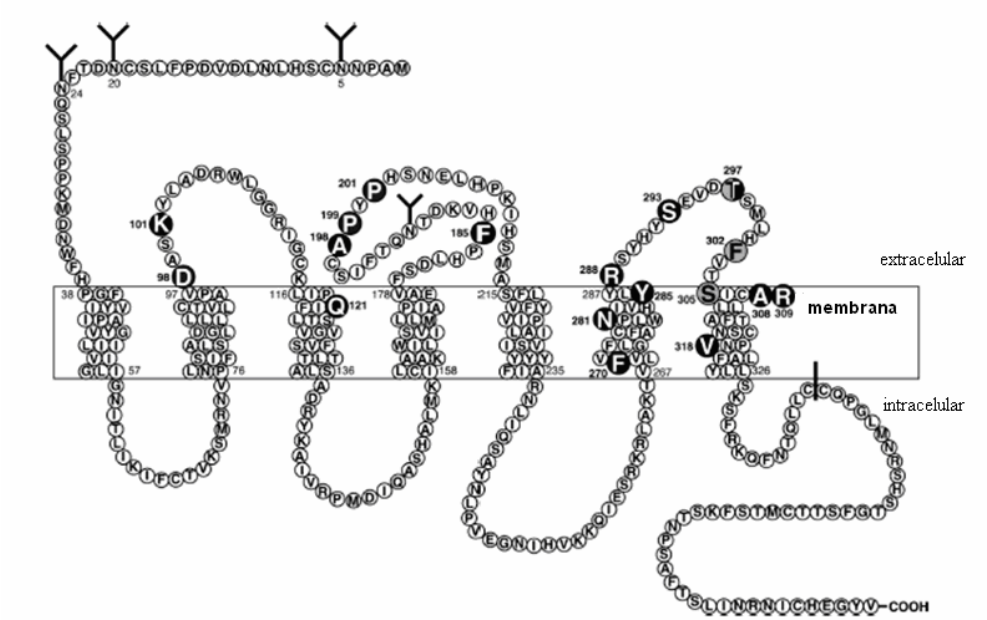

Figura 4. Estrutura do receptor de GRP (adaptado de Jensen et al., 2008).

O gene de GRPR humano está localizado no Xp22 (Maslen e Boyd, 1993; Xiao et al., 2001) e o gene de GRPR murino está no cromossomo X entre os *loci* Pdha-1 e Amg (Maslen e Boyd, 1993). O GRPR humano possui três éxons interrompidos por dois íntrons com 20 e 2 Kb de comprimento (Corjay et al., 1991; Weber et al., 2000; Xiao et al., 2001). O éxon 1 codifica os

primeiros três domínios transmembrana do receptor e a área de processamento está localizada próxima à segunda alça intracelular (resíduo 137). O éxon 2 codifica as regiões 4 e 5 transmembrana da terceira alça intracellular, o sítio localizado no resíduo 254. O éxon 3 codifica o domínio transmembrana 5 bem como a região carboxi-terminal do receptor (Xiao et al., 2001).

3.3.1 A ativação da transcrição do gene de GRPR

Weber et al. (2000) sugerem que a regulação da transcrição do gene de GRPR é dependente dos motivos elemento de resposta ao AMPc (CRE, *cAMP response elemen*t) e TTTAAA. Em GRPR de murinos, foi encontrada uma seqüência homóloga a CRE a montante ao início do sítio de transcrição do gene e esse elemento foi essencial para a atividade desse gene em células Swiss 3T3. Para a atividade transcripcional basal do gene de GRPR humano, foi requerida uma região entre 97 e 247 pb a montante do início da transcrição de RNA em células de próstata e de carcinoma gastrointestinal (Qu et al., 2004). Xiao et al. (2001) sugeriram que um motivo CRE localizado a 112 pb a montante do maior sítio de transcrição é requerido para conferir a atividade basal do promotor em células de câncer de duodeno. Mais recentemente, Chinnappan et al. (2008) mostraram que fatores de transcrição ligados em dois distintos sítios CRE são requeridos para que o gene de GRPR humano promova a transcrição em células de câncer gastrointestinal. Eles ainda sugerem que a regulação do gene de GRPR humano é dependente da via de sinalização induzida por cAMP e ativada pelos fatores de transcrição ATF-1

(*activating transcription factor 1*) e a proteína que se liga a CRE (CREB, *CRE binding protein)*.

3.3.2 A expressão de GRPR

A expressão de GRPR tem sido bastante estudada em roedores, enquanto que o panorama da expressão de GRPR em outros mamíferos como macacos e em humanos vem sendo desenhado mais lentamente. Em macacos, foi encontrada expressão do gene que codifica para o GRPR no pâncreas, próstata, músculo esquelético, SNC e níveis de expressão mais baixos no estômago. No SNC, é expresso no hipocampo, hipotálamo e amígdala (Sano et al., 2004).

A hibridização *in situ* em ratos mostrou que GRPR é expresso amplamente durante o desenvolvimento embrionário nos sistemas nervoso, respiratório, urogenital, gastrointestinal e que ocorre um pico nos níveis de expressão na hipófise posterior em torno do vigésimo dia dos embriões seguindo por um rápido declínio durante o período pós-natal (Battey et al., 1994). Durante a vida adulta, em roedores, é predominantemente expresso no trato digestivo e cólon, mas não no estômago (Battey et al., 1991) e amplamente expresso no SNC (Moody e Merali, 2004; Kamichi et al., 2005).

A expressão de GRPR em humanos (hGRPR) tem sido mais estudada em tecidos e linhagem tumorais (veja item 3.3.5). Em tecidos normais GRPR é fortemente expresso em pâncreas e mais fracamente no estomago e córtex adrenal e não é expresso em medula adrenal, tireóide, timo, intestino delgado e mucosa epitelial exceto no antro gástrico (Ferris et al., 1997; Xiao et al., 2001).

3.3.3 O GRPR e suas funções fisiológicas

As primeiras funções nas quais foi identificada a atuação de GRPR foram no trato gastrointestinal incluindo regulação da secreção gástrica estimulando a liberação de gastrina das células G e somatostantina das células D (Schubert et al., 1991; 2002); regulação da mobilidade gastrointestinal (Degen et al., 2001; Yegen et al., 2003); estimulação da secreção pancreática (Niebergall-Roth e Singer , 2001); liberação de insulina (Persson et al., 2002).

O GRPR é amplamente expresso no SNC e na medula espinhal de roedores, e numerosos efeitos têm sido descritos por sua ativação. O GRPR tem função sobre a memória aversiva e emocional. A estimulação do receptor induz um melhoramento da memória em camundongos e em ratos tratados de forma sistêmica ou intra-cerebral, enquanto que a inibição do receptor promove um prejuízo na formação deste tipo de memória (Roesler et al., 2006b; Preissler et al., 2007). Além disto, o GRPR está envolvido com a mediação da extinção de memória aversiva (Luft et al., 2006). A expressão de GRPR no SNC também tem sido relacionada ao envolvimento deste receptor em diversas doenças psiquiátricas e desordens neurológicas como ansiedade, esquizofrenia, autismo, demência, desordem de pânico e desordens alimentares incluindo anorexia nervosa, bulimia e depressão (Roesler et al., 2006a; Hodges et al., 2009). Comportamentos modulados por GRPR em roedores incluem "*grooming*", ingesta de comida, comportamento estereotipado e comportamento social (Taylor e Garcia, 1985; Piggins e Merali, 1989; Flynn et al., 1997; Meller et al., 2004; Moody e Merali, 2004; Presti-Torris et al., 2007). Além de efeitos mais comportamentais, o GRPR no SNC apresenta função sobre a termoregulação, metabolismo e saciedade (Spindel et al., 1986). Está

bem estabelecido que administração de bombesina suprime a ingesta de alimento em numerosas espécies, incluindo ratos (Gibbs et al., 1979), camundongos (Taylor e Garcia,1985) e humanos (Muurahainen et al., 1993).

Trabalhos mais recentes vêm mostrando que a expressão de GRPR no cordão espinhal está relacionada com a mediação no comportamento de coçar mediante estímulo ao prurido (Sun e Chen, 2007; Swain, 2008) e com o reflexo peniano (Sakamoto et al., 2008).

O GRPR também tem sido implicado no processo inflamatório (Dal-Pizzol et al., 2006; Petronilho et al., 2007). Dal Pizzol et al. (2006) mostraram que RC-3095, um antagonista seletivo de GRPR, atenua a liberação de citocinas pro-inflamatórias *in vitro* e *in vivo* melhorando a sobrevida de ratos com sepsis estabelecida. A ativação de GRPR promove a estimulação de células natural *killer* (De la Fuente et al., 1993) por aumentar a expressão de Hsp72 (*heat shock protein 72*) (Taglia et al., 2008). Outros estudos mostraram que a ativação do GRPR promove a quimioatração de leucócitos (Ruff et al., 1985; Del Rio et al., 1994).

3.3.4 Camundongos deficientes em GRPR

Camundongos deficientes em GRPR desenvolvem-se e reproduzem-se normalmente e não mostram qualquer anormalidade fenotípica bruta (Hampton et al., 1998). Wada et al. (1997) mostraram que camundongos *knockouts* para GRPR quando o tratamento com GRP não induzem hipotermia, diferentemente deste mesmo tratamento no tipo selvagem. Além disto, eles mostraram que os animais deficientes em GRPR apresentaram significante aumento na atividade locomotora durante a noite e na interação social, enquanto que não geraram

comportamento agressivo contra animal intruso. Eles ainda analisaram os efeitos de monoaminas como dopamina e serotonina no cérebro dos animais mutantes e não encontram diferenças em relação ao tipo selvagem. Apesar de muitos estudos farmacológicos mostrarem que GRP regula as secreções exócrina e endócrinas relacionadas à digestão, o metabolismo e o comportamento dos animais deficientes em GRPR não apresentam diferenças nos níveis de açúcar, gastrina, amilase, insulina, hormônio do crescimento (GH, *growth hormone*) e o hormônio estimulante da tireóide (TSH, *thyroid-stimulating hormone*) em seu sangue em relação ao tipo selvagem. No estudo de Hampton et al. (1998) esses animais demonstraram deficiência na resposta à saciedade, pois quando tratados com bombesina mantiveram a ingesta de glicose ao passo que nos tipo selvagem suprimiram a ingesta.

Outro estudo mostrou que os camundongos *knockouts* para GRPR têm aumentada sua preferência por odores con-específicos (por outros animais) e aumentam seu comportamento social investigatório (Yamada et al., 2000b). Camundongos mutantes para GRPR não mostraram diferenças comparadas ao tipo selvagem em relação à resposta termal, mecânica, inflamatória e dor neuropática, mas mostraram diferença no estímulo ao prurido (Swain et al., 2008). Finalmente, o receptor de GRP tem uma importante função no crescimento celular não neoplásico e neoplásico (Moody et al., 1992; Jensen et al., 2008).

3.3.5 O GRPR e seu envolvimento no câncer (GRPR como fator de crescimento no câncer)

GRP e bombesina induzem proliferação e crescimento celular. Em ratos neonatos, a bombesina administrada subcutaneamente estimula o crescimento das células da mucosa gastrointestinal e do pâncreas (Lehy et al., 1986; Puccio et al.,1989), também estimula o crescimento de fibroblastos de ratos e células normais de epitélio bronquial humano (Rozengurt e Sinneth-Smith, 1993; Willey et al., 1984). Em porcos, a bombesina e o GRP têm efeitos regenerativos depois da pancreatectomia (Fiorucci et al., 1998). Em diversos tumores, a bombesina e o GRP têm efeito mitogênico. A bombesina e o GRP induziram a proliferação em linhagens tumorais gástricas, pancreáticas, de próstata, mama, de câncer colorretal, neuroblastoma, pulmão, cabeça e pescoço (Iishi et al., 1992; Hoosein et al., 1993; Avis et al., 1993; Bold et al., 1998; Lango et al., 2002; Kang et al. 2007; Ishola et al., 2007). Em carcinoma de pequenas células de pulmão, a porção carboxi-terminal de GRP e não a porção GRP_{1-16} apresentam efeito mitogênico (Weber et al., 1985).

Diversos estudos vêm mostrando a expressão de GRPR nas mais variadas neoplasias. Já foi demonstrado a expressão de GRPR em câncer de próstata, renal, mama, ovário, pancreático, cólon, pulmão, cabeça e pescoço, gástrico, uterino, esôfago, gastrinoma, coloretal e neuroblastoma (revisado por Patel et al., 2006; Cornélio et al., 2007).

Além da expressão diferenciada dos tecidos normais e do efeito mitogênico, alguns trabalhos indicam envolvimento de GRPR no processo de metástase e angiogênese. Células do epitélio vascular responderam ao

estímulo com bombesina induzindo a ativação de MAPKs (Short et al., 1998). A estimulação de linhagens de neuroblastoma com bombesina aumenta a expressão da molécula-1 de adesão celular endotelial a plaquetas (PECAM-1, *platelet endothelial cell adhesion molecule-1*) e VEGF, enquanto que uso de um antagonista seletivo de GRPR, RC-3095, ou o silenciamento de GRPR atenua a angiogênese em neuroblastoma *in vivo*, inibindo significativamente a expressão de VEGF (Kang et al., 2007). Em câncer de pequenas células de pulmão um antagonista seletivo de GRPR, RC-3940-II, diminuiu os níveis de VEGF e VEGFR em camundongos xenografados (Kanashiro et al., 2007).

3.3.5.1 O GRPR em gliomas

Apesar de a expressão de GRPR já ter sido avaliada em quase todos os tipos tumorais, em gliomas essa expressão somente foi avaliada em linhagens celulares como de rato e algumas humanas como: U-373MG, U-87MG, D-247MG, U-118MG, U-251MG, D-245MG, U-105MG, D-54MG, A-172MG, and D-270MG e pediátricas SJ-S6 and SJ-G2 (Moody et al., 1989; Sharif et al., 1997).

Um análogo citotóxico da bombesina, AN-215, tem sido testado em modelos animais de camundongos *nude* implantados com linhagens humanas. No estudo de Szereday et al. (2002), com U-87, o agente citotóxico inibiu o volume tumoral em cerca de 69%. O efeito anti-tumoral de AN-215 pode ser bloqueado com pré-tratamento com excesso do antagonista da bombesina, indicando que o efeito citotóxico no análago é mediado pelo receptor. Nos animais implantados com U-138, houve uma diminuição nos níveis de VEGF e do volume tumoral em aproximadamente de 50% (Kanashiro et al., 2005a).

Neste mesmo modelo animal, RC-3940et, um antagonista seletivo de GRPR, diminui 72%, 28% e 35% o volume tumoral, os níveis de VEGF e a expressão de PKCα, respectivamente (Kanashiro et al., 2005b).

O RC-3095, um antagonista seletivo de GRPR, foi desenvolvido pelo grupo de Schally (Figura 5) e colaboradores e vem sendo testado como agente anti-tumoral para vários tipos tumorais e modelos animais. Em gliomas esse mesmo grupo demonstrou o efeito desse agente em camundongos *nudes* implantados com U-87 e com U-373, além do efeito *in vitro* com estimulação do receptor com o agonista, GRP. Animais implantados com U-87MG e animais implantados com U-373MG tratados com RC-3095 tiveram diminuição do volume tumoral 79 e 72% respectivamente (Pinski et al., 1994). Em outro estudo, animais tratados independentemente com RC-3095 e com RC-3940-II tiveram os volumes tumorais diminuídos em 60 e 74%, respectivamente, e tiveram os níveis de c-fos diminuídos em 30-40%. Em cultura de U-373, o GRP_{14-27} induziu com o efeito máximo em duas horas a expressão de mRNA dos fatores de transcrição e este aumento foi revertido quando as células foram pré-tratadas com RC-3095 (Kiaris et al., 1999).

Em relação à sinalização celular que o GRPR desencadeia em gliomas, foi observado que a estimulação do receptor promove o aumento da concentração de Ca^{++} intracelular, e ativa PKC e as MAPKs, ERK_1 e ERK_2 (Shartif et al., 1997; Kanashiro et al., 2005b).

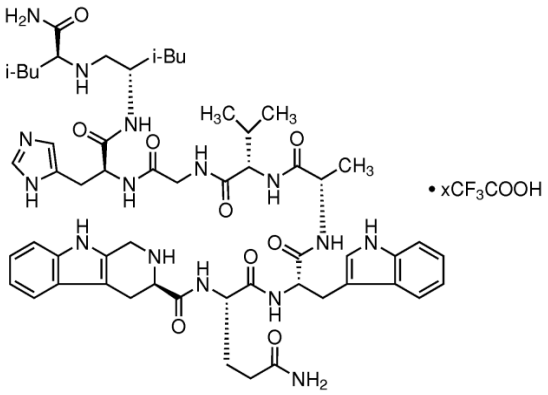

Figura 5. Estrutura química do RC-3095.

4. A sinalização celular de PI3K

A fosfatidilinositol-3-hidroxicinase (PI3K- do inglês, "*phosphatidylinositol-3-hydroxikinase*") é uma cinase pertencente a uma família que fosforila a posição três do anel de inositóis de membrana. PI3K regula vários procesos intracelulares como: proliferação, crescimento, apoptose e rearranjamento do citoesqueleto. Estas cinases são divididas em três classes baseadas em sua estrutura primária e especificidade ao substrato. A classe I contém um grupo de proteínas heterodiméricas consistindo de uma subunidade catalítica e uma regulatória. Essa classe fosforila fosfatidilinositol (PI – do inglês, *phosphatidylinositol*), fosfatidilinositol-4-monofosfato (PIP – do inglês, *phosphatidylinositol 4-phosphate*) e fosfatidilinositol-4,5-bifosfato (PIP_2 – do inglês, *phosphatidylinositol 4,5- trisphosphate*). A classe II consiste de três membros $PI3KC2_{\alpha}$, $PI3KC2_{\beta}$, $PI3KC2_{\gamma}$, essa classe fosforila PI e PIP. A classe III contém somente um membro, Vps34 que fosforila somente PI (Kong e Yamori, 2008).

A classe I é a mais estudada. Ela é dividida nas subclasses IA e IB, baseada em sua subunidade regulatória e o substrato ativado. A classe IA é

composta pela subunidade regulatória p85 e subunidade catalítica p110 que pode ser uma das três isoformas existentes em mamíferos: $p110_{\alpha}$, $p110_{\beta}$, $p110_{\delta}$ (conhecidas como: $PI3K_{\alpha}$, $PI3K_{\beta}$, $PI3K_{\delta}$). A subunidade regulatória liga-se a vários receptores de tirosina cinases para ativar a subunidade catalítica p110. Em adição, p110 pode ser ativada por se ligar diretamente a Ras. A classe IB corresponde a $PI3K_{\gamma}$ que é ativada diretamente pela subunidades βγ dos receptores acoplados à proteínas G (GPCRs). A $PI3K_{\gamma}$ é composta pela subunidade regulatória p101 e catalítica $p110_{\gamma}$. A $PI3K_{\alpha}$ e $PI3K_{\beta}$ são amplamente expressas, enquanto as expressões de $PI3K_{\delta}$ e $PI3K_{\gamma}$ parecem ser mais restritas. O regulador negativo da sinalização produzida por PI3K é a fosfatase PTEN que desfosforila PIP_3 em PIP_2 (revisado por Fan e Weiss, 2006; Kong e Yamori, 2008). A desregulação da via desencadeada por PI3K, assim como mutações em PTEN tem sido encontrada em vários tipos de câncer (Vivanco et al., 2002).

5. As Células-tronco em glioma

Evidências recentes sugerem que uma sub-população de células nos tumores, chamadas de células-tronco do câncer, podem dirigir o crescimento e a formação de metástases destes tumores e são caracterizadas por sua habilidade de iniciar e gerar um novo tumor**.** Essas células tanto geram clones de si próprias como geram células diferenciadas (Marx, 2007).

Em alguns tipos tumorais, têm sido identificada esta sub-população, com propriedades de tronco, por diferentes marcadores de superfície. Em tumores de cólon, as CT são positivas para e CD44 e/ou CD166 e CD133; câncer de mama positivas para CD24 e negativas para CD44; tumores de cabeça e

pescoço positivas para CD44; tumores pancreáticos positivas para CD44 e CD14 e para meduloblastomas e glioblastomas são positivas para CD133 (revisado por Cho e Clarke, 2008).

Há menos de uma década vem sendo identificada uma sub-população de células com características de CT em tumores do SNC. Uchida et al. (2000) isolaram CT de tecido cerebral de feto humano utilizando vários marcadores. Estas células foram fenotipicamente identificadas como $CD133^{+}$, $CD34^{-}$, $CD45^{-}$, $CD24^{-}$ e foram capazes de formar neuroesferas em culturas e gerar células diferenciadas.

Em 2003 Singh et al. identificaram CT em tumores cerebrais pediátricos por meio do marcador de superfície prominina-1, CD133. Em seguida, outro grupo isolou de glioblastomas multiformes de adultos uma sub-população de CT (Yuan et al., 2004). Várias linhagens tumorais apresentam uma sub-população de CT (Lee et al., 2005; Gal et al., 2007). Em C6, uma linhagem de glioma de rato, esta sub-população origina neurônios e células gliais (Kondo et al., 2004).

