KELLEN KETTY DE SOUZA

IRINOTECANO ATIVA A VIA PI3-QUINASE/AKT/mTOR EM LINHAGEM DE ADENOCARCINOMA DE CÓLON

Este exemplar corresponde à versão final da **Dissertação de Mestrado** apresentada ao Programa de Pós-Graduação Clínica Médica da Faculdade de Ciências Médicas da UNICAMP, para obtenção do título de Mestre em Clínica Médica, área de concentração Ciências Básicas do(a) aluno(a) **KELLEN KETTY DE SOUZA**

Campinas, 28 de agosto de 2007.

Prof(a). Dr(a). José Barreto Campello Carvalheira
Orientador (a)

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ORIENTADOR: Prof. Dr. José Barreto Campello Carvalheira

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αPy	Antifosfotirosina
6-MP	6-mercaptopurina
AKT/PKB	Proteína quinase B
ATP	Adenosina Trifosfato
CPT11	Camptotecina
DNA	Ácido desoxirribonucleico
DTT	Ditiotreitol
EDTA	Ácido etilenodiaminotetracético
ERK	Quinase regulada por sinal extracelular
GTP	Guanosina trifosfato
IR	Receptor de insulina
IKK	Complexo de quinases que ativam o NFκB
IκB	Inbidor do NFκB no citoplasma
IRS-1	Substrato 1 do receptor de insulina
IRS-2	Substrato 2 do receptor de insulina
JAK	Janus kinase
JNK	Quinase da c-jun
kDa	Quilo Dalton
KI-67	Anticorpo mouse monoclonal – clone MIB-1
MOPP	Regime quimioterápico composto por: vincristina, procarbazine e predinisona
mTOR	*Mammalian Target of Rapamycin*
NFκB	Fator de transcrição *nuclear factor kappa B*

p-c-jun	Substrato fosforilado da JNK
PI	fosfatidilinositol
PIP	fosfoinositíodeos fosforilados
$PI_{3,4}$-P_2	fosfatidilinositol-bifosfato
$PI_{3,4,5}$-P_3	fosfatidilinositol-trifosfato
PI3-kinase	fosfatidilinositol 3-quinase
PDK	Quinase dependente de fosfatidilinositol
PMSF	Fluoreto de fenilmetil sulfonila
POMP	Regime quimioterápico composto por: metotrexato, vincristina, 6-MP e predinisona
PTEN	Fosfatase e homólogo tensina
PKC-α	Proteína quinase C alfa
p85	Subunidade da PI3-quinase
Raf-1	Proteína da cascata de ativação da MAP quinase
Ras	Proteína originalmente identificada como oncogene, têm participação na regulação do metabolismo e crescimento celular
RNA	Ácido ribonucléico
Scid	*Severe combined immunodeficiency*
SDS-PAGE	Eletroforese em gel de poliacrilamida com dodecil sulfato de sódio
ser	Serina
Src	Oncogene originalmente definido como produto do sarcoma vírus Rous
Shc	Molécula adaptadora e substrato do receptor de insulina
Topo I	Topoisomerase I
Tris	Tri (hidroximetil)-aminometano

TNFα	Fator de necrose tumoral
TSC1/2	Complexo da esclerose tubero (*Tuberous sclerosis complex*)
Tunel	*Terminal desoxynucleotidyl transferase bloting UTP nick end labeling*

Pág.

RESUMO

A resistência dos tumores aos quimioterápicos é um problema clínico comum. Recentemente, foi demonstrado que o bloqueio da via de sinalização da PI3-quinase aumenta a apoptose induzida pelo SN-38, um metabólito ativo do irinotecano. Entretanto, os mecanismos moleculares destas mudanças ainda não são esclarecidos. Para investigar os eventos moleculares envolvidos no aumento da sinalização na via da PI3-quinase associada ao irinotecano, aspirina e rapamicina foram utilizadas para modular esta via de sinalização. Nós observamos que a aspirina é capaz de inibir a fosforilação do IRS-1, através de seus alvos JNK e IKK, e aumentar o crescimento dos tumores em camundongos Scid previamente injetados com linhagem de adenocarcinoma de cólon (HT29).

Adicionalmente, demonstramos que o bloqueio da mTOR reduz a proliferação celular induzida pelo irinotecano. Assim, a ativação da via PI3-quinase/Akt/mTOR pode contribuir para a quimiorresistência induzida pelo irinotecano.

ABSTRACT

Resistance of tumors to chemotherapeutic agents is a common clinical problem in human cancer. Recently, the blocking of PI3-kinase signaling pathway was shown to enhance apoptosis induced by SN-38, an active form of irinotecan. To gain further insight into the molecular events of irinotecan-associated increase in PI3-kinase signaling pathway, aspirin and rapamycin were used to modulate this signaling pathway. We describe here that aspirin is able to further inhibit IRS-1 serine phosphorylation induced by irinotecan through targeting JNK and IKK, thus agonist activation of IRS-1/PI3-kinase pathway blocked the growth-inhibitory effect of irinotecan in HT-29 colon cancer xenografts; our data also demonstrate a synergistic effect of mTOR inhibition and irinotecan on tumor growth. Activation of PI3-kinase/Akt/mTOR pathway may thus contribute to refractoriness to treatment with irinotecan.

INTRODUÇÃO

1- Histórico da quimioterapia

Dentre os muitos desafios da medicina, nenhum teve um início mais controverso que o do tratamento do câncer. Embora os processos neoplásicos sejam reconhecidos há séculos, pouco se sabia a respeito dos mecanismos biológicos de transformação e progressão tumoral até os adventos da medicina molecular na metade do século vinte (Chabner and Roberts 2005)

O início da era moderna da quimioterapia pode ser vinculado diretamente com a descoberta do gás mostarda. Em maio de 1942, Louis Goodman e Alfred Gilman, ambos farmacologistas, convenceram seus colaboradores a tratar um paciente portador linfoma não-Hodgkin com o gás mostarda (Gilman 1963). Propuseram que este reagente deveria destruir o tumor, baseado em achados da autópsia de soldados que morreram na Primeira Guerra Mundial ao entrarem em contato com o gás mostarda. Estas vítimas tinham uma profunda hipoplasia medular e mielossupressão. Razões pelas quais as doses de um reagente similar deveriam causar a regressão de linfomas e leucemias. O tratamento de fato resultou em regressão tumoral, entretanto em poucas semanas a doença voltou a progredir, mas o paradigma de que as drogas poderiam ser administradas sistemicamente para induzir a regressão tumoral estava estabelecido (Chabner and Roberts 2005).

Nos estudos seguintes, os mesmos cientistas, definiram a ação molecular do componente do gás mostarda, demonstrando a formação de um agente alquilante intermediário, o anel etileimonium, o qual reagia com os sítios doadores de elétrons nas proteínas e ácidos nucléicos. Os resultados destas pesquisas permitiram que novo princípio fosse estabelecido: os tumores são mais suscetíveis às toxinas do que os tecidos normais. A descoberta de que o reagente formava uma ligação covalente com o DNA foi feita através de estudos posteriores que demonstraram os sítios específicos de alquilação nas bases purínicas, levando a um *"crosslinking"* de extremidades e induzindo apoptose. Outros agentes alquilantes foram desenvolvidos nos 20 anos que se seguiram. Ciclofosfamida, Clorambucil e outros se tornaram drogas "*standard*" para o tratamento de pacientes com linfomas e leucemias. Infelizmente, Goodman e seus colaboradores notaram nestes primeiros experimentos que os tumores tornavam-se rapidamente resistentes a estas drogas,

uma observação que antecipou a experiência clínica com o uso da monoterapia (Chabner and Roberts 2005)

Uma segunda abordagem para o tratamento do câncer teve início logo após a segunda guerra mundial, quando Sydney Farber, um patologista da escola de medicina da Harvard, investigou o efeito do ácido fólico em pacientes com leucemia. Esta vitamina parecia estimular a proliferação de células de leucemia linfoblástica aguda (LLA) quando administrada a crianças com este câncer (Wills, Clutterbuck et al. 1937). Este fato levou a síntese de agentes análogos do ácido fólico, aminopterina e depois o metotrexate, que foram administrados a crianças com LLA. As remissões foram de curta duração, mas o princípio era claro: antifolatos podiam suprimir a proliferação de células malignas, e podiam restabelecer o funcionamento normal da medula óssea.

1.1 - Início da quimioterapia moderna

Outras drogas antileucêmicas surgiram em 1950, com os trabalhos de George Hitchings e Gertrude Elion, que estudaram os análogos purínicos como o 6-mercaptopurina 6-MP (Hitchings and Elion 1954; Johnson, Armstrong et al. 1963; Skipper, Schabel et al. 1964) Demonstraram que uma pequena mudança num componente necessário para as células poderia inibir o crescimento das células tumorais através da inibição dos passos que precediam à síntese de RNA e DNA. Paralelamente foram descobertos os alcalóides da vinca, originalmente descritos como agentes antidiabetes, estas drogas eram capazes também de bloquear a proliferação das células tumorais (Johnson, Armstrong et al. 1963).

O efeito antitumoral dos alcalóides da vinca decorre da habilidade dessas drogas em inibir a polimerização do microtúbulo, portanto, a divisão celular. Finalmente, em 1965, a combinação do metotrexato (um antifolato), a vincristina (alcalóide da vinca), 6-MP (6-mercaptopurina) e predinisona – os quais juntos foram referidos como o regime POMP (Frei, Karon et al. 1965). A combinação das drogas, cada uma com diferentes sítios de ação, provou ser mais efetiva na prevenção da resistência das células tumorais às drogas.

Em 1960, Frank Schabel e Howard Skipper analisaram a cinética do crescimento tumoral, bem como criaram ensaios *in vivo* para quantificação da citotoxidade (Skipper and Griswold 1984). Eles mostraram que diferentes doses da droga anticâncer destruia uma fração de células do tumor e que, dependendo da droga, a célula deveria ser exposta ao quimioterápico durante um período particular do ciclo celular. Assim, inibidores da síntese de DNA, e o metotrexato foram mais efetivos durante a divisão celular, enquanto drogas que danificavam fisicamente o DNA, como os agentes alquilantes, matavam as células em todas as fases do ciclo celular. Estas pesquisas também mostraram que a citotoxidade é dose dependente, e demonstraram que a combinação das terapias é eficaz na prevenção da resistência às drogas. Finalmente, Schabel e Skipper foram os primeiros a sugerir que altas doses de quimioterápicos poderiam ser usadas para curar pacientes com tumores refratários.

1.2 - Combinação de drogas em quimioterapia

Clinicamente a combinação quimioterápica começou a ser usada no tratamento de crianças com LLA. Esta abordagem foi estendida para o tratamento dos linfomas na década de 60 quando o gás mostarda, a vincristina, procarbazine e a prednisona – regime quimioterápico conhecido como regime MOPP – foram usadas conjuntamente no tratamento de linfoma de Hodgkin e não-Hodgkin com êxito (Moxley, De Vita et al. 1967; Devita, Serpick et al. 1970).

De acordo com os resultados obtidos em modelos animais, os agentes quimioterápicos são mais efetivos se usados combinados em pacientes com tumores de pequeno volume. De forma que mesmo drogas com atividade mais modesta como o 5-fluorouracil, um inibidor da síntese de DNA, pode diminuir a taxa de recidiva se usada como adjuvante ao tratamento de pacientes com câncer de cólon (Moertel, Fleming et al. 1990). Dados semelhantes foram obtidos no tratamento de tumores de outros sítios como no caso do câncer de mama (Bonadonna, Brusamolino et al. 1976). Em geral, a combinação quimioterápica das drogas provou ser mais eficiente do que a monoterapia tanto no tratamento do câncer metastático quanto no tratamento adjuvante.

1.3 - Os produtos naturais

Em 1956, C. Gordon Zubrod, estabeleceu um programa amplo nos Estados Unidos para a coleta e teste de atividade anticâncer de plantas e algas marinhas. Este programa resultou na descoberta dos taxanos (em 1964) e das camptotecinas (em 1966). Ambas as drogas encontraram grandes dificuldades no seu desenvolvimento. Paclitaxel, um dos principais taxanos usados atualmente cuja promoção de morte celular ocorre através dos seus efeitos em microtúbulos, inicialmente era difícil de ser sintetizado e virtualmente insolúvel, só tendo sido possível seu uso clínico a partir de 1991.

A camptotecina também encontrou problemas durante seu desenvolvimento. Derivada de uma planta ornamental chinesa age através da inibição da topoisomerase I. Inicialmente, o agente tinha pequena atividade antitumoral e alta toxicidade renal. Em 1996, o irinotecano, análogo estável da camptotecina, foi aprovado pela *Food and Drug Administration* (FDA) para o tratamento do câncer de cólon (Saltz, Cox et al. 2000).

1.4 - 1970 a 1980 sucesso na descoberta de novos quimioterápicos

Nesse período vários compostos que são usados como agentes quimioterápicos foram descritos: a cisplatina, essencial na cura do câncer de testículo e pedra angular no tratamento de carcinomas epidermóides; as nitrosuréias, tratamento de gliomas; a fludarabina, tratamento de leucemia linfocítica crônica; a doxorrubicina, droga em que se baseia o tratamento do câncer de mama.

1.5 - A necessidade de mudança das estratégias

Na década de 80 o progresso no desenvolvimento do tratamento quimioterápico tornou-se mais lento. Os estudos clínicos realizados com as drogas já existentes mostravam apenas ganhos marginais, além disso, os modelos animais de leucemia e tumores sólidos, que eram essenciais para o *screening* de drogas na época não eram bons preditores das respostas clínicas. Em 1985, começou-se a produzir grupos monotônicos de

antimetabólitos, alquilantes, antimitóticos e inibidores de topoisomerase. Análogos destas drogas determinaram aumento na eficácia dos tratamentos, gerando entusiasmo para clínicos e pacientes, atentos para novos tipos de agentes.

Em resposta a esse período os testes de screening de drogas passaram a ser feitos com 60 diferentes linhagens de tumores humanos. Componentes químicos de plantas, organismos marinhos e agentes descobertos na natureza tiveram seus extratos testados nessas linhagens tumorais. Houve sucesso em parte deles, a maioria agentes antimitóticos e inibidores da topoisomerase, resultando em aumento do número de agentes anti-câncer.

1.6 - Terapia alvo – Revolução no tratamento quimioterápico

Enquanto as atenções estavam voltadas para a descoberta dos agentes citotóxicos, no final de 1980, conhecimentos sobre a genética e a biologia molecular propiciaram entendimento sobre as vias de sinalização e atividades celulares reguladas, tais como a proliferação e sobrevivência. Muitas destas vias foram encontradas radicalmente alteradas em células de câncer. Pesquisas para reparar esses defeitos moleculares em células de câncer deram início a era da terapia alvo, que incluíam novos alvos como: fatores de crescimento, moléculas de sinalização, proteínas do ciclo celular, moduladores de apoptose e moléculas que promoviam a angiogênese (Hanahan and Weinberg 2000).

