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Caracterização Molecular e Atividade Citotóxica sobre Células Tumorais da Lectina do Veneno da Serpente *Bothrops jararacussu*

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas, para obtenção do título de Doutor em Biologia Funcional e Molecular na área de Bioquímica.

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On ne voit bien qu'avec le coeur. L'essentiel est invisible pour les yeaux.

« Só vemos bem com o coração. O essencial é invisível para os olhos. »

(Le Petit Prince, Antoine de Saint-Exupéry)

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Resumo

As lectinas são proteínas ligantes de carboidratos, de origem não-imune, encontradas numa grande variedade de organismos. Aquelas isoladas de venenos de serpentes são constituídas de cadeias polipeptídicas relativas à região molecular correspondente ao domínio de reconhecimento de carboidrato (CRD) de lectinas. Devido às interações lectina – carboidrato serem reversíveis, e não covalentes, estas moléculas podem ser utilizadas como importantes ferramentas bioquímicas.

Este estudo tem como objetivo: (i) a caracterização da estrutura primária da lectina do veneno da serpente *Bothrops jararacussu* (BJcuL); (ii) a investigação do efeito de BJcuL sobre a adesão de células tumorais a proteínas da matriz extracelular, (iii) e sobre a proliferação destas e de células endoteliais.

O seqüenciamento N-terminal de BJcuL revelou a presença de uma única seqüência, indicando que esta proteína é composta de cadeias idênticas. A determinação da estrutura primária da lectina realizada por meio de análise dos peptídeos obtidos pela fragmentação da proteína com clostripaína e protease SV-8, demonstrou que BJcuL possui os 18 resíduos invariantes que caracterizam o tipo C de lectinas animais. Este fato indica que BJcuL pertence à família das lectinas tipo-C ligantes de β-galactosídeos e apresenta similaridades estruturais com a região C-terminal do CRD das lectinas animais de membrana.

Nos primeiros testes em cultura de células tumorais, a viabilidade celular foi avaliada após 5 dias de cultivo na presença da lectina. Nestas condições, a BJcuL inibiu 50% do crescimento das células de carcinoma de rins ou pâncreas em concentrações entre 1 e 2 μM. Em testes posteriores, foi observado que as células de carcinoma de mama ou de ovário foram capazes de aderir fracamente a BJcuL. Entretanto, esta adesão não inibiu a fixação destas células à proteínas da matriz extracelular tais como; fibronectina, laminina e colágeno tipo I. Após proliferação de linhagens tumorais por 4 dias na presença de BJcuL, foi observado um efeito inibidor dose–dependente da lectina em células tumorais de glioma e carcinomas de mama ou ovário. No intuito de verificar as propriedades anti-angiogênicas da lectina, foi realizado um ensaio de proliferação de células endoteliais na presença de BJcuL. Os resultados mostraram que a BJcuL, numa concentração de 0,09 μM, foi capaz de reduzir em 50% a viabilidade destas células.

Os resultados aqui mostrados fornecem base para estudos acerca da estrutura molecular da BJcuL e suas funções, da caracterização da ligação lectina-carboidrato, e dos mecanismos envolvidos na ligação da lectina às superfícies celulares.

Abstract

Lectins are carbohydrate-binding proteins of non-immune origin found in a diverse array of organisms. Snake venom lectins are malleable molecules, and their molecular structure comprises the carbohydrate recognition domain (CRD) characterized in other Ca^{2+}-dependent animal lectins. They could be used as interesting tools since lectin-glycan interactions are reversible and non-covalent.

The aim of this study was (i) to characterize the molecular structure of lectin from the venom of the snake *Bothrops jararacussu* (BJcuL); (ii) to investigate the effect of BJcuL on the adhesion properties of tumor cells to the extracellular matrix proteins, (iii) and on the proliferation of cancer and endothelial cells.

The determination of the single N-terminal sequence has shown that BJcuL is a homodimer. The analysis of the complete amino acid sequence of the peptides obtained by enzymatic BJcuL digestion (clostripain and SV-8 protease), showed that this lectin displays 18 invariant amino acid residues characteristics of C-type lectins. This fact implies that BJcuL possesses structural similarities to the C-terminal region of the animal membrane lectins CRD, belonging to the C-type β-galactoside binding lectin family.

In the first evaluation in a tumor cell system, cell viability was evaluated after 5 days cultivation. This lectin was a potent inhibitor of growth in human renal or carcinoma cells, with 50% inhibitory concentrations (IC50) of cell growth between 1 and 2 μM of BJcuL. In the second evaluation, cells of human metastatic breast cancer or human ovarian carcinoma cell lines weakly adhere to BJcuL. However, BJcuL was not capable of inhibiting adhesion of these cells to the extracellular matrix proteins such as fibronectin, laminin and type I collagen. It was also observed a dose-dependent inhibitory effect of BJcuL in proliferation assays with glioma, breast or ovarian carcinoma cells after 4 days of incubation. Proliferation assays were performed on bovine brain and endothelial cells in order to verify the antiangiogenic properties of the BJcuL These experiments revealed that BJcuL was capable to reduce cell proliferation (IC_{50} of 0,09 μM).

Taken together, our findings could be helpful to further studies regarding the lectin structure and function, lectin-glycan binding characterization, as well as, the mechanisms involved in BJcuL-binding to cell surface molecules, which influence cell proliferation and angiogenesis.

Introdução Geral

1. Lectinas

As lectinas são proteínas ou glicoproteínas que possuem em comum a característica de ligação não covalente a carboidratos (Drickamer & Taylor, 1993). Por não transformarem o carboidrato ligante não são consideradas enzimas. O termo LECTINA foi proposto em 1954 por Boyd & Sharpleigh, e generalizado em 1972 para todas as proteínas ligantes de açúcar, aglutinantes de células, que não possuem origem imune. Estas proteínas estão presentes em plantas, animais e em vários outros organismos (Sharon, 1993). Quanto maior o número de lectinas isoladas, maior é o número de funções atribuídas a estas moléculas (Singh e cols, 1999).

As lectinas possuem uma ou mais regiões moleculares ligantes de carboidratos (Singh e cols, 1999). As ligações das lectinas aos glicoconjugados localizados nas membranas biológicas lhes conferem a propriedade de aglutinar eritrócitos, células normais e tumorais, assim como microrganismos (Sharon, 1993). O uso das lectinas como ferramentas biológicas vem sendo amplamente estudado devido à capacidade reversível de ligação aos carboidratos.

Segundo Drickamer, 1995, as lectinas animais podem ser divididas em cinco grupos: (i) As lectinas tipo-C, que são dependentes de íons cálcio para sua atividade e possuem estruturas moleculares diversas, às quais incluem-se as proteínas ligantes de manose. (ii)

As lectinas tipo-S (galectinas), que requerem grupos tióis livres para manutenção de sua atividade e, em sua maioria, se ligam a β-galactosídeos. (iii) As lectinas tipo- I, envolvidas nas interações célula-célula, localizadas na membrana celular, e que apresenta região de ligação ao carboidrato homóloga às estruturas encontradas nas imunoglobulinas. (iv) As lectinas presentes no retículo endoplasmático, responsáveis pelo mecanismo de "folding" e de transporte protéico na célula. (v) Por último, as lectinas tipo-L, relacionadas em seqüência com as lectinas de plantas leguminosas.

A região molecular das lectinas que interage com o carboidrato já foi identificada e caracterizada como um domínio que reconhece a seqüência específica de resíduos de açúcar. Esta região foi denominada de domínio de reconhecimento de carboidrato - CRD (do inglês "Carbohydrate Recognition Domain"), e está presente em cada subunidade da lectina (Drickamer, 1993).

Algumas proteínas possuem um único sítio de ligação a carboidratos. Este sítio único não lhes confere a característica funcional de lectinas, como a hemaglutinação; entretanto, não as exclui de pertencerem a este grupo. Estas interpretações estendem o termo "Lectina" para moléculas que não foram, inicialmente, caracterizadas com tais (Harisson, 1991).

Dentro da grande diversidade existente de lectinas animais dois aspectos em sua organização são comuns; a ligação aos açúcares através de um CRD de aproximadamente 200 resíduos de aminoácidos (Kishore e cols, 1997) e a íntima relação estrutural entre as seqüências representativas do CRD nas diversas proteínas ligantes de açúcar, formando um grupo de alta homologia (Weis & Drickamer, 1996).

As propriedades das ligações das lectinas aos açúcares se mostram comuns aos diversos CRDs. Estes domínios interagem com os carboidratos reconhecendo-os e ajustando-os de acordo com o modelo chave e fechadura, através de um complexo sistema de pontes de hidrogênio. A formação do complexo carboidrato-proteína envolve o deslocamento da molécula de água associada com o grupo polar da proteína e em torno do carboidrato altamente polar, com o estabelecimento de novas pontes de hidrogênio. Essas últimas ligações e as forças de "van der Waals" existentes entre a superfície hidrofóbica do açúcar e das cadeias laterais de aminoácidos aromáticos, são fundamentais na estabilização da interação lectina - carboidrato (Weis & Drickamer, 1996).

A seletividade por diferentes açúcares resulta, por sua vez, de um complexo sistema de ligações de hidrogênio e coordenações com metais (Drickamer, 1997).

A especificidade por galactose nas lectinas tipo-C é determinada por alguns resíduos de aminoácidos, enquanto que a seletividade por galactose é imposta por uma região rica em resíduos de glicina (Kolatkar & Weis, 1996). A ligação do cálcio no CRD deste tipo de lectina, estabiliza o sítio de ligação e fixa as posições dos aminoácidos que interagem com os galactosídeos (Weis & Drickamer, 1996).