O CD133 é um membro da família de glicoproteínas de superfície celular, que possui cinco domínios trans-membranas, e para o qual a sua função ainda não foi esclarecida (Shmelkov et al., 2004). Tem sido sugerido que CD133 é um marcador específico para várias populações de CT e células progenitoras. Ele é expresso em células indiferenciadas de epitélio, retinoblastoma e teratocarcinomas, leucemias e tumores cerebrais (Shmelkov et al., 2004; Lee et al., 2005).

A sub-população de glioblastomas $CD133^{+}$ apresenta maior resistência aos quimioterápicos temozolamida, carboplatina, paclitaxel e etoposídeo, que a

sub-população $CD133^-$ (Liu et al., 2006). Marcadores de CT têm sido correlacionados com prognósticos e sobrevidas de pacientes. A expressão de nestina, um marcador de célula neuronal indiferenciada, é significativamente maior em pacientes com menor sobrevida (Strojnik et al., 2007).

Singh et al. (2004) mostraram que as CT são essenciais para a formação do tumor em modelo animal. Eles implantaram em camundongos *nude* 100 células $CD133^+$, originadas de gliomas de pacientes, e houve a formação de uma massa tumoral, enquanto que, nos animais onde eles implantaram 10^5 células $CD133^-$ não houve a formação do tumor.

Zeppernick et al. (2008) analisaram a expressão de CD133 em 95 gliomas de vários graus. Eles encontraram que tanto a proporção de células $CD133^+$ quanto à organização destas em agrupamentos (clusters; quando mais de cinco células vizinhas são positivas para CD133) foram significativas como fator de mau prognóstico em relação à sobrevida, independentemente do grau ou idade do paciente (Zeppernick et al., 2008). Os autores deste trabalho consideraram gliomas $CD133^+$ os tumores que apresentaram mais de 1% da população positiva. A proporção de células $CD133^+$ em gliomas é variável, mas em sua maioria é muito pequena cerca de 2% de toda a população de células do tumor.

Muitas linhagens humanas de gliomas apresentam em cultura morfologia de neuroesferas. Essas já foram identificadas e isoladas nas linhagens A172, U373 e U87 (Patrawala et al., 2005; Kang et al., 2006; Gal et al., 2007). Kang e colaboradores identificou uma sub-população de 0,05 a 2% em U87. Yu e colaboradores mostraram que a linhagem U87 crescendo sem soro fetal bovino e com alguns fatores de crescimento específicos crescem e enriquecem a

formação de neuroesferas. Essas neuroesferas foram positivas para CD133, para nestina e marcador de astrócito, GFAP (Yu et al., 2008).

Os estudos com a sub-população $CD133^{+}$ dos tumores são recentes e pouco é conhecido sobre a biologia destas células. Será que receptores de fatores de crescimento e genes que levam a proliferação celular são expressos diferentemente nesta população? O quanto os receptores que são superexpressos no câncer podem induzir a proliferação destas células? Quais vias de sinalização celulares são ativadas nestas células? Muito ainda tem a ser explorado sobre esta sub-população.

6.OBJETIVOS

6.1 OBJETIVO GERAL

Avaliar a expressão de GRPR em gliomas, sua influência no crescimento celular e a relação com vias de sinalização intracelular.

6.2 Objetivos específicos:

- Avaliar a expressão dos genes codificadores de GRP e seu receptor em linhagens tumorais humanas e de ratos;
- Avaliar a expressão protéica do GRPR em linhagens tumorais humanas e de ratos;
- Avaliar a expressão protéica do GRPR em amostras de tumores de pacientes provenientes de biopsias e em cérebro humano sadio;
- Avaliar se a expressão do GRPR está relacionada com a sobrevida do pacientes;
- Avaliar o efeito do agonista e antagonista do GRPR sobre a proliferação celular em linhagens tumorais humana e de rato;
- Avaliar se o efeito de indução da proliferação induzida pelo agonista é dependente de PI3K em linhagem de rato C6;
- Avaliar o efeito do agonista do GRPR sobre a formação de neuro-esferas em linhagem de glioma humano;
- Avaliar a expressão do marcador de células tronco, CD-133, em tumores de pacientes provenientes de biopsias;

Capítulo I

Gastrin-releasing peptide receptor signaling in cancer

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Gastrin-releasing peptide receptor signaling in cancer

Review Article

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Summary

The gastrin-releasing peptide (GRP), a mammalian homolog of the amphibian peptide bombesin (BB), is a major growth factor in many types of human cancer. The GRP receptor (GRPR) is overexpressed in different malignancies and its activation stimulates tumor growth. Thus, GRPR antagonists have been developed as candidate anticancer agents and shown to display antiproliferative activity in experimental cancer models. Here we review the cell signaling mechanisms associated with the GRPR in cancer cells, and discuss the implications for the development of novel targeted therapies.

I. Bombesin-like peptides and their receptors

Bombesin (BB) is one of the biologically active peptides purified from amphibian skin in 1971 by Anastasi and colleagues (Anastasi et al, 1971). The first mammalian BB-like peptide (BLP) was isolated from porcine gastric tissue and named gastrin-releasing peptide (GRP) because of its potent gastrin releasing action (McDonald et al, 1978; 1979). Thereafter in 1983 a novel BLP was identified in porcine spinal cord and named neuromedin B (NMB). BB is a 14-amino acid peptide while GRP and NMB contain 27 and 32-amino acid residues in the mature form respectively (reviewed in Ohki-Hamazaki et al, 2005; Patel et al, 2006; Cornelio et al., 2007; Gonzalez et al, 2008; Jensen et al, 2008). GRP and BB share a highly conserved 7-amino-acid COOH-terminal sequence, which is required for immunogenicity and for high-affinity binding to the GRP-preferring receptor (GRPR, BB2 receptor) (Chu et al, 1995; Jensen et al, 2008). BB and GRP act by activating the GRPR, while NMB has higher affinity for the NMB receptor (NMBR, BB1 receptor) (Sharif et al, 1997; Roesler et al., 2006a; Jensen et al, 2008). The GRPR gene encodes for a 384-amino-acid protein with a predicted molecular mass of 43 kDa, which demonstrates 48% identity with the mouse BRS-3 (the orphan BB receptor subtype 3) protein and 53% identity with the mouse NMBR protein. By DNA sequencing and Southern blot analyses, Weber and colleagues determined the protein-coding region as contained in three exons interrupted by two introns 20 and 2kb in length. Both the TTTAAA motif and the intact cAMP response element (CRE) are necessary for activation of transcription of mGRPR in Swiss 3T3 cells (Weber et al, 2000).

GRPR mediates a variety of cellular responses, including modulation of smooth muscle contraction, secretion of gastric acid, secretion of pancreatic enzymes, regulation of body temperature, stimulation of cell growth. GRP serves as a neurotransmitter in the central nervous system and has also been implicated in inflammatory

processes (Rozengurt et al, 1983; Battey et al, 1991; Lin et al, 2000; Fang et al, 2004; Dal-Pizzol et al, 2006; Cornelio et al, 2007; Oliveira et al, 2008; Roesler et al, 2006a; Weber, 2009). More recently Sakamoto et al. showed that pharmacological stimulation of spinal GRPRs restores penile reflexes in male rats (Sakamoto et al, 2008).

II. Signaling mechanisms associated with GRPR activation in cancer cells

GRP and BB have been shown to display mitogenic activities, increasing proliferation and growth of a variety of cellular types including lung (Weber et al, 1985), prostate (Hoosein et al, 1993; Nagakawa et al, 2001), breast (Bold et al, 1998), gastric (Bold et al, 1994), pancreatic (Avis et al, 1993), and colorectal cancers (Iishi et al, 1992), neuroblastoma (Ishola et al, 2007), and glioma (Pinski et al., 1994; Kiaris et al, 1999; Flores et al, 2008; de Oliveira et al., 2009). In addition, through competitive RT-PCR experiments in messenger RNA (mRNA) on fresh-frozen specimens as well as by immunohistochemistry in sections of paraffin-embebed tumors, aberrant expression of both GRP and GRPR has been reported in a variety of human malignancies (reviewed in Patel et al, 2006; Cornelio et al, 2007). Synthetic GRPR antagonists have been developed as candidate anticancer compounds, displaying antitumor activity both *in vitro* and *in vivo* in various murine and human tumors (Radulovic et al., 1991; Zhou et al, 2004; Schwartsmann et al, 2006).

A. Phospholipase C activation and Ca^{++} mobilization

The GRPR is a G protein-coupled receptor (GPCR). After agonist binding, GPCRs expose intracellular sites involved in the interaction with the G-protein heterotrimer, which contains α, β and γ subunits. The α subunits of G proteins heterotrimer are divided into four subfamilies: Gαs, Gαi, Gαq and Gα12 (Dorsam et al, 2007). From these subfamilies, GRPR seems to interact only with the Gαq and Gα12 subfamilies. Cells expressing GRPR catalyzed the activation of squid retinal Gαq and mouse Gαq but not bovine retinal Gαt or bovine brain Gαi/o (Jian et al, 1999). The *in situ* reconstitution of GRPR with purified G protein subunits shows explicitly that GRPR activates Gαq but not the pertussis toxin-sensitive Gαi/o or retinal specific Gαt (Gαt is a member of the subfamily Gαi) (Hellmich et al, 1997). In oocytes, GRPR showed to be coupled with $G\alpha_q$ and $G\alpha_{11}$, but not $G\alpha_{14}$ (Shapira et al, 1998). However, stimulation with BB induces responses in prostate cancer and Swiss 3T3 cells that are mediated by the Gα13 subunit, G (12) subfamily (Sinnett-Smith et al, 2000; Zheng et al, 2006). GRPR is likely to have specificity for two Gαq subfamily members ($G\alpha_q$ and $G\alpha_{11}$), that stimulate phosphoinositide hydrolysis by activating phospholipase C-β, and for one Gα12 subfamily member (Gα13) that activates Rho-GTP.

Two residues are critical for the coupling of GRPR to $G\alpha_q$, the GRPR residue (D98) at the extracellular boundary of transmembrane domain II and a conserved arginine residue (R309) near the extracellular boundary of transmembrane domain VII (Donohue et al, 1999). In addition, other two amino acids residues F(270) and N(281) at the sixth transmembrane segment of the mouse GRPR are important for receptor activation (Lin et al, 2000).

The phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol 4,5-biphosphate produces two second messengers: inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to its intracellular receptor, a ligand gated Ca^{++} channel located in the endoplasmic reticulum membrane, and triggers the release of Ca^{++} from internal stores. Piiper and co-workers showed that BB activates PLC-beta 1 and PLC-beta 3, in rat pancreatic acinar cells, and this activation is mediated by Gq/11 (Piiper et al, 1997).

In the last two decades several studies have shown that activation of BB receptors promotes an increase in cytosolic Ca^{++} and generation of inosotol phosphates. This has been shown in many animal and human cell lines and different tumor types. BB gradually increased cytoplasmic Ca^{++} concentration and generation of inosotol phosphates, but not in the absence of extracellular Ca^{++}, in C6 glioma cells (Wang et al, 1992; Takemura et al, 1999). GRP mobilizes Ca^{++} from a thapsigargin-sensitive intracellular Ca^{++} pool through activation of PLC in HIT-T15 cells (Karlsson et al, 1996). BB induces rapid calcium mobilization in both PC-3 (> 200% of baseline) and DU-145 (> 100% of baseline) cells, but not in LNCaP cells (Aprikian et al, 1996). BB administration leads to an immediate increase in free intracellular Ca^{++} concentration in the human pancreatic adenocarcinoma cell line HPAF (Burghardt et al, 2001). GRP treatment results in intracellular Ca^{++}, mobilization in two neuroblastoma cell lines (SK-N, LAN-1), and the specific GRPR antagonist BIM26226 blocked this effect (Kim et al, 2002). BB stimulated the release of intracellular Ca^{++} in adult (U-373MG, D-247MG, U118MG, U-251MG, D-245MG, U-105MG, D-54MG, A-172MG, and D-270MG lines) and pediatric (SJ-S6 and SJ-G2 lines) glioblastoma cell lines. BB and GRP showed similar potencies on stimulation of intracellular Ca^{++} in U-373MG cells (Sharif et al, 1997). BB-induced gastrin release from canine G cells is stimulated by Ca^{++} but not by protein kinase C (PKC), as well as by disruption of rho/cytoskeletal pathways (Seensalu et al, 1997). In PC-3 prostate cancer cells, BB enhances the Ca^{++} concentration cytokeratin expression and cytoskeleton morphology (Wu et al, 2007). The BB efficacy to increase the [^{3}H] inositol phosphates correlates with receptor number in mouse embryonic fibroblast cell line, BALB 3T3 (Tsuda et al, 1997).

B. PKC

PKC comprises a multigene family of related serine/threonine kinases that sits at the crossroads of many signal transduction pathways and is implicated in a wide

range of G protein-coupled receptor and other growth factor-dependent cellular responses. PKCs are modular enzymes comprised of a regulatory domain (that contains the membrane-targeting motifs that respond to lipid cofactors, and in the case of some PKCs calcium) and a relatively conserved catalytic domain that binds ATP and substrates. These enzymes are coexpressed and respond to similar stimulatory agonists in many cell types (Steinberg et al, 2008).

PKCs play a critical role in transducing GRPR signals into activation of protein kinase cascades (Rozengurt et al, 2002). This role of PKC stems from multiple lines of evidence in different systems, as shown in **Table 1**. GRPR activation seems to regulate the expression of PKCs and these enzymes seem to be important in the negative feedback, regulating downergulation of the GRPR. Several biological effects of GRP and BB, such as stimulation of secretion of chromogranin-A, neurotensin, cholecystokinin, and modulation of neuronal function, are mediated by PKC. GRPR antagonists cause a reduction in the expression of PKC isoforms alpha (α), eta (η), and zeta (ζ), and increase expression of the delta (δ) isoform, while BB or GRP significantly reduced the expression of beta II (βII) and delta (δ), but not of alpha (α) and eta (η) PKC isoforms (**Table 1**). PKCα mediates feedback inhibition of EGFR transactivation induced by BB. PKC inhibition enhanced EGFR transactivation in BB-stimulated Rat-1 fibroblasts stably transfected with the BB receptor. Transfection of siRNAs targeting PKCα also enhanced BB-induced EGFR tyrosine phosphorylation in Rat-1 cells (Santiskulvong and Rozengurt, 2007).

Table 1: Effects of gastrin-releasing peptide receptor (GRPR) activation on protein kinase C (PKC) isoforms in different biological systems

Agent/Treatment	PKC isoform	Effect	Biological system	Reference
BB	PKCα	↑ PKCα translocation to the membrane	Swiss 3T3 cells	Florin-Christensen et al, 1996
BB + siRNA	PKCα	↑ transactivation of EGFR	Rat-1 fibroblasts	Santiskulvong et al, 2007
GRPR antagonists	PKCα	↓ expression	U-118MG - glioma; PC-3 *in vivo*	Stangelberger et al, 2005
BB + inhibitor	PKCβ II	↓ expression	H-69 human small cell lung carcinoma	Kanashiro et al, 2004
BB + inhibitor	PKCδ	↓ expression	H-69 human small cell lung carcinoma	Kanashiro et al, 2004
BB + inhibitor	PKCα	no ↓ expression	H-69 human small cell lung carcinoma	Kanashiro et al, 2004
BB + inhibitor	PKCη	no ↓ expression	H-69 human small cell lung carcinoma	Kanashiro et al, 2004
GRPR antagonists	PKCη	↓ expression	PC-3 human prostate	Stangelberger et al, 2005
GRPR antagonists	PKCζ	↓ expression	PC-3 human prostate	Stangelberger et al, 2005
GRPR antagonists	PKCδ	↑ expression	PC-3 human prostate	Stangelberger et al, 2005

BB + inhibitor	PKCα	↓ BB-mediated neurotensin secretion	BON/GRP-R cells	Li et al, 2002
BB + inhibitor	PKCδ	↓ BB-mediated neurotensin secretion	BON/GRP-R cells	Li et al, 2002
BB + inhibitor	general	↓ cholecystokinin release	STC-1 cell line - the mouse intestinal	Takahashi et al, 2000
BB + inhibitor	general	no secretion of chromogranin-A	BON/GRP-R cell line	Hellmich et al, 1999
BB + inhibitor	general	↓ BB-mediated AP-1 gene induction	Human gastric cancer SIIA	Kim et al, 2000
BB + inhibitor	PKCα	↓ BB-mediated neurotensin secretion	BON/GRP-R cells	Li et al, 2002

Although PKCs have a role in GRPR downstream signaling, its involvement in receptor phosphorylation is not clear. Benya and colleagues suggested that activation of the distal carboxyl-terminal region of GRPR, that selectively replaced the Ser and Thr of the protein kinase C consensus sequence (PKC-CS), is essential for chronic desensitization and down-regulation of this receptor (Benya et al, 1995). Some studies found that short exposures to 12-O-tetradecanoylphorbol 13-acetate (TPA) caused desensitization of BB-induced inositol phosphate generation without diminishing ^{125}I-Tyr^{4}-BB binding, implying that PKC can cause acute desensitization without reducing GRPR density on the cell surface (Brown et al, 1987; Swope and Schonbrunn, 1990). Other studies showed that inhibition of PKC prevented phorbol ester- but not BB-induced desensitization, suggesting that PKC activation may not be a physiologically relevant mechanism for agonist-induced acute desensitization of the GRPR and raising the possibility that other kinases may be involved in acute desensitization of GRPR (Frankel and Viallet, 1993; Walsh et al, 1993). Kroog and colleagues (1995) showed that GRPR is rapidly phosphorylated on serine and threonine residues in response to agonist exposure and this phosphorylation is not caused by PKC (Kroog et al, 1995). The same authors have demonstrated that an intact carboxyl terminal domain (CTD) is required for GRPR phosphorylation by either agonist stimulation or activation of PKC, but acute desensitization processes are independent of CTD because desensitization can precede ligand binding in intact GRPR and in the mutated receptor. However, GRPR is phosphorylated in vitro by GPCR kinase2 (GRK2) and multiple PKC isoforms and PKC phosphorylation reduces GRPR-Gq coupling by approximately 80% in vitro. They conclude that PKC may be activated by any number of stimuli, all of which may result in impaired GRPR signal transduction. GRPR-stimulated PKC activation may play a role in GRPR desensitization (Ally et al, 2003). In addition, the activation of protein kinase D (PKD) by BB can depend or not of PKC (Jacamo et al, 2008). However, the PKC activation is not essential for BB to mediate the activation of praline-rich tyrosine kinase (Pyk2), its association with Src kinases that critically depends on the elevation of Ca^{++} (Roelle et al, 2008).

C. PKD

PKD is a family of serine/threonine protein kinases that is classified as a subfamily of the Ca^{2+}/calmodulin-dependent kinase (CaMK) superfamily. Three members of the PKD family have been identified so far: PKD1 (which includes mouse PKD and its human homolog PKCμ), PKD2, and PKD3 (originally named PKCn) (Wang, 2006). PKD structure include an N-terminal regulatory region containing a tandem repeat of cysteine-rich zinc finger-like motifs that confers high affinity binding to phorbol esters and DAG. PKD not only is a direct DAG target, but also lies downstream of PKCs in novel signal transduction pathway that is increasingly implicated in regulation of multiple fundamental biological process, including membrane trafficking (Rozengurt et al, 2005; Rozengurt, 2007).

BB rapidly induces PKD activation in Swiss 3T3 cells, in Rat-1 cells stably transfected with GRPR and in COS-7 cells transiently co-transfected with PKD and GRPR. BB activates PKD through a PKC-dependent signal transduction pathway in Swiss 3T3 (see **Table 2**) (Zugaza et al, 1997; Rey et al, 2001b). BB induces a rapidly reversible plasma membrane translocation of green fluorescent protein-tagged PKD, an event that can be divided into two distinct mechanistic steps. The first step, which is exclusively mediated by the cysteine-rich domain in the N terminus of

PKD, involved its translocation from the cytosol to the plasma membrane. The second step, the rapid reverse translocation of PKD from the plasma membrane to the cytosol, requires its catalytic domain and surprisingly PKC activity (Rey et al, 2001b). BB induces a transient redistribution of PKD from the cytoplasm to the nucleus of quiescent cells (Rey et al, 2001a). BB induced activation PKD3 in COS-7 cells co-transfected with PKD3 and Gα13 or Gα12 and with a constitutively active Rac, but not when co-transfected with active Rho, Cdc42, or Ras. The PDK3 activation by Rac was dependent of PKC (Yuan et al, 2006). A recent study has shown that BB induced PKD activation mediated by early PKC-dependent and late PKC-independent mechanisms. PKC-dependent phosphorylation of the activation loop ser711 and Ser748 is the primary mechanism involved in early phase PKD activation, whereas PKD autophosphorylation on Ser748 is a major mechanism contributing to the late phase of PKD activation (Jacamo et al, 2008). Sinnett-Smith and colleagues demonstrated that PKD2 facilitates mitogenesis and supports the hypothesis that an increase in the duration of the extracellular signal-regulated kinase (ERK) signaling leading to accumulation of immediate gene products is one of the mechanisms by which isoforms of the PKD family enhance re-initiation of DNA synthesis by BB activation (Sinnett-Smith et al, 2007; 2009).