Inovações na tecnologia aumentaram o sucesso dos inibidores para alvos específicos. A química combinatória gerou milhares de estruturas para *screening* de inibidores *in vitro*, além disso, as características das drogas anticâncer tornaram-se mais bem delineadas de forma que um agente quimioterápico deveria ser metabolicamente estável, ter meia vida longa em modelo animais e em humanos e ter uma baixa taxa de depuração por enzimas da família do citocromo P450. As moléculas candidatas deveriam ser bem absorvidas por via oral, o que não era uma característica típica dos quimioterápicos descobertos entre 1970 e 1980.

Um dos eventos que marcaram a era da terapia alvo foi o desenvolvimento do mesilato de imatinib (glivec®). O Imatinib é um inibidor potente da quinase BCR-ABL, que está envolvida na patogênese da leucemia mielóide crônica. O Imatinib também inibe a tirosina quinase c-KIT e o receptor fator de crescimento derivado de plaquetas (PDGFRβ). Estas características do Imatinib permitiram seu uso no tratamento da leucemia mielóide crônica e de tumores do estroma gastrointestinal (Hughes, Kaeda et al. 2003).

Uma segunda classe de drogas são aquelas que inibem o fator de crescimento epidérmico (EGFR), que apesar de apresentarem uma menor atividade antitumoral que o imatinib fazem parte do arsenal de quimioterapias. O geftinib (Iressa®) está em testes no tratamento do câncer de pulmão (Kris, Natale et al. 2003) e o cetuximab (Erbitux®) indicado na terapia combinada do câncer de cólon (Cunningham, Humblet et al. 2004). Além dessas classes, os inibidores da angiogênese estão em franco desenvolvimento na terapia clínica e o bevacizumab (Avastin®) atualmente é usado na terapia combinada para câncer de cólon; o SU-11248 (sutent®) usado no tratamento do câncer renal e o Bayer 43-9006 (Sorafenib ®) usado no tratamento do câncer renal e hepatocarcinoma (Motzer and Bukowski 2006).

Há mais de 60 anos, clínicos dependem da classificação histológica dos tumores para ditar as escolhas terapêuticas. A tendência atual é de que o tratamento seja específico, isto é, os pacientes são cada vez mais selecionados para determinado tipo de tratamento baseado nas características moleculares do tumor. Esta abordagem confere vantagens enormes na eficiência e custo (Roberts and Chabner 2004).

1.7 – As vias de sinalização intracelular

A complexidade do desenvolvimento e crescimento de organismos, pode em parte, ser atribuído às interações dinâmicas e diversas entre hormônios, fatores de crescimento, contatos entre as células e outros estímulos externos que coordenam o destino de cada célula através de seus receptores de membrana. A explosão da pesquisa em transmissão do sinal intracelular nos últimos 10 anos vem decifrando os mecanismos

básicos de sinalização intracelular de um grande número desses receptores de membrana (Gough and Ray 2002). Entretanto, embora um grande progresso tenha sido realizado em identificar e descrever algumas das funções de moléculas intermediárias da sinalização celular, nós estamos longe de um entendimento completo de muitas dessas vias de sinalização. O seqüenciamento do genoma humano permitiu a identificação de diferentes membros de várias vias de sinalização. Nesse sentido, 11,2% dos genes que podem ter sua função predita estão diretamente envolvidos na transmissão do sinal (Venter, Adams et al. 2001). Isto é um número subestimado do total de genes envolvidos porque 41,7% dos genes no genoma humano não têm sua função predita, e muitos genes são importantes em múltiplas funções, tais como ancoramento celular e participação da matrix extracelular, que não foram classificados na categoria de genes envolvidos na sinalização celular (Venter 2000).

Um dos mais interessantes aspectos da transmissão do sinal intracelular descoberto nos últimos cinco anos é a constatação de que muitas vias de sinalização com diversas ações podem interagir em múltiplos níveis – isto é freqüentemente chamado de *cross-talk*. Uma procura pelo termo *"cross-talk"* no Medline mostra que ele aparece pela primeira vez em 1991 e tem sido usado em mais de 3500 artigos desde então (Carvalheira, Calegari et al. 2003; Carvalheira, Ribeiro et al. 2003; Carvalho, Carvalheira et al. 2003). É cada vez mais claro que a maioria das vias de sinalização, são intricadas e complexas de forma que, para entender uma única via de sinalização é necessário que se entenda globalmente a rede de interação entre as vias de sinalização (Arkin 2001; Levchenko 2001).

A elucidação das vias de crescimento celular e a observação de que essas vias estão alteradas no câncer humano levou a procura de inibidores específicos dessas vias (Zwick, Bange et al. 2001). Um dos principais desafios para descoberta de inibidores de sinalização é que vias distintas têm *cross-talk* em múltiplos níveis e, além disso, existe redundância de vias de sinalização usadas com o mesmo objetivo. Embora múltiplos inibidores de sinalização estejam atualmente em desenvolvimento ou já em ensaios clínicos, apenas alguns mostraram eficácia. Uma razão óbvia para isso é que vias distintas apresentam *cross-talk*. Assim, uma via compensatória pode emergir ou ganhar força após o bloqueio de uma via específica. Diferentes vias de sinalização podem controlar ou afetar a

mesma função celular, e uma única via de sinalização pode regular diferentes funções celulares. Esta rede de sinalização celular redundante parece refletir um mecanismo de ajuste fino para as células responderem e se ajustarem a efeitos combinados ou estímulos seqüenciais de muitos sinalizadores extracelulares ou sinais internos e controlar a duração e a intensidade de cada sinal.

Embora a rede de sinalização seja de alta complexidade e redundante existem obstáculos a essas vias, isto é, existem proteínas onde o sinal converge e se bloqueados podem impedir diversos processos celulares ao mesmo tempo correspondendo às diversas capacidades da célula cancerosa como: auto-suficiência de fatores de crescimento, defeito de apoptose, insensibilidade a sinais que inibem o crescimento e metástase (Cui and Lee 2004).

Proteínas adaptadoras, isto é, que se ligam a múltiplos elementos de uma cascata de sinalização e coordenam a sinalização celular, bem como quinases intracelulares podem ser candidatos ideais como alvos para o bloqueio da sinalização celular. Nesse sentido a via de sinalização IRS/PI3-quinase/Akt/mTOR e o NFkB aparecem como alvos para o bloqueio de crescimento e indução de apoptose de células tumorais.

1.8 - Os substratos do receptor de Insulina

O IRS-1 e o IRS-2 são substratos imediatos do IGF-IR, IR, RET e JAKs (através dos receptores de interleucinas e interferons) (Sun, Rothenberg et al. 1991; Myers, Sun et al. 1993; Saad, Carvalho et al. 1996; Burfoot, Rogers et al. 1997; Melillo, Carlomagno et al. 2001) (Figura 1). Os IRSs não possuem atividade tirosina quinase, mas são fosforilados após se ligar aos seus receptores ativados. A fosforilação em tirosina das proteínas IRS cria sítios de reconhecimento para moléculas contendo domínios com homologia a Src 2 (SH2) incluindo a PI3–quinase, Fyn, Grb2, Nck, Csk e SHP2, cada uma dessas moléculas iniciará uma via de sinalização diferente que contribuirá para o efeito biológico final das proteínas IRS (Carvalheira, Zecchin et al. 2002).

A ativação das proteínas IRS é conhecida por ser crítica para a mitogênese celular. Células 32D murinas que não expressam IRS-1 perdem sua capacidade de proliferar quando tratadas com interleucina-4 (IL-4) ou insulina (Wang, Myers et al. 1993). A expressão de IRS-1 nessas células, entretanto, restabelece a resposta mitogênica a insulina ou IL-4. Em células de hepatoma (Taouis, Dupont et al. 1998) e células de ovário de hamster (Waters, Yamauchi et al. 1993) a expressão de um oligonucleotídeo antisense para o IRS-1 reduz a taxa de crescimento e a incorporação de timidina em resposta à insulina.

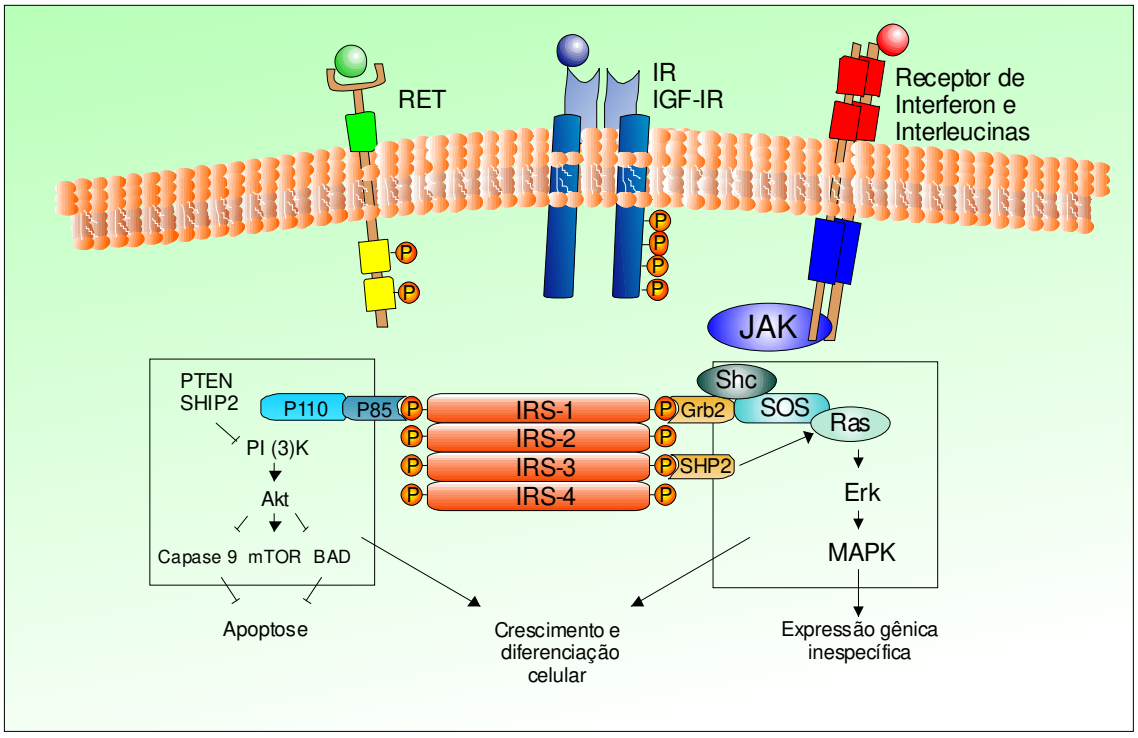

Figura 1- As proteínas IRS são centrais para várias funções celulares.

Vários estudos demonstraram o potencial de transformação para o IRS-1. O aumento da expressão de IRS-1 em células NIH3T3 induz transformação neoplásica através da interação com o Grb2 e SHP2, que ativam a via de sinalização da *mitogen-activated protein kinase* (MAPK) (Ito, Sasaki et al. 1996; Tanaka, Ito et al. 1996). Fibroblastos

derivados de embrião de camundongos são resistentes a transformação por oncogenes como o antígeno do SV40 T. A coexpressão de IRS-1 e o antígeno SV40 T induz a transformação neoplásica do fibroblasto (D'Ambrosio, Keller et al. 1995). Além disso, o IRS-1 está associado com o antígeno SV40 T nas células transformadas (Fei, D'Ambrosio et al. 1995). A expressão de IGF-IR induz a diferenciação e subseqüente morte de células de câncer de próstata (LNCaP). A coexpressão de IRS-1 e IGF-IR nestas células, entretanto, inibe efetivamente a diferenciação e restabelece o fenótipo transformado da célula LNCaP (Reiss, Wang et al. 2000).

Recentemente foi descrito que a ativação constitutiva das proteínas IRS é um fenômeno comum em vários tumores humanos (Chang, Li et al. 2002) e que os IRSs são também importantes mediadores da angiogênese tumoral em células de câncer de pâncreas (Neid, Datta et al. 2004). Além disso, a inter-relação entre a ativação do IRS-1 com oncoproteínas já foi estabelecida. As oncoproteínas BCR-ABL (Traina, Carvalheira et al. 2003), TRK-T1 (Miranda, Greco et al. 2001) e ETV6-NTRK3 (Lannon, Martin et al. 2004) se ligam ao IRS-1 e estão associados com o aumento da fosforilação em tirosina do IRS-1. Assim, a ativação das proteínas IRS pode ser um mecanismo mais geral da mediação da transformação tumoral mediada por oncogenes.

2 - Quimiorresistência

A resistência dos tumores à quimioterapia é um problema clínico comum em cânceres humanos (Fisher 1994). A resistência a quimioterápicos pode já existir antes do início da terapia ou ser adquirida. A efetividade do tratamento quimioterápico nos diferentes tipos de câncer é bastante distinta sendo a quimiorresistência um dos principais responsáveis. Dessa forma, os diferentes tipos de câncer são subdivididos de acordo com os padrões das respostas aos tratamentos e sensibilidade tumoral da seguinte maneira:

1- Tumores intrinsecamente sensíveis às drogas. Exemplo: leucemia linfoblástica, linfoma de Hodgkin e câncer testicular.

2- Tumores que respondem inicialmente aos tratamentos, mas depois tornam-se refratários à terapia. Exemplo: câncer de mama, câncer de pequenas células de pulmão e carcinoma de ovário.

3- Tumores intrinsecamente resistentes à maioria dos agentes quimioterápicos. Para este grupo, o número de agentes antioneoplásicos ativos é pequeno e as respostas quimioterápicas significantes são afetadas na minoria dos casos. Exemplo: melanoma maligno e câncer de cólon.

A seleção experimental de resistência às drogas por repetidas exposições a um único agente antineoplásico geralmente resulta em resistência cruzada a outros agentes quimioterápicos. Este fenômeno é explicado com base nos transportadores da droga, vias de metabolismo da droga e alvos citotóxicos intracelulares. Assim, agentes antineoplásicos de diferentes classes frequentemente dividem as mesmas vias metabólicas, sistemas de transporte de efluxo ou sítios de ação citotóxica.

2.1 - Diminuição do acúmulo de quimioterápicos

Diminuição dos níveis intracelulares de agentes citotóxicos é um dos mecanismos mais comuns de resistência às drogas. Drogas solúveis em água não podem penetrar a bicamada lipídica da membrana celular e requerem mecanismos específicos para entrar na célula. Como exemplo podemos citar a diminuição do influxo intracelular causado pela alta afinidade da droga ao sistema de sistema de transporte (Antony, Kane et al. 1985), bem como a redução do transporte (Dixon, Lanpher et al. 1994) ,estes mecanismos são causas de resistência ao metotrexato (Hill, Bailey et al. 1979; Sirotnak, Moccio et al. 1981). Um sistema de transporte deficiente pela membrana tem sido identificado em células resistentes ao gás mostarda (Goldenberg, Vanstone et al. 1970). Drogas apolares podem facilmente atravessar a membrana celular, entretanto a concentração intracelular destas drogas pode ser reduzida com o aumento da atividade do efluxo da droga. O aumento da glicoproteína-P (codificada pelo gene MDR1 associado à resistência a múltiplas drogas) constitui um dos principais mecanismos responsáveis pelo efluxo da droga e é um

importante exemplo deste mecanismo de resistência (Endicott and Ling 1989; Gottesman and Pastan 1993). Assim, a resistência associada às antraciclinas, antibióticos e agentes antimicrotúbulos, são frequentemente associadas ao aumento da expressão de glicoproteína-P.