De acordo com as características da região molecular de ligação ao carboidrato, as lectinas podem ser divididas em dois grandes grupos: o grupo I, onde o sítio de ligação é interno e escondido, englobando completamente o ligante em seu interior, e o grupo II onde o sítio de ligação é raso, na forma de uma depressão na superfície da proteína. No grupo II se enquadram os três grupos clássicos de lectinas: as lectinas de leguminosas, as lectinas tipo-C e as galectinas (Elgavish & Shaanan, 1997).

As lectinas tipo-C apresentam um importante mecanismo de reconhecimento de oligossacarídeos, tanto de membranas celulares, quanto de ligados à proteínas circulantes ou à matriz extracelular. A ligação destas lectinas aos açúcares específicos media vários eventos biológicos, como adesão célula-célula, renovação ("turnover") de glicoproteínas do soro e respostas imunes inatas contra diversos patógenos em potencial (Drickamer, 1999). É esperado, conseqüentemente, que a ligação aos carboidratos componentes das membranas biológicas resulte em diferentes efeitos sobre a proliferação das células, como indução de divisão celular (mitose) e proliferação, ou de morte celular programada (apoptose) ou ainda, morte celular por necrose, entre outras.

A proteína ligante de manose isolada de ratos (MBP-A) é uma proteína sérica envolvida na defesa do organismo contra patógenos. O CRD desta proteína já foi caracterizado e possui uma seqüência consenso. Esta região é composta de 32 aminoácidos altamente conservados, localizados em intervalos fixos, incluindo duas pontes dissulfeto, distribuídos num intervalo de aproximadamente 120 resíduos de aminoácidos. Estes resíduos conservados em intervalos fixos caracterizam a estrutura do CRD tipo-C (Weis e cols, 1991a).

Em termos de estrutura molecular, o CRD da MBP-A (Figura 1) tem sua estrutura tridimensional definida nas seguintes proporções de estruturas secundárias: um quinto em α-hélice, um quarto em fita β (faltam estruturas tipo "folhas pregueadas"), um quinto em voltas β, e o resto constitui-se em estruturas irregulares (Weis e cols, 1991b).

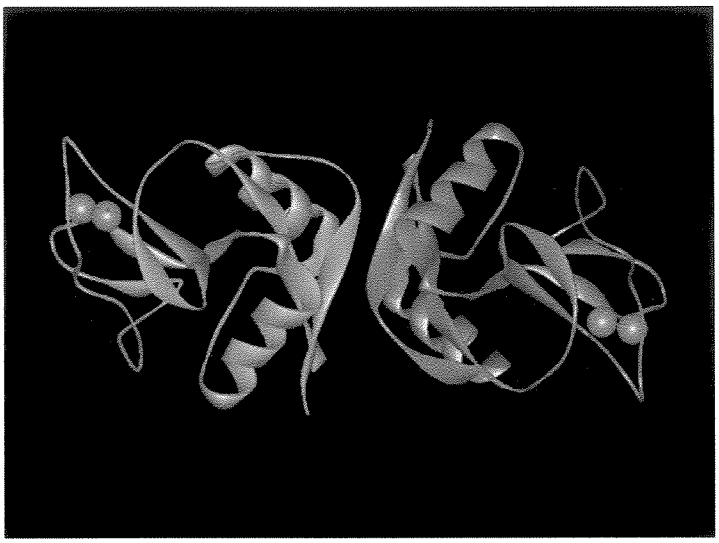

Figura 1- Estrutura tridimensional do CRD da proteína ligante de manose de rato - MBP-A. Esta proteína possui resíduos altamente conservados entre as lectinas do tipo-C, às quais se incluem as lectinas isoladas de venenos de serpentes (retirado de Weis e cols, 1991b)

Os sítios onde estão os resíduos conservados do CRD não devem ser vistos como entidades independentes, pois a maioria das lectinas possui domínios adicionais. Assim sendo, a capacidade da lectina em se ligar aos carboidratos é modulada por vários outros domínios presentes na molécula (Gabius, 1994). A combinação do CRD com outros domínios, assim como os domínios adicionais destas moléculas, determinam as funções das lectinas (Drickamer & Taylor, 1993).

Postula-se que as proteínas que apresentam o domínio CRD do tipo-C tenham evoluído de um ancestral comum, que teria divergido em proteínas diversas, tendo uma variedade de funções, não necessariamente ligante de carboidrato, e que hoje formam uma grande família (Drickamer, 1999). Outros CRD tipo-C talvez tivessem surgido por evolução convergente, como o inibidor de angiogênese endostatina (Hohenester e cols, 1998) e a molécula de adesão bacteriana intimina (Kelly e cols, 1999). Haja visto que ambas possuem dobramento e estrutura terciária semelhantes à da MBP-A, não sendo relacionadas com as lectinas tipo-C no nível de seqüência primária (Drickamer, 1999).

2. Lectinas de Venenos de Serpentes

Os venenos são importantes fontes de proteínas, uma vez que 90 a 95 % do peso seco do veneno é composto de proteínas. Cinqüenta a sessenta diferentes tipos de proteínas com diferentes atividades já foram caracterizadas em um só veneno (Tu, 1996). Este fato o torna uma importante fonte de estudo das proteínas e das relações estrutura-função destas macromoléculas.

Na complexa mistura de peptídeos ativos e proteínas presentes nos venenos de serpentes, o grupo de proteínas relacionadas com as lectinas tipo–C (CLPs) se destaca por ser constituído de proteínas estruturalmente homólogas. Entretanto, essas proteínas desempenham funções distintas, como atividade de hemaglutinação, efeitos anticoagulantes, ou efeitos na aglutinação e agregação de plaquetas (Kini, 1996).

As proteínas hemaglutinantes isoladas de venenos de serpentes incluem-se no grupo das CLPs. Estas lectinas possuem características intermediárias entre os grupos S e C de lectinas animais; possuem massa molecular de aproximadamente 14 kDa por subunidade, são solúveis e específicas para açúcares com β-galactosídeos e requerem Ca^{2+} para sua atividade (Komori e cols, 1999, Carvalho e cols, 1998).

As lectinas encontradas nos venenos de serpentes podem se tornar moléculas maleáveis, pois as cadeias polipeptídicas constituintes da estrutura deste tipo de proteína compreendem a região molecular relativa ao CRD de outras lectinas do tipo-C. São

constituídas de aproximadamente 130 resíduos de aminoácidos, e são caracterizadas como oligômeros solúveis de CRDs do tipo–C, não possuindo domínios acessórios (Rini, 1995).

Em trabalho recente, a oligomerização destas proteínas foi citada usando como modelo a lectina do veneno da cascavel "Western diamond back". A cristalização desta lectina mostrou que ela forma agregados de 5 dímeros, ou seja, forma decâmeros através de ponte dissulfeto e interações não covalente entre os CRD (Drickamer, 1999).

Os avanços moleculares na caracterização de lectinas de venenos estão bem representados no trabalho de Zeng e cols (1999). Neste trabalho a lectina de veneno, TSL, a primeira glicoproteína de veneno de serpente descrita apresentando atividade hemaglutinante, foi caracterizada a partir da seqüência do cDNA e clonagem, espectrometria de massa e degradação de Edman da proteína expressa. A utilização de várias técnicas de bioquímica e biologia molecular possibilitou análises das modificações pós-traducionais, como a N-glicosilação encontrada no quinto resíduo amino terminal desta lectina (Zeng e cols, 1999).

O seqüenciamento dos 135 resíduos da lectina purificada do veneno da cascavel *Crotalus atrox* (RSL), caracterizou a região molecular do CRD. Este se encontra entre os resíduos 31 e 131, onde estão 15 resíduos conservados. A variação dos resíduos não conservados garante a especificidade para o reconhecimento de diferentes açúcares por parte das diferentes lectinas. Entre os resíduos conservados, oito destes são encontrados na maioria das proteínas que contêm este domínio de reconhecimento de carboidrato: Cys (posição 31), Gly (69), Trp (92), Pro (97), Cys (106), Asp (120), Cys (123) e Cys (131). A

posição relativa destes resíduos garante a caracterização do CRD (Hirabayashi e cols, 1991).

Na proteína ligante de manose (MBP-A), os resíduos acima especificados se encontram, respectivamente, nas seguintes posições: Cys (145), Gly (176), Trp (169), Pro (174), Cys (183), Asp (193), Cys (196) e Cys (204). Além destes, mais quatro resíduos são conservados entre RSL e MBP-A: Glu (48), Asp (72), Glu (104) e Trp (118) na RSL (Hirabayashi e cols, 1991); localizando-se nas respectivas posições na proteína ligante de manose; Glu (160), Asp (179), Glu (181) e Trp (191) (Drickamer e cols, 1986).

Os resíduos conservados são também encontrados na lectina do veneno da serpente *Bitis arietans* (PAL) que apresenta 93% de homologia seqüencial com a RSL (Nikai e cols, 1995), na lectina da serpente *Lachesis muta s.*, que possui 92% de homologia com RSL (Aragón-Ortiz e cols, 1996), e na lectina do veneno da serpente himebabu (*Trimeresurus okinavensis*) que possui 86% de homologia com RSL (Nikai e cols, 2000).