Table 2. Effects of gastrin-releasing peptide receptor (GRPR) activation on protein kinase D (PKD) isoforms in different biological systems

Agent/Treatment	Effect	Biological system	PKC-dependent	Reference
BB	PDK activation (14-fold above)	Swiss 3T3 cells	yes	Zugaza et al, 1997
BB	PKD translocation from the membrane	Swiss 3T3 cells	no	Rey et al, 2001b
BB	PKD translocation to the cytosol	Swiss 3T3 cells	yes	Rey et al, 2001b
BB	PKD translocation to the nucleus	fibroblasts and epithelial cells	yes	Rey et al., 2001a
BB and/or Rac	PDK3 activation	COS-7 cells	yes but Rac dependent	Yuan et al, 2006
BB	PDK activation	Swiss 3T3 cells	yes (early) or no (late)	Sinnett-Smith et al, 2007; 2009

D. PKA

Protein kinase A (PKA) involvement in GRPR-activated signaling remains unclear. Some studies have shown that BB or GRP do not alter cAMP levels. It seems, however, that BB or GRP might exert synergic effects with activators of PKA signaling (Benya et al, 1994; Farias et al, 2008). The GRPR is not directly coupled to Gαs or Gαi proteins, as discussed above, but when these dimers are affected by other activator they might act synergically with GRPR to activate downstream cascades. The effect of BB stimulation in Swiss 3T3 cells preincubated with forskolin is different of BALB/3T3 fibroblasts transfected with the coding sequence for the GRPR. In the Swiss 3T3 cells, with native receptor, there is a 9-fold increase in cAMP levels, but not in the cells with transfected receptor (Benya et al, 1994). Our group has demonstrated that, in human gliobastoma U-138 cells, GRP acts synergistically with agents that stimulate the cAMP/PKA pathway to promote cell proliferation (Farias et al, 2008). The inhibition of PKA blocked increases in c-fos levels and [^{3}H]thymidine incorporation induced by BB in Swiss 3T3 cells (Benya et al, 1994). The inhibition of proliferation produced by GRPR blockade was associated with a decrease of cAMP levels in human pancreatic adenocarcinoma cells (Qin et al, 1995). GRP did not stimulate increases in cAMP in mouse, rat or guinea pig pancreatic acini or in SCLC cells alone, either with IBMX or forskolin, while in mouse Swiss 3T3 cells TPA significantly increased cAMP stimulated by BB or GRP (Garcia et al, 1997).

Our group has demonstrated that the BB-induced regulation of brain function in the rat hippocampus was blocked by an otherwise ineffective dose of a PKA inhibitor and potentiated by activators of the PKA pathway (Roesler et al, 2006b). We suggested that the Ca^{2+} signal from GRPR might synergistically potentiate the dopamine D_1R-triggered cAMP elevation when the two receptors were stimulated simultaneously (Roesler et al, 2006b). Chan and Wong showed that cAMP serves as a suppressor of BB-mediated c-jun N-terminal kinase (JNK) activation in green monkey kidney fibroblast, COS-7 cells (Chan and Wong, 2005). However, neither GRP nor BB cause increases in cAMP levels in C6 rat glioma cells and human pancreatic adenocarcinoma cells (Wang et al, 1992; Burghardt et al, 2001). BB-mediated activation of the protein-1 (AP-1) gene induction is not dependent on PKA in human gastric cancer. Moreover, pretreatment of these cells with PKA inhibitor exhibited only minimal effects on the induction of c-jun and jun-B mRNAs in response to BB (Kim et al, 2000).

It is possible that activation of adenylate cyclase is not carried through GRPR, but when it is activated it contributes

to an increase in cellular responses to GRPR activation. Chinnappan and colleagues showed recently that in gastrointestinal cancer, CRE binding protein (CREB) is a critical regulator of human GRPR expression and might be activated through different upstream intracellular pathways (Chinnappan et al, 2008). This suggests a mechanism for synergistic effects between GRPR agonists and agents, which stimulate the cAMP/PKA pathway. CREB can be stimulated through the cAMP/PKA pathway, which in turn increases the GRPR expression. Moreover, cAMP signaling might be synergistically potentiated by Ca^{++} induced by BB-stimulation leading to increased activation of PKA (Wong et al, 1999; Kim et al, 2000; Roesler et al, 2006b).

E. Pyk2

Roelle and colleagues have suggested that BB mediated the activation of Pyk2 and its association with Src kinases. This activation depends on the elevation of Ca^{++}, but is independent of PKC (Roelle et al, 2008).

F. Src

Src is the most widely studied member of the largest family of nonreceptor protein tyrosine kinases, known as the Src family kinases (SFKs). Other SFK members include Lyn, Fyn, Lck, Hck, Fgr, Blk, Yrk, and Yes (Finn, 2008). BB stimulates phosphorylation of Src in Swiss 3T3 cells through a mechanism that does not depend on either PKC or Ca^{++} (Rodriguez-Fernández and Rozenburg, 1998). BB induces growth of prostate cancer LNCaP cells, which requires BB-induced activation of androgen receptors (AR). Src kinase is critical for BB-induced AR-mediated activity and is required for translocation and transactivation of AR (Desai et al, 2006). Cell proliferation and invasion processes induced by GRP in head and neck squamous cell carcinomas depend on Src activity. Activation of Src by GRP was more pronounced than other kinases of the Src family and was higher following treatment with GRP than EGF (Zhang et al, 2004). Src does not contribute to BB induced focal adhesion kinase (FAK) activation in Swiss 3T3 cells (Rodriguez-Fernández and Rozenburg, 1998). Src kinase is an important mediator in BB-induced EGFR transactivation, as demonstrated in prostate cancer cells, lung carcinoma cells, and squamous cell carcinoma of head and neck (Xiao et al, 2003; Zhang et al, 2004; 2006). GRP stimulates Src-activation, followed by extracellular release of the EGFR ligand, amphiregulin and TGF-α leading to the activation of EGFR (Zhang et al, 2004; 2006; Liu et al, 2007). Zhang and colleagues have suggested that after GRPR stimulation Src is activated, leading to downstream induction of phosphatidylinositol 3-kinase (PI3K) (Zhang et al, 2006). After PI3K activation, PDK1 could phosphorylate TNF-α converting enzyme (TACE) leading to translocation of TACE and EGFR proligand cleavage and subsequent EGFR phosphorylation. In addition, the phosphorylation of TACE may result in formation of a functional complex of Src kinases contribute to GRP-induce EGFR phosphorylation.

G. EGFR transactivation

GRP stimulation leads to EGFR activation in many cells types. EGFR ligands, TGF-α and heparin binding-EGF (HB-EGF), are present on the membrane surface as proligands. Cleavage of these proligands releases activated ligands into the extracellular milieu. Preincubation of neutralizing antibodies with EGFR ligands, TGF-α, HB-EGF, in a cell line of non-small cell lung carcinoma (NSCLC), decreased GRP-mediated mitogen-activated protein kinase (MAPK) activation, in the cell lysates and analyzed by immunoblotting. TGF-α neutralizing antibody had no effect on EGF stimulation of MAPK. These results imply that, upon GRP stimulation, both EGFR proligands, TGF-α and HB-EGF, are cleaved from the membrane surface and released into the extracellular milieu. Once released from the membrane surface, the ligands bind to the extracellular domain of EGFR in an autocrine fashion, activating the receptor. In addition, pretreatment with matrix metalloproteinase (MMP) inhibitors abrogated GRPR-mediated, but not EGFR-mediated, MAPK activation. This suggests that upon GRP stimulation of NSCLC cells, MMPs are activated and cleave the EGFR proligands from the membrane surface to facilitate EGFR activation (Thomas et al, 2005).

GRP induced rapid phosphorylation of EGFR and failed to stimulate proliferation in EGFR-deficient cells or with specific inhibition of EGFR in head and neck squamous cell carcinomas (Lui et al, 2003). Zhang and colleagues have shown that the EGFR phosphorylation of by GRP is dependent of Src, PI3K, TACE and release amphiregulin, suggesting that a signaling cascade including GRP-Src-PI3K-PDK1-TACE-amphiregulin-EGFR and TACE phosphorylation may result in the formation of a functional complex of Src and TACE on the membrane (Zhang et al, 2004; 2006). Liu and colleagues showed that GRP leads to EGFR stimulation through Src and amphiregulin release in lung carcinoma (Liu et al, 2007). Prenzel and colleagues showed that EGFR transactivation upon GPCR stimulation involves proHB-EGF and a MMP activity that is rapidly induced upon GPCR-ligand interaction (Prenzel et al, 1999). These authors have shown that inhibition of proHB-EGF processing blocks GPCR-induced EGFR transactivation and downstream signals. In addition in COS-7 cells transfected with VSV-tagged proHB-EGF together with BB receptor, stimulation by BB led to a rapid breakdown of the growth factor precursor, proHB-EGF, and proteolytic cleavage was concomitant with the appearance of the 9K VSV-tagged HB-EGF fragments containing the transmembrane anchor. Yan and colleagues showed that ADAM-10, an integral membrane metalloprotease also known as Kuzbanian, is a mediator of BB-induced EGFR transactivation (Yan et al, 2002). Upon stimulation of BB receptors, KUZ increases the docking and activation of adaptors Src homology 2 domain–containing protein and Gab1 on the EGFR, and activation of Ras and ERK. In contrast, blocking endogenous KUZ by morpholino antisense oligonucleotides, suppresses the

transactivation. The effect of KUZ on shedding of HB-EGF and consequent transactivation of the EGFR depends on its metalloprotease activity (Yan et al, 2002). In primary head and neck cancer tumors, GRPR expression correlated with TGF-α and EGFR expression raising the possibility of transactivation of EGFR by GRPR through TGF-α (Seethala et al, 2008). In prostate cancer cells, the process of cellular migration induced by BB is mediated by transactivation of EGFR (Madarame et al, 2003).

Zhang and colleagues investigated the effects of a GRPR antagonist (PD176252) in conjunction with an EGFR tyrosine kinase inhibitor (erlotinib) (Zhang et al, 2007). They showed that combining GRPR and EGFR pathways blockade caused a more pronounced effect inhibitory on proliferation, invasion, and colony formation and decreased phospho-EGFR, but not normal mucosal epithelial cell proliferation. The increased IC_{50} values of the EGFR and GRPR antagonists in the normal mucosa epithelial cells may be due to the lack of a requirement in these cells for EGFR or GRPR expression. The results of Santiskulvong and colleagues indicated that a functional EGFR pathway is necessary for the stimulation of cell cycle progression induced by BB in both Rat-1 cells and Swiss 3T3 cells. In addition, they demonstrated that four structurally different inhibitors of EGFR tyrosine kinase decreased DNA synthesis induced by the GRPR receptor (Santiskulvong et al, 2004). Treatment with RC-3940-II, GRPR antagonist, in NSCLC cell lines xenografted into nude mice resulted in significantly decreased protein expression of members of the EGFR/HER family (Kanashiro et al, 2007). The GRPR antagonist RC-3095 induced a prolonged decrease in levels and mRNA expression of EGFR in mice, rats and hamsters with MXT mouse mammary cancers (Szepeshazi et al, 1997). RC-3095 enhanced the effect of luteinizing hormone-releasing hormone antagonist Cetrorelix on diminishing EGFR expression, in animals xenografted with ES-2 human ovarian cancer (Chatzistamou et al, 2001). Either PKC inhibitors or transfection of siRNAs targeting PKCalpha increased EGFR tyrosine phosphorylation induced by BB in Rat-1 cells, supporting the hypothesis that conventional PKC isoforms, especially PKCα, mediate feedback inhibition of GPCR-induced EGFR transactivation (Santiskulvong et al, 2007).

H. PI3K

The PI3K pathway regulates various cellular processes, such as proliferation, growth, apoptosis and cytoskeletal rearrangement. Several components of the PI3K/AKT pathway are deregulated in a wide spectrum of human cancers (Vivanco et al, 2002). Few studies have shown mutations in the genes that encode the catalytic subunit, PIK3CA, and the regulatory subunit, PIK3R1, while PTEN (phosphatase and tension homolog deleted on chromosome 10, the counterpart of PI3K) mutation or silencing has been reported in many tumors including glioblastoma, ovarian, breast, endometrial, hepatocellular carcinoma, melanoma, digestive tract, lung and others (Wang et al, 1997; Zhang et al, 2008). More recently a study has shown the involvement of the PI3K/AKT pathway in the transduction of GPCR initiated signals. This activation can be direct or mediated by transactivation of receptor tyrosine kinases, class IA (Jia et al, 2009).

Transfection with a dominant negative mutant of p85 regulatory subunit of PI3K did not affect BB-stimulated cell proliferation in human colorectal carcinoma cell line, but inhibited BB-stimulated cell migration (Patel et al, 2004). GRP and BB increased phosphorylation of AKT and GSK-3β in neuroblastoma cell lines and the blockade of the GRPR, while the antagonist GRP-H2756 or GRPR siRNA attenuated the effect of BB (Ishola et al, 2007). Neuroblastoma cells transfected with siRNA directed to the GRPR (siGRPR) decreased AKT phosphorilation. BB promotes AKT phosphorylation *in vivo* in an experimental neuroblastoma model (Kang et al, 2007). GRP-mediated ERK1/2 activation and proliferation was reduced with use of a PI3K inhibitor (wortmannin) in the insulin secreting cell line (Böcker and Verspohl, 2001). RC-3040-II, GRPR antagonist, reduces protein levels of pAKT in non-smal-cell lung cancer xenografted into nude mice (Hohla et al, 2007). We have recently shown that GRPR regulates proliferation of C6 glioma cells through a PI3K-dependent mechanism (Flores et al, 2008). Liu and colleagues also showed that GRP stimulates AKT activation in NSCLC primarily through Src-activation, followed by extracellular release of the EGFR ligand, amphiregulin, leading to the activation of EGFR and PI3K cells in NSCLC cells (Liu et al, 2007). PI3K is required for GRP-induced TACE phosphorylation and EGFR ligand release in head and neck carcinoma cells (Zhang et al, 2007). In addition, neuroblastoma cells over-expressing GRPR present a decreased gene and protein expression of PTEN (Qiao et al, 2005). PTEN is a non-redundant negative regulator of the PI3K/Akt pathway, acting by dephosphorylating position 3 of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) (Salmena et al, 2008). These studies suggest that PI3K can be act both upstream and downstream of EGFR phosphorylation by GRPR.

PIP3 corecruits PDK1 and AKT to the membrane, resulting in the phosphorylation and activation of AKT by PDK1. The mammalian target of rapamycin (mTOR) is wired to the PI3K pathway through the tuberous sclerosis proteins TSC1 (hamartin) and TSC2 (tuberin). TSC1 and TSC2 act as a heterodimer that negatively regulates mTOR signaling. TSC2 is phosphorylated and functionally inactivated by AKT (Wullschleger et al, 2006). mTOR is a branch downstream of the PI3K/AKT pathway important in regulation of translation and cell growth (Manning, 2004). Neuroblastoma cell line transfected with siGRPR decreased m-TOR phosphorylation (Kang et al, 2007), suggesting that GRPR-initiated signals impinge not only on the proliferation and survival branches of the PI3K/AKT cascade but also on the metabolism and translation control branch of this important signaling.

I. GTPases

The GTPases are a large family of monomeric proteins. In mammals, there are over 60 GTPases, which fall into five major groups: Ras, Rho, Rab, Arf e Ran.

1. Ras family

Rap1 belongs to the superfamily Ras, sharing about 50% homology with Ras and a similar effector domain (Bos et al, 1997; Posern et al, 1998). BB increases activity of endogenous Rap1 in NIH3T3 fibroblast cells. In contrast, the activity of endogenous Ras was not affected by BB, despite increased MAPK/ERK phosphorylation and proliferation (Posern et al, 1998). In Swiss 3T3 fibroblast cells BB did not significantly activate Ras or Raf-1 above basal levels (Mitchell et al, 1995). The GRPR antagonist RC-3040-II reduced the protein levels of K-Ras in H460 NSCLC cells xenografted into nude mice (Hohla et al, 2007). BB increased the proportion of p21-Ras in the GTP bound (active) form from 28 to 43% in Rat-1 cells. Interestingly, cells treated with EGF the proportion of active Ras was increased from 28 to 61% by BB (Charlesworth and Rozengurt, 1997).

2. Rho family

Twenty members of the Rho family have been described so far and these can be further subdivided according to their sequence and function. Rho, Rac and Cdc42 are the three best-characterized members of the family. There are three isoforms of Rho (A, B, C) and Rac (1,2,3). The subgroups include proteins that are most similar to RhoA, those that are most similar to Rac1 and CDC42, and those that lack GTPase activity (Etienne-Manneville and Hall, 2002; Sahai et al, 2002). BB-stimulated PC cell migration via G alpha 13 subunit leads to the activation of RhoA, and Rho-associated coiled-coil forming protein kinase (ROCK). In addition, focal adhesion kinase, which activates PDZ-RhoGEF and LARG, is required for BB-stimulated RhoA activation (Zheng et al, 2006). In Isreco1 colon carcinoma cells, BB stimulates invasion and migration by Rho-dependent manner (Saurin et al., 2002). Of notice, the induction of the activation PKD3 in COS-7 cells co-transfected with PKD3 and Gα13 or Gα12, is mediated by Rac (Yuan et al., 2001; 2006).

J. Paxillin and FAK

BB induced rapid and coordinated tyrosine phosphorylation of a set of focal adhesion proteins, including the nonreceptor tyrosine kinase p125Fak, the adaptor proteins p130cas and paxillin, and this is critically dependent on the integrity of the actin cytoskeleton as well as on a functional Rho (Rozengurt, 1998). This stimulation could be dissociated from PKC activation and the mobilization of Ca^{++} from intracellular stores and presence of Src kinases, but was completely blocked by treatment with the actin polymerization blocker cytochalasin D or by placing the cells in suspension, suggesting that FAK activation requires an intact actin cytoskeleton (Zachary et al, 1993; Seufferlein et al, 1996; Taglia et al, 2007). In human colon cancer, GRP mediates morphogenic properties by upregulating intracellular adhesion protein-1 (ICAM-1) via FAK (Guo et al, 2001). The motility in prostate cancer caused by BB was reduced by FAK inhibitors (Lacoste et al, 2005). The tyrosine phosphorylation of FAK and paxillin induced by BB is not required for EGFR transactivation (Salazar et al, 2003).

K. MAPK

GRPR-induced MAPK activation has been evaluated in a variety of human malignancies. The majority of the studies have examined BB involvement in MAPK/ ERK activation, but some have shown that BB also regulates the JNK and/or p38. BB and GRP stimulated the activation of ERK-1 and -2 in many tumors and cell types as shown in **Table 3**. The activation of ERK-1/2 was dependent on EGFR activation in some tumors (Lui et al, 2003; Thomas et al, 2005).

The regulation of secretion chromogranin-A by GRPR in the BON/GRPR cell line is dependent on activation of mitogen-activated extracellular-regulated kinase (MEK), the kinase upstream of ERK. MEK activity is important for maintaining elevated Ca^{++} levels induced by GRPR activation (Hellmich et al, 1999). In addition, it remains unclear whether the ERK activation by BB is mediated depends on PKC. ERK-2 phosphorylation by BB-stimulation in Swiss 3T3 fibroblasts was decrease by over 90% after down-regulation of PKC induced by prolonged exposure to PMA (Pang et al, 1993). However in other studies using Rat-1 cells and the insulin secreting cell line, the increase in ERK-1/2 phosphorylation mediated by BB or GRP was not influenced by the inhibition of PKC (Charlesworth and Rozengurt, 1997; Böcker and Verspohl, 2001). These studies suggest that a novel PKC-independent pathway might be involved in the action of Gq-coupled receptors and imply that these receptors have the potential to activate ERK1/2 through multiple pathways. GRP-mediated effects on MAPK were reduced in the presence of PI3K inhibitor (wortmannin) in the insulin secreting cell line. Hence, it is possible that ERK1/2-activation by GRP can depend or not on PKC.

Studies using animal models have shown activation of MAPK by GRP stimulation. The GRPR antagonist RC-3940-II decreases the expression and protein levels of pERK1/2 in prostate carcinomas xenografted into nude mice as well as in cells cultured *in vitro* (Stangelberger et al, 2005; Hohla et al, 2007). BB stimuled the activation of $p38^{MAPK}$, but not JNK in intestinal epithelial cells (Guo et al, 2001). However BB induced JNK activation in Cos-7 cells (Zachary et al, 1993; Chan et al, 2004). Zhang and colleagues showed that phosphorylated JNK and p38 level were decreased upon combined targeting of GRPR and EGFR when compared with treatment with either agent alone, in head and neck squamous cell carcinoma (Zhang et al, 2007).