2.2 - Metabolismo alterado dos quimioterápicos

A inativação de drogas ou cofatores podem conferir resistência a agentes antineoplásicos. Por exemplo, vários antimetabólitos e alguns agentes alquilantes (ex. ciclofosfamida), são administrados como pró-drogas, as quais devem ser ativados em seus metabólitos citotóxicos pelo tumor, que é o alvo, ou por outros tecidos. A resistência a muitas drogas está associada com a diminuição da conversão destas drogas em suas formas ativas. A sensibilidade celular ao CPT-11 (um inibidor da enzima topoisomerase I) é em parte governada pela enzima carboxilesterase - enzima necessária para converter o CPT-11 no seu metabólito ativo SN-38 (Kanzawa, Sugimoto et al. 1990; Haaz, Rivory et al. 1997). Além disso, aumento dos níveis intracelulares das deaminases levando ao aumento da inativação dos análogos das pirimidinas e purinas é o elo para a resistência a estes agentes (Steuart and Burke 1971; Hunt and Hoffee 1983).

2.3-Tolerância celular e reparo aumentado aos danos induzidos pelos quimioterápicos

As células contêm um sistema complexo múltiplo envolvido no reparo de danos causados à membrana celular e ao ácido desoxirribonucléico (DNA). Como estes danos podem decorrer da ação direta ou secundária das drogas citotóxicas, mecanismos de reparo intrínsecos alterados podem influenciar a sensibilidade ao quimioterápico (Perez 1998).

Apesar das drogas anticâncer terem suas citotoxidades mediadas através de uma infinidade de alvos moleculares elas terminam por afetar as vias de morte celular associadas com a morte celular programada ou apoptose (Hickman 1996; Zunino, Perego et al. 1997). Os quimioterápicos podem levar à várias respostas celulares alternativas,

incluindo atrasos no ciclo celular e ativação de processos de reparo ou ativação de células suicidas por apoptose. Mutações ou níveis de expressões alterados dos genes que regulam estas respostas alternativas ao estresse induzido pela droga estão frequentemente associados à quimiorresistência. Por exemplo, a expressão de genes p53, p21 e genes da família bcl-2 por influenciar profundamente a apoptose modificam a sensibilidade celular ou resistência às drogas anticâncer.

2.4 - Alvos intracelulares modificados

O mecanismo de vários agentes antineoplásicos para destruir as células envolve interações entre as drogas e as enzimas intracelulares. Estas interações resultam numa alteração ou inibição das funções normalmente exercida pelas enzimas. Mudanças quantitativas ou alternativas nestas enzimas alvos das drogas antineoplásicas podem comprometer a eficácia das drogas. Estas mudanças têm sido demonstradas em várias enzimas associadas com células resistentes às drogas incluindo as topoisomerases (Vassetzky, Alghisi et al. 1995).

2.5 - Expressão alterada dos genes

Os mecanismos celulares de resistência às drogas citados anteriormente, dependem dos níveis alterados ou da função dos produtos dos genes alvos. Estas alterações podem resultar a partir de mudanças que ocorrem em qualquer ponto ao longo da via de expressão gênica e de regulação. Processos moleculares múltiplos estão envolvidos na resistência às drogas, incluindo mutações no DNA, deleção ou amplificação e controles transcricionais dos níveis de RNA. As prevalências destas mudanças refletem nas instabilidades genéticas e fenotípicas das células de câncer, talvez pressões mutagênicas de toxinas xenobióticas e exposição às drogas.

Em resumo, as células tumorais têm uma necessidade premente de sobreviver, de forma que qualquer alteração genética que favoreça a sobrevida em condições adversas será selecionada. A conseqüência, certamente, será a sobrevivência de alguns tumores ao mais potente agente terapêutico. Entretanto, o crescimento do conhecimento das vias envolvidas na sobrevivência, trouxe a esperança de que bloqueando moléculas específicas aumente a sensibilidade das células tumorais aos diferentes quimioterápicos – numa abordagem combinatória que sobrepuje a quimiorresistência (McCormick 2004).

2.6 - Via PI3-quinase/Akt/mTOR

Uma das principais moléculas ativada pelas proteínas IRS é a PI3-quinase, esta enzima é importante na regulação da mitogênese, diferenciação celular e transporte de glicose estimulado pela insulina (Folli, Saad et al. 1992; Saad, Folli et al. 1993; Shepherd, Nave et al. 1995). A PI-3 quinase foi originalmente identificada como um dímero composto de uma subunidade catalítica (p110) e uma subunidade regulatória (p85). A ligação dos sítios YMXM e YXXM (onde Y = tirosina, M = metionina e X = qualquer aminoácido) fosforilados das proteínas IRS ao domínio SH2 da subunidade p85 da PI3 –quinase ativa o domínio catalítico associado (Backer, Myers et al. 1992). A enzima catalisa fosforilação dos fosfoinositídeos na posição 3 do anel de inositol produzindo fosfatidilinositol 3 fosfato, fosfatidilinositol 3,4 difosfato e fosfatidilinositol 3,4,5 trifosfato (Lietzke, Bose et al. 2000).

A produção do fosfatidinositol 3,4,5 trifosfato (PIP3) pela PI3-quinase recruta as serinas treoninas quinases PDK1 e Akt para a membrana plasmática, onde a Akt é ativada pela PDK1 mediando a fosforilação (Lawlor and Alessi 2001). A via de sinalização iniciada após a ativação da PI 3-quinase também é importante para a prevenção de apoptose. A Akt destaca-se como uma das principais proteínas alvo da PI 3-quinase para esse efeito (Carvalheira, Ribeiro et al. 2003; Thirone, Carvalheira et al. 2004). Esforços para delinear a base molecular dos efeitos antiapoptose da Akt tomaram várias direções (Vivanco and Sawyers 2002) (figura 2). Como uma quinase, a tendência natural da Akt é adicionar um grupamento fosfato em substratos, assim, Bad e caspase 9, proteínas que levam a morte celular, foram os primeiros alvos fosforilados pela Akt descritos (Basu,

Totty et al. 2003). Em seguida vieram outros reguladores da morte celular como o IKKα, a família dos fatores de transcrição tipo forkhead, o Mdm2 e YAP (Basu, Totty et al. 2003). Assim bloqueando a Akt seria esperada a indução de morte celular em vários níveis (figura 2). Entretanto, se algum desses candidatos pudesse ser responsabilizado pela a ação antiapoptose da Akt na sobrevida de células cancerosas ainda não é claro.

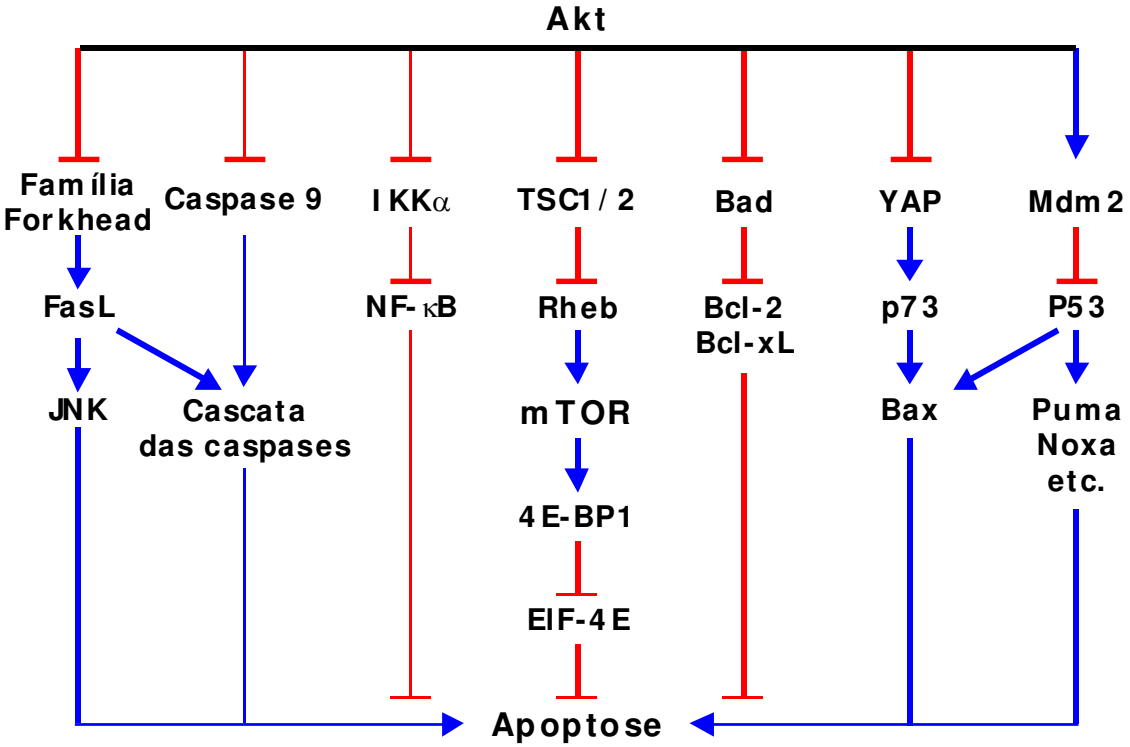

Figura 2- A complexidade da via de antiapoptose emergindo da Akt.

Mais recentemente, a atenção foi direcionada para um diferente ramo da via PI3-quinase/Akt, na qual figura a mTOR. Akt fosforila muitas proteínas com importantes papéis fisiológicos, incluindo TSC2, inibindo-se as GTPases ativa-se as proteínas através das proteínas-G Rheb (Inoki, Li et al. 2003). A mTOR é regulada pela proteína Rheb, que por sua vez é controlada pelos supressores tumorais TSC (Manning and Cantley 2003). Usando um modelo animal de linfoma de células B para explorar as conseqüências da inibição da Akt, estabeleceram a sinalização da Akt através da mTOR como um importante mecanismo de oncogênese e resistência a drogas quimioterápicas convencionais (Wendel,

Stanchina Ed et al. 2004). A via PI3-quinase/Akt, também foi descrita como alvo de quimioresistência em carcinoma hepatocelular (Jin, Yim et al. 2003; Knuefermann, Lu et al. 2003) . Assim, a Akt tem sua expressão aumentada em muitos tumores malignos (Cheng, Godwin et al. 1992) e sua ativação leva à resistência a diversas drogas (Knuefermann, Lu et al. 2003).

2.7 - Via de sinalização do NFκB

O NFκB ativado pode ser crucial no desenvolvimento da resistência das células de câncer às drogas. Estudos recentes indicam que o NFκB tem um sítio de ligação para os genes de resistência à múltiplas quimioterapias (MDR1) (Nakanishi and Toi 2005).

O NFκB é ativo no núcleo e inibido no citoplasma onde é mantido através do seu inibidor κB (IκB) (Jobin and Sartor 2000). IκB contém um domínio anquirina, ao qual se liga ao NFκB mantendo-o no citoplasma. O NFκB torna-se ativo quando é liberado do IκB. IκB é alvo de uma bem caracterizada cascata que ativa o IκB quinase (IKK). As subunidades IKKα e o IKKβ preferencialmente formam heterodímeros e ambos podem fosforilar diretamente os resíduos S32 e S36 do IκB requerido para a degradação. IκB fosforilado resulta na ubiquitinação e degradação pelo proteossoma (Senftleben, Cao et al. 2001).(Figura 3)

Um importante fator que influencia a apoptose das células tumorais é o fator de transcrição NFκB. Estudos recentes demonstram que a inibição do NFκB potencializa a terapia antitumoral através do aumento da apoptose (Wang, Cusack et al. 1999). Além disso, a inibição do NFκB pelo super-repressor (IκBsr) ou um inibidor de proteossoma sensibiliza as células de câncer de cólon a apoptose (Cusack, Liu et al. 2000), enquanto que a ativação constitutiva é observada no carcinoma hepatocelular (Tai, Tsai et al. 2000).

A morte celular programada pode ser tanto através de apoptose como de necrose. A apoptose pode ser caracterizada pela ruptura na membrana e condensação da célula e de suas organelas (Kerr, Wyllie et al. 1972; Wyllie, Kerr et al. 1980). Duas vias bem estabelecidas levam a apoptose: uma via extrínseca ligada ao domínio de morte, e

outra intrínseca ligada à via mitocondrial. Ambas dependem da ativação das caspases. Além do mais, a ativação das caspases nem sempre leva à morte celular, e a caspase-8 também tem função de pró-sobrevivência. A necrose é bem caracterizada pelo aumento de volume da célula e de suas organelas, culminando na ruptura da membrana e lise celular, normalmente acompanhada pela inflamação. Falhas no metabolismo energético e geração maciça de espécies reativas de oxigênio são causas de necrose (Fiers, Beyaert et al. 1999).

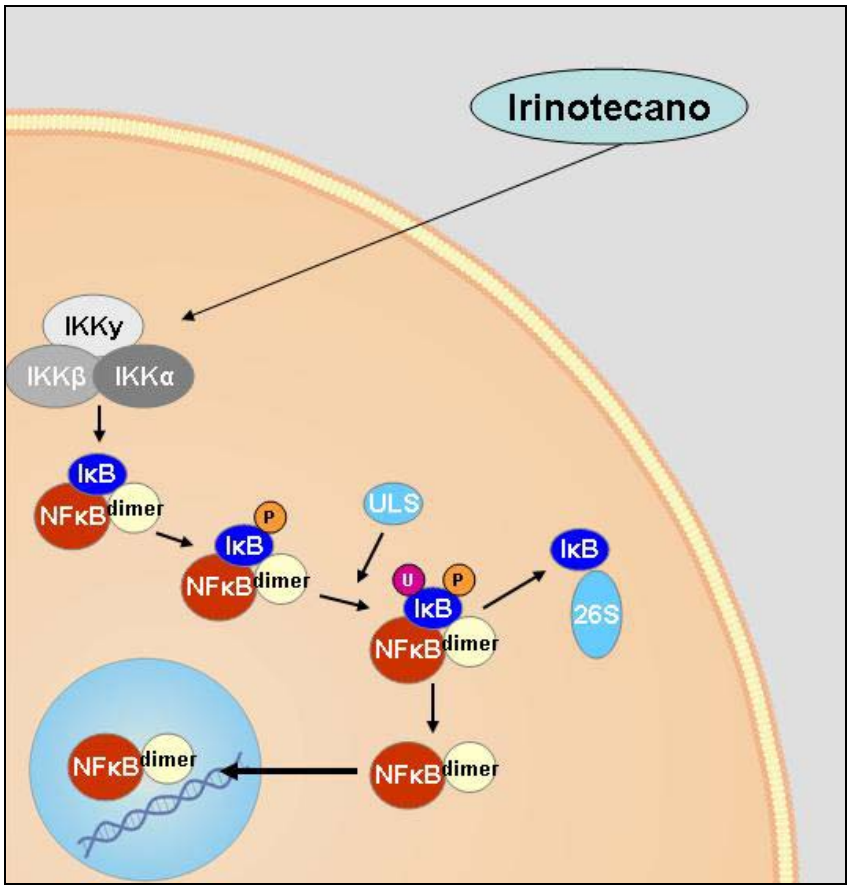

Figura 3- A via de ativação do NFκB (figura adaptada do artigo de Chikashi Nakanishi e Masakazu Toi)

O NFκB suprime os dois tipos de morte celular e, sendo um fator de transcrição, induz genes cujos produtos previnem a morte celular programada. Um iniciador da ativação do NFκB é o TNFα. Este inicia o processo de morte celular programada somente quando uma nova proteína ou síntese de RNA é inibida ou em células

deficientes em NFκB. Esta proteína enxerta uma atividade pró-sobrevivência através de várias proteínas antiapoptóticas, incluindo FLIP, XIAP e outros (Karin and Lin 2002; Kucharczak, Simmons et al. 2003). FLIP inibe a apoptose por interferir na ativação da caspase-8 (Micheau and Tschopp 2003). XIAP liga-se diretamente e inibe o efetor das caspases.