A análise da composição de aminoácidos de algumas lectinas de veneno de serpentes mostrou alta quantidade de resíduos de ácido glutâmico, ácido aspático, leucina e lisina. Sendo que a presença de resíduos de glicina restringiu-se a 4 % dos aminoácidos dessas proteínas (Hirabayashi e cols, 1991 e Aragón-Ortiz e cols, 1996). Por outro lado, a alta quantidade de resíduos de glicina (37%) foi observada em lectinas do veneno de outras três espécies de serpentes (Ogilvie e cols, 1986) e pode estar associada com a região de ligação seletiva da galactose verificada por Kolatkar & Weis (1996) na proteína mutante de MBP-A.

3. Atividades Biológicas das Lectinas de Venenos de Serpentes

Apesar de várias lectinas de serpentes aglutinarem células, o papel fisiológico destas proteínas não está bem estabelecido. Ainda não se sabe ao certo a função das lectinas no envenenamento, e a principal atividade característica destas moléculas é a atividade hemaglutinante, um fenômeno observado somente em ensaios realizados *in vitro* (Carvalho e cols, 1998, Kassab e cols, 2001).

De acordo com a literatura, diversos efeitos biológicos das lectinas presentes nos venenos de serpentes já foram reportados. No veneno da serpente *Agkistrodon rhodostoma*, uma lectina específica para galactose mostrou grande capacidade em estimular a mitose em linfócitos humanos dos tipos T e B (Helmbold e cols, 1986). De fato, lectinas purificadas do veneno de algumas serpentes apresentam atividade mitogênica comparável à da concanavalina A, lectina de origem vegetal conhecida como estimuladora da mitose em linfócitos (Mastro e cols, 1986).

Algumas lectinas isoladas de veneno de serpentes estimulam a secreção e agregação plaquetária, como as lectinas isoladas do veneno de *Lachesis muta* (BML), *Ancistrodon piscivorous leukostoma* (CML) e *Crotalus atrox* (RSL) (Ogilvie e cols, 1989).

Ohkura e cols, (1996), mostraram que a lectina do veneno da serpente *Bitis arietans* induz liberação de cálcio do retículo sarcoplasmático de músculo esquelético de coelho, e que esta indução é cerca de 200 vezes mais potente que a indução por cafeína. Este fenômeno parece não estar envolvido com a atividade de fosfolipase A_2 observada em

frações de venenos, e tem efeito nos mecanismos que regulam a liberação do cálcio, especificamente sobre os canais de liberação de cálcio nas células musculares esqueléticas.

Da mesma forma, a atividade de indução de liberação de cálcio do retículo sarcoplasmático de músculo esquelético foi reportada para a lectina do veneno da serpente *Trimeresurus okinavensis*, lectina himehabu (HHL). Os resultados sugerem que esta indução é parcialmente mediada pelos canais de liberação de cálcio (Hirata e cols, 1999).

4. BJcuL - Lectina do Veneno da Serpente *Bothrops jararacussu*

A lectina do veneno da serpente *Bothrops jararacussu* foi isolada em trabalho anterior em cromatografia de afinidade em galactose (Carvalho e cols, 1998). A análise do perfil de massa molecular em SDS-PAGE a 15% mostrou que BJcuL é um homodímero composto de subunidades de 15 kDa. Quando submetido à coloração para carboidratos após SDS-PAGE, a proteína mostrou reação negativa, sugerindo que BJcuL não é uma glicoproteína.

BJcuL aglutina eritrócitos tripsinizados de porco e boi, e esta atividade é inibida especificamente por lactose, galactose e rafinose. EDTA e EGTA também inibem a atividade hemaglutinante de BJcuL, o que revela a dependência de cátions bivalentes. A associação das subunidades da lectina de *B. jararacussu* é essencial para sua atividade hemaglutinante, pois BJcuL reduzida com DTT não exibe esta atividade sobre eritrócitos porcinos (Carvalho e cols, 1998).

5. Células Tumorais

Numerosos estudos têm demonstrado que os carboidratos agem como determinantes moleculares responsáveis por mediarem interações moleculares e celulares (Varki, 1997). A ligação de proteínas aos carboidratos de superfície celular contribui para as interações célula-célula durante o desenvolvimento, na imunidade e em vários processos fisiológicos. Estas interações são direcionadas não só pela estrutura química do carboidrato receptor, mas também refletem as propriedades da proteína, e do microambiente no qual a interação proteína-carboidrato ocorre (Lin e cols, 1998).

As glicoproteínas são importantes constituintes das membranas plasmáticas. Experimentos com lectinas de diferentes especificidades têm demonstrado que os grupos carboidratos das glicoproteínas ligadas à membrana celular são invariavelmente localizados na superfície exterior da membrana, o que os fazem importantes receptores e marcadores biológicos (Lis & Sharon, 1993).

O uso de lectinas no isolamento de células parece estar relacionado com o agrupamento das lectinas ligadas a receptores de superfície. Nas células tumorais, devido às alterações do padrão de glicosilação, tais agrupamentos ocupam regiões restritas da superfície, enquanto nas células normais a distribuição das lectinas ligadas aos receptores é homogênea (Schallier e cols, 1988).

A observação de que as células cancerosas são mais susceptíveis à aglutinação por lectinas do que as células normais, permitiu o descobrimento de diferenças na distribuição e na natureza dos carboidratos de superfície celular entre células cancerosas e normais (Kobata, 1998).

Nas células tumorais, os glicoconjugados de superfície celular encontram-se modificados, tanto estruturalmente, como na sua localização na superfície celular, determinando parte do fenótipo tumoral (Kim & Varki, 1997). As modificações dos oligossacarídeos de receptores glicoproteicos localizados na superfície de células tumorais são de especial interesse, pois alteram muitas das funções essenciais da célula, como a adesão às proteínas da matriz extracelular (Moshfegh e cols, 1998).

A existência de diferenças significativas nos receptores carboidratos de células tumorais, faz com que a possibilidade de ligação seletiva de lectinas nas células tumorais altere a viabilidade destas células. Vários grupos descreveram lectinas de origem vegetal capazes de influenciar no crescimento e na proliferação de células cancerosas. Dentre estes podemos citar, GS1B4 (*Griffonia simplicifolia* 1-B4), WGA (gérmen de trigo) e KML-C (*Viscum album coloratum*) que atuam induzindo apoptose e também a lise celular (Kim e cols, 1993; Yoon e cols, 1999). Diversas lectinas de origem vegetal foram reportadas influenciando a proliferação de linhagens tumorais de próstata, LNCaP, PC-3 e DU 145 (Camby e cols, 1996), de células tumorais de mama (Marth & Daxenbichler, 1988), de tumores de cólon e reto (Ryder e cols, 1994), e de células de glioma de rato (Lenartz e cols, 1998).

De fato, muitas lectinas vegetais têm mostrado efeitos antitumorais diretos ou indiretos. Como efeito direto, foi observado em experimentos com a lectina da "mistletoe" Coreana (*Viscum album coloratum)* sobre várias células tumorais, um fenômeno que causou a morte celular via apoptose (Yoon e cols, 1999). E como efeitos indiretos, a atividade imunoestimulatória, observada na lectina da "mistletoe" Européia - ML-1 (*Viscum album* L.) (Beuth, e cols, 1992; Lenartz e cols, 1996), mais especificamente, a indução da produção de citocinas em cultura de monócitos humanos pelas lectinas ML-I, II e III (Ribereau-Gayon e cols, 1996).

Foi demonstrado que o extrato do *Viscum album* Europeu possui atividade antitumoral, e é usado na Europa como terapia alternativa do câncer (Stoffel e cols, 1997). Recentes achados indicam que este extrato induz a morte celular típica por apoptose, sendo esta indução mediada principalmente pelas lectinas, estando fortemente correlacionada com o envolvimento de outros componentes, que provavelmente, modulem a citotoxicidade da lectina (Bussing & Schietzel, 1999).

Por outro lado, as alterações na afinidade de ligação de lectinas às células malignas, permitem o estudo destas proteínas como marcadores em transformações neoplásicas. Os estudos imunohistoquímicos derivados das diferentes propriedades de ligação de lectinas às células tumorais e endoteliais, têm como objetivo a caracterização morfológica da microvasculatura de carcinomas humanos (Ohtani & Sasano, 1989), de cultura de células endoteliais de porco (Fischer & Kissel, 2001), e também, o reconhecimento de células capilares endoteliais e seus processos durante a angiogênese (Williams e cols, 1989).

O uso da lectina de *Helix pomatia* como indicador do prognóstico de cânceres de mama, cólon e gástrico demonstra o reconhecimento dos carboidratos presentes nas células de adenocarcinomas pela lectina (Mitchell & Schumacher, 1999). As diferenças existentes no perfil de glicosilação das proteínas integrinas $\alpha 3\beta 1$ de diferentes linhagens de células de bexiga puderam ser analisadas com a utilização das aglutininas de *Phaseolus vulgaris* (feijão) e *Datura stramonium* (Litynska e cols, 2000).

6. Integrinas na Adesão de Células Tumorais

As integrinas são receptores glicoproteicos de superfície celular responsáveis pela adesão e migração das células na matriz extracelular (Ruoslahti, 1991). A expressão destas proteínas se encontra modificada em células tumorais, provocando profundas alterações na adesão e migração destas células, representando papel fisiológico significativo no processo angiogênico e no espalhamento do câncer (Varner & Cheresh, 1996).