Table 3. Effects of gastrin-releasing peptide receptor (GRPR) activation on mitogen-activated protein kinase (MAPK) isoforms in different biological systems

Agent/Treatment	Effect	Biological system	Reference
BB	↑ pERK 1 /2	Intestinal epithelial cells	Guo et al, 2001
BB	↑ pERK 1 /2	Lung cancer cells	Thomas et al, 2005
BB	↑ pERK 1 /2	Head and neck squamous cell carcinomas	Lui et al, 2003
BB	↑ pERK 1 /2	Insulin secreting cell line	Böcker and Verspohl, 2001
BB	↑ pERK 1 /2	U-373 cell line (glioblastoma)	Sharif et al, 1997
BB	↑ pMEK	BON/GRP-R cell line (chromogranin-A secretion)	Hellmich et al, 1999
BB	↑ pERK 1 /2	Rat-1 cells (rodent fibroblasts)	Charlesworth and Rozergurt, 1997
BB	↑ pERK 1 /2	Swiss 3T3 fibroblasts	Wang et al, 1997
RC-3040 II	↓ pERK 1 /2	Prostate carcinoma model	Stangelberger et al, 2005
RC-3040 II	↓ pERK 1 /2	Small cell lung carcinoma, *in vivo* and *in vitro*	Kanashiro et al, 2004

L. Cyclooxigenase 2

BB stimulated an increase of cyclooxygenase-2 (COX-2) expression and activity in RIE/GRPR cells, and this increase was dependent on Ca^{++}-induced activation of MAPK dependent pathways and increased expression and activation of the AP-1 transcription factor (Guo et al, 2001). BB stimulated the expression of COX-2 m-RNA and protein, resulting in enhanced release of prostaglandin E2 in human colon adenocarcinoma Caco-2 cells (Corral et al, 2007). Accordingly, the GRPR antagonist, RC-3040-II reduced the levels of COX-2 protein in H460 NSCLC cells xenografted into nude mice (Hohla et al, 2007).

M. Transcription factors and apoptotic agents

A cytotoxic analog of BB reduce the levels of Bcl-2 while the levels of Bax protein were significantly increased in U-118MG human glioblastomas xenografed into nude mice (Kanashiro et al, 2005). Smad protein is a transcription factor that acts in cooperation with other transcription factors to regulate the expression of target genes that are involved with cell growth. GRP treatment decreased Smad DNA binding activity in neuroblastoma cells leading to effect anti-apoptotic and cell growth (Qiao et al, 2006).

Transcription factors activated by the MAPK pathway, such as, c-fos, c-jun and c-myc are induced by BB or GRP. BB stimulated c-fos, c-myc and high mobility group protein IY (HMG-I(Y)) expression and these effects were blocked by a GRPR antagonist, but not by an NMBR antagonist (Burghardt et al, 2001). BB induced increase in the expression of c-jun and jun-B at 1 h after treatment in SHA cells (Kim et al, 2000). The GRPR antagonists RC-3095 and RC-3940-II decreased by 30-45% the level of m-RNA of c-fos in animal model of gliomas. In addition, GRP induced the expression of c-fos and c-jun mRNA in U-373 and U-87 gliomas xenografts (Kiaris et al, 1999). In animal models of prostate cancers DU-145 and PC-3 the level of c-fos was not affected, but there was a tendency for the inhibition of jun mRNA expression in both tumour models treated with RC-3940-II (Stangelberger et al, 2005). The GRPR antagonists RC-3095 and RC-3940-II decreased the expression of the ErbB-2_HER-2 oncoprotein and c- jun and c- fos oncogenes, leading to inhibition of MDA-MB-435 estrogen-independent breast cancer growth (Bajo et al., 2002). BB induced a striking accumulation of c-fos protein in Swiss 3T3 cells overexpressing PKD2 as compared with cultures of control cells (Sinnett-Smith et al, 2007). BB also showed to stimulate AP-1 gene expression in SHA human gastric cancer. This induction was mediated by both PKC and protein tyrosine kinase-dependent signal transduction pathways, with only minimal involvement of PKA (Kim et al, 2000).

N. Angiogenic factors

RC-3095 and RC-3940-II inhibit the experession of proangiogenic factors in human experimental breast cancers. These GRPR antagonists decreased the expression of mRNA, protein, and secretion into the medium of basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF)-II, and vascular endothelial growth factor (VEGF) in oestrogen-independent breast carcinoma cell lines (Bajo et al, 2004). In addition, a GRPR antagonist reduced NGF secretion in HT-29 human colon cancer cells (Farias et al., 2009). A cytotoxic analog of BB reduced the levels of VEGF by 45% in U-118MG human glioblastomas xenografed into nude mice (Kanashiro et al, 2005). BB significantly increased expression of angiogenic markers, platelet endothelial cell adhesion molecule (PECAM)-1 and VEGF, whereas RC-3095 attenuated this effect, in an animal model of neuroblastoma (Kang et al, 2007). Levine and colleagues observed that treatment with GRP stimulated pro-angiogenic factors NF-$\kappa\beta$, IL-8 and VEGF in prostate cancer (Levine et al, 2003). The GRPR antagonist RC-3940-II decreased IGF-II expression in PC-3 human prostate cancer (Plonowski et al, 2000). Heuser et al. analyzed the effects of GRP receptor blockade on neoangiogenesis in renal cell carcinoma. The neoangiogenesis was measured by intravital microscopy in mice implanted with A498 spheroids. They found GRPR expression in implanted tumor cell spheroids

and spheroid microvessels, and spheroid neoangiogenesis was significantly inhibited by RC-3095 (Heuser et al, 2005). RC-3940-II decreased expression of MMP-2 isoforms, but did not affect MMP-9 in the MDA-MB-435 cell line (Bajo et al, 2004).

GRPR was expressed in vessels of the various classes of ovarian tumors. Generally, malignant tumors had a higher incidence of GRPR positive vessels compared to their benign counterparts. The prevalence of such vessels was particularly high in ovarian carcinomas (16/19) and their metastases (5/5) (Fleischmann et al, 2007). The increase of pro-angiogenic markers induced by GRP and the expression of GRPR in peritumor vessels indicate that the GRPR is involved in angiogenesis.

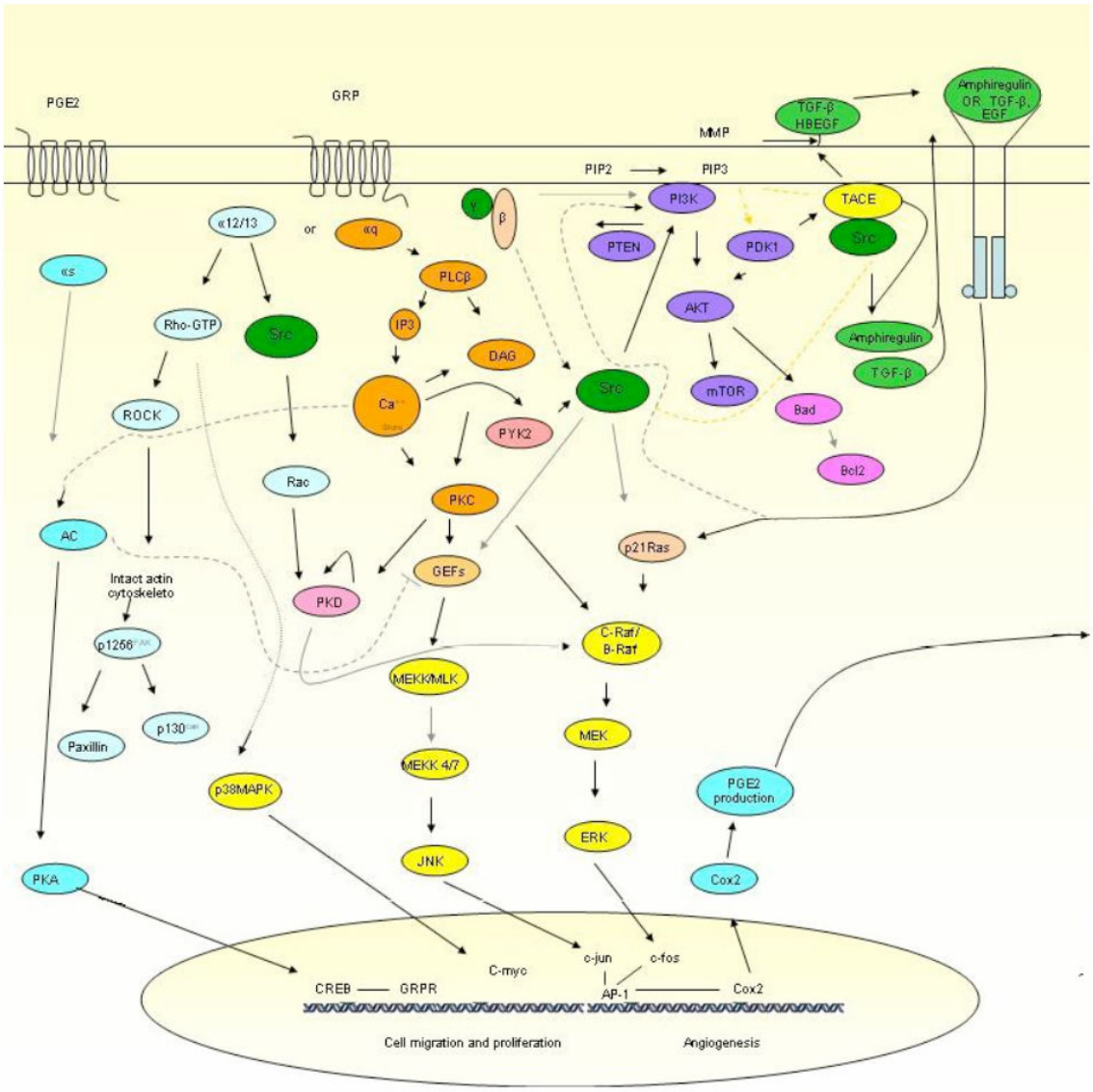

Figure 1: Signaling pathways associated with the gastrin-releasing peptide receptor (GRPR) in cancer. Gray lines indicate possible functional interactions. Black lines indicate established pathways activated upon GRPR stimulation

III. Concluding remarks

Due to its high expression in many cancers, the GRPR is a major target for novel anticancer therapies and a better understanding of GRPR signaling mechanism can contribute to the development of more efficient strategies. GRPR interacts with Gαq and Gα12 G protein subfamilies. Gαq activation leads to an increase of cytoplasmic Ca^{++} concentration and generation of inositol phosphate and DAG. Ca^{++} and DAG in turn activate PKC. The MAPKs can be activated by PKC, and/or by downstream EGFR transactivation. In addition, ERK activation can be sustained by PKD activation through a PKC-independent pathway. Pyk2 is also dependent of Ca^{++} elevation and can be associated with Src, which can be activated by the βγ subunit of G proteins. Src mediates MMP activity and release of EGFR ligands. The activation of Src leads to downstream induction of PI3K. After PI3K activation PDK1 can phosphorylate TACE. TACE phosphorylation may result in its translocation to its targets or formation of a functional complex of Src and TACE on the membrane, and subsequent the EGFR ligand release. PI3K mediates the proliferation and migration induced by BB and GRP. In addition, it is not clear whether PI3K is an upstream or downstream event to EGFR transactivation. PI3K activation induces subsequent activation of Akt and mTOR. The α12/13 subunit of G protein activates Rho, FAK and adaptador protein p130cas and paxilin, but this subunit seems not to mediate EGFR transactivation. A diagram of relevant signaling pathways associated with the GRPR in cancer cells is shown in **Figure 1**.

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Capítulo II

Gastrin-releasing peptide receptors regulate proliferation of C6 glioma cells through a phosphatidylinositol 3-kinase-dependent mechanism

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Gastrin-Releasing Peptide Receptors Regulate Proliferation of C6 Glioma Cells through a Phosphatidylinositol 3-Kinase-Dependent Mechanism

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Abstract: Gastrin-releasing peptide (GRP) has been proposed as a major growth factor in brain tumors, and GRP receptor (GRPR) antagonists show antiproliferative effects in experimental gliomas. However, the underlying molecular events downstream of GRPR activation remain poorly understood. In the present study, we examined the role of the GRPR in regulating proliferation of glioma cells *in vitro* and its possible interaction with the phosphatidylinositol 3-kinase (PI3K) signaling pathway. Expression of GRPR mRNA and protein in C6, U-87MG, and U-373MG glioma cells was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry. Proliferation of C6 and U-87MG, but not U-373MG cells was significantly inhibited by the GRPR antagonist RC-3095, whereas the GRPR agonist bombesin (BB) significantly enhanced proliferation of C6 cells. The BB-induced stimulatory effect on cell proliferation was prevented by either RC-3095 or the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002. Our results provide the first evidence that the GRPR regulates proliferation of C6 glioma cells and suggest that PI3K is required for GRPR-mediated stimulation of glioma growth.

Key Words: Bombesin-like peptides, gastrin-releasing peptide receptor, RC-3095, phosphoinositide 3-kinase, glioma, brain tumor.

INTRODUCTION

Gliomas are the most common primary malignant brain tumors and are classified into four clinical grades according to their histological appearance. Surgical resection and radiotherapy are the standard of care, with an increasing role for adjuvant chemotherapy. However, the prognosis for gliomas remains poor despite optimal clinical treatment, and traditional chemotherapy for these cancers has a marginal impact on patient survival (reviewed in Prados and Levin, 2000; Carpentier, 2005; Norden and Wen, 2006; Schwartzbaum *et al*., 2006). Thus, the development of novel, molecularly targeted therapeutic agents for the treatment of gliomas presents a major current challenge in oncology (Rich and Bigner, 2004; Mullholland *et al*., 2005).

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Molecular targets for the development of novel potential anticancer agents include growth factor receptors. A number of growth factor signaling pathways is abnormally activated in cancers, giving tumor cells a survival advantage over surrounding non-neoplastic tissues (Rich and Bigner, 2004; Sanson *et al*., 2004). Gastrin-releasing peptide (GRP), a mammalian bombesin (BB)-like peptide, has emerged as a major autocrine growth factor involved in the development of a variety of human cancers, and aberrant expression of both GRP and its receptor (GRPR, also known as BB2 receptor) has been reported in many types of tumors (reviewed in Patel *et al*., 2006; Cornelio *et al*., 2007; Gonzalez *et al*., 2008; Jensen *et al*., 2008). GRPR expression and stimulatory actions on mitogenesis have been characterized in human glioma cell lines (Moody *et al*., 1989; Staley *et al*., 1993; Pinski *et al*., 1994; Sharif, 1997; 1998). The evidence indicating that GRP and the GRPR play a role in cancer growth has led to the development of selective GRPR antagonists as potential targeted anticancer drugs (Zhou *et al*., 2004; Cornelio *et al*., 2007). Such synthetic GRPR antagonists include [D-Tpi^{6}, Leu^{13} psi(CH_2NH)-Leu^{14}] bombesin (6-14) (RC-3095), developed by Schally and colleagues and evalu-

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ated as a potential anticancer drug in a variety of experimental cancer models (Radulovic *et al*., 1991; Pinski *et al*., 1994; Szepeshazi *et al*., 1997).

The intracellular signaling mechanisms underlying GRP-induced cell proliferation in gliomas remain unclear. Evidence indicates that GRPR is coupled to the G_q family of G proteins, which directly activates the phospholipase C (PLC)/ protein kinase C (PKC) pathway. Intracellular responses to GRPR activation involve an increase in $[Ca^{2+}]$ and PKC activation, which, in turn activates the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) cascade (Hellmich *et al*., 1999; Kim *et al*., 2000; Xiao *et al*., 2003; Stangelberger *et al*., 2005). More recently, studies have suggested that phosphatidylinositol 3-kinase (PI3K) is critically involved in mediating GRP-triggered responses (Ishola *et al*., 2007; Liu *et al*., 2007). PI3K regulates cancer cell growth by inducing phosphorylation of its downstream effector Akt, and the PI3K/Akt pathway has emerged as a major target for the development of novel candidate anticancer agents (reviewed in Vivanco and Sawyers, 2002). PI3K activity is often upregulated in brain tumors, and loss of function of the tumor suppressor gene phosphatase tensin homolog on chromosome ten (PTEN) also frequently contributes to upregulation of PI3K/Akt (Castellino and Durden, 2007; Merlo, 2003; Newton, 2004). It has been shown that PI3K is required for GRP-mediated cell cycle progression in neuroblastoma cells (Ishola *et al*., 2007), as well as for GRP-induced Akt phosphorylation in non-small cell lung carcinoma (NSCLC) cells (Liu *et al*., 2007). However, previous studies have not examined whether PI3K is involved in mediating GRPR-triggered growth in gliomas.

In the present study, we examined GRPR mRNA and protein expression and the effects of GRPR blockade on cell proliferation in three glioma cell lines. We also verified whether GRPR-mediated stimulation of cell proliferation depends on PI3K activity in C6 rat glioma cells.

METHODS

Cell Culture

The rat C6, human U-87MG, and human U-373MG cell lines were obtained from American Type Culture Collection (Rockville, Maryland, USA). The cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Carlsbad, USA) containing 2.5 mg/ml (C6) or 0.1 % (U-87MG and U-373MG) Fungizone® and 100 U/l gentamicin, and supplemented with 10% (C6) or 15% (U-87MG and U-373MG) (v/v) fetal bovine serum (FBS; Sorali, Campo Grande, Brazil). Cells were kept at a temperature of 37 °C, a minimum relative humidity of 95% and an atmosphere of 5% CO_2 in air.

Cell Proliferation Assay and Treatments

For the experiment examining the effects of GRPR antagonism on cell proliferation, cells were seeded in 24-well plates at densities of 1 X 10^4 (C6) or 2 X 10^4 (U-87MG and U-373MG) cells/well in DMEM/10% FBS (C6) or DMEM/ 15% FBS (U-87MG) into 24-well plates and allowed to grow for 24 h. the medium was replaced and RC-3095 (0.01, 0.1, 1.0, or 10 μM; Zentaris GmbH, Frankfurt, Germany) was added to the culture. Cell counting was carried out either 24 or 48 h after treatment. The medium was removed, cells were washed with Hank's Balanced Salt Solution (HBSS; Invitrogen, São Paulo, Brazil) and 100 μl of 0.25% trypsin/EDTA solution was added to detach the cells, which were counted immediately in a hemocytometer.

For experiments examining the effects of BB in cells pretreated or not with RC-3095 or the PI3K inhibitor LY-294002, C6 cells were seeded in 24-well plates at a density of 2.0 x 10^4 cells/wel. Cells grew for 24 h in DMEN with 5% FBS, and quiescence was induced by reducing FBS to 0.5% for 24 h. The cells were treated with BB (0.1, 1, 10, or 100 nM), then after 48 h the medium was removed, cells were washed with HBSS and 70 μl of 0.25/% trypsin/EDTA solution was added to detach the cells, which were counted immediately with trypan blue in a hemocytometer. To evaluate whether BB-induced effects depend on the GRPR and PI3K, the cells were treated with RC-3095 (0.1 μM) or LY294002 (20 μM) for 20 min prior to stimulation with BB (100 nM). RC-3095 was dissolved in 4% dimethylsulphoxide (DMSO; Sigma) in saline (NaCl 0.9%), and LY294002 was dissolved DMSO to a final medium concentration of 0.5%. A control experiment showed that 4% DMSO by itself did not affect cell proliferation (data not shown). Cell counting was carried out as described above 48 h after treatments. All experiments were performed at least 3 times with 4–7 replicates for each drug concentration.

RT-PCR Analysis of GRPR mRNA Expression

Total RNA was extracted from C6, U-87MG, and U-373MG cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and RNA concentration was determined spectrophotometrically. For the RT reaction, primer poli-T 0,5 μg was mixed with 5 μg of RNA and H_2O miliQ to a total volume of 14 μl, for 5 minutes at 70ºC. After the addition of 5 μl enzyme buffer, 5 μl of dNTP, and 1 μl of MMLV-RT (Promega, Madison, USA) the misture was heated at 42ºC for 60 min.

GRPR mRNA was amplified using primers 5'-GTCAA GTCCATGCGAAACG-3' (nucleotides 855-873, Genbank sequence NM_005314), and 5'-GGGTGTCTGTCTTCACA CT-3' (reverse and complement of nucleotides 1027-1045) was used as a hybridization probe. The GRPR probes represent a 190-bp c-DNA fragment of GRPR. For each sample 2μL of enzyme buffer 10X, 0.4μL dNTPs, 0.2μL Taq polimerase (Promega), 0.6μL $MgCl_2$ 50mM, 1μL cDNA, 0.9 μL primers (F+R), and 14.8 μl H_2O milliQ were used. Amplification consisted of 1 minute at 95°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and extension of primers at 72°C for 45 s. The products were then held at 72°C for 10 minutes for DNA extensions to occur. A 10-μl aliquot of the RT-PCR reaction was analyzed through by gel eletrophoresis through a 2% agarose gel, and the products were visualized by staining with ethidium bromide. GRPR mRNA bands of approximately 190 bp were visualized by ultraviolet illumination.

Immunohistochemistry

Expression of the GRPR protein in C6, U-87MG, and U-373MG was accessed by immunohistochemistry. The

primary antibody used was a rabbit polyclonal antibody against GRPR (OPA1-15619, Affinity Bioreagents, Golden, CO, USA), corresponding to the second extracellular loop of GRPR.

Cells were seeded in flasks of 25 cm³. The cells grown until confluency and were then detached with a trypsin/EDTA solution. After centrifugation, the cell pellet was resuspended in 3 ml formol and embedded into paraffin wax.

Four-µm-thick sections were mounted on organosilane-coated slides and dried overnight at 37°C. Sections were deparaffinized in stove, rehydrated in graded alcohols, and washed with distilled water. The procedure to antigenic recuperation was performed in the microwave, the inactivation of the endogenous peroxidase through immersion in hydrogen peroxide and blocking cross-reaction with normal serum. The primary antibody diluted in solution (1:50) was incubated for 12 hours, at 4°C, followed by an application of the complex biotin streptavidin-biotin-peroxidase (LSAB, Dako) and the revelation with diaminobenzidine tetrahidroclore (Kit DAB, Dako). Cells were lightly counterstained with hematoxylin-eosin as a control.