A associação do NFκB com a inflamação associada à promoção tumoral, progressão e metástase é bem documentada e demonstrada em modelos animais (Greten, Eckmann et al. 2004; Luo, Maeda et al. 2004; Pikarsky, Porat et al. 2004). A ativação do NFκB dependente do IKKβ estabelece uma ligação entre o câncer de cólon e a inflamação também em modelos animais (Greten, Eckmann et al. 2004). A ativação do IKKβ aumenta o componente maligno deste tumor, suprime a apoptose de células pré-neoplásicas, onde a ativação destas promove a produção de várias citocinas que servem como fator de crescimento e transformação. IL-6 interfere no crescimento tumoral, mas não afeta a sobrevivência celular (Becker, Fantini et al. 2004), enquanto que a inativação do IKKβ resulta em uma diminuição dramática das células tumorais por aumentar a apoptose, mas sem efeito na proliferação ou crescimento tumoral (Greten, Eckmann et al. 2004).

3- Mecanismos de contra-regulação da PI 3-quinase/AKT/mTOR

Muitos mecanismos podem contribuir para contra-regular a via da PI3–quinase, incluindo as fosfatases e a fosforilação em serina das proteínas IRS pelas proteínas serina quinases, como a c-jun-N-terminal kinase (JNK) (Lee, Giraud et al. 2003; Barreiro, Prattali et al. 2004; Prada, Zecchin et al. 2005) e a mTOR.

3.1 - Cross-talk entre o NFκB e a JNK.

O papel da JNK na morte celular programada tem sido controverso, porque tem efeitos tanto na sobrevivência quanto na morte celular. As evidências de que a JNK é um regulador da morte celular programada vêm a partir de uma análise de camundongos

knockout para JNK1 ou JNK2. Esses animais são relativamente resistentes à indução de hepatite fulminante em resposta à concanavalina A (Maeda, Chang et al. 2003). A atividade pró-sobrevivência do NFκB depende da habilidade de se prevenir à ativação prolongada da JNK. Então, NFκB pode ser um regulador crítico da sobrevivência e morte celular através de sua habilidade de controlar a duração da ativação da JNK (De Smaele, Zazzeroni et al. 2001; Sakon, Xue et al. 2003; Kamata, Honda et al. 2005). Esta ativação sustentada em células deficientes em NFκB implica que o NFκB induz a expressão de inibidores da JNK, tais como o GADD45β (Papa, Zazzeroni et al. 2004) e XIAP (Meng, Buckley et al. 2004).

3.2 - Feedback da mTOR

Estudos recentes demonstram que muitas quinases, incluindo enzimas sensíveis à rapamicina, promovem a fosforilação em serina do IRS-1 e consequentemente inibem sua função e promovem sua degradação (Mothe and Van Obberghen 1996; Rui, Fisher et al. 2001). A fosforilação do Akt pode ser resgatada com o tratamento com rapamicina, fenômeno que coincide com o aumento dos níveis protéicos de IRS-1. O resgate da fosforilação do Akt mediado pela rapamicina pode ser bloqueado com a redução da expressão de IRS-1 Assim, a ativação crônica da mTOR através da inativação do TSC1-TSC2 estimula componentes de vias da síntese de proteínas, enquanto inibe a sinalização da cascata de sinalização da insulina/IGF-1 através do IRS-1 (Harrington, Findlay et al. 2004; Shah, Wang et al. 2004; Ueno, Carvalheira et al. 2005).

3.3 - Proteínas fosfatases

As proteínas fosfatases regulam negativamente os efeitos metabólicos e promotores de crescimento da atividade da PI3-quinase. As fosfatases PTEN e SHIP-1 e 2 podem remover os fosfatos do PIP3 (Steelman, Bertrand et al. 2004). Mutações nestas fosfatases, as quais eliminam suas atividades, podem levar à progressão do tumor. Consequentemente, os genes que codificam estas fosfatases são referidos como genes supressores tumorais (McCubrey, Steelman et al. 2006). Dessa maneira, sinais de

sobrevivência ativam a PI3-quinase cuja subunidade catalítica está amplificada em alguns tipos de câncer, enquanto que a fosfatase homóloga da tensina presente no cromossomo 10 (PTEN), um antagonista da PI3-quinase, está frequentemente mutada em cânceres avançados.

3.3.1 - Serinas quinases

JNK

JNK é uma serina treonina quinase responsável pela ativação dos fatores de transcrição c-jun e ATF2 (Derijard, Hibi et al. 1994; Minden, Lin et al. 1995). Recentemente, a JNK tem sido associada à regulação da sinalização através da PI3–quinase em vários estudos (Aguirre, Uchida et al. 2000; Rui, Aguirre et al. 2001; Aguirre, Werner et al. 2002; Hirosumi, Tuncman et al. 2002; Lee, Giraud et al. 2003). Estes estudos demonstraram que a JNK contribui para a redução da sinalização da PI 3–quinase através da fosforilação do IRS-1 em Ser^{307} e esta fosforilação leva à inibição da função do IRS-1 (Aguirre, Uchida et al. 2000; Rui, Aguirre et al. 2001; Aguirre, Werner et al. 2002; Lee, Giraud et al. 2003). A aspirina inibe a atividade da JNK (Gao, Zuberi et al. 2003; Jiang, Dallas-Yang et al. 2003) e consequentemente reduz a fosforilação do IRS-1 em Ser^{307}. Ser^{307} está localizada próximo ao domínio fosfotirosina (PTB) no IRS-1 e sua fosforilação inibe a interação do domínio PTB com o NPEY fosforilado, modificado em receptor ativo, causando redução da sinalização através da via da PI3–quinase (Aguirre, Werner et al. 2002).

A anisomicina, um ativador específico da JNK, induz a diminuição da mobilidade eletroforética do IRS-1. A JNK se liga diretamente ao IRS-1 e fosforila seus resíduos de Serina, além disso a fosforilação da Ser^{307} do IRS-1 está reduzida em células desprovidas de JNK1 e JNK2. Assim, conclui-se que os efeitos da anisomicina na ativação da PI3-quinase são mediados pela habilidade da JNK de fosforilar o IRS-1 (Harrington, Findlay et al. 2004).

4 - Irinotecano

O irinotecano (CPT-11) é um análogo semisintético da camptotecina, originalmente isolado na China de uma planta ornamental conhecida como *Camptotheca acuminata*. É um agente quimioterápico que causa morte celular especificamente na fase S por ligar-se com a topoisomerase I (Topo I). A descoberta e síntese dessa droga ocorreram no Japão em 1983, e só recentemente ficou clara a potente atividade antitumoral do irinotecano contra inúmeros tumores (Rothenberg 2001) .

CPT-11 é uma pró-droga convertida pela carboxilesterase em sua forma ativa SN-38, que tem sido usado em pacientes com câncer. Estudos de fase II mostraram taxa de resposta objetiva em 32% dos pacientes com câncer colorretal. Posteriormente estudos de fase III consolidaram o papel do CPT-11 no tratamento de primeira linha em combinação com o fluoracil e o ácido fólico, para o tratamento do câncer colorretal metastático (Conti, Kemeny et al. 1996; Saltz, Cox et al. 2000) .

4.1 - Mecanismo de ação

O irinotecano interage num complexo topoisomerase I – DNA e tem uma citotoxidade específica na fase S (Liu 1989). As topoisomerases reduzem a torção do DNA e o *supercoiled* que ocorre em regiões específicas do DNA possibilitando sua transcrição e replicação. O irinotecano cliva a ligação de fosfodiester do DNA, e forma uma ligação covalente da enzima com o DNA, os quais permitem a passagem de uma outra fita (dupla ou única) do DNA pelas extremidades do DNA. A topoisomerase I se liga nesta extremidade clivada do DNA e o complexo formado TopoI – irinotecano – DNA, mas sozinho não é letal para as células. Entretanto, após as colisões com os *forks* de replicação avançado, provocam quebras na fita do DNA, levando a um atraso irreversível na replicação e morte celular (Liu 1989). A colisão do complexo do irinotecano com a topoisomerase I, com o processo de replicação também resulta em atraso na fase G2 por sinalizar a presença de danos no DNA para o mecanismo de checkpoint na fase S do ciclo celular (Shao, Cao et al. 1999). Altas concentrações de irinotecano, em células que não

estejam na fase S, também destroem as células e este mecanismo de morte celular é transcricionalmente mediado pelo dano no DNA através de apoptose (Morris and Geller 1996).

4.2 - Mecanismos de resistência ao irinotecano

4.2.1 - Diminuição do nível intracelular de irinotecano por aumento do efluxo da droga

A resistência a múltiplas drogas é o maior obstáculo encontrado pela quimioterapia no câncer, é caracterizado pelo aumentado da expressão de transportadores transmembrana ligados ao ATP (ABC – ATP *binding Cassete*) tais como a glicoproteina-P e a proteína associada à resistência a múltiplas drogas (MRP), os quais transportam os agentes quimioterápicos para fora da célula (Loe, Deeley et al. 1996). A glicoproteína-P e o MRP estão envolvidos no efluxo ativo do SN-38 e irinotecano (Chen, Furukawa et al. 1999; Chu, Suzuki et al. 1999).

4.2.2 - Níveis de topoisomerase I

A topoisomerase I é o alvo celular do irinotecano, e é aceitável que os níveis celulares da topoisomerase I sejam proporcionais aos efeitos citotóxicos. Esta idéia deriva de experimentos com linhagens celulares de mamíferos (Kanzawa, Sugimoto et al. 1990; Reid, Benedetti et al. 1998). As linhagens celulares resistentes ao irinotecano tornaram-se gradualmente resistentes através de tratamentos contínuos com irinotecano, e a atividade total da topoisomerae I foi reduzida quando comparada com linhagens celulares sensíveis ao irinotecano (Giovanella, Stehlin et al. 1989). Teoricamente a expressão da topoisomerase I em vários tumores pode ser com um indicativo para a sensibilidade ao irinotecano. Níveis aumentados da topoisomerase I são demonstrados em câncer de cólon (Giovanella, Stehlin et al. 1989; Husain, Mohler et al. 1994). Os níveis de topoisomerase I encontrados em tumores colorretais são altos quando comparados com a mucosa colônica

normal sugerindo uma relação causal com a terapêutica favorável (Giovanella, Stehlin et al. 1989).

4.2.3 - Mutações da topoisomerase I

Mutações da topoisomerase I são encontradas em células resistentes ao irinotecano (Rubin, Li et al. 1996; Saleem, Edwards et al. 2000). Estas mutações são encontradas em áreas que podem alterar a clivagem do DNA ou as interações do DNA com o irinotecano.

4.3 - O NFκB e o irinotecano

A maioria dos quimioterápicos atua através da indução da via apoptótica. A resistência na indução da apoptose é o principal mecanismo pelo qual a célula de câncer protege-se contra a morte celular (Fisher 1994). A ativação do NFκB pelo TNF-α, radiação ionizante e quimioterápicos, leva a inibição da reposta apoptótica induzida por estes estímulos em células de fibrosarcoma (Wang, Mayo et al. 1996). Recentemente, foi descrito que o inibidor da topoisomerase I CPT-11/SN-38 ativa a via do NFκB em células de fibrosarcoma e hepatocarcinoma (Cusack, Liu et al. 2000).

4.4 - PI 3-quinase e irinotecano

Estudos iniciais, em células de hepatoma humano, indicam que o bloqueio da PI3–quinase aumenta a apoptose induzida pelo SN-38, (Koizumi, Hatano et al. 2005). Entretanto, os mecanismos moleculares envolvidos na ativação da PI3-quinase ainda são desconhecidos.

OBJETIVOS

- Caracterizar a via de sinalização IRS/PI3-quinase/Akt/mTOR em linhagem de células tumorais de adenocarcinoma de cólon (HT29).

- Investigar a ação conjunta de bloqueadores (rapamicina) ou ativadores (aspirina) na via IRS/PI3-quinase/Akt/mTOR e o irinotecano no crescimento e apoptose de células tumorais.

- Investigar a ação conjunta de bloqueadores (rapamicina) ou ativadores (aspirina) da via IRS/PI3-quinase/Akt/mTOR e irinotecano no desenvolvimento de tumores em camundongos da linhagem Scid.

ARTIGO

CHRONIC TREATMENT WITH IRINOTECAN ACTIVATES THE PI3K/AKT/mTOR PATHWAY IN HT-29 COLON CANCER XENOGRAFTS

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KEYWORDS: HT-29; mTOR; IRS-1; Irinotecan, aspirin

ABSTRACT

Resistance of tumors to chemotherapeutic agents is a common clinical problem in human cancer. Recently, the blocking of the PI3-kinase signaling pathway was shown to enhance apoptosis induced by SN-38, an active form of irinotecan. To gain further insight into the molecular events of the irinotecan-associated increase in the PI3-kinase signaling pathway, aspirin and rapamycin were used to modulate this signaling pathway. We herein report that aspirin is able to further inhibit IRS-1 serine phosphorylation induced by irinotecan through targeting of JNK and NFκB. Thus, agonist activation of the IRS-1/PI3-kinase pathway blocked the growth-inhibitory effect of irinotecan in HT-29 colon cancer xenografts; our data also demonstrate a synergistic effect of mTOR inhibition and irinotecan on tumor growth. Activation of the PI3-kinase/Akt/mTOR pathway may, thus, contribute to refractoriness for treatment with irinotecan.

INTRODUCTION

Resistance of tumors to chemotherapies is a common clinical problem in human cancer (Fisher 1994). Resistance to chemotherapies may already exist before the initiation of therapy due to the overexpression of the multidrug resistance gene product, MDR1, which functions in the export of a variety of chemotherapies (Baldini 1997). Moreover, chemoresistance (acquired or inducible) may develop in response to chemotherapies by mostly unknown mechanisms.

Irinotecan, a derivative of camptothecin, is a major antitumor agent known to cause cellular damage via inhibition of the nuclear enzyme, topoisomerase I (Hahn and Weinberg 2002; Schaid 2004). Over the last decades these compounds have been successfully used as anticancer agents in the clinic, in particular against metastatic colorectal cancer (Garcia-Carbonero and Supko 2002). Numerous reports on topoisomerase inhibitors show that irinotecan potently activates NF-κB and the cytotoxicity generated by irinotecan is enhanced considerably by inactivation of NF-κB (Piret and Piette 1996; Wang, Cusack et al. 1999). Recently, the blocking of the PI3-kinase/Akt pathway was shown to

enhance apoptosis induced by SN-38, an active form of irinotecan, in human hepatoma cells (Koizumi, Hatano et al. 2005). Activation of Akt leads to the stimulation of antiapoptotic pathways, promoting cell survival. Akt also regulates the mammalian target of the rapamycin (mTOR)-S6K-S6 pathway to control cell growth in response to growth factors and nutrients (Manning and Cantley 2007).