As diferenças na expressão de integrinas em células tumorais são bem estudadas em melanomas, e os seus produtos aparecem como importantes antígenos responsáveis pela progressão e invasão de vários tipos de células tumorais. Aumentos na expressão de certos tipos de integrinas associadas com a expressão de oligossacarídeos modificados, como o proteoglicano NG2, estão correlacionados com o comportamento invasor e conseqüente metástase de tumores primários (Burg e cols, 1998). Por outro lado, a prevenção da ligação das integrinas às proteínas da matriz extracelular suprime o crescimento celular ou induz morte celular por apoptose (Meredith e cols, 1993).

Várias proteínas isoladas de veneno de serpentes denominadas desintegrinas, possuem a seqüência de aminoácidos RGD (responsável pela interação aos sítios receptores das integrinas), e desta maneira, inibem a ação de integrinas (Zhou e cols, 2000). Por outro lado, o recente estudo de Marcinkiewicz e cols (2000), demonstrou pela primeira vez que uma proteína derivada do veneno da serpente *Echis multisquamatus*, não pertencente à família das desintegrinas, foi capaz de inibir potente e seletivamente a integrina $\alpha 2\beta 1$, bloqueando a agregação plaquetária induzida por colágeno.

Nas integrinas, os carboidratos também apresentam funções regulatórias. Os gangliosídeos (moléculas compostas de lipídeos e carboidratos, localizadas na membrana celular) são capazes de regular as funções das integrinas, e o ácido siálico, importante constituinte das membranas biológicas, tem papel fundamental nesta função reguladora (Cheresh e cols, 1987).

A atividade de desintegrina foi também demonstrada na galectina-8, uma lectina específica para galactosídeos. Esta lectina foi capaz de inibir a adesão de células de carcinoma aos ligantes de integrinas induzindo apoptose celular. A ligação de gal-8 modula as interações das integrinas com a matriz extracelular e assim regula a adesão e sobrevivência celular (Hadari e cols, 2000).

Tomando em conjunto as modificações estruturais, de localização dos glicoconjugados e das integrinas de superfície celular nas células tumorais, assim como a presença de carboidratos reguladores das funções das integrinas; a ligação seletiva da lectina do veneno da serpente *Bothrops jararacussu* na superfície destas células poderia alterar a proliferação destas células, ou inibir a adesão destas células a proteínas da matriz extracelular. Este processo ocorreria por meio da desestabilização da conformação das integrinas ou de proteínas reguladas pela presença de oligossacarídeos.

Objetivos

Estabelecer a estrutura molecular da Lectina do veneno da serpente *Bothrops jararacussu*, determinando sua composição de aminoácidos e sua estrutura primária. Estudar o efeito da lectina do veneno da serpente *Bothrops jararacussu* (BJcuL) na adesão de células tumorais de carcinoma de ovário e mama, a proteínas da matriz extracelular. Verificar o potencial citotóxico "in vitro" de BJcuL sobre o crescimento de células tumorais humanas e células endoteliais.

Capítulo 1

Caracterização da Estrutura Primária da Lectina do Veneno da Serpente *Bothrops jararacussu*

Artigo Aceito para Publicação

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Primary Structure Characterization of *Bothrops jararacussu* Snake Venom Lectin

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The complete amino acid sequence of the lectin from *Bothrops jararacussu* snake venom (BJcuL) is reported. The sequence was determinated by Edman degradation and amino acid analysis of the S-carboxymethylated BJcuL derivative (RC-BJcuL), and from its peptides originated from enzymatic digestion. The sequence of amino acid residues showed that this lectin displays the invariant amino acid residues characterized in C-type lectins. Amino acid analysis revealed a high content of acidic amino acids and leucine. These findings suggest that BJcuL, as other snake venom lectins, posses structural similarities to the carbohydrate recognition domain (CRD) of calcium-dependent animal lectins, belonging to the C-type β-galactoside binding lectin family.

Key words: snake venom, *Bothrops jararacussu*, C-type lectin, amino acid sequence.

1. INTRODUCTION

Lectins are proteins, or glycoproteins, found in a diverse array of organisms. They consist in a large group of proteins with ability of binding specifically, reversibly, and non-covalently to carbohydrates (Kishore, 1997). Some of these molecules may also contain a second binding site that interacts with a noncarbohydrate ligand. As many as the numbers of lectins are, many are the different functions they have (Singh *et al.*, 1999).

Although the number of animal lectins continues to increase, it is possible to classify them into five major groups: (i) The C-type or calcium dependent lectins, (ii) the galactose-binding galectins, (iii) the I-type lectins (including sialoadhesins and other immunoglobulin-like sugar-binding proteins), (iv) the lumenal proteins of the ER (endoplasmic reticulum) that interacts transiently with glycoproteins, (v) and the L-type lectins related in sequence to the leguminous plant lectins (Drickamer, 1995).

The sugar-binding activity can be ascribed to a limited portion of most lectins molecules, typically a globular carbohydrate-recognition domain of less than 200 amino acids. This molecular region is designated CRD, and many of them are related in amino acid sequence to each other, having high sequential homology (Weis & Drickamer, 1996)

Carbohydrate-binding to CRD is due to a combination of hydrogen bonding to the sugar hydroxyl groups, metal coordination, van der Waals packing; often including packing of a hydrophobic sugar face against aromatic amino acid side chains (Elgavish & Shaanan, 1997). In C-type lectins, galactose specificity is imposed by a glycine-rich loop (Kolatkar & Weis, 1996), and calcium forms direct coordination bonds with the sugar ligand (Weis & Drickamer, 1996).

Several lectins have been isolated from snake venoms (Hirabayashi *et al.*, 1991; Nikai *et al.*, 1995; Komori *et al.*, 1999; Nikai *et al.*, 2000). They are shown to be composed of two identical subunits with invariant amino acids residues, which are also find in the carbohydrate recognition domain of the C-type lectins (Weis *et al.*, 1991). They also posses similarity in sequence with other snake venom proteins, like glycoprotein Ib-binding proteins (Polgár *et al.*, 1997) and botrocetin, a von Willebrand factor-binding protein (Usami *et al.*, 1993).

The role of the lectins in envenomation has not yet been clarified. Many biological effects of snake venom lectins have been reported, like erythrocyte agglutination under *in vitro* assays (Kassab *et al.*, 2001), mitogenic activity on lymphocytes (Mastro *et al.*, 1986), platelet aggregation (Ogilvie *et al.*, 1989), induction of edema (Lomonte *et al.*, 1990), induction of Ca^{2+} release from rabbit skeletal muscle sarcoplasmic reticulum (Ohkura *et al.*, 1996), and inhibition of cancer cell proliferation (Pereira-Bittencourt *et al.*, 1999).

Snake venom lectins are interesting lactoside-binding molecules because their molecular structure comprises the carbohydrate recognition domain characterized in other calcium-dependent animal lectins, being soluble oligomers of C-type CRDs not linked to accessory domains (Rini, 1995).

Bothrops jararacussu lectin (BJcuL), a galactoside-specific lectin (Carvalho *et al.*, 1998) has shown to be a very effective inhibitor of human renal and human pancreatic carcinoma cell proliferation (Pereira-Bittencourt *et al.*, 1999), and also, BJcuL inhibited glioma and endothelial cell proliferation (de Carvalho *et al.*, 2001).

The aim of this work is determine precisely the complete amino acid sequence of the lectin from *Bothrops jararacussu* snake venom (BJcuL). For this purpose, Edman automatic degradation, amino acid analyses of the S-carboxymethylated BJcuL derivative (RC-BJcuL) and peptides originated from enzymatic digestion were carried out.

2. MATERIALS AND METHODS

Materials

Lyophilized crude venom from *Bothrops jararacussu* was purchased from Enzifarma, São Paulo, Brazil. Affinity column of immobilized D-lactose was acquired from Sigma Chemical Co, St. Louis-MO, USA. Sequencing grade Endoproteinase Glu-C from *Staphylococcus aureus* and clostripain were purchased from Promega, Madison, WI, USA. The reagents used for automatic protein sequencing were supplied from Perkin Elmer Applied Biosystems. All other reagents and solvents were of analytical or HPLC grade.

2.1. Purification

The lectin from *Bothrops jararacussu* snake venom was purified as described in Carvalho *et al.*, 1998, with little modifications in the method. Briefly, BJcuL was isolated from the crude venom by affinity chromatography on absorbed lactose column; the lectin was extensively dialyzed against water and lyophilized.

2.2. Reduction and Carboxymethylation

One milligram of purified and lyophilized BJcuL was reduced with 5 mM dithiothreitol in 6 M guanidine 0.6 M Tris 0.001M EDTA buffer, pH 8.2, for 1 h at 37°C. After reduction, the protein was alkylated for 1 h at room temperature with 11 mM iodoacetic acid. The protein was desalted on a 0.5 x 9 cm column of Sephadex G-25, which was previously equilibrated with ammonium bicarbonate buffer 0.1 M pH 7.8, then eluted with the same solvent and lyophilized.

2.3 Purity assay

Reduced and carboxymethylated-BJcuL (RC-BJcuL) was assayed for purity in a reverse-phase HPLC on a C-18/μ Bondapak column with the following eluent system (A) 0.1% TFA and (B) acetonitrile 66% in 0.1% TFA.