Statistics

Data for cell proliferation are shown as mean ± SEM. Differences between mean values were evaluated by one-way analysis of variance (ANOVA) followed by least multiple comparisons using the least significant difference test when appropriate. In all comparisons, $p < 0.05$ was considered to indicate statistical significance.

RESULTS

RT-PCR Detection of GRPR mRNA in C6, U-87MG, and U-373MG Glioma Cells

We first aimed to confirm GRPR expression in C6, U-87MG, and U-373MG glioma cells. RT-PCR analyses demonstrated that all three cell lines express mRNA for GRPR. A transcript size of 190 bp, representing a fragment of the GRPR, was identified in all three cell lines (Fig. **1**).

Immunohistochemical Detection of GRPR in C6, U-87MG, and U-373MG Glioma Cells

GRPR protein expression in C6, U-87MG, and U-373MG was analyzed by immunohistochemistry using a synthetic rabbit polyclonal antibody against GRPR corresponding to the second extracellular loop of GRPR. The results confirmed that C6, U-87MG, and U-373MG cells express GRPR (Fig. **2**).

The GRPR Antagonist RC-3095 Inhibits Proliferation of C6 and U-87MG, but not U-373MG Glioma Cells

Few studies have evaluated the antiproliferative effects of different concentrations of GRPR antagonists in cancer cells *in vitro* in the absence of stimulation by exogenous GRP or BB. We thus verified whether a GRPR antagonist could affect proliferation of C6, U-87MG and U-373MG glioma cells. The proliferation of C6 glioma cells *in vitro* was significantly inhibited 48 h, but not 24 h after treatment, by RC-3095 at 0.1 µM (Fig. **3**). The number of C6 cells was decreased by about 45% 48 h after treatment with RC-3095 at 0.1 µM compared to cells not exposed to this antagonist ($p < 0.05$). Lower or higher concentrations of RC-3095 did not induce alteration in the number of C6 cells 48 h after treatment (RC-3095 at 0.01 µM *versus* control, $p = 1.00$; RC-3095 at 1.0 µM *versus* control, $p = 1.00$; RC-3095 at 10 µM *versus* control, $p = 0.69$) (Fig. **3B**). RC-3095 did not significantly affect cell proliferation 24 h after treatment, although the comparison of number of cells among groups by ANOVA fell short of significance ($F_{(4,20)} = 2.44$, $p = 0.08$) (Fig. **3A**).

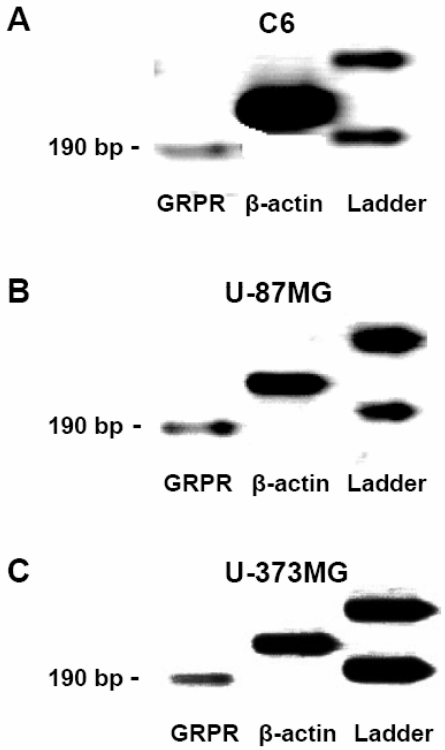

Fig. (1). Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of gastrin-releasing peptide receptor (GRPR) mRNA expression in (**A**) C6, (**B**) U-87MG, and (**C**) U-373MG glioma cells. RNA was extracted from rat C6, human U-87MG and human U-373MG cells and RT-PCR analysis was performed. A transcript size of 190 bp, representing a fragment of the GRPR, was identified in all three cell lines.

RC-3095 significantly inhibited the proliferation of U-87MG cells at either 0.1 or 1.0 µM 48 h, but not 24 h after treatment (Fig. **3**). The number of U-87MG cells was significantly reduced 48 h after exposure to RC-3095 at 0.1 (24% decrease, $p < 0.01$), 1.0 (26% decrease, $p < 0.01$), or 10 µM (19% decrease, $p < 0.05$) as compared to control cells, whereas the lower dose of RC-3095 did not affect proliferation ($p = 0.97$ compared to controls) (Fig. **3B**). Proliferation of U-87MG cells 24 h after treatment was not affected by RC-3095 ($F_{(4, 30)} = 0.97$, $p = 0.44$) (Fig. **3A**).

In contrast to C6 and U-87MG cells, the proliferation of U-373MG glioma cells was not affected by RC-3095 (Fig. **3**). There was no significant difference in cell number among

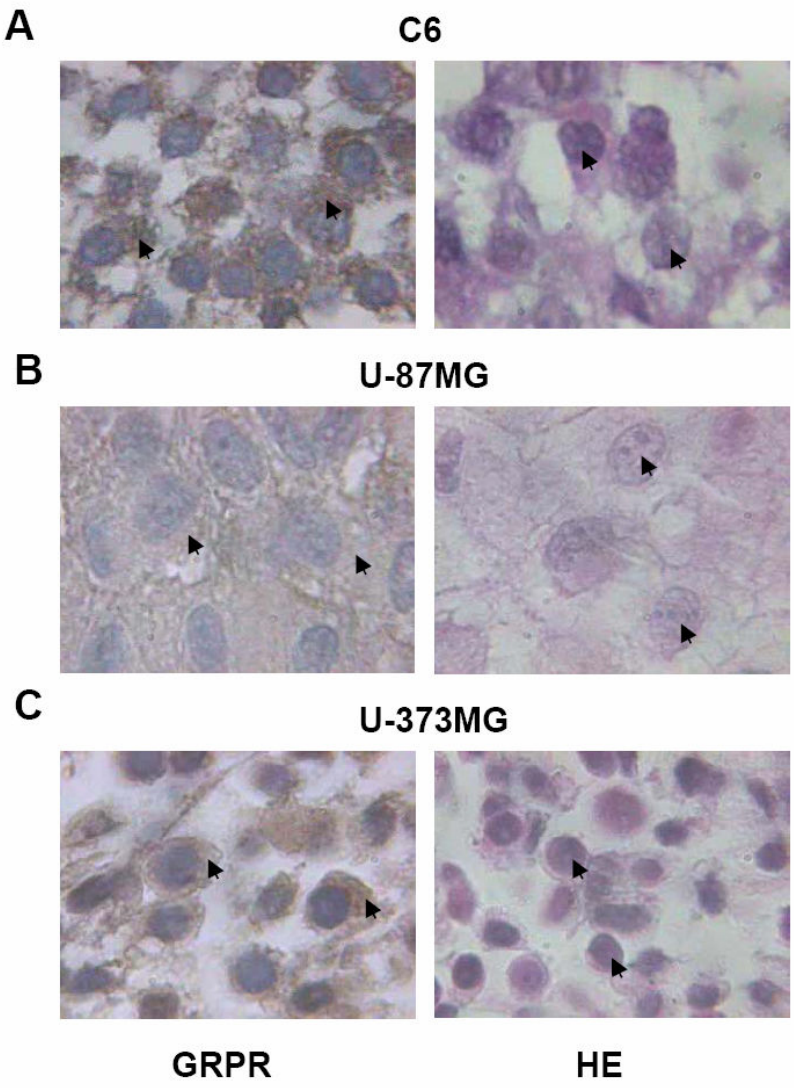

Fig. (2). Immunohistochemical analysis of gastrin-releasing peptide receptor (GRPR) expression in (**A**) C6, (**B**) U-87MG, and (**C**) U-373MG glioma cells. Sections were incubated with anti-GRPR antibody, sequentially treated with biotinylated anti-rabbit IgG and streptavidin-biotin-peroxidase solution, and then developed with diaminobenzidine as chromogen (*brown*/left panels, GRPR expression on the membranes of two cells of each line are indicated by arrowheads, x 1,000). Cell nuclei were lightly counterstained with hematoxilin-eosin (HE) as a control (*blue*/right panels, two cells of each line are indicated by arrowheads, x 1,000).

groups at either 24 ($F(4,25) = 0.20$, $p = 0.94$) (Fig. **3A**) or 48 h ($F_{(4,15)} = 0.86$, $p = 0.51$) (Fig. **3B**) after treatment.

Bombesin-Induced Stimulation of C6 Glioma Cell Growth is Prevented by GRPR Blockade

We decided to focus on the C6 cell line for further experiments because it is an increasingly important cell line for experimental studies on gliomas (Benda *et al*., 1968; Barth, 1998; Grobben *et al*., 2002; Lind *et al*., 2006), which, nevertheless, has not featured in previous studies on the involvement of the GRPR in proliferation. This rat glioma transformed cell line consistently mimics pathological features of human glioblastoma when implanted in rats and has been widely used as a model in studies on the biology of brain tumors (Barth, 1998; Grobben *et al*., 2002; Lind *et al*., 2006). The proliferation of C6 glioma cells *in vitro* was significantly increased by BB at 10 (34% increase, $p < 0.05$ compared to control cells) or 100 nM (50% increase, $p < 0.01$ compared to control cells) 48 h after treatment (Fig. **4A**). RC-3095 did not have an effect by itself ($p = 0.45$ compared to controls), but prevented the BB-induced increase in cell proliferation ($p = 0.37$, comparison between cells treated with RC-3095 plus bombesin *versus* control cells) (Fig. **4B**).

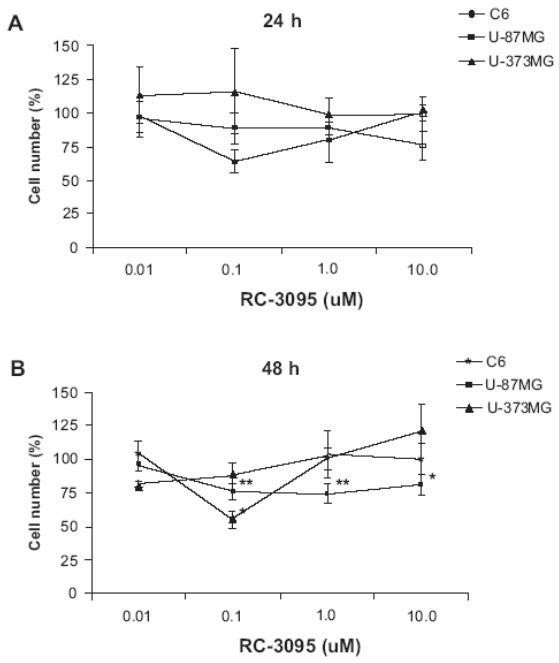

Fig. (3). Influence of the gastrin-releasing peptide receptor (GRPR) antagonist RC-3095 on proliferation of C6, U-87MG, and U-373MG glioma cells. Data are expressed as mean ± SEM percentage of the number of cells found (**A**) 24 or (**B**) 48 h after treatment with RC-3095 (0.01, 0.1, 1.0, or 10.0 μM) compared to the number of cells found with fetal bovine serum (FBS) alone, which was accepted as 100%; n = 4–7 replicates; * $p < 0.05$ and ** $p < 0.01$ compared to control cells.

Bombesin-Induced Stimulation of C6 Glioma Cell Growth is Prevented by PI3K Inhibition

Previous studies have not examined whether PI3K is required for GRPR-mediated glioma cell growth. The BB-induced increase in C6 cell proliferation was prevented by the PI3K inhibitor LY294002 (Fig. **5**). Proliferation was increased by about 106 % by BB ($p < 0.01$ compared to control cells treated with DMSO alone). Treatment with LY294002 by itself did not significantly affected cell proliferation, although the comparisons of number of cells between cells treated with LY294002 and controls fell short of significance (comparisons between control cells and cells treated with LY294002 alone or LY294002 plus BB, both ps = 0.06).

DISCUSSION

GRPR antagonists have previously been shown to be effective in inhibiting the growth of human U-87MG and U-373MG gliomas xenografted into nude mice (Pinski *et al.*, 1994; Kiaris *et al.*, 1999). Nevertheless, previous studies have not examined GRPR expression and the effects of GRPR antagonism in the C6 rat cell line, and relatively few studies have evaluated the antiproliferative effects of different concentrations of GRPR antagonists in cancer cells *in vitro* in the absence of stimulation by exogenous GRPR agonists. Moreover, previous studies have not examined whether GRPR-triggered stimulation of glioma growth requires the PI3K/Akt pathway. We thus decided to examine the possible antiproliferative effects of RC-3095 in C6 cells *in vitro*, and compare these effects with those observed in U-87MG and

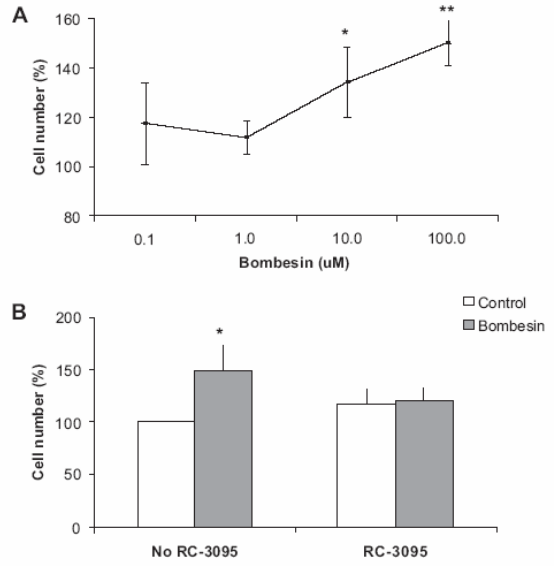

Fig. (4). Bombesin (BB)-induced stimulation of proliferation of C6 glioma cells is prevented by pretreatment with the gastrin-releasing peptide receptor (GRPR) antagonist RC-3095. (**A**) Cells were treated with BB (0.1, 1, 10, or 100 nM); (**B**) Cells were pretreated with RC-3095 (0.1 μM) for 20 min alone or prior to stimulation with BB (100 nM). Data are expressed as mean ± SEM percentage of the number of cells found 48 h after treatment compared to the number of cells found with fetal bovine serum (FBS) alone, which was accepted as 100%; n = 3-4 replicates; * $p < 0.05$ and ** $p < 0.01$ compared to control cells.

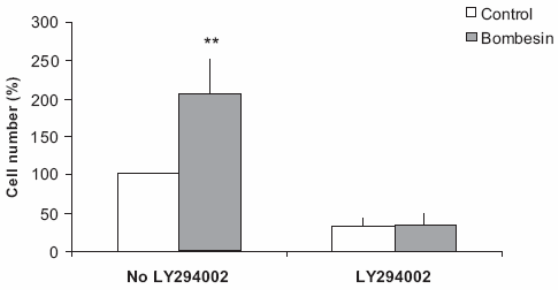

Fig. (5). Bombesin (BB)-induced stimulation of proliferation of C6 glioma cells is prevented by pretreatment with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002. Cells were pretreated with LY294002 (20 μM) for 20 min alone or prior to stimulation with BB (100 nM). Data are expressed as mean ± SEM percentage of the number of cells found 48 h after treatment compared to the number of cells found with fetal bovine serum (FBS) alone, which was accepted as 100%; n = 3 replicates; ** $p < 0.01$ compared to control cells.

U-373MG, two human glioma cell lines previously featured in *in vitro* experiments on RC-3095 and cell proliferation (Pinski *et al*., 1994). We then went on to verify whether BB would affect proliferation of C6 cells, and whether the BB-induced increase in proliferation of C6 cells would be prevented by GRPR antagonism and PI3K inhibition.

Our findings that C6 glioma cells express GRPRs and RC-3095 significantly inhibits, whereas BB enhances C6 proliferation demonstrate the usefulness of the C6 cell line in experimental studies on the biological effects and anticancer activities of GRPR antagonists. The lack of effect of RC-3095 at the concentrations used in the present study on proliferation of U-373MG cells indicate that GRPR antagonists induce differential effects in different glioma cell lines. Also, the lack of effect of RC-3095 in U-373MG cells is overall consistent with previous evidence that RC-3095 inhibits U-373MG proliferation *in vitro* at lower concentrations compared to those effective in inhibiting proliferation of U-87MG cells, but not at higher doses (Pinski *et al*., 1994). However, in contrast to our results, Pinski *et al*., (1994) found a 21% decrease in the number of U-373MG cells exposed to RC-3095 at 0.01 μM *in vitro* compared to controls. Differences in experimental procedures (e.g., interval between drug treatment and cell counting) may explain this discrepancy between the two studies. Also, it should be pointed out that our finding that RC-3095 inhibited proliferation of C6 and U-87MG cells in the absence of stimulation by addition of exogenous GRP or BB suggests that endogenous GRP plays a role in promoting proliferation of those cells lines. However, previous experiments have failed to detect GRP expression in U-87MG cells (Kiaris *et al*., 1999).

In both C6 and U-87 cells, intermediate concentrations of RC-3095 decreased cell proliferation, whereas lower and higher doses were ineffective. A similar dose-response pattern for the effects of RC-3095 on proliferation of U-87 and U-373 cells was cursorily observed by Pinski *et al*., (1994). Moreover, in some of our previous *in vivo* studies on the effects of systemic or intracerebral administration of RC-3095 in rodent models of brain function, we have found that intermediate doses of RC-3095 induced significant biological effects whereas high doses were ineffective or induced opposite effects (Roesler *et al*., 2003; 2004; Dantas *et al*., 2006). Together, these findings provide a consistent body of evidence suggesting that high doses of GRPR antagonists might display low efficacy. It has been proposed that the lack of effect of high doses of GRPR antagonists on cancer cell proliferation might be related to an intrinsic agonist activity of these compounds on the GRPR (Casanueva *et al*., 1996). This is a crucial issue because it raises the possibility that in a clinical setting low doses of GRPR antagonists could be more beneficial than high doses in patients with cancer. A phase I trial of RC-3095 in cancer has been recently carried out by Schwartsmann and colleagues (2006), and larger trials are now warranted. The findings from preclinical studies reporting differential effects of low and high doses of GRPR antagonists should be taken into account when designing such clinical trials.

Conversely to the inhibitory effect of RC-3095 on proliferation, BB induced a significant stimulation of proliferation of C6 cells. The effect of BB was prevented by an otherwise ineffective dose of RC-3095, suggesting that it was mediated by GRPR activation. The stimulatory effect of BB was also completely prevented in cells pretreated with a PI3K inhibitor, suggesting that the PI3K/Akt signaling pathway is required for GRPR to promote mitogenesis. Although recent studies have indicated that PI3K is importantly involved in mediating GRP-induced cell growth in neuroblastoma cells (Ishola *et al*., 2007) and inducing Akt phosphorylation in non-small cell lung carcinoma (NSCLC), the present study provides the first evidence for the involvement of PI3K in GRPR-stimulated growth in glioma cells. Other possible molecular mechanisms mediating the antiproliferative actions of RC-3095 in gliomas include drug-induced down-regulation of receptors for epidermal growth factor (EGF) (Pinski *et al*., 1993; Halmos and Schally, 1997; Szepeshazi *et al*., 1997; Koppan *et al*., 1998), decreased expression of c-fos and c-jun oncogenes (Bajo *et al*., 2002; Kiaris *et al*., 1999; Ishola *et al*., 2007), and inhibition of proangiogenic factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and insulin-like growth factors (IGFs) (Bajo *et al*., 2004).

Investigating the role of PI3K in mediating cellular responses to growth factors is particularly important in gliomas because the PTEN gene is altered by somatic mutations in many malignant gliomas. PTEN is a tumor suppressor gene that leads to inhibition of the PI3K/Akt pathway. Inactivation of PTEN in gliomas can lead to uncontrolled cell proliferation, escape from apoptosis, and aberrant angiogenesis (Castellino and Durden, 2007; Knobbe *et al*., 2002; Merlo, 2003). Our results suggest that GRPR inhibition should be further investigated as a potential strategy to develop novel targeted molecular therapies aimed at inhibiting the PI3K/Akt pathway in PTEN mutant glioma cells.

Overall, our results are consistent with the view that GRPR antagonists could be potentially useful for the treatment of malignant gliomas, and novel therapies targeted at the GRPR may prove an effective strategy to inhibit intracellular signaling pathways such as the PI3K cascade in gliomas.

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Capítulo III

Gastrin-releasing peptide receptor expression in gliomas

Manuscrito submetido à Cancer Science

Cancer Science

Report

TUMOR BIOLOGY AND PATHOLOGY

Gastrin-releasing peptide receptor expression in gliomas

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The gastrin-releasing peptide receptor (GRPR) has been put forward as a therapeutic target in brain tumors. Here we evaluated GRPR expression in specimens from patients as well as in normal human brain samples. Sections of paraffin-embedded brain tumors and non-neoplasic control brain tissue were analyzed with immunohistochemistry for GRPR expression. Digital image analysis revealed that 100% of glioma samples showed GRPR expression, with a mean index of 4972 pixels. In normal brain tissue, GRPR was expressed in neurons, but not glial cells. This study is the first to confirm GRPR expression in human glioma specimens and the normal human brain.