A number of mechanisms may contribute to the deregulation of the PI3-kinase signaling pathway, including serine phosphorylation of IRS proteins by protein kinases such as c-jun N terminal kinase (JNK) (Lee, Giraud et al. 2003; Barreiro, Prattali et al. 2004; Prada, Zecchin et al. 2005) and IKK. Ser^{307} is located next to the phosphotyrosine-binding (PTB) domain in IRS-1 and its phosphorylation inhibits the interaction of the PTB domain with the phosphorylated NPEY motif in the activated receptor, causing a reduction in the signaling through the PI3-kinase pathway (Aguirre, Werner et al. 2002).

To further understand the molecular mechanism underlying chemoresistance to irinotecan, we examined the serine kinases listed above in HT-29 xenografts after irinotecan chronic treatment. Our results show that IKK is activated by irinotecan, in addition, there is a reduction in JNK activation and these alterations are correlated to a reduced phosphorylation of IRS-1 serine residues that leads to an increase in PI3-kinase/Akt/mTOR pathway signaling; moreover, our data also demonstrate a synergistic effect of mTOR inhibition and irinotecan on tumor growth.

MATERIALS AND METHODS

Antibodies, Chemicals and Buffers

The reagents and apparatus for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were from Bio-Rad (Richmond, CA). Tris-[hydroxymethyl]amino-methane (Tris), phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT), Triton X-100, Tween 20, glycerol and aspirin were from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was from Calbiochem (La Jolla, CA, USA). Aprotinin was from Bayer (São Paulo, SP, Brazil). Ketamin was from Parke-Davis (São Paulo, SP, Brazil), Diazepam and sodium thiopental were from Cristália

(Itapira, SP, Brazil), Irinotecan was from Novartis and rapamycin was from LC Laboratories (Woburn, MA, USA). Protein A-Sepharose 6 MB, nitrocellulose membrane (Hybond ECL, 0.45μm) and [^{125}]I-Protein A were from Amersham (Buckinghamshire, UK). Anti-phospho-JNK, anti-phospho-c-jun, anti-AKT antibody for immunoblotting was from Santa Cruz (CA, USA). Anti-IRS-1, anti-IRS-2, anti-PI3K, antiphosphoserine IRS-1 307, antiphosphotyrosine antibody for immunoblotting was from Upstate Biotechnology (NY, USA). P-p70S6K, p70S6K, eIF4E, p-eIF4E and p-AKT antibodies for immunoblotting were from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

The human colon cancer cell line HT-29 was obtained from ATCC, Philadelphia, PA, USA. Cells were cultured in RPMI containing 10% fetal calf serum and glutamine with the addition of penicillin/streptomycin and amphotericin B. Cell lines were maintained at 37°C, 5% CO2.

Human Tumor Xenograft Models

SCID mice were provided by the State University of Campinas - Central Breeding Center (Campinas, SP, Brazil). SCID mice, 5–6 weeks old and with a body weight of approximately 20–25 grams were implanted with 1 x 10^6 HT-29 cells into the dorsal subcutis of male mice. When the tumors were between 50 and 100 mm^3, the animals were pair matched into treatment and control groups. Each group contained eight mice, each of which was ear tagged and followed individually throughout the experiment. Initial doses were given on the day of pair matching (day 0). Aspirin (294mg/kg) was administered via gavage daily, rapamycin (25μg) was given once a day by i.p injection and irinotecan (50 mg/kg) once a week by i.p injection. Tumor measurements were taken by calipers daily, starting on day 0. These tumor measurements were converted to tumor volume (V) using the formula ($V = W^2 \times L \times 0.52$), where W and L are the smaller and larger diameters, respectively, and plotted against time. Upon termination, the mice were weighed and sacrificed, and their tumors were excised. Treatment-related toxicity was evaluated by means of serial weight measurements. All experiments were approved by the Ethics Committee of the State University of Campinas.

Tumor extracts

Mice were anesthetized with sodium amobarbital (15 mg/kg body weight, i.p.) and were used 10-15 min later, i.e. as soon as anesthesia was assured by the loss of pedal and corneal reflexes. Tumors were removed, minced coarsely and homogenized immediately in extraction buffer (1% Triton-X 100, 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg of aprotinin/ml) at 4 ºC with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed for 30 sec. The extracts were centrifuged at 15,000 rpm and 4°C in a Beckman 70.1 Ti rotor (Palo Alto, CA) for 45 min to remove insoluble material, and the supernatants of these tissues were used.

Protein analysis by immunoblotting

The whole tissue extracts or 1.0 x 10^6 cells were treated with Laemmli sample buffer (Laemmli 1970) containing 100 mM DTT and heated in a boiling water bath for 4 min after which they were subjected to SDS-PAGE (6% bis-acrylamide) in a Bio-Rad miniature slab gel apparatus (Mini-Protean). For total extracts, similar sized aliquots (200µg protein) were subjected to SDS-PAGE.

Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean), as described by Towbin *et al*. (Towbin, Staehelin et al. 1979), except for the addition of 0.02% SDS to the transfer buffer to enhance the elution of high molecular mass proteins. Non-specific protein binding to the nitrocellulose was reduced by pre-incubating the filter overnight at 4° C in blocking buffer (5% non-fat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The nitrocellulose blot was incubated with the indicated antibodies, diluted in blocking buffer (0.3% BSA instead of non-fat dry milk) overnight at 4º C and then washed for 60 min with blocking buffer without milk. The blots were subsequently incubated with 2.0 µCi of ^{125}I-protein A (30 µCi/µg) in 10 ml of blocking buffer for 2 h at room temperature and then washed again for 30 min as described above. ^{125}I-Protein A bound to the antiphosphotyrosine and anti-peptide antibodies was detected by

autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at -80°C for 12-48 h. Band intensities were quantitated by optical densitometry (Hoefer Scientific Instruments, San Francisco; model GS 300) of the developed autoradiographs.

Determination of NF κB activation

NF κB p50 activation was determined in nuclear extracts from tumor tissue by ELISA (Pierce Biotechnology-89858), according to the recommendations of the manufacturer.

Immunohistochemistry.

To detect Ki-67, microwave postfixation was carried out using a domestic oven (Panasonic Junior) at 700 W, which was delivered to slides immersed in 0.01 mol/l citrate buffer, pH 6.0, in two 7-min doses separated by a 2-min break, which allowed for buffer replenishment. The slides were allowed to cool to room temperature before being removed from the oven. Sections were then incubated at room temperature for 1 h with primary monoclonal mouse anti-human Ki-67 clone MIB-1 (Dako Cytomation) (diluted 1:150). The antibodies for Ki-67 were applied overnight. The slides were then incubated with avidin-biotin complex LSAB+ Kit (Dako Cytomation) for 30 min followed by the addition of diaminobenzidine tetrahydrochloride (DAB) (Sigma Aldrich) as a substrate-chromogen solution. After hematoxylin counterstaining and dehydration, the slides were mounted in Entellan (Merck, Darmstadt, Germany). The experiments were performed at least in triplicate for each mouse.

TUNEL assay

Terminal deoxynucleotidyl Transferase Biotin –d UTP Nick End Labeling (TUNEL) staining was performed using a commercial apoptosis detection kit (Upstate- 17 141), according to the recommendations of the manufacturer.

Statistical analysis

All groups of animals were studied in parallel. Comparisons between different groups were performed by employing ANOVA, as appropriate. The level of significance adopted was $P<0.05$.

RESULTS

Aspirin and irinotecan synergize to increase the PI3-kinase signaling pathway

Immunoblotting experiments were conducted to determine the effects of aspirin, irinotecan, and the combination of both on IRS-1 tyrosine phosphorylation in HT-29 xenografts. We observed that treatment with aspirin alone induced a significant increase in IRS-1/2 tyrosine phosphorylation when compared to the control group. Irinotecan alone also induced IRS-1 tyrosine phosphorylation. The association of both treatments acted synergistically in the increase of IRS-1 phosphorylation, which was significantly greater than for each compound when utilized alone (Fig. 1A and B upper panels). The same membranes, used to detect tyrosine phosphorylation of IRS-1 and IRS-2, were reblotted with antibodies against the p85 subunit of PI3-kinase. The PI3-kinase association with IRS-1 and IRS-2 paralleled the changes in the phosphorylation of these proteins (Figure 1A and B middle panels). The protein expressions of IRS-1 and IRS-2 in the HT-29 xenografts were quantitated by immunoprecipitation and immunoblotting with anti-IRS-1 or anti-IRS-2 antibodies and we did not detect differences in the protein expression between the different treatments (Figure 1A and B lower panels). Consistent with these results, we observed an increase in Akt phosphorylation after treatment with aspirin and irinotecan and a synergistic effect of this association (fig. 1C).

Mechanisms of synergy between aspirin and irinotecan

It has been reported that aspirin could inhibit IRS-1 serine phosphorylation (Yuan, Konstantopoulos et al. 2001), but the effect of irinotecan has not been determined. To address this issue, we tested Ser^{307} phosphorylation in HT-29 xenografts that were chronically treated with these drugs. As shown in figure 2A, there was a significant

reduction in IRS-1 serine phosphorylation in mice treated with aspirin and irinotecan, with no alterations in IRS-1 protein levels (Fig. 2A lower panel) in HT-29 xenografts. The combined used of aspirin and irinotecan enhanced the effect of either drug used alone.

JNK activation was determined by monitoring phosphorylation of JNK (Thr^{183} and Tyr^{185}) and c-Jun (Ser^{63}), which is a substrate of JNK. JNK phosphorylation was detected in control animals, however a significant decrease was observed in animals treated with aspirin or irinotecan (Fig 2B). JNK phosphorylation was reduced by 65-80% in mice treated with aspirin plus irinotecan. Consistent with JNK inactivation, c-Jun phosphorylation was reduced by aspirin and irinotecan treatment in a similar fashion to JNK phosphorylation (Fig 2C). On the other hand, NFκB was induced by irinotecan treatment and reversed by aspirin (Fig. 2D).

Immunoblotting experiments were conducted to determine the effects of aspirin, irinotecan, and the combination of both on p70S6K and eIF4E phosphorylation in HT-29 xenografts. We observed that treatment with aspirin alone induced a significant increase in the phosphorylation of these kinases tyrosine when compared to the control group. Irinotecan alone also induced p70S6K and eIF4E tyrosine phosphorylation. The association of both treatments acted synergistically to increase p70S6K and eIF4E phosphorylation, which was significantly greater than for each compound when used alone (Fig.3 A and B). Conversely, rapamycin treatment blocked the effects of irinotecan on p70S6K phosphorylation (Fig. 3C).

mTOR antagonist and irinotecan synergistic effect on colon cancer xenografts

To further extend these studies, we investigated whether modulating the IRS-1/PI3-kinase/mTOR pathway could change the effects of irinotecan on colon cancer xenografts. As shown in Figure 4A, exposure of HT-29 xenografts to aspirin or irinotecan treatment resulted in a decrease in tumor growth during the experimental period. However, the association of aspirin with irinotecan enhanced tumor growth, related to the treatment with the isolated drugs in the frame shift period of study. No substantial weight loss was observed in any of the groups throughout the thirty days of analysis. We then treated the

mice with rapamycin and irinotecan at doses that reduce tumor growth *in vivo* for each agent alone. Although tumor growth was reduced in mice treated with each agent alone, tumors still increased by 5-fold (figure 4B). In striking contrast, growth of the tumors in animals treated with the combination was completely arrested. It is important to note that the combination did not appear to have an overall increased toxic effect, since a difference in weights of the mice was not observed (data not shown). These data demonstrate that combining the mTOR inhibitor rapamycin with irinotecan dramatically reduces tumor growth *in vivo*.

To support the results observed for the growth of HT-29 xenografts, we performed TUNEL staining on paraffin-embedded sections from the xenografted tumors of mice treated with aspirin, irinotecan and rapamycin. Figure 4C and D shows that irinotecan, aspirin and rapamycin alone increased the number of TUNEL-positive cells compared to sections from control-treated-tumors. However, the combinations did not change the number of TUNEL- positive cells compared to either treatment alone. Next we performed immunohistochemistry to detect Ki-67 (Figs. 4C and 4E). Following inoculation of HT29 colon cancer cells and establishment of tumors (50-100mm^3), mice were treated with aspirin, irinotecan and rapamycin alone, or in combination, for 30 days. While aspirin alone did not have a significant effect on the Ki-67-positive cells, compared to sections from control-treated-tumors, there was a significant increase in the Ki-67-positive cells in the sections from mice treated with the combination of aspirin and irinotecan, compared to sections from irinotecan-treated-tumors. In striking contrast, there was a significant decrease in the Ki-67-positive cells in the sections from mice treated with the combination of rapamycin and irinotecan, compared to sections from irinotecan-treated-tumors.

DISCUSSION

Many cancer cells either show chemo- or radioresistance or acquire resistance after therapy (Hickman 1992; Kerr, Winterford et al. 1994). Increased concentrations of cytotoxic drugs and higher dosages of irradiation fail to improve the pharmacotherapeutic response in resistant cancer cells. Thus, research efforts are aimed at determining the

regulatory events involved in chemo- and radioresistance. We herein describe that agonist activation of the IRS-1/PI3-kinase pathway blocked the growth-inhibitory effect of irinotecan in HT-29 colon cancer xenografts; our data also demonstrate a synergistic effect of mTOR inhibition and irinotecan on tumor growth. Activation of the PI3-kinase/Akt/mTOR pathway may, thus, contribute to refractoriness to treatment with irinotecan.

Although there may be many mechanisms by which chemoresistance is achieved in tumors, our data indicate, in accordance with a previous report (Koizumi, Hatano et al. 2005), that a main pathway involved in inducible resistance to irinotecan, besides the activation of the transcription factor NF-κB within tumor cells, is the activation of the PI3-kinase signaling pathway. Our results show that decreased serine phosphorylation of IRS-1 may be one of the mechanisms implicated in the activation of PI3-kinase signaling. Serine phosphorylation of IRS proteins is believed to be a major mechanism of suppression of IRS-1 activity (Saltiel and Olefsky 1996; Saltiel and Kahn 2001).

Three recent studies provide clear evidence that aspirin enhances the signaling through PI3-kinase (Kim, Kim et al. 2001; Yuan, Konstantopoulos et al. 2001; Hundal, Petersen et al. 2002). In these studies, aspirin was found to reduce serine phosphorylation of IRS-1. Our results show a marked reduction in IRS-1 serine phosphorylation after aspirin or irinotecan treatment in parallel with an increase in Akt and p70S6k phosphorylation. Taken together, these data suggest that irinotecan mediates an increase in the PI3-kinase signaling pathway, at least in part, by reducing IRS-1 serine phosphorylation and increasing IRS-1 tyrosine phosphorylation and that this effect is amplified by aspirin. These three studies also provided the first evidence that IKK may be a target for aspirin enhancing the signaling through the PI3-kinase pathway (Kim, Kim et al. 2001; Yuan, Konstantopoulos et al. 2001). We observed that aspirin inhibited irinotecan-induced IKK and NFκB activity in HT-29 xenografts, and that this inhibition contributed to the prevention of IRS-1 serine phosphorylation.