2.4. Amino acid analysis

Amino acid analysis was performed in a Pico-Tag amino acid analyzer (Waters System). The purified sample was hydrolyzed with 6 HCl containing 1% phenol (v/v) at 106°C for 24 hr in evacuated tubes. Hydrolysates were reacted with 20 ml of fresh derivatization solution (v/v, 7:11:1:1; ethanol: triethylamine: water: phenylisothiocyanate) for 1 hr at room temperature. PTH-amino acids were identified in a model 120-A PTH amino acid analyzer, according to the retention times of a 20 PTH-amino acid standard. Evaluation of free thiol groups was performed by S-pyridylethylation of BJcuL in the absence of a reductant and subsequent amino acid analysis.

2.5. Enzymatic Digestions

Isolated and lyophilized RC-BJcuL was dissolved in the reaction buffer (10 mM Tris-HCl, pH 7.5, containing 50 mM $CaCl_2$ and 2.5 mM DTT) and mixed with a clostripain solution in 10 mM Tris-HCl containing 10 mM DTT. The digestion was carried out for 17h or 3 h at 37°C, using enzyme: protein ratio of 1:30 or 1:25, respectively. *S. Aureus* V8 protease digestion was carried out using an enzyme: protein ratio of 1:50, in 50 mM ammonium bicarbonate, pH 7.8, at 37°C for 17 hours. Both digestions were stopped by lyophilization.

The resulting peptides were separated by reversed-phase chromatography HPLC on a 0.39 x 30 cm μBondapack C18 column (Waters System) with the eluent system cited in 2.3. A linear gradient from 0 to 100% of solvent B over 40 min at a flow rate of 1 ml/min was applied. When necessary, the fractions obtained were repurified using a stepwise gradient covering the same concentration range as the first purification. The peptides were detected by their absorbance at 220 nm.

2.6. Sequence analysis

Isolated peptides were submitted to Edman automatic sequential degradation (Edman & Begg, 1967) in an Applied Biosystems 477A protein gas-liquid sequenator. Overlapping sequences were obtained to compose the complete sequence.

2.7. Sequence homology search

Sequence homology search was performed using the protein sequence database, BLAST and SWISS-PROT - Protein Data base accessible by Internet (www.ncbi.nlm.nih.gov/Blast/ or www.expasy.ch/sprot/)

2.8. Carbohydrate analyses

Neutral sugars were measured by the phenol-sulfuric acid method of Dubois *et al.*, (1956).

3. RESULTS

3.1. Amino acid sequence of BJcuL

Reduced and carboxymethylated-BJcuL (RC-BJcuL) was separated as a single peak on the described eluent system; and has shown to have a single N-terminal amino acid sequence determined on an automated Edman degradation amino acid sequenator. These both results indicate that BJcuL is a homodimer.

In order to obtain the complete amino acid sequence of BJcuL, clostripain and SV-8 digestions of the lectin were performed. Fragmentation of the protein by Clostripain for 17 hours and 3 hours, generated peptides designed CL-1 to CL-6 (Figure 1A), and peptides CP-1 to CP-7 (Figure 1B), respectively. All of them were submitted to Edman automatic degradation. *S. aureus* V8 protease digestion of BJcuL, with the system used, resulted in Glu-C cleavage. Eight peptides designated SA-1 to SA-8 were isolated by chromatography (Figure 2) and sequenced by Edman degradation.

The complete sequence of BJcuL and the overlapping regions of the peptides are showed in Figure 3. This lectin is composed by 135 amino acid residues and the calculated molecular mass from the sequence data is 16,079.59 (Table1).

3.2. Amino acid and Carbohydrate Compositions

The amino acid composition of BJcuL is shown in table 1. Whereas amino acids compositions of each peptide submitted to Edman degradation were shown in Table 2 and 3. This protein contains relatively large amounts of aspartic acid, glutamic acid and lysine; together they constituted 25.9 % of the total amino acid residues. The high content of leucine was also observed in other snake venoms lectins (Table 4).

Nine half-cystines were identified in the complete amino acid sequence of BJcuL. Since any pyridyl-cysteine was found in the intact lectin, these half-cystines should be involved in either intra or intersubunit disulfide bridges (data not shown). No neutral sugar was detected by the procedure employed.

3.3. Homology Search

Protein databases showed that BJcuL are included in the C-type lectin family. BJcuL are very homologous to other venom snake lectins (Figure 4). By comparison with amino acid sequence of BJcuL, 10 variant amino acid were observed for APL (Komori *et al.*, 1999) and for PAL (Nikai *et al.*, 1995); 11 for LmsL (Aragón-Ortiz *et al.*, 1996), 13 for RSL (Hirabayashi *et al.*, 1991), 16 for TSL (Zeng *et al.*, 1999) and 25 for HHL (Nikai *et al.*, 2000).

4. DISCUSSION

The lectins isolated from snake venoms are very homologous to the lectins found in C-type animal lectin group (Drickamer, 1999). The family of calcium-dependent carbohydrate-binding proteins, designed C-type animal lectins, includes endocytic receptors of hepatocytes and macrophages, the selectin cell-adhesion molecules and secreted molecules found in the extracellular matrix and in serum (Weis *et al.*, 1992).

In snake venoms, the appearance of C-type lectins is interpreted as an aggressive mode for the benefit of the predator as strong agglutinins (Nikai e cols, 2000) and inhibitors of coagulation or inflammatory responses (Chen & Tsai, 1995).

In this work, the lectin from *Bothrops jararacussu* snake venom (BJcuL) was submitted to automatic Edman degradation and amino acid analysis in order to determine its complete amino acid sequence. The primary sequence obtained was compared to other snake venom lectins homologous to the C-type lectins CRDs.

BJcuL is composed by 135 amino acids residues. The molecular weight of the monomeric chain of this lectin calculated from the amino acid sequence corresponds to 16,079; and is closed to the previous value (15,000) estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Carvalho *et al.*, 1998)

Since the primary structures of the snake venoms lectins are homologous (87 a 97% identity of amino acids residues) and with conserved positions of cysteines, it is suggested that the intermolecular and intramolecular disulfide linkages must possesses similarities.

By comparison of the disulfide bridges in the RSL molecule (Hirabayashi *et al.*, 1991), we can estimate a disulfide link between Cys (position 86) of lectin monomers and

other half-cystine residues forming four intramolecular bridges as follows: Cys (3) to Cys (14); Cys (31) to Cys (131); Cys (38) to Cys (133) and Cys (106) to Cys (123).

As observed for other snake venom lectins, amino acid analyses of BJcuL have showed a high content of aspartic and glutamic acids and lysine (Hirabayashi *et al.*, 1991). These lectins are also characterized by high contents of tryptophan and half – cystines (Aragón-Ortiz *et al.*, 1996).

BJcuL was found to be homologous to type-C CRD containing proteins, since it showed to have the invariant amino acid residues characterized in this type of domain (Weis *et al.*, 1991). C-type CRDs are characterized by a sequence motif of 32 conserved amino acids at fixed intervals, including two disulfide bonds, distributed over approximately 120 amino acids. The conserved residues appear to form a general calcium-dependent carbohydrate–binding framework. Specificity for particular sugars is presumably encoded in some of the non-conserved residues, as different C-type lectins show a wide range of distinct carbohydrate specificities (Weis *et al.*, 1991)

High conserved amino acids were found in the primary sequence of BJcuL: Cys (position 3), Gly (12), Cys (14), Trp (24), Cys (31), Glu (48), Trp (67), Ile (68), Gly (69), Leu (70), Trp (92), Pro (97), Glu (104), Cys (106), Gly (114), Trp (118), Asp (120), Cys (123) e Cys (131). The position of Gln-Pro-Asp (QPD) sequence (96-98), esssential for galactose binding, is in accord with other hemagglutination snake venom lectins (Komori *et al.*, 1999).

BJcuL has 29% of similarity in sequence to rat asialoglycoprotein receptor, and all of the high conserved aminoacids in BJcuL is present in the sequence of this receptor (Drickamer *et al.*, 1984). The CRD of rat mannose-binding protein has 26 amino acids residues in common with BJcuL sequence, and 11 of these amino acids residues are well conserved within C-type lectin group (Drickamer *et al.*, 1986).

The homology of BJcuL primary structure with C-type lectins was expected since the majority of the lectins from snake venoms are high homologous and share homology with the C-type CRD. Snake venom lectins consist in a homologous group of proteins functionally distinct. Due to the variation of their target and biological activities, the structure-function relationship of these proteins is subtle, complex and interesting (Kini, 1996).

The results showed that BJcuL is homologous to other snake venom lectins and belongs to the C-type lectin family. Further analyses are needed for the determination of the complete 3D structure of BJcuL.

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Figure Legends

Figure 1 - Purification of clostripain derived peptides by high performance chromatography. One mg of reduced and carboxymethylated BJcuL was hydrolyzed with clostripain for 17 hours (A) or 3 hours (B); and submitted to a reverse phase C18 column with 0.1% TFA (solvent A) and 60% acetonitrile in TFA (solvent B). A linear gradient of 0-100% (dashed line) was applied for 35 min at room temperature. The peptides, which were sequenced, are showed in Fig. 3.