Malignant tumors intrinsic to the central nervous system (CNS) are among the most difficult neoplasms to treat effectively. The survival rate may range from a few months to several years, which, together with the poor prognosis, points to the need for new independent prognostic factors that may enable better treatment modalities of patients with higher risk of death.[1-2] Gastrin-releasing peptide (GRP) is a mammalian bombesin-like peptide that regulates many biological responses through the activation of the GRP receptor (GRPR). Biological functions influenced by the GRPR include modulation of smooth muscle contraction, secretion of gastric acid, regulation of body temperature, glucose intake, secretion of neuropeptides and hormones and regulation of synaptic transmission.[3,4] GRPR has also been linked to development of human cancer: it is highly expressed in many tumor types, and GRPR agonists (bombesin or GRP) promote cancer cell proliferation. GRPR expression has been shown in prostate cancer, gastrinoma, breast, ovarian, pancreatic, colon, renal, lung, head and neck, neuroblastoma, esophagenal, GI-carcinoid, gastric, and uterine cancers.[5,6] In gliomas, GRPR expression has been demonstrated to date only in cell lines, such as U-87, U138, 373, C6, and A172.[7-9] Several studies have evaluated the effect of GRPR antagonist in glioma cells lines implanted in nude mice, showing that the GRPR antagonist RC-3095 inhibits tumor growth.[10,11] We have recently shown that RC-3095 inhibits the growth of rat experimental models of glioma both in vitro and in vivo.[8,12] However, GRPR expression in glioma samples from patients has not been examined by previous studies. In the present study we used immunohistochemistry to evaluate GRPR expression in astrocytomas of different WHO-grades as well as in the normal human brain.

Materials and Methods

Tissue specimens. Astrocytoma samples of different WHO grades were obtained from the Pathology Department, University Hospital, Federal University of Rio Grande do Sul (HCPA-UFRGS; Porto Alegre, Brazil) between January 2004 and June 2008. As controls, 9 samples from normal brain tissue obtained from autopsies were selected. All samples were obtained in accordance with approved ethical standards of the institutional research ethics committee (document number GPPG 08-259, HCPA-UFRGS). Paraffin wax blocks were collected from 34 biopsies from patients with glioma and 9 autopsies.

Immunohistochemistry. Immunohistochemical analysis was performed as previously described.[8,13] The primary antibody used was a rabbit polyclonal antibody against GRPR (Ab13339; Abcam, Cambridge, MA). For antibody control glioma cell line were used. The samples were lightly counterstained with hematoxylin-eosin (HE) as a control.

Quantification by image analysis. The images were captured using AxisVision AC Real 4.5 program; inverted microscope with a 40X objective; and an AxioCam IC Zeiss camera. The selection was done manually trying to include all regions of the tumor. Five to ten photos of each tumor were taken. To analyze the image the Image J software was used, (obtained from

http://www.uhnresearch.ca/facilities/wcif/fdownload.html). For each image colour functions and colour deconvolution, H&E DAB vectors: colour 3 R: 0.2614752, G: 0.57031375, B: 0.77642715 were applied. The colour 3 (brown) image was inverted and measure was obtained as percentage of DAB chromogen. For intensity analysis, point selections were applied to many regions of each photo. For each tumor the mean percentage minus the background (80) and the mean intensity of all tumor images was calculated. To obtain the expression index, percentage was multiplied by intensity. All these procedures were repeated for two different examiners.

Statistics. Cox regression analysis and Kaplan-Meier for survival and Pearson Chi-square for correlation with grades were used. A probability value (*P*) of less than 0.05 was taken to indicate statistical significance. All statistic comparisons were done using Statistical Package for the Social Sciences (SPSS).

Results and Discussion

Thirty-four cases of gliomas were included, three of which corresponded to astrocytomas WHO grade I, four to grade II, three to grade III and twenty four were glioblastoma multiforme. The median age was 39 years old, and gender distribution was: 16 female and 18 male. The survival median for glioblastoma multiforme was 10 months (Table 1). The survival of patients with astrocytoma grade I, II and III was significantly higher than patients with glioblastoma multiforme (log-rank $P = 0.0028$).

Table 1 should be inserted here

GRPR expression was identified in 100% of the glioma samples analyzed. High GRPR expression was observed also in endothelial cells. However, GRPR expression was not identified in glial cells found in normal brain tissue samples. In all nine normal brain samples, GRPR expression was identified in neurons (Fig. 1). GRPR expression was observed in 10 to 50% of neuron cells, varying from weak to strong intensity (Table 2).

Figure 1 should be inserted here

There was no correlation between expression index (P = 0.398), intensity (P = 0.293), or area ratio (P = 0.779), and tumor grade. In addition, the GRPR expression index was not significantly related to patient survival (P = 0.915) (Fig. 2).

Table 2 should be inserted here

Figure 2 should be inserted here

GRPR expression has been previously examined in many tumor types and cancer cell lines[5,9], and GRPR antagonists have been investigated in experimental glioma models as potential anticancer agents.[8,10-12] However, GRPR expression in glioma biopsies has not been evaluated by previous studies. In addition, GRPR expression in the CNS has been recently described by immunohistochemistry in mice [14], but not in humans. The present study is the first to demonstrate that GRPR is abundantly expressed in human gliomas and human neurons, but not in normal glial cells. Consistent with these findings, Kamichi and colleagues[14] detected GRPR immunoreactivity in MAP-2 positive cells and NeuN–positive cells, but not in GFAP-positive cells or in OX42-positive cells, in the mouse brain, and their results indicated that GRPR is expressed mainly on the cell body of neurons and weakly in the neuritis, and not in astrocytes and microglial cells. In addition, our findings are consistent with data showing that GRPR signaling regulates several aspects of CNS function.[3]

GRPR expression is more commonly found in malignancies than in normal tissues. Pansky and colleagues[15] demonstrated that GRPR expression is found in renal cell carcinoma, but not in normal kidney tissues. Non-neoplastic prostatic tissue only rarely expressed a measurable amount of GRPR, while prostate cancer specimens were found to be 100% positive for GRPR.[16] Our group has recently shown that GRPR was detected in 99% (87/88) of cervical cancer samples, but not in normal cervical

tissues.[13] In contrast, in breast and colorectal cancers, GRPR might be expressed in normal tissue next to the tumor. [17,18] It is possible that a change in the pattern of GRPR expression is related to the tumor's niche.

GRPR expression index did not have a significant correlation with either glioma grade or patient survival. Data from The Cancer Genome Atlas (TCGA), a project carried out by many centers that evaluated more than 200 samples of glioblastomas for mRNA expression, showed no significant difference in survival between samples expressing higher (n = 7) or lower (n = 56) levels of GRPR mRNA (p = 0.162).[19] Lango and colleagues[20] found no correlation between GRPR expression and survival when analyzing the level of mRNA for GRPR in 24 patients with squamous cell carcinoma of the head and neck. Overall, our findings are consistent with evidence from preclinical models indicating that the GRPR is highly expressed in gliomas and represents a molecular target for the development of novel target therapies for the treatment of brain tumors.

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Legends for figures

Fig. 1. Immunohistochemical analysis of GRPR expression from biopsies of patients with astrocytomas and normal brain samples. Sections were incubated with anti-GRPR antibody, sequentially treated with biotinylated anti-rabbit IgG and streptavidinbiotin-peroxidase solution, and then developed with diaminobenzidine as chromogen. GRPR expression is shown in the right column (brown, 400x) and HE in the left column (400x). (A, B) astrocytoma grade I; (C, D) astrocytoma grade II; (E, F) astrocytoma grade III; (G, H) astrocytoma grade IV; (I, J) normal brain.

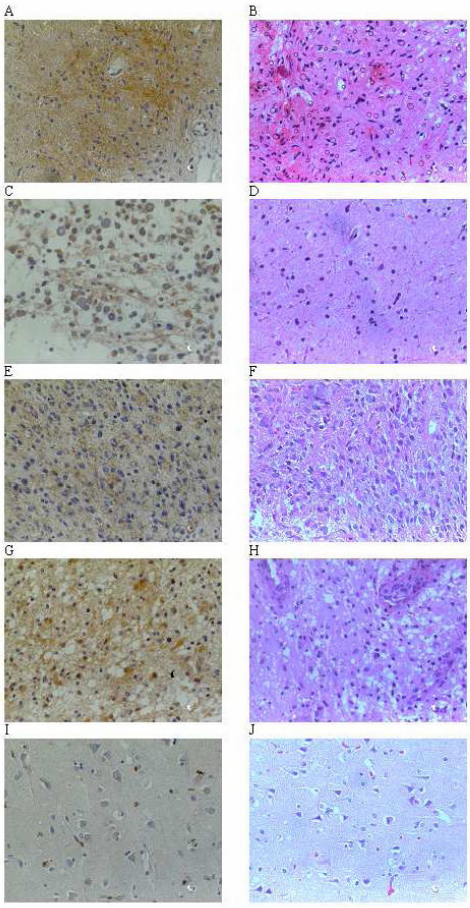
Fig. 2. Survival of 34 patients with astrocytomas grade I-IV. Cox regression analysis of GRPR expression was performed; $P = 0.915$.

Table I. Results of digital image analysis for GRPR expression

Sample number	Gender	Age	Histology	Survival months	GRPR expression		
					Intensity	Area ratio	Expression index
1	F	11	I	> 37 alive	185	102	4.144
2	M	45	I	> 4 alive	169	96	2.772
3	M	11	I	> 8 alive	222	132	11.633
4	F	35	II	> 62 alive	177	91	1.903
5	F	45	II	> 62 alive	144	99	2.776
6	M	43	II	> 9 alive	181	104	4.344
7	F	19	II	11	152	92	1.824
8	F	51	III	> 8 alive	178	118	6.764
9	M	35	III	> 23 alive	165	96	2.574
10	M	31	III	> 32 alive	161	104	3.784
11	M	9	IV	1	177	100	3.505
12	M	34	IV	17	159	101	3.260
13	M	31	IV	> 32 alive	174	111	5.359
14	F	64	IV	10	117	91	1.264
15	F	45	IV	> 33 alive	230	130	11.500
16	F	12	IV	13	203	114	6.851
17	F	64	IV	4	155	116	5.518
18	M	60	IV	6	174	99	3.236
19	M	54	IV	14	168	95	2.520
20	M	76	IV	5	217	126	10.025
21	M	5	IV	6	120	97	2.016
22	M	76	IV	7	184	106	4.784
23	F	71	IV	15	200	120	7.932
24	F	53	IV	12	193	96	3.022
25	F	23	IV	36	161	97	2.705
26	M	26	IV	> 23 alive	163	118	6.194
27	F	35	IV	> 13 alive	159	86	954
28	F	83	IV	1	180	112	5.729
29	M	62	IV	0,1	195	109	5.558
30	F	85	IV	52	193	116	6.871
31	M	12	IV	1	159	93	2.003
32	M	78	IV	1	231	127	10.894
33	M	31	IV	1	169	116	6.050
34	F	14	IV	> 46 alive	221	120	8.796

Table 2. GRPR expression in normal brain specimens

Sample	Neuron			Glia	Expression index
	Weak	Moderate	Strong		
1		10%		0	1881
2		10%		0	136
3	50%			0	2784
4	50%			0	2360
5			10%	0	1920
6		20%		0	4040
7	20%			0	2676
8	20%			0	2535
9		20%		0	3840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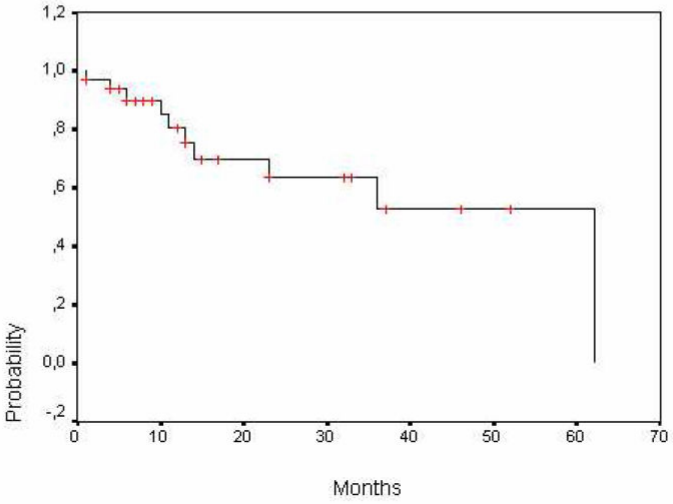

Capítulo IV

Cancer stem cells and the biology of brain tumors

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Cancer Stem Cells and the Biology of Brain Tumors

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Abstract: There is now compelling evidence that brain tumors harbor a small population of cells characterized by their ability to undergo self-renewal and initiate tumors, termed cancer stem cells (CSCs). The development of therapeutic strategies targeted towards CSC signaling may improve the treatment of brain tumors such as malignant gliomas and medulloblastomas. Here we review the role of cancer stem cells in glioma and medulloblastoma and some of the signaling mechanisms involved in brain tumor stem cell (BTSC) biology, and discuss how these signaling pathways may represent new stem cell targets for the treatment of brain tumors. In addition, we provide illustrative immunohistochemical data on the presence of BTSCs in human gliomas and medulloblastomas, and show preliminary findings suggesting the involvement of a GPCR, the gastrin-releasing peptide receptor (GRPR), in the expansion of BTSCs in vitro.

Keywords: Cell signaling, neural stem cells, cancer stem cells, glioma, medulloblastoma, brain tumors.

INTRODUCTION

Gliomas and medulloblastoma are the most frequent malignant brain tumors in adults and children, respectively. The most prevalent and aggressive form of glioma is grade IV glioma (glioblastoma multiforme, GBM), which is highly malignant and resistant to standard chemotherapy. The prognosis of this tumor type remains poor despite optimal clinical treatment, with overall survival of less than 20% after one year and less than 5% after two years [1-3]. Medulloblastoma is a primitive neuroectodermal tumor that represents the most common pediatric primary malignant intracranial neoplasm. In spite of significant advances in chemo- and radiation therapy, about 30% of patients still have a low chance of being cured and survivors experience long-term neurocognitive and/or neuroendocrine sequelae [4, 5]. The development of novel therapies based on the understanding of the basic biology of brain tumors is clearly needed to improve the treatment of both gliomas and medulloblastoma.

In recent years, several studies have reported the presence of a subpopulation of tumor cells with stem-cell like properties, termed cancer stem cells (CSCs), in both gliomas and medulloblastoma. These brain tumor stem cells (BTSCs) display self-renewal properties, unlimited growth, a high migration rate, resistance to chemotherapy, and are capable of recapitulating the whole tumor cell population [6-8]. BTSCs provide a target for the treatment of malignant brain tumors, and understanding the signaling pathways involved in BTSC proliferation may contribute to the development of novel therapies. In this review, we discuss the role of BTSCs in the initiation and progression of brain tumors. We summarize some of the signaling pathways critically implicated in the proliferation of BTSCs, including G-protein coupled receptors (GPCRs) as emerging targets to inhibit BTSC propagation and reduce brain

tumor progression. Finally, we present original data illustrating the presence of BTSCs in glioma and medulloblastoma samples from patients and the possible role of a GPCR in stimulating the expansion of BTSCs in culture.

STEM CELLS IN THE CENTRAL NERVOUS SYSTEM

Our understanding of BTSC biology has been enhanced by studies of normal stem cells in the central nervous system (CNS). Almost every known mammalian tissue contains a population of stem cells, which play a crucial role in tissue development and repair. Stem cells are defined as cells capable of undergoing self renewal and generating all major cell types found in the host tissue, coordinating tissue generation, maintenance, and regeneration. In the adult CNS, stem cells differentiate into distinctive mature cell types. Thus, multipotent neural stem cells (NSCs) can originate neurons, astrocytes and oligodendrocytes via the generation of lineage-committed progenitor cell populations. Astrocytes may be derived from either oligodendrocyte type 2 astrocyte (O-2A) cells (which in turn derive from glial-restricted precursor cells) or astrocyte precursor cells, whereas oligodendrocytes derive from O-2A cells and neurons are generated from neuron-restricted precursor cells. In discrete areas of the adult brain (namely, the dentate gyrus in the hippocampus and the subventricular zone of the lateral ventricles in the forebrain), NSCs are believed to originate new neurons, a process known as neurogenesis (reviewed in [6, 9]).

ON THE DEFINITION OF "CANCER STEM CELL"

Normal stem cells are characterized by their undifferentiated nature, high self renewal capacity and asynchronous divisions, therefore producing differentiated cells types of a particular lineage as well as maintaining a population of stem cells in a given tissue. Although CSCs can present all these features, it has been argued that the most important, and perhaps the only feature that really matters, is the tumor-forming ability of an undifferentiated cancer cell [10]. Considering the genetic instability of cancer cells, it might well be that some cells in the population express stem cell markers that are expressed by normal stem cells, which are of no practical value if these cells are not able to form tumors. Therefore, the most important defining property of CSCs should be their tumor-initiation capacity, and a single *bona fide* CSCs should sufficient to form a tumor [11]. CSCs have been also called tumor-initiating cells (TICs), which is a more strict definition of CSCs.

This is not to say that other features may not have practical and clinical relevance, as shown in so many studies that linked tumor-initiating capacity and patient survival with the presence of stem cell markers, such as CD133 in gliomas [12]. CSCs can also present features that are peculiar for the cancerous counterpart of stem cells, such as enhanced radio- and chemoresistance [13] and induction of angiogenesis [14], which are features that increase tumorigenesis.

BRAIN TUMOR STEM CELLS (BTSCs) AND BTSC MARKERS

CSCs were originally reported in acute myeloid leukemia [15], but CSCs populations were soon identified in several solid tumors [16]. In order for a cell to be considered a BTSC, the following characteristics must be present: 1) the ability to generate tumors in xenotransplantation experiments that resemble the original tumor and that persist after serial in vivo transplantation rounds; 2) formation of neurospheres; 3) ability to undergo self renewal; and 4) expression of markers of both glial cells (astrocytes, oligodendrocytes) and neurons [6-8].

CSCs are commonly identified by the expression of the stem cell marker CD133. This marker is expressed in only a subpopulation of glioma [17, 18] and medulloblastoma [19] cells. $CD133^+$ glioma cells alone are capable of forming multipotent neurospheres in vitro and initiate tumors in vivo [12, 18], and $CD133^+$ cells from medulloblastoma cell lines show increased ability to originate tumor xenografts [20]. However, other studies have indicated that $CD133^-$ tumor cells can propagate experimental brain tumors as well. Thus, $CD133^-$ glioma cells fulfilled stem cell criteria and were tumorigenic in vivo, although $CD133^-$ glioblastomas showed a lower proliferation rate compared $CD133^+$ xenografts [21].

The use of CD133 as a bona *fide* marker of glioma BTSCs was established since the first studies indicating the presence of CSCs in gliomas were published. As mentioned above, this first round of studies showed that xenografts of $CD133^-$ gliomas in immunocompromised mice did not form tumors, while implantation of 1000 times less $CD133^+$ cells produced large tumors [12, 18]. CD133 expression in gliomas varies widely, with frequencies of $CD133^+$ cells increasing with tumor grade [22; 23]. In addition, CD133 expression is a significant prognostic

factor, but only when a threshold of 1% or 2% of $CD133^+$ cells (or organization in clusters) is considered. On the other hand, CD133 mRNA expression is significantly associated with overall survival of patients [24, 25]. However, no direct correlation between the absolute percentage of $CD133^+$ cells and patient survival has been demonstrated [24]. CD133 expression in gliomas varies from less than 1% to over 80% [26, 27]. It is quite risky, however, to define a threshold in flow cytometry experiments above which a glioma cell is considered positive for CD133. Therefore, the percentage reported often depends on the control used. Overlay of the supposedly negative and positive most often leads to the best perception of the difference between groups.

Other studies have examined CD133 expression in cultured medulloblastoma. Medulloblastoma stem cells selected by CSC medium can form 3D spheroids and display enhanced self-renewal. $CD133^+$ Daoy medulloblastoma cells restore native population within 18 days, whereas $CD133^-$ cells remained negative [28]. A frequency of about 25% of $CD133^+$ tumor spheres is formed in medulloblastoma cell cultures [18]. Ptc+/- medulloblastomas in a mouse model express about 5% of $CD133^+$ cells, but some tumors contain as many as 56% $CD133^+$ cells [29].

Expression of CD133 seems to be related to considerable differences in the genetic profiles of cells, as shown by microRNA (miRNAs) profiles of $CD133^+$ and $CD133^-$ cell populations. miR-451, miR-486, and miR-425 were among the miRNAs upregulated in $CD133^+$ cells when compared to $CD133^-$ cells. Some of these miRNAs may be involved in regulation of brain differentiation and tumorigenesis [30]. The presence of CD133+ cells in samples of human glioma and medulloblastoma is illustrated in Fig. **1.**

Fig. (1) should be inserted here

Other markers have been reported for BTSCs. In experimental medulloblastoma, tumors from the Patched mutant mouse model of medulloblastoma were propagated not by $CD133^+$ cells but by cells expressing the normal NSC marker CD15 [29, 31]. CD15 expression is also found in a subset of human medulloblastomas, and expression of a CD15-associated gene signature predicts survival. Although the $CD15^+$ cells that coexpress Math1 are unable to form neurospheres, suggesting that these are not stem-like cells, the isolated $CD15^+$ cells are progenitor-like cells with a unique capacity for tumor propagation [29]. The expression of CD15 in human medulloblastoma is shown in Fig. **2**.