JNK is a serine kinase that is responsible for activation of the transcription factors, c-Jun and ATF2, by phosphorylating these two proteins (Derijard, Hibi et al. 1994; Minden, Lin et al. 1995) and are implicated in cell-death pathways stimulated by

environmental stress and TNF-α. Recently, JNK has been linked to the regulation of signaling through PI 3-kinase by several studies (Aguirre, Uchida et al. 2000; Rui, Aguirre et al. 2001; Aguirre, Werner et al. 2002; Hirosumi, Tuncman et al. 2002; Lee, Giraud et al. 2003). It has been suggested that JNK contributes to reduced signaling through PI3-kinase by phosphorylating IRS-1 at serine 307, and this phosphorylation leads to the inhibition of IRS-1 function (Aguirre, Uchida et al. 2000; Rui, Aguirre et al. 2001; Aguirre, Werner et al. 2002; Lee, Giraud et al. 2003). It has recently been observed that aspirin inhibits JNK activity (Gao, Zuberi et al. 2003; Jiang, Dallas-Yang et al. 2003). In this study, we observed that aspirin amplified the effect of chronic treatment in JNK activity, and that this activation was accompanied by an increase in IRS-1 serine phosphorylation at Ser^{307}, indicating that this serine kinase is one of the causes of irinotecan-induced chemoresistance.

The mechanism by which chronic treatment with irinotecan results in a reduced activation of JNK is not completely known. Major recent discoveries have revealed that the NF-κB cell-survival pathway cross-talks with the c-Jun N-terminal kinase (JNK) pathway, substantially blunting this pathway (De Smaele, Zazzeroni et al. 2001; Tang, Minemoto et al. 2001). Two JNK inhibitory proteins have been discovered. In one model, activation of NF-κB mediates transcriptional increase of a protein called gadd45b/myd118, which then lowers JNK signaling induced by the TNF-α receptor (De Smaele, Zazzeroni et al. 2001). The other protein (NF-κB-induced X-chromosome-linked inhibitor of apoptosis) is switched on by TNF-α in an NF-κB-dependent manner and also blunts JNK activation (Tang, Minemoto et al. 2001). Another possibility is the presence of a direct cross-talk between PI3-kinase/Akt and JNK pathways. The ability of Akt to suppress the JNK pathway has been observed in a variety of cell systems (Okubo, Blakesley et al. 1998; Levresse, Butterfield et al. 2000; Galvan, Logvinova et al. 2003). Akt has been shown to suppress the JNK pathway by phosphorylating and negatively regulating ASK1 (Kim, Khursigara et al. 2001), MLK3 (Barthwal, Sathyanarayana et al. 2003) and MKK4 (Park, Kim et al. 2002). In addition, Akt has also been suggested to prevent JNK activation by directly interacting with JIP1 and preventing the recruitment of upstream kinases to JNK (Kim, Yano et al. 2002). These results may be clinically relevant, since one of the

mechanisms proposed for the proapoptotic effect of irinotecan is the activation of JNK (Catley, Tai et al. 2004; Malathi, Paranjape et al. 2004) and, even with inhibition of irinotecan-induced NFκB activity by aspirin the combined treatment with aspirin, and irinotecan results in an increased growth of HT-29 xenografts and a reduction in IRS-1 serine phosphorylation.

The effectiveness of the combination of irinotecan and rapamycin in HT-29 colon cancer xenografts suggests that blocking mTOR may circumvent irinotecan resistance by inhibiting the PI3-kinase/Akt/mTOR signaling pathway. Cells acquire irinotecan resistance by several mechanisms, mTOR inhibition by rapamycin may overcome this resistance by encoding major components of protein synthesis machinery and controlling translation of mRNA with secondary structure (often GC rich) (Chung, Kuo et al. 1992; Jefferies, Fumagalli et al. 1997; Gingras, Raught et al. 2001). These mRNAs are known to encode proteins related to proliferation. In this study, we have shown that, in HT-29 xenografts, the phosphorylation of p70S6K is increased by irinotecan alone, but decreased with the combined treatment of irinotecan and rapamycin, indicating that rapamycin can block the translational initiation. These results correspond with our growth inhibitory findings, which show that the addition of mTOR inhibitor significantly augments the growth inhibitory effect of irinotecan HT-29 xenografts. Overall, our findings support the notion that the inhibition of protein translational machinery by rapamycin may overcome irinotecan resistance.

In summary, aspirin treatment blocked the effect of irinotecan inhibition of tumor growth by increasing IRS/PI3-kinase/Akt/mTOR pathway activity. In contrast, mTOR inhibition and irinotecan synergizes to suppress tumor growth in colon cancer xenografts. Overall, these results provide important new insights into the mechanism of irinotecan-induced chemo-resistance.

FIGURE LEGENDS

Figure 1. Aspirin and irinotecan synergize to induce the activation of the IRS/PI3-kinase/Akt pathway. After thirty days of treatment, HT-29 xenografts were prepared, as described in Methods. (A) The lysates were immunoprecipitated (IP) with anti-IRS-1 antibodies and immunoblotted (IB) with antiphosphotyrosine (αPY), with anti PI 3-kinase or with anti-IRS-1 antibodies. (B) The lysates were immunoprecipitated (IP) with anti IRS-2 antibodies and immunoblotted (IB) with antiphosphotyrosine (αPY), with anti PI 3-kinase or with anti IRS-2 antibodies. (C) The lysates were immunoblotted (IB) with pAkt (A-upper panel) and Akt (A-lower panel). Data (mean ± SEM; n = 5) are expressed as relative to control. # P<0.05, *vs.* control; * P<0.05, *vs.* other groups.

Figure 2. Effects of aspirin and irinotecan on IRS-1 serine phosphorylation and on JNK and NFκB activities. Mice were treated with either vehicle or aspirin plus vehicle or irinotecan plus vehicle or aspirin. HT-29 xenografts were then lysed and the proteins were separated by SDS-PAGE and blotted with phosphoserine-specific IRS-1 antibodies (A-upper panel), with IRS-1 antibodies (A-lower panel), phospho-JNK (B-upper panel), JNK (B-lower panel), or phospho-c-jun (C), and NF κB p50 activation was determined in nuclear extracts from tumor tissue by ELISA (D). Data (mean ± SEM; n = 5) are expressed as relative to control. # P<0.05, *vs.* control; * P<0.05, *vs.* other groups.

Figure 3. Irinotecan activates mTOR pathway. (A-B) Mice bearing HT-29 xenografts were treated with irinotecan or aspirin alone or in combination. Activation of mTOR was determined by phospho-p70S6K (A-upper panel), p70S6K (A-lower panel), and peIF4E (B-upper panel), eIF4E (B-lower panel). (C) Mice bearing HT-29 xenografts were treated with irinotecan or rapamycin alone or in combination. Activation of mTOR was determined by phospho-p70S6K (C-upper panel), p70S6K (C-lower panel). Data (mean ± SEM; n = 5) are expressed as relative to control. # P<0.05, *vs.* control; * P<0.05, *vs.* other groups.

Figure 4. Rapamycin and irinotecan synergize *in vivo* to reduce tumor growth. 1.0 x 10^6 HT-29 cells were injected subcutaneously into the flank of Scid mice. Once the tumor reached 50-100 mm^3, treatments were initiated. (A) Irinotecan, aspirin, or the association of aspirin with irinotecan was injected in mice bearing HT-29 xenografts. (B) Control, irinotecan, rapamycin or in combination. Control mice received a similar schedule of the vehicle solution. Tumor growth was measured daily. n = 8 mice per group, mean ± SEM. (C-E) Average TUNEL and Ki-67-positive cells per fields, 4 fields per tumor section, mean ± SEM.

FIGURE 1.

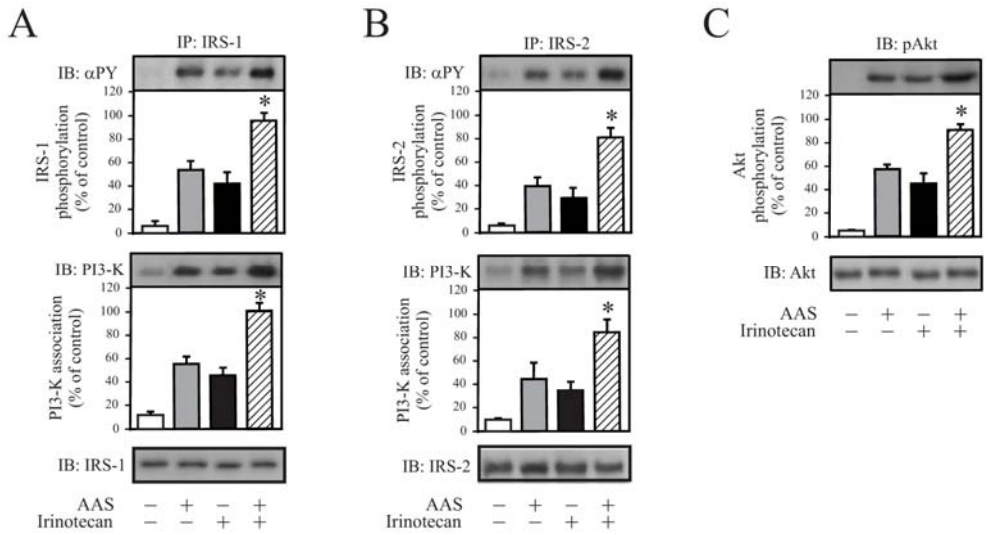


FIGURE 2.

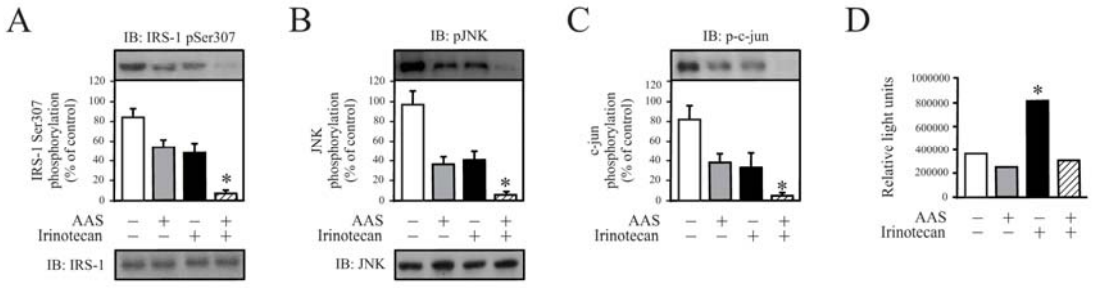


FIGURE 3.

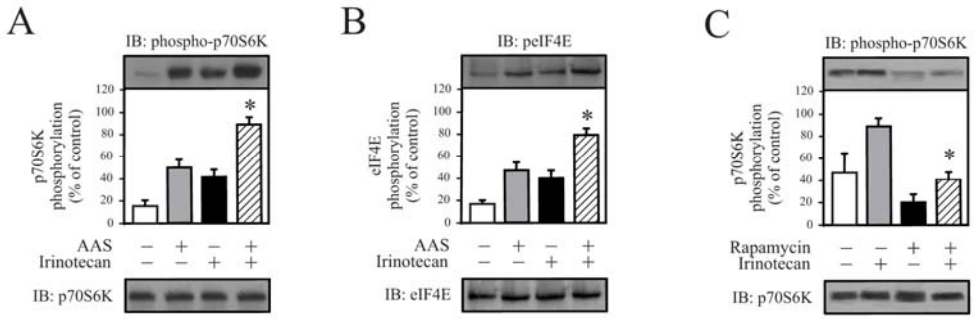
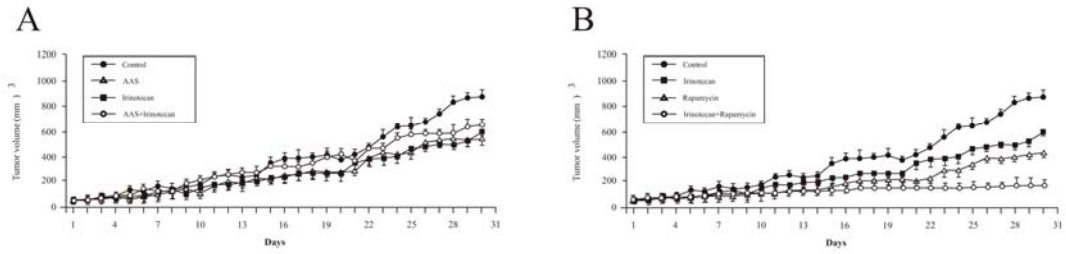
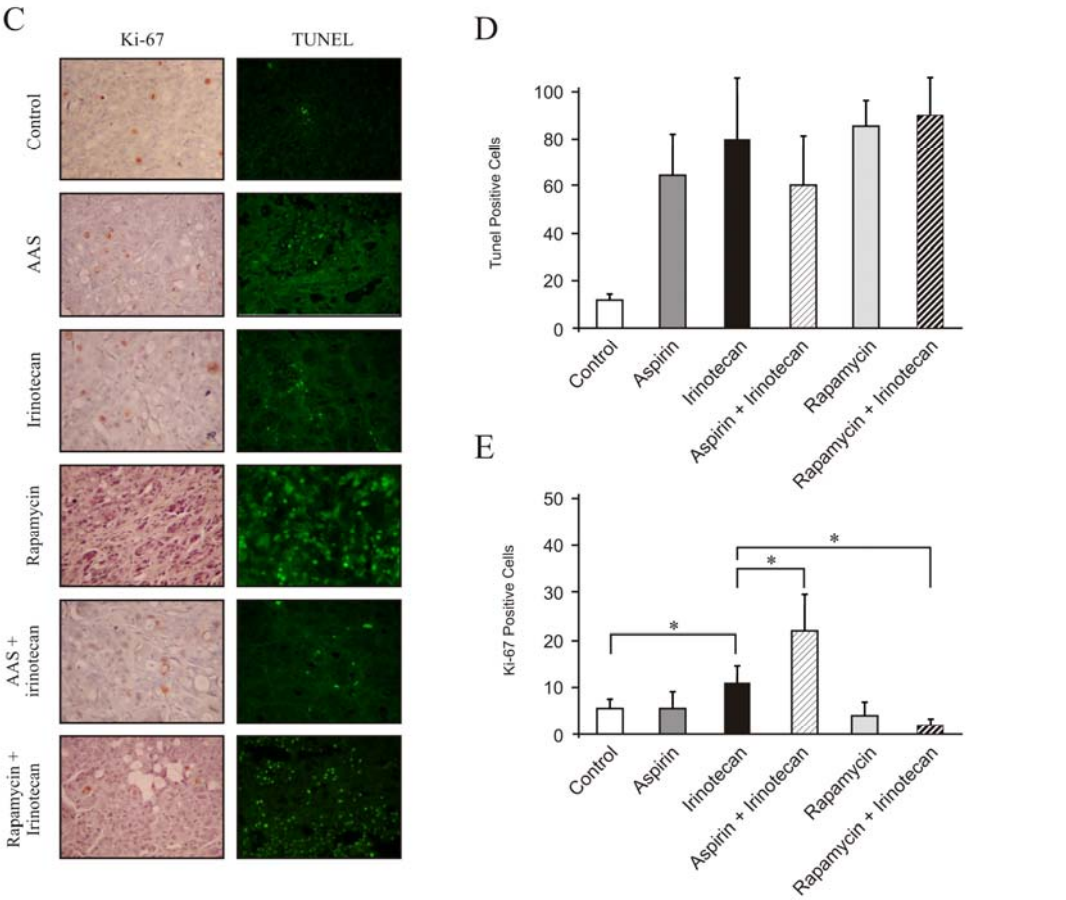


FIGURE 4.