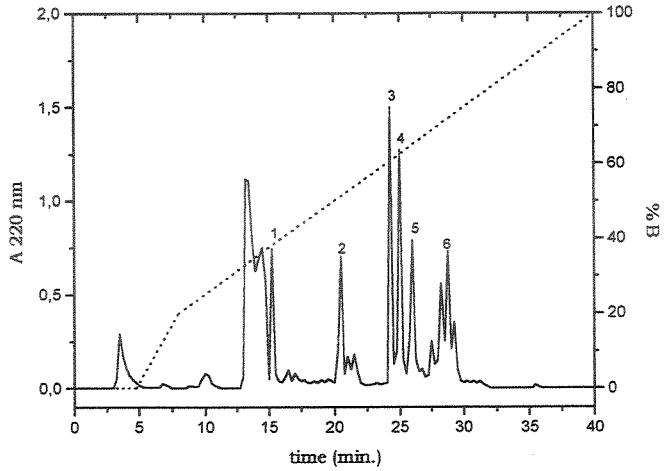
Figure 2 - Purification of SV-8 digest of BJcuL by high performance chromatography. One mg of reduced and carboxymethylated BJcuL was hydrolyzed with Protease SV-8 for 17 hours; and submitted to a reverse phase C18 column with 0.1% TFA (solvent A) and 60% acetonitrile in TFA (solvent B). A linear gradient of 0-100% (dash line) was applied for a period of 35 min at room temperature. The peptides sequenced are showed in Fig. 3.

Figure 3 - Amino acid sequence of *Bothrops jararacussu* venom lectin. The complete sequence and overlapping regions are shown. N –terminal stands for the region directly sequenced in the native lectin. CL and CP denote for the peptides derived from clostripain digestion of RC-BJcuL for 17 and 3 hours, respectively. SA denotes for protease SV-8 digestion of RC-BJcuL. A and B came from the repurification of the peptides.

Figure 4 - Comparison of amino acid sequence of hemagglutinating snake venom lectins with CRD from rat asialoglycoprotein receptor (Rasgpr, residues 150-279) (Drickamer *et al.*, 1984), and rat mannose-binding protein (RMBP, residues 115-238) (Drickamer *et al.*, 1986). Aligments of sequences were made according to the half-cystine residues (shady). An asterisk (*) indicates identical residues with BJcuL, gaps (-) and dots (...) were introduced to conserve homology. High invariant residues are boxed. PAL, puff-adder lectin (Nikai *et al.*, 1995); RSL, rattlesnake lectin (Hirabayshi *et al.*, 1991); LmsL, *Lachesis muta* lectin (Aragón-Ortiz *et al.*, 1996); APL, *Agkistrodon p. piscivorous* lectin (Komori *et al.*, 1999); TSL, *Trimeresurus stejnegeri* lectin (Zeng *et al.*, 1999), and HHL, Himehabu lectin (Nikai *et al.*, 2000).

Figure 1

A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% B
time (min.)
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100
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30
35
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1
2
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6

B

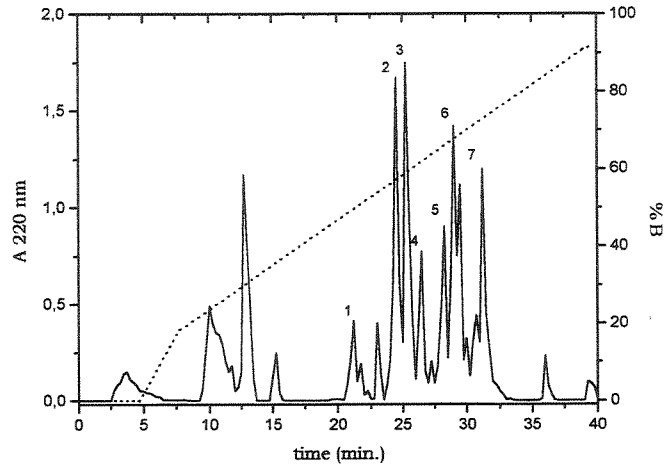
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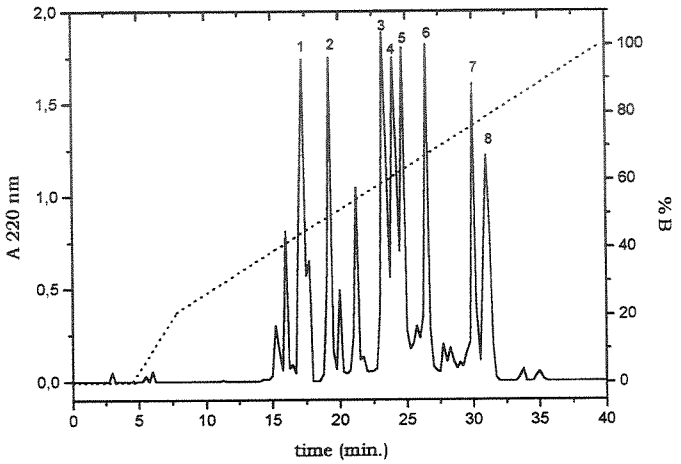
Figure 2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A 220 nm
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time (min.)
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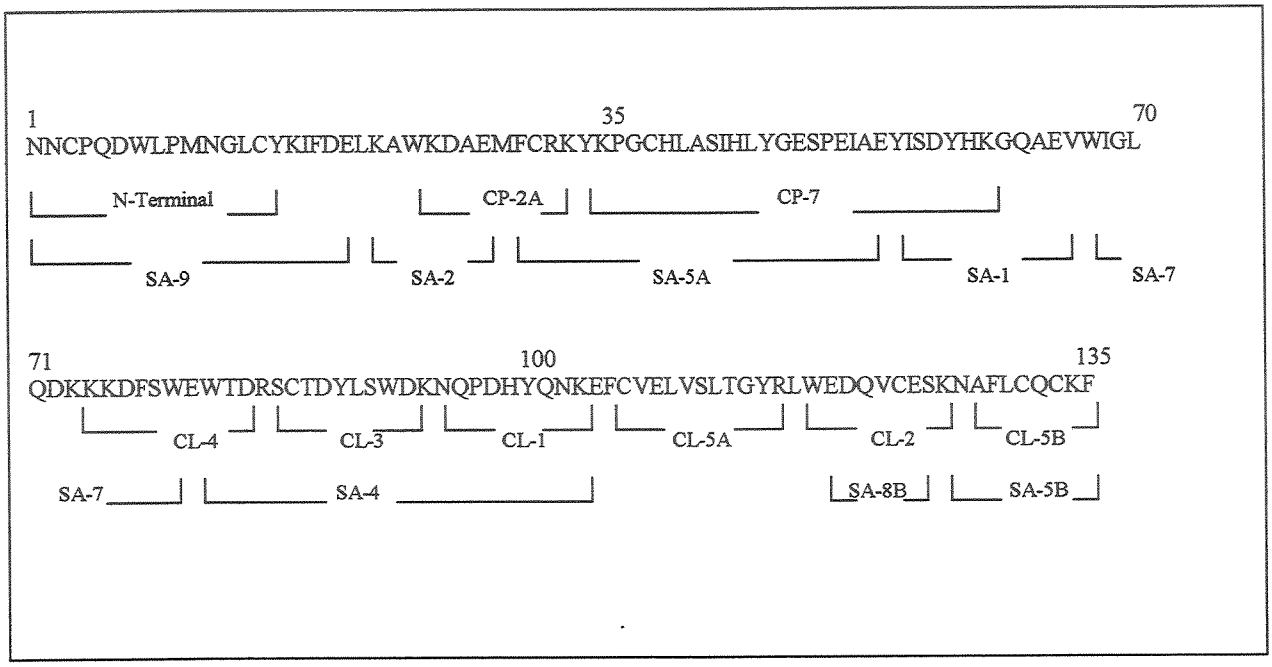
Figure 3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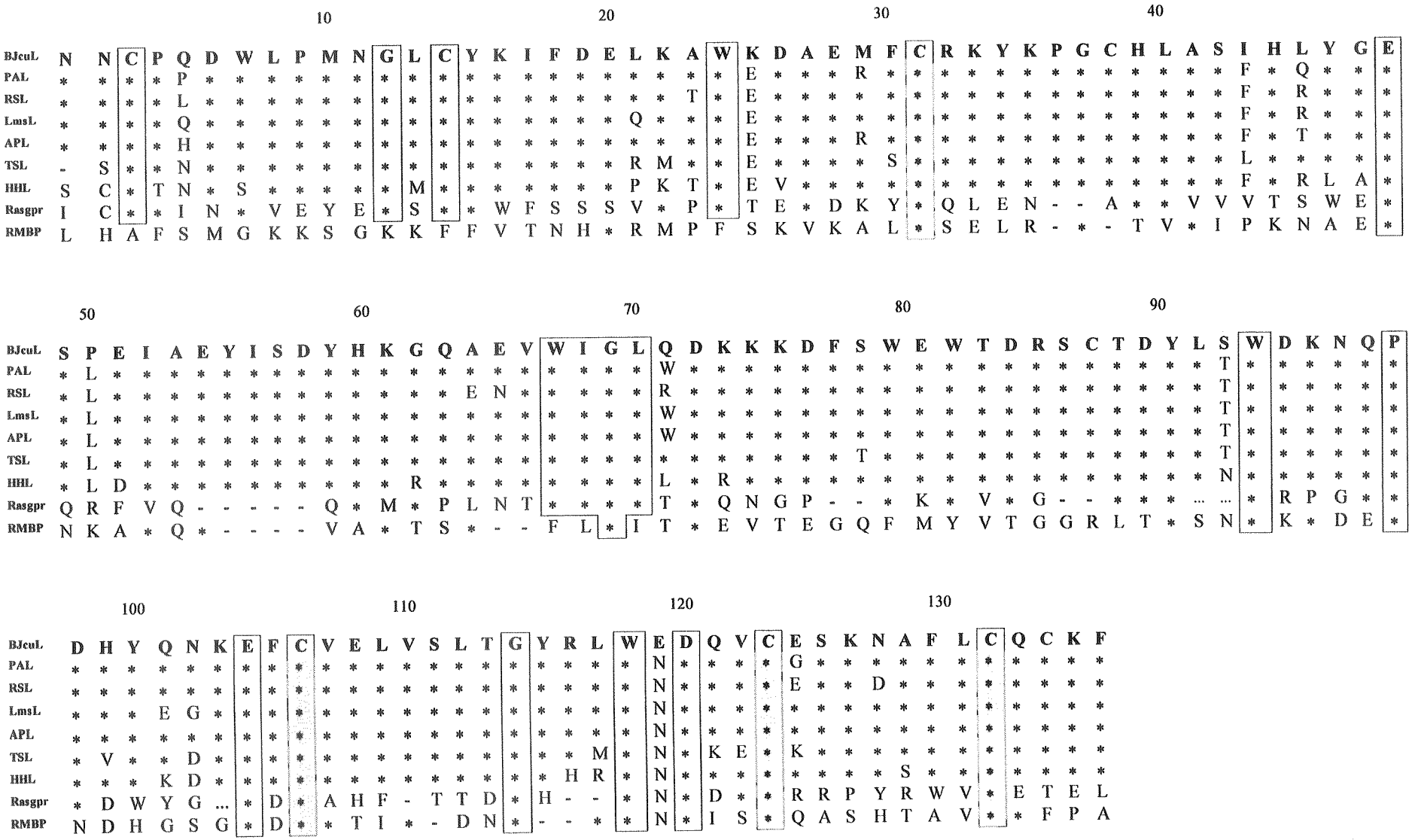
CP-7
SA-9
SA-2
SA-5A
SA-1
SA-7
71
100
135
QDKKKDFSWEWTDRSCTDYLSWDKNQPDHYQNKEFCVELVSLTGYRLWEDQVCESKNAFLCQCKF
CL-4
CL-3
CL-1
CL-5A
CL-2
CL-5B
SA-7
SA-4
SA-8B
SA-5B