Neurospheres derived from medulloblastomas express many genes characteristic of NSCs and other stem cells, including CD133, Sox2, musashi-1, bmi-1, Oct-4, Nanog maternal embryonic leucine zipper kinase, phosphoserine phosphatase and repressor element 1-silencing transcription factor (REST) [19, 32, 33]. Membrane type-1 matrix metalloproteinase (MT1-MMP) and MMP-9 contribute to the invasive phenotype during $CD133^+$ neurosphere-like formation in medulloblastoma cells. Specific small interfering RNA gene silencing of either MT1-MMP or MMP-9 reduces the capacity of the Daoy monolayers to generate neurospheres [34].

Fig. (2) should be inserted here

THE RELATIONSHIP BETWEEN BTSCs AND NSCs

It is still debated how BTSCs play a role in the formation of brain tumors, since the cellular origin of these kind of tumors and BTSCs is not completely clear. Although it is likely that BTSCs derive from normal stem cells, it is also possible that differentiated cells giving rise to tumors acquire stem cell-like properties after transformation. The first experiments that tackled this problem using transgenic mice strongly supported the hypothesis that brain tumors originated from transformation of NSCs. In these experiments, a higher incidence of tumors were observed in mice transgenic for the oncogenes Akt and Ras (K12D) regulated by the nestin promoter, which is active in NSCs, when compared to the GFAP promoter, which is active predominantly in differentiated reactive astrocytes [35]. However, after the proposal of this early model, several reports showed that GFAP-expressing cells can indeed be the origin of tumors when Akt and Ras are overexpressed together with c-Myc [36] or with deletion of Ink4a-Arf [37]. Interestingly, loss of Ink4a-Arf and overexpression of c-Myc can lead to loss of differentiation in astrocytes in culture and are common genetic alterations found in human gliomas [38].

If NSCs were the sole cell type of origin of brain tumors, one would expect that brain regions rich in NSCs would be more often affected by tumors than regions which present fewer NSCs. Brain tumors are found in virtually all regions of the brain with no clear preference for

NSC-rich areas, which may argue against the NSC origin hypothesis, unless brain tumor initiating cells are highly mobile. Lentiviral mediated expression of H-Ras and Akt driven by the GFAP promoter in a mouse heterozygous for p53 produced tumors when lentivirus were injected into the subventricular zone and hippocampus, but not into the cortex, arguing in favor of the requirement of NSCs for the formation of brain tumors [39]. The findings from transgenic models and viral mediated expression of oncogenes or deletion of tumor suppressor genes induced by promoters can have multiple interpretations, mainly due to the lack of certainty about the cells in which a given promoter is actually active. In spite of the evidence that NSCs are the cells of origin of brain tumors, it remains unclear whether the transformation of NSCs is an absolute requirement for brain tumor formation.

SIGNALING PATHWAYS IN BTSCs

CSCs show alterations in signaling pathways involved in differentiation, such as the Notch, hedgehog and Wnt pathways. However, other pathways that are more commonly associated with tumorigenesis, such as the Ras/mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTor) cascade, are altered in CSCs and seem to play a role in the maintenance of the undifferentiated phenotype (reviewed in [7, 40, 41]). In addition, recent evidence has indicated that metabotropic glutamate receptors (mGluRs) play a role in the proliferation of brain tumor cells and suggested that GPCRs might emerge as regulators of BTSC proliferation [42]. Here we will present a few selected signaling pathways in BTSCs.

Notch

The Notch pathway is involved in several developmental processes, including CNS development. Notch is activated by activators found in neighboring cells, leading to the cleavage of its intracellular domain, which then diffuses to the nucleus, regulating gene expression. One of the main target genes of the Notch pathway is the transcription repressor Hes1, which has pleiotropic effects on several key developmental stages. Over-expression of Hes1 leads to inhibition of neurogenesis and maintenance of neural stem cells. Later in development, Hes genes promote gliogenesis [43].

Basal Notch signaling is higher in the $CD133^+$ population when compared to the $CD133^-$ population of medulloblastoma cells. Inhibition of gamma-secretase, the enzyme responsible for the cleavage of Notch, reduced viability and proliferation of medulloblastoma cells. The cell population that survived treatment was no longer able to form soft agar colonies and tumor xenografts. Notch inhibition also reduced by 5 fold the $CD133^+$ cell population [44].

Hedgehog

Sonic hedgehog (Shh) is a growth factor that binds to the Patched receptor (Ptc). When not activated by Shh, Ptc inhibits the activity of the GPCR Smoothened (Smo). The targets of Smo include Gli transcription. The Shh signaling has been shown to regulate neural stem cell development in many species and is present in glioblastoma, medulloblastoma, and, to a lesser extent, neuroblastoma [45]. Gli activation and Shh expression in glioma correlate with tumor grade. In addition, an intact tumor microenvironment or neurosphere conditions in vitro are

necessary for Gli activity in gliomas [46]. Yang and colleagues [47] have shown that activation of Shh signaling, by deletion of Ptc, in neuronal progenitors causes medulloblastoma in mice. Deletion of Ptc in multipotent stem cells leads to expansion of the stem cell population, but only stem cells that commit with the granule lineage continue to divide and go on to form tumors. Approximately 15% of human sporadic medulloblastomas show Ptc mutations and ~ 20% of mice heterozygous for Ptc develop medulloblastoma [7].

Wnt

The Wnt signaling pathway regulates proliferation of stem and progenitor cells in the fetal ventricular zone, as well as the post natal subventricular zone and hippocampus [48]. Wnt signaling has also been shown to play an important role in the progression of medulloblastoma, and, more recently, gliomas. Mutations in the Wnt pathway have been identified in medulloblastoma patients with Turcot's syndrome as well as in sporadic medulloblastoma [49-52]. However, activation of Wnt is seen predominantly in classic tumors not showing signs of Shh signaling or chromosome 17 aberrations, suggesting they represent a unique subset of medulloblatomas [7, 53].

Some members of the Wnt family are overexpressed in astrocytomas. Experiments in vitro and in vivo using siRNA for Wnt2 and beta-catenin inhibited cell proliferation and invasive ability, and delayed tumor growth. In addition, downregulation of Wnt2 and beta-catenin was associated with decreased PI3K/p-Akt expression, indicating the interplay between Wnt/beta-catenin and PI3K/Akt signaling cascades [54]. The expression of CD133 and Wnt-1 increased in CSCs from glioblastoma compared with adherent monolayer cells [55]. The up-

regulation of Axin, a negative regulator of Wnt/β-catenin signaling, significantly inhibited proliferation of glioma cells [56]. Immunohistochemical analyses have revealed that Wnt5a expression was higher in human GBM than in normal brain tissue and low-grade astrocytoma. The overexpression of Wnt5a increased the proliferation of primary culture from patient and U87MG line. In contrast, the downregulation of Wnt5a expression as the result of RNA interference reduced proliferation in these cultures and reduced tumorigenicity in vivo [57].

PI3K, MAPKs and myc oncogenes

Activation of the PI3K/mTor pathways is downstream of several receptors such as Shh, Wnt, tyrosine kinase receptors and GPCRs [58]. PI3K signaling pathway plays an important role in the renewal of embryonic stem cells (ESCs). PI3K inhibition by LY294002 induces differentiation of mouse ESCs [59]. Loss of PTEN, the negative regulator of the PI3K/Akt pathway, results in disorganization of the brain, probably due to a flaw in cell migration. Experiments with adult $PTEN^{+/-}$ mice demonstrate that PTEN regulates migration of precursor cells in the subventricular zone to the olfactory bulb [60]. Zheng and colleagues have shown that p53 and PTEN control neural and glioma stem/progenitor cell renewal and differentiation. Concomitant deletion of p53 and PTEN in NSC increased proliferation and self-renewal reflected by neurosphere formation capacity, compared to wild-type or NSCs null for PTEN or p53 only [61]. NSCs infected with EGFRvIII (which is a constitutively activated receptor) and $PTEN^{-/-}$ showed increased cell proliferation, centrosome amplification, colony formation in soft agar, self-renewal, CD133 expression, and resistance to oxidative stress and ionizing radiation. In addition, these cells showed increase activity of the RAS/mitogen-activated

protein kinase (ERK) and PI3K/Akt pathways. Injecting these cells in mice decreased tumor latencies and additional chromosomal aberrations [62]. Hambardzumyan et al. have demonstrated that radiation activates Akt/mTOR signaling in the perivascular nestin-expressing stem cells from medulloblastomas. These cells re-enter the cell cycle at 72 h and are responsible for the tumor recurrence [38]. In other tumors types such as prostate, the PI3K/Akt pathway is critical for CSC maintenance [63]. However, PTEN was highly expressed in $CD133^{+}$ compared to $CD133^{-}$ cells in the U251-MG glioma cell line [13].

In astrocytes from p53-deficient mice, the MAPK/extracellular-regulated kinase (ERK) pathway was shown to mediate neurosphere formation [64]. Both fibroblast growth factor (FGF) and epidermal growth factor (EGF), which are used to expand NSCs and BTSCs, lead to activation of Ras/ERK pathways. FGF inhibits transcription of Shh target genes and prevents activation of a Gli-responsive promoter in fibroblasts, suggesting that it blocks Shh signaling upstream of Gli-mediated transcription. FGF-mediated inhibition of Shh responses requires activation of FGF receptors as well as ERK and JNK kinases. FGF promotes differentiation of granule cell precursors in vitro and in vivo and halts proliferation of tumor cells from patched (ptc) mutant mice, a model of medulloblastoma [65]. In contrast, Schwindt and colleagues demonstrated that culturing neurospheres in suspension for 2 weeks without EGF and FGF-2 significantly increases neuronal differentiation and neurite extension when compared to cells cultured using these factors [66].

N-myc and c-myc ongenes downstream of Wnt, Shh and MAPK respectively are altered in stem cells and brain tumors. c-myc is highly expressed in glioma CD133+ relative to non-stem glioma cells [67]. N-myc plays an important role in the growth of the cerebellum and the

cerebral cortex [68]. In medulloblastoma, N-myc is amplified, it can dramatically alter the fate of preneoplastic cells, preventing differentiation and driving tumor progression [69].

GPCRs

Although GPCRs have not featured among the main molecular regulators of BTSC proliferation, recent evidence indicates that these receptors might represent emerging BTSC targets. Glutamate, the main neurotransmitter in the CNS, is an extracellular signal regulating the proliferation and fate of NSCs in the developing and adult brain. Glutamate regulation of NSC proliferation is mediated by metabotropic glutamate receptors (mGluRs), which are GPCRs (reviewed in [42]). The expression of the $mGlu_4$ subtype of mGluR in medulloblastoma has been found to be inversely related to tumor severity, spreading, and recurrence, and a selective $mGlu_4$ enhancer inhibited the proliferation of medulloblastoma cell lines [70].

Other GPCRs, such as neuropeptide receptors mediating autocrine signaling in tumors, might prove to be new cancer stem cell targets in brain tumors. The gastrin-releasing peptide receptor (GRPR), a GPCR activated by the neuropeptide GRP or the amphibian peptide bombesin, is overexpressed in many types of cancer and is importantly involved in regulating the proliferation of cancer cells (reviewed in [71, 72]). We have recently shown that GRPR stimulation by bombesin enhances proliferation of C6 glioma cells in vitro through a mechanism dependent on PI3K signaling [73]. Here we present our finding that bombesin induced an increase in neurosphere formation in cultured human U-87 glioma cells, suggesting that GRPR stimulation might lead to an increase BTSC expansion (Fig. **3**). Further studies

might reveal that the GRPR and other GPCRs activated by neuropeptides are regulators of BTSC proliferation.

Fig. (3) should be inserted here

CONCLUDING REMARKS

BTSCs represent an increasingly important target for the development of more effective targeted therapies for the treatment of glioma and medulloblastoma. These stem cells are highly resistant to traditional chemotherapy, and the development of effective treatments will require the understanding of the biological mechanisms regulating BTSC proliferation and differentiation, so that signaling pathways critical for cell survival, expansion, and "stemness" can be targeted. In addition to the better understood pathways active in BTSCs, it is possible that other, less explored mechanisms such as GPCRs and neuropeptide signaling will provide new promising stem cell targets.

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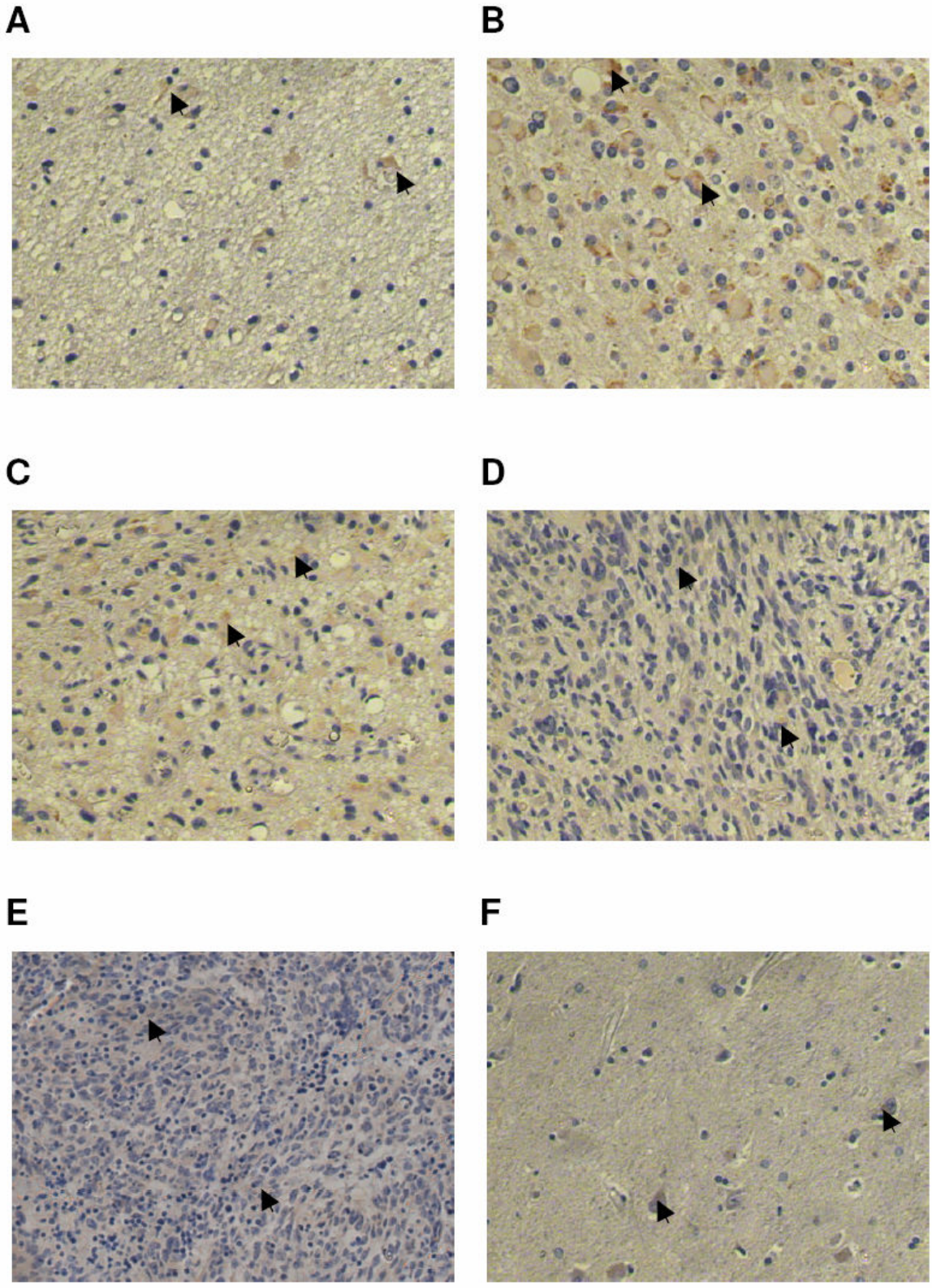
LEGENDS FOR FIGURES

Fig. (1). CD133 expression in brain tumors. Immunohistochemical analysis of CD133 expression from biopsies of patients with astrocytomas, medulloblastoma, or normal brain samples (brown, two CD133-positive cells are indicated by arrowheads, 400 X). Paraffin wax blocks from biopsies of astrocytoma of different WHO grades, medulloblastoma, and normal brain samples were obtained from the Pathology Departament, University Hospital (HCPA), Federal University of Rio Grande do Sul, Porto Alegre, Brazil). All samples were obtained in accordance with approved ethical standards of the institutional research ethics committee (document number GPPG 08-259). The expression of CD133 was examined by immunohistochemistry as previously described [75]. The primary antibody was a rabbit monoclonal antibody against CD133 (C24B9; Cell Signaling, 1:100). The samples were lightly counterstained with hematoxylin-eosin (HE) as a control. **(A)** Astrocytoma grade I; **(B)** astrocytoma grade II; **(C)** astrocytoma grade III; **(D)** astrocytoma grade IV; **(E)** medulloblastoma; **(F)** normal brain.

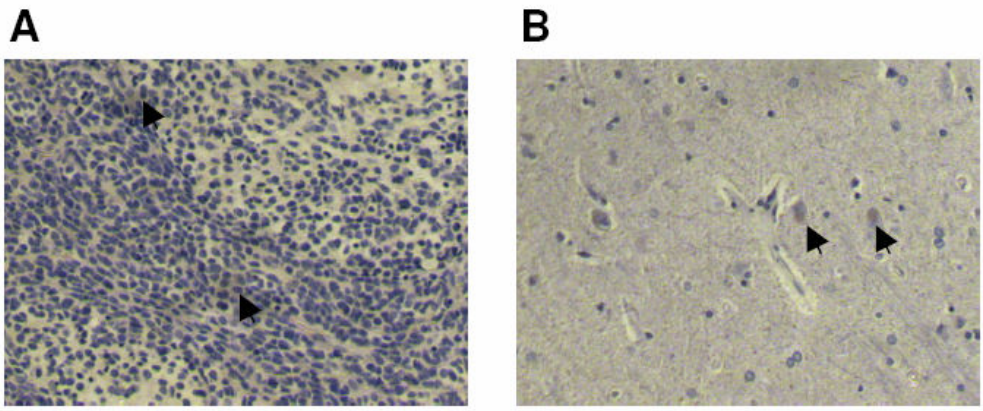
Fig. (2). CD15 expression in medulloblastoma. Immunohistochemical analysis of CD15 expression from biopsies of patients with medulloblastoma or normal brain samples (brown, two CD15-positive cells are indicated by arrowheads, 400 X). Paraffin wax blocks from biopsies of medulloblastoma and normal brain samples were obtained from the Pathology Departament, University Hospital (HCPA), Federal University of Rio Grande do Sul, Porto Alegre, Brazil). Samples were obtained in accordance with approved ethical standards of the institutional research ethics committee (document number GPPG 08-259). The expression of

CD15 was examined by immunohistochemistry as previously described [75]. The primary antibody was a rabbit monoclonal antibody against CD15 (Dako M-0733, 1:100). The samples were lightly counterstained with hematoxylin-eosin (HE) as a control. **(A)** Medulloblastoma; **(B)** normal brain.

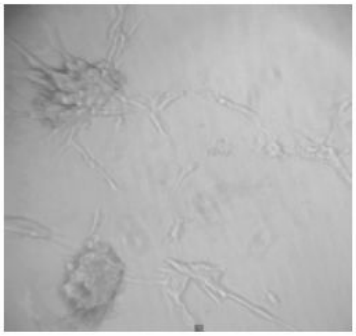
Fig. (3). Stimulation of a G-protein coupled receptor (GPCR) increases neurosphere formation in cultured human glioma cells. U-87MG cells (1,000) were grown in a 96 well plate for 7 days and spheres were counted from days 3 to 7 in the absence or presence of the gastrin-releasing peptide receptor (GRPR) agonist bombesin (100 nM). **(A)** Photomicrograph of neurosphere in cultured U-87MG cells. **(B)** Bombesin-induced increase in neurosphere formation in the 6th and 7th days; 6th day, $P = 0.004$; 7th day, n = 3 replicates, $P = 0.008$ compared to control cells.