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ANEXOS

Antineoplastic Effect of Rapamycin is Potentiated by Inhibition of IRS-1 Signaling in Prostate Cancer Cells Xenografts

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KEYWORDS: Protein Kinases; Phosphatidylinositol 3-Kinase; insulin receptor substrate 1; Rapamycin; Prostate Neoplasms

ABSTRACT

Proper activation of phosphoinositide 3-kinase-Akt pathway is critical for the prevention of tumorigenesis. Recent data have characterized a negative feedback loop wherein mammalian target of rapamycin (mTOR), blocks additional activation of the Akt/mTOR pathway through inhibition insulin receptor substrate 1 (IRS-1) function. However, the potential of IRS-1 inhibition during rapamycin treatment has not been examined. Herein, we show that IRS-1 antisense oligonucleotide and rapamycin synergistically antagonize the activation of mTOR *in vivo* and induced tumor suppression, through inhibition of proliferation and induction of apoptosis, in prostate cancer cell xenografts. These data demonstrate that the addition of agents that blocks IRS-1 potentiate the effect of mTOR inhibition in the growth of prostate cancer cell xenografts.

INTRODUCTION

Prostate cancer is a major health problem in the world and the available treatment options have proven to be inadequate in controlling the mortality and morbidity associated with this disease (Petrylak, Tangen et al. 2004; Tannock, de Wit et al. 2004; Jemal, Siegel et al. 2007). Research efforts in the last decade have shown that molecular targeted-therapy is a promising approach that could expand the arsenal against prostate cancer.

The mammalian TOR (mTOR) pathway is a key regulator of cell growth and proliferation, and increasing evidence suggests that its deregulation is associated with human diseases, including cancer and diabetes (Ueno, Carvalheira et al. 2005; Sabatini 2006). The mTOR pathway integrates signals from nutrients, energy status and growth factors to regulate many processes, including autophagy, ribosome biogenesis and metabolism (Dennis, Jaeschke et al. 2001; Sabatini 2006). Thus, rapamycin and several analogs, such as CCI-779 and RAD001, are currently undergoing clinical evaluation as anticancer agents (Huang and Houghton 2002; Majumder, Febbo et al. 2004).

The insulin and IGF-1 receptors are tyrosine kinases which phosphorylates the Insulin Receptor Substrate (IRS) upon ligand binding. Phosphorylated IRS, in turn, acts as a protein scaffold that activates the phosphatidylinositol (PI) 3-kinase/Akt cascade (Yenush and White 1997). The production of phosphatidylinositol 3,4,5-triphosphate (PIP3) by PI 3-kinase recruits the serine/threonine kinases PDK1 and Akt to the plasma membrane, by binding to its N-terminal pleckstrin homology (PH) domain. At the membrane, where Akt is phosphorylated by PDK1-mediated phosphorylation (Lawlor and Alessi 2001). Akt phosphorylates many proteins with important physiological roles, including TSC2, inhibiting its GTPase activating protein effect towards the small G-protein Rheb (Inoki, Li et al. 2003). The accumulation of the GTP-bound Rheb leads to the activation of mTOR through an as yet unknown mechanism.

Inactivation of the tumor-suppressor gene, PTEN, occurs in glioblastoma multiforme, endometrial cancer and prostate cancer, among others (Sansal and Sellers 2004). The suppressor-tumor function of PTEN is linked to its lipid phosphatase activity; loss of this activity leads to accumulation of its substrate, PIP3, and activation of the PI 3-kinase signaling pathway (Maehama and Dixon 1998). One consequence of PTEN loss is hyper-phosphorylation downstream Akt substrates (Cross, Alessi et al. 1995; del Peso, Gonzalez-Garcia et al. 1997; Kops, de Ruiter et al. 1999; Nakamura, Ramaswamy et al. 2000). Phosphorylation of these proteins can lead to enhanced cell survival, increase cell proliferation and altered cellular metabolism.

Recent studies have shown that many kinases, including rapamycin-sensitive enzymes, promote serine/threonine phosphorylation of IRS-1 that inhibits their function and promotes their degradation (Mothe and Van Obberghen 1996; Rui, Fisher et al. 2001). Insulin or IGF stimulated Akt phosphorylation could be rescued by rapamycin treatment, coincident with restored IRS protein levels. The rapamycin-mediated rescue is blunted by reducing IRS-1 expression with specifc siRNAs, and rendered unnecessary by overexpression of IRS-1. Thus, chronic hyperactivation of mTOR by inactivation of TSC1-TSC2 stimulates components of the protein synthesis pathway, while inhibiting the IRS branch of the insulin/IGF-1 signaling cascade (Harrington, Findlay et al. 2004; Shah, Wang et al. 2004; Ueno, Carvalheira et al. 2005).

To clarify the role of mTOR *feedback* in prostate cancer, we studied the potential of the IRS-1 antisense oligodeoxinucleotide (ASO) as an inhibitor of proliferation during rapamycin treatment, using the PC-3 cell line, which is reported to be PTEN negative.

MATERIALS AND METHODS

Antibodies, Chemicals and Buffers

The reagents and apparatus for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were from Bio-Rad (Richmond, CA). Tris-[hydroxymethyl]amino-methane (Tris), phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT), Triton X-100, Tween 20 and glycerol were from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was from Calbiochem (La Jolla, CA, USA). Aprotinin was from Bayer (São Paulo, SP, Brazil). Ketamin was from Parke-Davis (São Paulo, SP, Brazil), Diazepam and sodium thiopental were from Cristália (Itapira, SP, Brazil). Protein A-Sepharose 6 MB, nitrocellulose membrane (Hybond ECL, 0.45μm) and ^{125}I-Protein A were from Amersham (Buckinghamshire, UK). Anti-IRS-1 and anti-IRS-1 phosphoserine 307 specific, anti-Erk and anti-Erk phosphotyrosine specific antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-Akt (Ser473) and anti-phospho-p70 S6K (Thr421/Ser424) antibodies were from New England Biolabs (Beverly, MA, USA). Rapamycin was from LC Laboratories (Woburn, MA, USA).

Phosphorothioate-modifield oligonucleotides

Sense and antisense phosphorothioate oligonucleotides specific for IRS-1 (IRS-1 AS) (sense, 5´-ACC CAC TCC TAT CCC G-3´ and antisense, 5´-CGG GAT AGG AGT GGG T-3´) were produced by Invitrogen Corp. (Carls-bad, CA). The antisense oligonucleotide sequences were submitted to BLAST analyses (www.ncbi.nlm.nih.gov) and matched only for the Human IRS-1 coding sequence.

Cell culture

The human prostate cancer cell line PC-3 was obtained from ATCC, Philadelphia, PA, USA. Cells were cultured in RPMI containing 10% fetal bovine serum and glutamine without addition of antibiotcs or fungicides, they were maintained at 37°C, 5% CO2.

Human Tumor Xenograft Models

SCID mice were provided by the State University of Campinas - Central Breeding Center (Campinas, SP, Brazil). SCID mice, 5–6 weeks old and weighing approximately 20–25 grams, were implanted with 1,0 x 10^6 PC-3 cells into the dorsal subcutis of male mice. Mice were weighed twice weekly, and tumor measurements were taken by calipers daily, starting on day 0. These tumor measurements were converted to tumor volume (V) using the formula ($V = W^2 \times L \times 0{,}52$), where W and L are the smaller and larger diameters, respectively, and plotted against time. When the tumors were between 50 and 100 mm^3, the animals were pair matched into treatment and control groups. Each group contained eight mice, each of which was ear tagged and followed individually throughout the experiment. Initial doses were given on the day of pair matching (day 0). Rapamycin and IRS-1 ASO were administered via i.p. injection daily at the doses indicated. On termination, the mice were weighed and sacrificed, and their tumors were excised. Treatment-related toxicity was evaluated by means of serial weight measurements. All experiments were approved by the Ethics Committee of the State University of Campinas.

Tumor extracts

Mice were anesthetized with sodium amobarbital (15 mg/kg body weight, i.p.), and were used 10-15 min later, i.e. as soon as anesthesia was assured by the loss of pedal and corneal reflexes. Tumors were removed, minced coarsely and homogenized immediately in extraction buffer (1% Triton-X 100, 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg of aprotinin/ml) at 4 ºC with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35) operated at maximum speed for 30 seconds. The extracts were centrifuged at 15,000 rpm and 4°C in a Beckman 70.1 Ti rotor

(Palo Alto, CA) for 45 min to remove insoluble material, and the supernatants of these tissues were used.

Protein analysis by immunoblotting

The whole tissue extracts was treated with Laemmli sample buffer (Laemmli 1970) containing 100 mM DTT and heated in a boiling water bath for 4 min after which they were subjected to SDS-PAGE (6% bis-acrylamide) in a Bio-Rad miniature slab gel apparatus (Mini-Protean). For total extracts, similar sized aliquots (200µg protein) were subjected to SDS-PAGE.

Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean), as described by Towbin *et al*. (Towbin, Staehelin et al. 1979), except for the addition of 0.02% SDS to the transfer buffer to enhance the elution of high molecular mass proteins. Non-specific protein binding to the nitrocellulose was reduced by pre-incubating the filter overnight at 4° C in blocking buffer (5% non-fat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The nitrocellulose blot was incubated with the indicated antibodies, diluted in blocking buffer (0.3% BSA instead of non-fat dry milk) overnight at 4º C and then washed for 60 min with blocking buffer without milk. The blots were subsequently incubated with 2 µCi of ^{125}I-protein A (30 µCi/µg) in 10 ml of blocking buffer for 2h at room temperature and then washed again for 30 min as described above. ^{125}I-Protein A bound to the antiphosphotyrosine and anti-peptide antibodies was detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at -80°C for 12-48 h. Band intensities were quantified by optical densitometry of developed autoradiographs (Scion Image software - *Scion Corporation*, Frederick, Md., USA).

Immunohistochemistry.

To detect proliferating cell nuclear antigen (PCNA), microwave post fixation was carried out using a domestic oven (Panasonic Junior) at 700 W, which was delivered to slides immersed in 0.01 mol/l citrate buffer, pH 6.0, in twice doses during seven minutes

separated by a break of two minutes, which allowed for buffer replenishment. The slides were allowed to cool to room temperature before being removed from the oven. Sections were then incubated at room temperature for one hour with primary antibody PC10 (Dako) diluted 1:150. Biotinylated horse mouse anti-human (Dako) antibodies for PC10 was applied for one hour at room temperature. The slides were then incubated with avidin-biotin complex (ABC) reagent (Vector) for 30 minutes followed by the addition of diaminobenzidine tetrahydrochloride (DAB) (Sigma) as a substrate-chromogen solution. After hematoxylin counterstaining and dehydration, the slides were mounted in Entellan (Merck, Darmstadt, Germany). The experiments were done, at least, in triplicate for each mouse.

A TUNEL apoptosis detection kit (Upstate Biotechnology Inc.) was used for DNA fragmentation fluorescence staining according to the manufacturer's protocol. Following the extraction, the tissue extracts were fixed with 4% paraformaldehyde in 0.1 M NaH_2PO_4, pH 7.4, and incubated with a reaction mix containing biotin-dUTP and terminal deoxynucleotidyl transferase for 60 minutes. Fluorescein-conjugated avidin was applied to the sample, which was then incubated in the dark for 30 minutes. Positively stained fluorescein-labeled cells were visualized and photographed by fluorescence microscopy.

Statistical analysis

All groups were studied in parallel. Comparisons between different groups were performed by employing ANOVA, as appropriate. The level of significance adopted was $P<0.05$.

RESULTS

Effects of treatment with rapamycin and/or IRS-1 ASO, on Akt/mTOR and MAPK signaling pathway in PC-3 xenografts

To evaluate IRS-1 ASO activity, we performed experiments using samples from PC-3 xenografts, immunoblotted with anti IRS-1 (Fig. 1A). There was a decrease in

IRS-1 protein levels within 24 hours after ASO treatment which was minimal at 48 hours and almost vanished at 72 hours after ASO treatment. The ASO-mediated IRS-1 inhibition in PC-3 xenografts was dose-dependent (Fig. 1B). The inhibition of IRS-1 protein levels was detected after the injection of 0.1 nmol/Kg, whilst maximal stimulation was observed with 0.8 nmol/Kg of IRS-1 ASO. There was no change in IRS-1 protein levels after IRS-1 sense oligonucleotide (SO) treatment.

Immunoblotting experiments were conducted to determine the effects of rapamycin, IRS-1 ASO, and the combination of both on IRS-1 serine phosphorylation in PC-3 xenografts (Fig 1C). Thus, with isolated rapamycin, there was a significant decrease in IRS-1 serine phosphorylation when compared to the control group. The isolated IRS-1 ASO inhibited partially the IRS-1 serine phosphorylation, whilst the simultaneous treatment with rapamycin and the IRS-1 ASO shown inhibition of IRS-1 serine phosphorylation similar to the treatment with rapamycin alone. There was an increase in IRS-1 protein levels with rapamycin treatment; the isolated administration IRS-1 ASO as well as the association strongly decreases IRS-1 protein levels. Isolated rapamycin induced increases in the serine phosphorylation of Akt, compared to control group. The IRS-1 ASO and the association induced decreases in the phosphorylation of Akt protein. Conversely, the isolated treatment with rapamycin induced a great decrease in p70S6k phosphorylation, ASO treatment decreased in a small extent p70S6k phosphorylation compared to rapamycin. The association of both treatments acted synergistically in the reduction of p70S6k phosphorylation, which was significantly slower than each compound alone. There were no changes in p70S6k protein levels. In order to evaluate the IRS-1-mediaded Erk activation, using antibodies against tyrosine-phosphorylated Erk-1/2, the levels of Erk-1/2 activation were examined in PC-3 xenografts. Isolated rapamycin increased Erk phosphorylation, when compared to the others treatments and control group. As expected there was no change in Erk-1/2 protein expression.

Effects of treatment with rapamycin and/or IRS-1 ASO on growth of PC-3 xenografts

As shown in Fig. 2, exposure of PC-3 xenografts to isolated IRS-1 ASO resulted in a weak decrease in tumor growth during the experimental period. Rapamycin

treated group shown an important decrease in growth, with a very low velocity of growth. Meanwhile, the association of IRS-1 ASO with rapamycin enhanced the inhibitory effect on tumor growth showing no important change in tumor volume in the frame shift period of study. No substantial weight loss was observed in any of the groups throughout the thirty days of analysis.