Figure 4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BJcuL N N C P Q D W L P M N G L C Y K I F D E L K A W K D A E M F C R K Y K P G C H L A S I H L Y G E
PAL * * * * P * * * * * * * * * * * * * * * * * E * * * R * * * * * * * * * * * * F * Q * * *
RSL * * * * L * * * * * * * * * * * * * * * * T * E * * * * * * * * * * * * * * * * F * R * * *
LmsL * * * * Q * * * * * * * * * * * * * * * Q * * E * * * * * * * * * * * * * * * * F * R * * *
APL * * * * H * * * * * * * * * * * * * * * * * * E * * * R * * * * * * * * * * * * F * T * * *
TSL - S * * N * * * * * * * * * * * * * * * R M * * E * * * * S * * * * * * * * * * * L * * * *
HHL S C * T N * S * * * * * M * * * * * * * P K T * E V * * * * * * * * * * * * * * * F * R L A *
Rasgpr I C * * I N * V E Y E * S * * W F S S S V * P * T E * D K Y * Q L E N - - A * * V V V T S W E *
RMBP L H A F S M G K K S G K K F F V T N H * R M P F S K V K A L * S E L R - * - T V * I P K N A E *
50 60 70 80 90
BJcuL S P E I A E Y I S D Y H K G Q A E V W I G L Q D K K K D F S W E W T D R S C T D Y L S W D K N Q P
PAL * L * * * * * * * * * * * * * * * * * * * W * * * * * * * * * * * * * * * * * T * * * * * *
RSL * L * * * * * * * * * * * * E N * * * * * R * * * * * * * * * * * * * * * * * T * * * * *
LmsL * L * * * * * * * * * * * * * * * * * * * W * * * * * * * * * * * * * * * * * T * * * * *
APL * L * * * * * * * * * * * * * * * * * * * W * * * * * * * * * * * * * * * * * T * * * * *
TSL * L * T * * * * * * * * * * T * * * * *
HHL * L D * * * * * * * * * R * * * * * * * * L * R * * * * * * * * * * * * * * N * * * * *
Rasgpr Q R F V Q - - - - - Q * M * P L N T * * * T * Q N G P - - * K * V * G - - * * * ... ... * R P G * *
RMBP N K A * Q * - - - - V A * T S * - - F L * I T * E V T E G Q F M Y V T G G R L T * S N * K * D E *
100 110 120 130
BJcuL D H Y Q N K E F C V E L V S L T G Y R L W E D Q V C E S K N A F L C Q C K F
PAL * N * * * * G * * * * * * * * * * *
RSL * N * * * * E * * D * * * * * * * *
LmsL * * * E G * * * * * * * * * * * * * * N * * * * * * * * * * * * * * * *
APL * N * * * * * * * * * * * * * * * *
TSL * V * * D * * * * * * * * * * * * * * M * N * K E * K * * * * * * * * * *
HHL * * * K D * * * * * * * * * * * * * * R * N * * * * * * * * * * * * * * *
Rasgpr * D W Y G ... * D * A H F - T T D * H - - * N * D * * R R P Y R W V * E T E L
RMBP N D H G S G * D * * T I * - D N * - - * * N * I S * Q A S H T A V * * F P A

Table 1- Amino acid composition of *Bothrops jararacussu* snake venom lectin (mol/mol).

Amino acids	Aa analysis	Edman Sequencing
Asx	17.4	17
Glx	18.5	18
Ser	8.3	8
Gly	6.5	6
His	4.3	4
Arg	3.2	3
Thr	3.2	3
Ala	5.7	6
Pro	4.9	5
Tyr	6.4	8
Val	4.7	4
Met	1.8	2
Cys	7.8	9
Ile	4.8	5
Leu	10.3	11
Phe	5.5	6
Lys	13.5	13
Trp	Nd	7
Total	126.7	135
Mw	14,550.98*	16,079

Data are expressed as integral molar, Trp was not determined

* Trp not included

Table 2 – Amino acid composition of the peptides* derived from clostripain digestion for 17 hours (CL) and for 3 hours (CP) of the lectin from the venom of the snake *Bothrops jararacussu* (BJcuL).

AAs	CL-1	CL-2	CL-3	CL-4	CL-5A	CL-5B	CL-6A	CP-2A	CP-2C	CP-3A	CP-3B	CP-7
Asp/Asn	2.8	1.1	2.0	1.9	-	1.0	1.7	1.1	2.0	1.8	3.2	0.8
Glu/Gln	2.0	2.7	-	0.8	1.8	1.2	1.1	0.9	-	1.0	2.3	2.9
Ser	-	1.0	2.1	1.1	0.8	-	0.9	-	2.3	1.2	-	3.1
Gly	-	-	-	-	1.3	-	-	-	-	-	-	2.0
His	1.3	-	-	-	-	-	-	-	-	-	1.0	3.2
Arg	-	-	-	1.0	0.9	-	-	1.0	-	0.8	-	-
Thr	-	-	0.8	-	1.4	-	1.0	-	0.9	1.2	-	-
Ala	-	-	-	-	-	0.7	-	2.3	-	-	-	2.2
Pro	0.9	-	-	-	-	-	-	-	-	-	1.3	1.7
Tyr	1.3	-	1.1	1.2	0.8	-	-	-	0.9	-	1.1	4.4
Val	-	0.9	-	-	2.0	-	-	-	-	-	-	-
Met	-	-	-	-	-	-	-	1.3	-	-	-	-
Cys	-	0.8	0.7	-	0.9	1.6	-	0.8	1.0	-	-	1.0
Ile	-	-	-	-	-	-	-	-	-	-	-	3.2
Leu	-	1.1	0.9	-	2.4	0.9	-	-	1.2	-	-	2.1
Phe	-	-	1.2	1.1	0.7	2.3	0.9	1.2	-	0.8	-	-
Lys	0.8	1.2	-	2.3	-	1.0	1.2	1.1	0.8	2.2	1.3	1.8

*The peptides are the same submitted to the automatic sequencing

Table 3 – Amino acid composition of the peptides* derived from SV-8 digestion of *Bothrops jararacussu* lectin for 17 hours.

AAs	SA-1	SA-2	SA-4	SA-5A	SA-5B	SA-7	SA-8B	SA-9
Asp/Asn	0.9	0.9	5.8	-	1.0	2.0	1.1	4.9
Glu/Gln	2.0	1.0	2.9	1.1	1.1	2.1	2.1	1.9
Ser	1.0	-	2.0	1.0	1.0	0.9	-	-
Gly	1.1	-	-	2.2	-	0.9	-	1.0
His	0.8	-	1.1	2.0	-	-	-	-
Arg	-	-	0.9	1.1	-	-	-	-
Thr	-	-	2.1	-	-	-	-	-
Ala	0.9	1.9	-	0.8	0.8	-	-	-
Pro	-	-	1.0	0.9	-	-	-	1.9
Tyr	2.1	-	1.8	1.9	-	-	-	1.1
Val	-	-	-	-	-	1.1	0.9	-
Met	-	-	-	1.1	-	-	-	0.9
Cys	-	-	1.0	2.1	2.1	-	0.8	1.8
Ile	1.2	-	-	1.0	-	1.0	-	1.1
Leu	-	1.2	1.1	1.8	1.1	0.8	-	2.1
Phe	-	-	-	1.1	1.9	1.1	-	-
Lys	0.9	2.1	2.0	1.9	1.8	3.0	-	2.1

*The peptides are the same submitted to the automatic sequencing

Table 4 - Comparison of amino acid composition of *Bothrops jararacussu* lectin (BJcuL) with amino acid composition of RSL - rattlesnake lectin, LmsL – *Lachesis muta s.* lectin, BJL – *Bothrops jararaca* lectin and BMooL – *Bothrops moojeni* lectin.