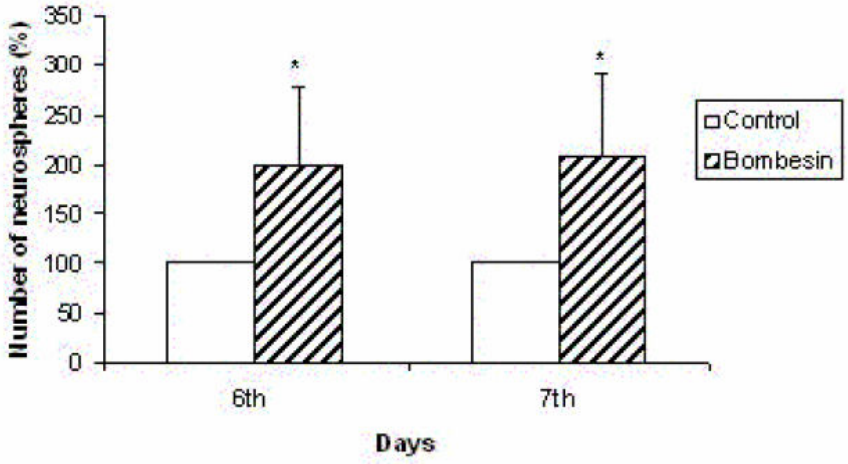
28


A
B

A



B


Number of neurospheres (%)
350
300
250
200
150
100
50
0
*
*
6th
7th
Days
Control
Bombesin

VI- DISCUSSÃO GERAL

Peptídeos da família da bombesina, como o GRP, vêm sendo estudados em muitos tipos e modelos tumorais, embora em gliomas esse estudo tenha ficado restrito à linhagens celulares. Quatro foram os pontos analisados pela primeira vez em gliomas neste trabalho: o efeito da bombesina e do antagonista de GRPR, sem pré-estímulo, sobre a proliferação celular em linhagens humana e de ratos; o envolvimento da via PI3K desencadeada pela ativação de GRP, e sua mediação no processo proliferativo; a expressão de receptores de GRP em tumores de pacientes e em cérebro humano sem neoplasia; a indução na proliferação de neuroesferas por bombesina.

O antagonista de GRPR, RC-3095, inibiu a proliferação celular na linhagem de rato C6 e na linhagem humana U-87, mas não na linhagem humana U-373, nas doses analisadas. Em células C6, essa droga foi analisada pela primeira vez, enquanto que o efeito sobre as linhagens humanas já tinha sido relatado na literatura. Porém, os autores haviam pré-estimulado com agonista, mimetizando o efeito parácrino (Pinski et al., 1994; Kiaris et al., 1999). Adicionalmente, o RC-3095 teve efeito em doses intermediárias e não em doses mais altas. Em estudos de função neuronal, RC-3095 também apresentou esse padrão de dose resposta, com efeito em doses intermediárias e não em altas. Na linhagem de U-373, Pinski et al. (1994) encontraram efeitos antiproliferativos em doses menores (10^{-11} a 10^{-8} M) a que analisamos e com pré-estimulação com GRP. RC-3095, em doses maiores do que 100 nM, pode apresentar um efeito estimulatório sobre a proliferação, possivelmente por uma ação como agonista parcial. O RC-3095, além de ser um antagonista seletivo para GRPR, diminui os níveis de expressão do EGFR em muitas linhagens tumorais, inclusive de glioma, e tem sido sugerido como um dos mecanismos

pelos quais o RC-3095 inibe a proliferação (Speshazi et al., 1992; Pinski et al., 1994a; 1994b). Provavelmente esse efeito de RC-3095 sobre os níveis de expressão de EGFR seja pela própria ação como antagonista de GRPR, pois muitos trabalhos vêm mostrando que a ativação de receptores acoplados a proteínas G, trans-ativam receptores tipo tirosina-cinases como EGFR. Essa trans-ativação dá-se via metalo-proteinases de membrana que clivam pré-ligantes de EGFR liberados pela ativação de GRPR. Além disto, a ativação de EGFR é retro-alimentada positivamente e vice-versa.

A atividade intrínseca de RC-3095 em doses mais altas como agonista foi sugerida pelo grupo de Schally, o mesmo grupo que desenvolveu a molécula. Eles avaliaram seis antagonistas sintéticos, desenhados pelo grupo, em linhagens de fibroblastos e colorretal e entre estes RC-3095 foi o que apresentou maior efeito inibitório sobre a proliferação, porém não em altas doses (Casanueva et al., 1996). Outros antagonistas de GRPR vêm sendo sugeridos como agonistas parciais, um exemplo é o BIM26226 (Ryan et al., 1996; Jensen et al., 2008). A atividade de agonista, agonista parcial e antagonista de um mesmo composto pode variar de acordo com a espécie ou mesmo em tecidos de uma mesma espécie (Coy et al., 1991; 1992; Jensen and Coy, 1991; Jensen et al., 2006). A densidade do receptor no qual o composto tem afinidade também pode contribuir para atividade de agonista parcial deste (Jensen et al. 2008). Tanto agonistas quanto antagonistas do GRPR promovem uma *down-regulation* do receptor (Millar ans rozengurt, 1990; Halmos e Schally, 1997). Porém, não foi estudado o efeito de um amplo espectro de doses de agonista nem de antagonistas sobre a regulação no número de receptores de bombesina na membrana. Esta regulação pode se tornar uma retro-

alimentação positiva dependendo da dose, pois Tsuda et al., (1997) mostraram que, quanto maior o número de receptores de GRP na membrana, menor é a regulação negativa (repressão). Neste caso, uma hipótese seria que o RC-3095, em altas doses, pode ter efeito diferente devido à densidade de GRPR na membrana, onde a inibição de GRPR por RC-3095 pode estimular o aumento do número de receptores, disponibilizando maior número destes para os ligantes autocrinos ou mesmo paracrinos.

Para uma possível aplicação clínica do RC-3095, um melhor entendimento sobre a diferença no efeito das doses é necessário, pois altas doses podem ter efeito inverso ao esperado, enquanto baixas doses podem produzir um bom resultado. Schwartsmann et al. (2006) realizaram um estudo clínico de fase I e não encontrarem efeito tóxico do RC-3095 nas doses analisadas. Porém altas doses a longo prazo pode ter uma efeito danoso.

O GRPR foi amplamente expresso nos diferentes graus de gliomas, mas não em células gliais de cérebros pós-morte de pacientes sem nenhum tipo de câncer diagnosticado. A superexpressão de GRPR vem sendo relatada nos mais diferentes tumores. Tanto tecido que já expressam constitutivamente GRPR em níveis mais baixos, quanto tecidos normais onde não há expressão, quando neoplásicos a expressão de GRPR geralmente está presente. Em próstata, a expressão de GRPR é menos intensa e menos freqüente em tecido de próstata não maligno (biopsias de próstata benignas) do que em carcinomas de próstata (Bartholdi et al., 1998; Markwalder e Reubi, 1999). No tecido mamário, GRPR é expresso tanto no tecido tumoral como no tecido sadio adjacente ao tumor (Gugger e Reubi, 1999). Em tecido colorretal, GRPR é expresso tanto em tecido tumoral quanto em mucosa de tecido sadio adjacente

ao tumor, porém com a expressão mais intensa no tecido tumoral (Chave et al., 2000). Em outro estudo em tumores colorretais, houve expressão de GRPR no tecido tumoral, mas não no tecido retirado a mais de 5 centímetros da área doente (Saurin et al., 1999). Em tecido de cólon e de rim, o GRPR é fortemente expresso no tumor, mas não no tecido adjacente a este (Carrol et al., 1999; Pansky et al., 2000). A maioria dos estudos de expressão de GRPR em tumores usa como controle o tecido sadio nas imediações do tumor do próprio paciente. Isso pode limitar a análise, já que a expressão no tecido como um todo pode estar alterada. Portanto, para análise comparativa mais abrangente da expressão do receptor e possíveis correlações, o melhor é a análise da expressão em uma outra população de pacientes que não tenham câncer.

Neste trabalho, encontramos a expressão de GRPR em cérebro humano sem neoplasia em neurônios e não em células gliais. Kamichi et al. (2005) encontraram expressão de GRPR somente em neurônios e não em células gliais em cérebro de camundongos. O GRPR é expresso em cérebro de macacos, mas não foi analisada a expressão quanto ao tipo celular, neurônio ou glia (Sano et al., 2004).

Esse achado, além de ser relevante nos estudos de tumores do SNC pode ser importante por ser o primeiro a mostrar a expressão de GRPR em cérebro humano, com isso contribuindo para a hipótese de que esse receptor tenha funções no SNC humano, assim como vem sendo mostrado, em muitos trabalhos, sua função no SNC de animais (Ohki-Hamazaki et al., 2005; Roesler et al., 2006b).

Considerando a hipótese das células tronco tumorais, alterações genéticas ocorridas nestas células levariam ao desencadeamento de um processo proliferativo, além da alta capacidade multipotente destas, que no caso do cérebro seria gerar tanto neurônios quanto células da glia. Uma hipótese a ser considerada e mais especulada é que se GRPR é expresso em cérebro humano normal em neurônios e não em células gliais, mas é amplamente expresso em células gliais tumorgênicas, em gliomas, essa mudança no padrão de expressão pode ter se dado em células tronco cerebrais.

A bombesina induziu a formação de neuroesferas na linhagem humana de glioma U-87. Muitos trabalhos vêm usando os fatores de crescimento, EGF e FGF, para induzir a formação de neuroesferas (Singh et al., 2003; Yuan et al., 2004; Gal et al., 2007). O uso destes fatores em meio de cultura sem soro fetal bovino (SFB) torna a morfologia da cultura quase exclusivamente formada por neuroesferas. Em nossa experiência com a cultura, além da análise da literatura, observamos que o SFB influencia diretamente na formação das esferas, quanto menor a quantia de soro mais neuroesferas a cultura forma. A principio, mesmo não sendo mencionado diretamente nos artigos, os fatores são necessários exclusivamente para que as células não morram, pois com maior presença de soro elas diferenciam, e a falta de soro resulta em morte celular. Portanto, a indução de neuroesferas na cultura pode ser devido à falta de estímulo para diferenciarem.

Neste trabalho, mostramos que a linhagem humana, U-87 plaqueada com 5% de SFB, bem menos do que a quantidade convencional, e que permanecem neste meio por sete dias, formam esferas, e esta formação foi

significativamente mais acentuada quando tratadas com bombesina. Yu et al. (2008) mostraram que as neuroesferas formadas em U-87 são positivas para CD-133. Eles sugerem que a indução com fatores seja uma boa forma de isolar CT tumorais. Nós também encontramos, através de citometria de fluxo, que U-87 crescida por uma semana com mudança gradual, diariamente, de meio com soro para meio sem soro e com fatores de crescimento apresentam uma população de células CD-133 positivas muito maior do que células crescidas sempre com 10% de SFB.

Adicionalmente, nós mostramos que os mesmos tumores originários de biopsias de pacientes que expressam GRPR, expressam fortemente CD-133 em uma sub-população de células. Isto é coerente com a possibilidade de que a mudança no padrão de expressão de GRPR se dê nesta sub-população de CT, CD-133^{+}, que geram tanto células diferenciadas como seus próprios clones, desencadeando assim um processo proliferativo. Além disto, uma mudança na estimulação do receptor, por indução parácrina dos ligantes de GRPR, poderia desencadear uma maior estimulação de CT que em conseqüência poderia levar a formação tumoral. Certamente, GRPR não seria o único agente com alterações de expressão nas CT, ou mesmo o desencadeador do processo, mas sim um dos multiplos agentes envolvidos nesta cascata de eventos.

Quanto à expressão de GRPR e GRP em células tumorais mais ou menos diferenciadas, não há um consenso para os diferentes tipos tumorais. Em carcinomas de ovário, a expressão de GRPR é maior em tumores menos diferenciados (Sun et al., 2000), enquanto a co-expressão do ligante e do receptor em câncer de ovário foi encontrada em tumores bem diferenciados e

não nos menos diferenciados (Carroll et al., 1999). Em tumores de próstata e pequenas células de pulmão, parece haver maior expressão de GRPR em células mais diferenciadas (Jensen et al., 2001).

A mudança de expressão de GRPR se daria de dentro do tumor para fora, a partir de CT. Talvez por isto, em alguns tumores o tecido adjacente já apresenta expressão de GRPR, embora menos intensa e por isso que a expressão se faz presente tanto nas células diferenciadas como nas indiferenciadas.

No capítulo I mostramos que a bombesina induz proliferação celular em gliomas e essa proliferação foi revertida por uma dose sem efeito por si mesma de RC-3095, um antagonista seletivo de GRPR. A bombesina mostrou efeito mitogênico em uma variedade de tipos tumorais. Em linhagens de gliomas a bombesina provocou um aumento nos níveis de Ca^{++} intracelular e induziu fosfatos de inositóis, porém o efeito proliferativo em si ainda não havia sido mostrado. A bombesina apresenta maior afinidade por GRPR do que por outros subtipos de receptores ao qual ela também tem afinidade. Além disso, a estimulação do GRPR é maior pela bombesina do que pelo próprio ligante endógeno, GRP (Lin et al.,1995). Pinski et al. (1994) mostraram que em linhagem humana de glioma, U-87, GRP estimula a proliferação.

Os reais mecanismos pelos quais a bombesina estimula a proliferação nos diversos tumores não são ainda bem esclarecidos, menos ainda em gliomas. Na busca de alvos terapêuticos mais eficazes, em gliomas, tem se dado atenção à via de PI3K. Muitos estudos mostram que receptores que sabidamente ativam PI3K estão superexpressos em gliomas. Além disto, alguns estudos indicam que o regulador negativo da via PI3K, a fosfatase

PTEN, está deletada em alguns gliomas. Alguns autores chegam a relatar que 60 a 70% de glioblatomas primários apresentam mutação para PTEN. Por este motivo, inibidores de PI3K estão sendo estudados como agentes anti-tumorais.

Neste trabalho, nós mostramos que o efeito proliferativo induzido pela bombesina em linhagem de glioma C6, é dependente da a atividade de PI3K. A ativação de PI3K depende de suas subunidades regulatória e catalítica. A forma mais comum e mais associada ao câncer é a subclasse IA que pode ser ativada por receptores tirosina-cinases. EGFR possui tirosinas fosforilavéis que podem se ligar a p85 (subunidade regulatória de PI3K), ou também à subunidade catalítica pode ser ativa por Ras. Depois de ativada, PI3K fosforila PIP_2 em PIP_3, que ativa PDK1, fosforilando AKT e ativando a cascata subseqüente que levará a proliferação, inibição de apoptose, síntese de proteínas, entre outros efeitos. Por outro lado, uma outra composição das subunidades de PI3K regulatória e catalítica é ativada pelas subunidades βγ dos receptores acoplados a proteína G. Então, neste caso, a ativação de PI3K requerida na indução de proliferação, pode se dar de forma direta por GRPR ou de forma indireta pela trans-ativação de EGFR por GRPR. A PI3K relacionada ao câncer tem sido PI3Kα, embora a grande maioria dos trabalhos relacionados à PI3K em tumores usem inibidores pan-seletivos, que inibem todas as formas de PI3K. A indução de PI3K por GPCRs não tem sido associada a proliferação. A hipótese mais provável é que a ativação de GRPR trans-ative EGFR que ativa PI3K e leva ao processo proliferativo. Estudos recentes mostram que PI3K medeia à proliferação induzida pela ativação de GRPR em neuroblastoma e tumores de pequenas células de pulmão. Em carcinoma de pequenas células de pulmão Liu et al. (2007) mostraram que GRPR induz a fosforilação de AKT e

que um pré-tratamento com inibidor de PI3K, LY-294002, bloqueou essa indução. Além disto, eles mostraram que GRP induz a liberação de amphiregulina, um ligante de EGFR, e a inibição de amphiregulina, por um anticorpo neutralizante, inibiu a fosforilação de AKT induzida por GRP. Adicionalmente, eles mostraram que a fosforilação de AKT por GRP depende da ativação de SRC, mas que a fosforilação de AKT por EGF não. SRC já vem sendo uma molécula candidata à mediação da trans-ativação de receptores tipo tirosin-cinases por GPCRs.

Um recente estudo multicentrico (com mais de 7 centros e 231 colaboradores envolvidos) denominado de *The Cancer Genome Atlas Research Network* (TCGA) analisou mais de 206 glioblastomas e concluíu que 86% destes tem alguma alteração que envolve a via de PI3K. Na maioria dos tumores ocorrem amplificações de receptores que ativam a via PI3K. Eles encontraram em 45% das amostras amplificação ou mutações ocorridas no receptor de EGF; 13% das amostras com o receptor de PDGF amplificado; 6% de mutações em ERBB2 e 4% de MET amplificada. Mutações ocorridas nos genes que codificam para as subunidades de PI3K também vêm sendo encontradas (Philp et al., 2001; Mizoguchi et al., 2004). Este estudo também encontrou mutações no gene que codifica para a subunidade regulatória p85, em 9 amostras de 91. Mutações no gene PIK3CA, que codifica para a subunidade $p110_{\alpha}$ de PI3K, eles encontraram em 6 amostras de 91 analisadas, embora mutações ocorridas neste gene são menos relatada na literatura. Além disto, 36% das amostras apresentavam deleção, homozigota para o regulador negativo de PI3K, PTEN.

Considerando que GRPR está superexpresso em gliomas; que ativa a via PI3K, a qual é extremamente importante no processo de indução da proliferação e inibição de apoptose; e que esta ativação pode ser por trans-ativação de EGFR, um receptor que também tem a expressão amplificada em gliomas, inibir o desencadeamento deste processo mais em cima na cascata de eventos pode ser uma boa estratégia para o tratamento destes tumores. Ainda, se considerarmos que o GRPR é expresso fisiologicamente em alguns poucos tecidos normais e nestes parece ser mais um mediador no processo ao qual tem função e não o agente exclusivo, pois os *knockouts* para GRPR não apresentam funções completamente comprometidas, sua inibição seria uma ação bem mais seletiva que a maioria quimioterápicos e menos agressiva. Por outro lado, tumores que apresentam amplificação para agentes mais a baixo na cascata, como EGFR (isso considerando que a ativação de PI3K por GRPR é via trans-ativação de EGFR), como deleção de PTEN, poderiam ter efeitos inibitórios sobre a proliferação reduzidos quando tratados com bloqueadores de GRPR como monoterapia. Nestes casos, outros agentes pertencentes a nova geração de quimioterapicos, como inibidores seletivos de PI3K, que poderiam ser usados em baixas doses, conjuntamente com bloqueadores de GRPR poderiam apresentar melhores resultados.

VI- CONCLUSÕES

- ✓ O gene que codifica para GRPR é expresso em linhagens celulares de gliomas humano e de rato;
- ✓ GRPR é expresso a nível protéico em linhagens celulares de gliomas humano e de rato;
- ✓ GRPR é amplamente expresso a nível protéico em astrocitomas oriundos de biopsias de pacientes;
- ✓ No tecido cerebral de pacientes sem neoplasia, o GRPR é expresso somente em neurônios e não em células gliais;
- ✓ O agonista de GRPR induz proliferação celular em linhagem de glioma de rato;
- ✓ O antagonista de GRPR, RC-3095, inibe a proliferação celular *in vitro* nas linhagens U-87 e C6, mas não na linhagem humana U373;
- ✓ A proliferação celular induzida pelo agonista de GRPR é dependente de PI3K.
- ✓ O agonista de GRPR aumenta a formação de neuroesferas, aumentando o número de células tronco tumorais.
- ✓ Os mesmos tumores de pacientes que expressam GRPR apresentam em uma subpopulações de células expressão de um marcador de células tronco tumoral cerebral, CD-133.

VII – PERSPECTIVAS

O fechamento deste trabalho deixou em perspectiva muitas outras possibilidades de pesquisa. Uma destas perspectivas seria avaliar se a isoforma de PI3K necessária para a proliferação celular induzida pela ativação de GRPR é ativada pela subunidade βγ da proteína G, acoplada ao próprio GRPR, ou ativada por EGFR, decorrente da trans-ativação. Para isso, utilizaríamos inibidores de PI3K mais seletivos, bloqueador de EGFR (cetuximab) e poderíamos tanto medir a proliferação quanto a atividade de AKT. Se a PI3Kα for necessária para induzir proliferação por bombesina, poderíamos investir na investigação de toda a via necessária para que GRPR trans-ative EGFR, como prováveis mediadores, Src e metaloproteinases. Se a isoforma de PI3Kγ for necessária, investiríamos em mostrar seu envolvimento no câncer, como em diversas linhagens.

Outro ponto que deve ser explorado é a expressão de GRPR em diferentes populações de células como. CT tumorais (positivas para CD-133), CT cerebrais e precursores neuronais (linhagens P27), células mais o menos diferenciadas, linhagens tratadas com diferentes concentrações de SFB o e sem este, mas com fatores de crescimento específicos. Superexpressar GRPR em CT cerebrais em cultura com transfecção de GRPR e avaliar o comportamento e morfologia celular em comparação com linhagem de glioma. Testar o efeito de bloqueadores de GRPR sobre a sub-população de células tronco formadoras de neuroesferas e CD-133 positvas. Avaliar se esta sub-população é mais resistente do que a população total do tumor ao efeito do bloqueador de GRPR, pois em alguns estudos elas apresentaram maior resistência a compostos que inibem proliferação que a população total. As vias

de sinalização ativadas nesta sub-população ainda não foram estudadas, sendo que poucos trabalhos foram publicados.

Co-relacionar a expressão de GRPR com EGFR em tumores de pacientes, além de testar o efeito combinado de bloqueadores destes receptores pode ser uma estratégia de tratamento. Sobre este aspecto, poderíamos testar a combinação de agentes tóxicos em doses mais baixas com antagonistas de GRPR.

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ANEXO

Trabalhos publicados durante o período de 2005-2009*

Luft T.; Flores D. G.; Vianna M. R.; Schwartsmann G.; Roesler R.; Izquierdo I. A role for hippocampal gastrin-releasing peptide receptors in extinction of aversive memory. Neuroreport. **2006**, *17*, 935-939.

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