Effects of treatment with rapamycin and/or IRS-1 ASO on proliferation and apoptosis of PC-3 xenografts

We performed immunohistochemistry to detect PCNA, a proliferation index. The observed percent of PCNA positive cells was 45.9±5.2% in the control group. IRS-1 ASO and rapamycin administration significantly reduced the percent of positive cells to 36.6±6.8% and 24.5±4.7%, respectively ($p<0.05$, for both). The group treated with the association rapamycin and IRS-1 ASO was found to be 4.3±3.2% positive cells. Compared to control group, the association treated group shown ±90% of reduction in positive cells ($p<0.001$). IRS-1 ASO plus rapamycin enhanced the effectiveness of rapamycin by 570%.

To analyze the mechanism responsible for the growth inhibition of PC-3 cells by rapamycin and IRS-1 ASO, the effects of rapamycin and/or IRS-1 ASO on programmed cell death was examined. Thus, DNA fragmentation was measured by TUNEL assay in PC-3 xenografts. In relation to the control group, the IRS-1 ASO and rapamycin treated groups shown about 4- and 23-fold greater positive cells, respectively ($p<0.05$, for both). The association of rapamycin and ASO IRS-1 promoted a 37-fold increase in apoptotic cells number, when compared to control group ($p<0.001$), and up to 2 fold compared with that of rapamycin ($p<0.05$).

DISCUSSION

PTEN alterations have been robustly implicated in human prostate cancer, with PTEN deletions and/or mutations of at least one allele in up to 60% of primary prostate cancers, while homozygous PTEN inactivation is more frequently associated with metastatic prostate tissues (Gray, Phillips et al. 1995; Cairns, Okami et al. 1997; Suzuki, Freije et al. 1998). In the present study, we show that IRS-1 ASO and rapamycin

cooperatively antagonize the activation of mTOR in vivo and act synergistically in tumor suppression in prostate cancer xenografts, which does not express PTEN. Our results show that IRS-1 ASO alone reduces the activation of PI 3-kinase pathway, whilst rapamycin alone reduces the activation of mTOR, with activation of MAPK pathway. Combined treatment with rapamycin and IRS-1 ASO leads to a quantitative inhibition of molecular signaling through the PI 3-kinase and MAPK pathway. In accordance with these data, the proliferation of PC-3 xenografts after treatment with IRS-1 ASO and rapamycin was more pronounced than when each one was administered alone.

Tyrosine-phosphorylated IRS-1 proteins are known to bind efficiently a number of SH2 domain-containing proteins involved in activation of downstream signaling pathways, including PI3-K p85, GRB2, SHP-2, Nck, and Crk (for review, see Ref. (Saltiel and Kahn 2001)). There is increasing interest in the potential role of IRS-1 in oncogenesis. Overexpression of IRS-1 in NIH3T3 fibroblasts leads to increased activation of the Ras-MAPK cascade and cell transformation (Ito, Sasaki et al. 1996; Tanaka, Ito et al. 1996). IRS-1 overexpression also contributes to the progression of hepatocellular carcinoma, possibly by inhibiting transforming growth factor 1-mediated apoptosis (Tanaka and Wands 1996). Although the LNCaP prostate cancer cell line does not express IRS-1 or IRS-2, introduction of either protein in combination with IGF-IR converts these cells to a more aggressive phenotype (Reiss, Wang et al. 2000). A recent study examining endogenous IRS-1 shows that it is constitutively tyrosine-phosphorylated in a wide range of human tumor samples, suggesting that IRS-1 activation may be a common phenomenon in tumors (Chang, Li et al. 2002). Moreover, a relationship between IRS-1 activation and fusion oncoproteins has already been established. TRK-T1 (Miranda, Greco et al. 2001) and BCR-ABL (Traina, Carvalheira et al. 2003) have both been shown to bind IRS-1 and to be associated with increased IRS-1 tyrosine phosphorylation. Therefore IRS-1 activation may be a more general mechanism for transformation. We found that inhibition of IRS-1 by IRS-1 ASO can lead to a decrease in the growth of PC-3 xenografts. Our data demonstrate that lowering IRS-1 content leads to a decrease in the number of docking sites for maximal activation of PI3-kinase-Akt-mTOR cascade as shown by a reduction of Akt and p70S6k phosphorylation, respectively; further suggesting that IRS-1 is important for PC-3 xenografts growth.

The primary pathway by which most growth factors and cytokines activate mTOR and its downstream targets appears to be the PI3-kinase/Akt pathway (for review see Ref (Fingar and Blenis 2004)). Recent reports have shown that in absence of TSC2 or in hiperinsulinemic situations, IRS-1 increases its serine phosphorylation in parallel with a reduction in its protein levels. Important, all the studies found that long term treatment with rapamycin completely restores IRS-1 protein levels and the insulin/IGF-1 responsiveness of PI3-kinase Akt pathway (Harrington, Findlay et al. 2004; Shah, Wang et al. 2004; Ueno, Carvalheira et al. 2005). In agreement with these studies our results show an increase in IRS-1 serine phosphorylation as well as a reduction in protein levels in PC-3 xenografts, moreover the treatment with rapamycin restored IRS-1 levels and activity in the PC-3 xenografts as demonstrated by an increase in Erk1/2 phosphorylation. Furthermore, PC-3 xenografts treated with both IRS-1 ASO and rapamycin exhibit a strong attenuation of PI3-kinase and Ras-MAPK pathways. Altogether these data suggest that loss of PTEN expression in PC-3 xenografts triggers a feedback inhibition of Akt signaling, which is reversed by rapamycin and IRS-1 ASO can overcome.

An increase in the activity of MAPK has been correlated with the progression of prostate cancer to advance disease in humans (Gioeli, Mandell et al. 1999). These results, together with observations that Ras activity regulates the androgen requirement of prostate tumor growth in xenografts, suggest that MAPK plays a role in prostate cancer proliferation (Bakin, Gioeli et al. 2003; Bakin, Gioeli et al. 2003). In agreement with these results or data show a great reduction in MAPK activity when IRS-1 ASO was associated with rapamycin, this effect occurs in parallel with the reduction in PC-3 xenografts proliferation.

In addition to down-regulation of PI 3-kinase/Akt pathway, continuous treatment with IRS-1 ASO for 4 weeks resulted in significant tumor growth delay and the treatment with IRS-1 ASO plus rapamycin strongly inhibited cell proliferation and induced apoptosis. The suppression of PI3-kinase/Akt pathway in these tumors might potentially result in reduction of protein synthesis, cell growth and proliferation downstream mTOR through inactivation of p70S6k and activation of 4E-BP1 (Jefferies, Fumagalli et al. 1997; Fingar, Salama et al. 2002; Fingar and Blenis 2004).

In conclusion, our data demonstrate that the addition of agents that blocks IRS-1 potentiate the effect of mTOR inhibition in the growth of PC-3 xenografts.

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LEGENDS

Figure 1. Phosphorylation status of IRS-1, Akt, p70S6k, and Erk proteins after treatment with rapamycin, IRS-1 antisense oligonucleotide (ASO), and the association IRS-1 ASO with rapamycin. (A) The time course was performed with SCID mice treated i.p. with 0,4nMol/Kg of IRS-1 ASO, as indicated. At the time-points indicated, after the treatment, they were anesthetized and a fragment from xenograft was removed. Similar treatments were carried out with the IRS-1 sense oligonucleotide (SO). (B) In a dose response experiment, SCID mice were treated i.p. with varying doses of IRS-1 ASO or with vehicle, as indicated. After 72 hours, they were anesthetized and fragments from xenograft and from the liver were removed. (C) Representative Western blots of five independent experiments showing PC-3 lysates. The lysates were immunobloted (IB) with anti-IRS-1 pSER, anti-IRS-1 antibodies, anti-phospho-Akt (pAkt), anti-Akt, anti-phospho-p70S6k (p-p70S6k), anti-p70S6k antibodies, anti-phospho-Erk1/2, anti-Erk1/2 antibodies.

Figure 2. Growth curves for xenografts derived from PC-3 cells. Rapamycin (4mg/kg *i.p.*), IRS-1 AS (0.4nMol), or association of rapamycin with IRS-1 AS (4mg/kg and 0.4nMol, respectively) were injected daily in mice bearing PC-3 xenografts.

Control mice received a similar schedule of the vehicle solution. Points and means for at least 8 tumors.

Figure 3. Effects of vehicle, rapamycin (4mg/kg *i.p.*), IRS-1 ASO (0.4nMol), or the association of rapamycin with IRS-1 ASO (4mg/kg and 0.4nMol, respectively) on cell proliferation (as indicated by PCNA immunostaining) and on apoptosis (as indicated by TUNEL) in PC-3 xenografts.

FIGURE 1.

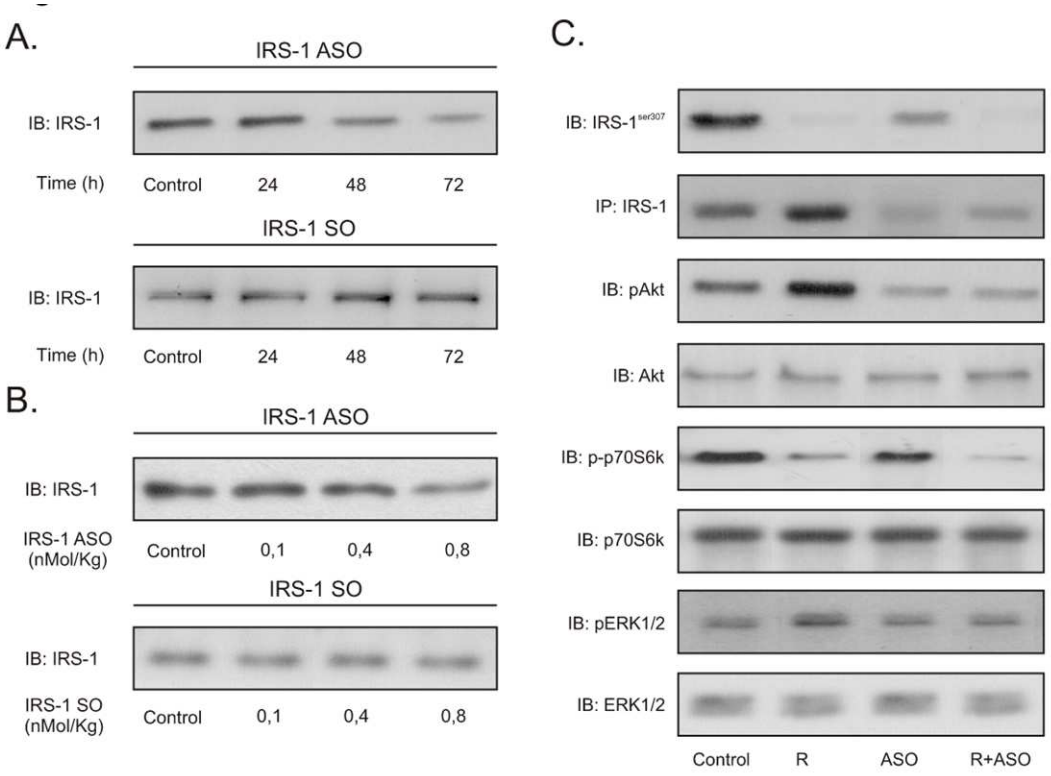


FIGURE 2.

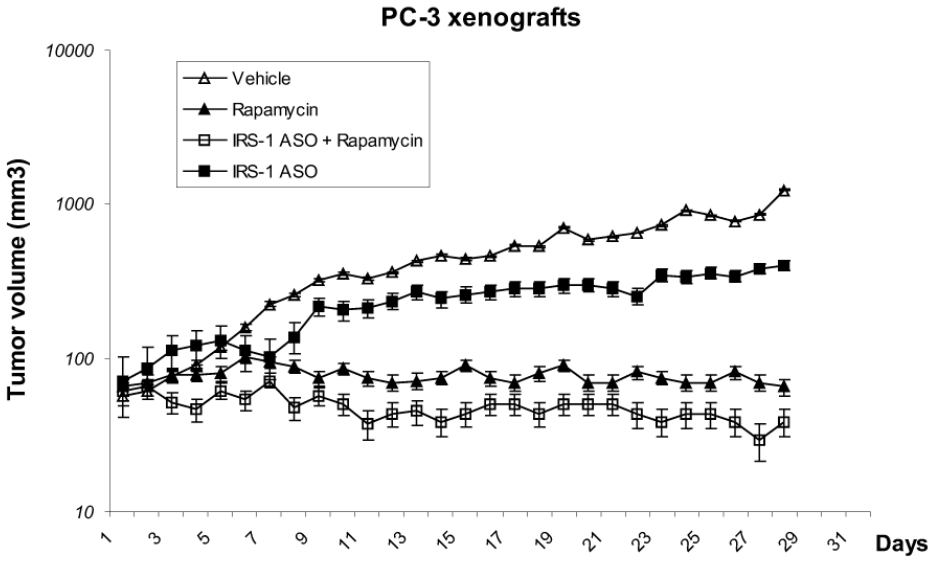
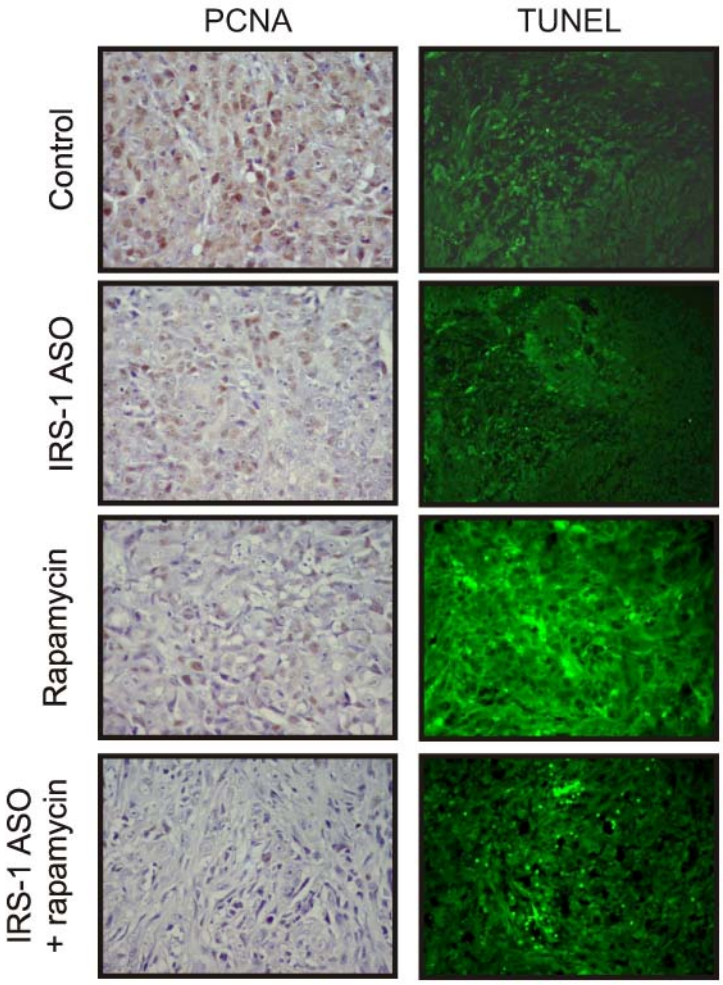


FIGURE 3.



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