Amino acids	BJcuL	RSL[a]	LmsL[b]	BJL[c]	BMooL[d]
Asx	17.4	19.0	17	19.0	18.8
Glx	18.5	17.2	18	18.1	17.0
Ser	8.3	6,8	7	8.9	5.7
Gly	6.5	6.8	7	6.6	6.1
His	4.3	3.9	4	4.1	4.8
Arg	3.2	4.8	4	3.1	4.4
Thr	3.2	5.0	4	2.9	4.2
Ala	5.7	4.4	6	5.3	7.0
Pro	4.9	3.9	6	5.4	4.6
Tyr	6.4	7.9	8	8.4	8.4
Val	4.7	4.4	4	3.9	3.4
Met	1.8	2.1	2	2.2	1.6
Cys	7.8	7.4	7	10.9	Nd
Ile	4.8	4.0	4	4.0	3.8
Leu	10.3	12.1	10	10.4	10.9
Phe	5.5	6.8	7	7.3	5.4
Lys	13.5	11.5	12	13.1	10.8

Taken from a- Hirabayashi *et al.*, 1991; b- Aragón-Ortiz *et al.*, 1996; c- Ozeki *et al.*, 1994, d - Kassab *et al.*, 2001.
Data are expressed as integral molar,
Nd- not determined

Capítulo 2

Efeito da Lectina do Veneno da Serpente *Bothrops jararacussu* na Proliferação de Células Tumorais

Artigo Publicado

Pereira-Bittencourt M., **Carvalho D.D.**, Gagliardi A.R. and Collins D.C. The effect of a lectin from the venom of the snake, *Bothrops jararacussu*, on tumor cell proliferation. **Anticancer Research 19 (5B): 4023-4025, 1999.**

The Effect of a Lectin from the Venom of the Snake, *Bothrops jararacussu*, on Tumor Cell Proliferation

MARISTELA PEREIRA-BITTENCOURT, DANIELA D. CARVALHO*, ANTONIO R. GAGLIARDI and DELWOOD C. COLLINS

*VA Medical Center and College of Medicine, University of Kentucky, Lexington, KY, USA; *IB-Departamento de Bioquímica, UNICAMP, Campinas-SP, Brazil*

Abstract *Lectins have been used extensively as histochemical probes to describe changes in tumor cell surface and are known to influence the growth of cancer cells. In this study, we determined the effect of a lectin from the venom of Bothrops jararacussu (BJcuL) on the proliferation of a number of established human cancer cell lines. The growth of eight cancer cell lines was inhibited in a dose-related manner in the presence of BJcuL lectin. This lectin was most potent as an inhibitor of growth in renal (Caki-1 and A-498) and pancreatic (CFPAC-1) cancer cell lines with 50% inhibitory concentrations (IC_{50}) of 1-2 mM. Melanoma (Wm115) and prostate (PC-3) cancer cells showed IC_{50} values of 7.9 and 8.5 mM, respectively, in the presence of BjcuL lectin whereas colon (Caco-2) and breast (MCF7) cancer cell lines showed no effect. Our results suggest that BJcuL lectin is an effective inhibitor of cell growth in some cancer cell lines.*

Lectins are polyvalent carbohydrate-binding proteins that are present in a wide range of plant and animal cells, including snake venoms (1-6). Studies of the effect of lectins on cancer have focused on the biological and biochemical characterization of endogenous lectins from tumor cells. Lectins also affect the growth of normal and cancer cells (3). Peanut lectin (PNA) binding sites have been reported in breast cancer cell lines (5). The binding of PNA to recognized cell surface glycoproteins inhibited cell proliferation of estrogen-sensitive human breast cancer cell lines (7). *Griffonia simplicifolia* lectin and wheat germ agglutinin (WGA) inhibited the growth of a number of tumor cell lines (8) and several plant lectins were effective inhibitors of cell proliferation in three prostatic cancer cell lines (LNCaP.FGC, PC-3. DU 145) (9).

Lectin-like compounds have been isolated from snake venoms (2). These proteins bind to lactose moieties and induce agglutination of erythrocytes, aggregation of platelets (10) and mitogenesis in lymphocytes (11). BJcuL, a lectin from the venom of the snake, *Bothrops jararacussu*, is a disulfide dimer composed of 15 kDa subunits with a high affinity for glycoproteins containing β-D-galactosides (2). In this study, we investigated the effect of BJcuL lectin on cell growth in eight human cancer cell lines (kidney, pancreas, prostate, and colon). Our results suggest that BJcuL lectin is an effective inhibitor of cancer cell growth in vitro, especially against renal and pancreatic cancer cell lines.

Materials and Methods

Lectin. The lectin (BJcuL) from *Bothrops jararacussu* venom was isolated by affinity chromatography on an immobilized D-galactose column, dialyzed exhaustively against distilled water and lyophilized as recently described (2). The BJcuL lectin was stored at -4°C until used.

Cells and media. The eight cell lines used in this study were obtained from the American Type Collection (ATCC, Rockville, MD). They were maintained as monolayers cultured at 37°C with 5% CO_2 in closed Falcon plastic dishes (GIBCO, Grand Island, NY) containing media supplemented with 5% or 10% heat inactivated fetal bovine serum (FBS) (HYCLONE LAB, Inc). Cells were routinely grown in their respective complete growth media; RPMI 1640 medium for PC-3 cells (human prostate carcinoma); DMEM medium for Caco-2 (human colon carcinoma), CFPAC-1 (human pancreatic adenocarcinoma), MCF7 (human breast carcinoma), A-498 (human kidney carcinoma) and Wm-115 (human melanoma); and McCoy's 5A medium for Caki-1 and Caki-2 cells (human renal carcinoma). DMEM was supplemented with 1 mM L-glutamine and 1% non-essential amino acids (NEAA); RPMI medium was supplemented with 1 mM sodium pyruvate and 1 mM L-glutamine. All media also contained antibiotics (10,000 units/L penicillin and 100 mg/L streptomycin).

The cells used in this study were harvested by trypsination (trypsin/EDTA for 2 min), resuspended in fresh medium and plated (3,000 – 5,000 cells/well) in 96-well flat microtiter plates. After 24 hr, various concentrations of BJcuL lectin (0-10 μM) were added and the cells were incubated for five days (37°C with 5% CO_2).

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

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Key Words: Snake venom, lectin, cancer cells, proliferation.

0250-7005/99 $2.00+.40

Table I. *The 50% inhibitory concentration (IC_{50}) of cell growth by BJcuL lectin (mM) in eight cancer cell lines.*

Cell line	IC50 (mM)
Renal	
Caki-1	1.06
Caki-2	7.10
A-498	1.63
Pancreas	
CFPAC-1	1.13
Prostate	
PC-3	7.90
Melanoma	
Wm-115	8.50
Colon	
Caco-2	>10.0
Breast	
MCF7	>10.0

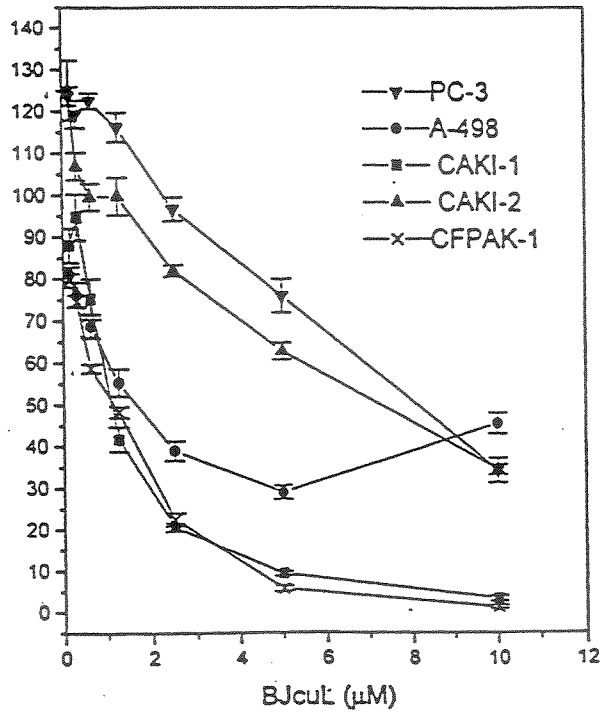

Figure 1. *The effect of various concentrations of BJcuL lectin (0-12 μM) on cell growth in five cancer cell lines: human renal carcinoma (A-498, Caki-1 and Caki-2), prostate carcinoma (PC-3) and human pancreatic adenocarcinoma (CFPAC-1). Results are expressed as percent of the control.*

bromide) assay measured cell growth as described by Carmichael *et al* (12). The effect of BJcuL lectin on cell growth was determined at each concentration from at least three different experiments with four replicate samples. This data was used to calculate the 50% inhibitory concentration (IC_{50}) for BJcuL lectin. A linear relationship between the MTT assay and cell number was established as previously described (12).

Statistical analysis. Data was analyzed by ANOVA and Dunnett's procedure. T tests were used to ascertain statistical differences. Statistical probability of $p \leq 0.05$ was considered significant.

Results

Figure 1 shows the effect of different concentrations of BJcuL lectin on cell proliferation in renal (Caki-1, Caki-2 and A-498), pancreas (CFPAC-1) and prostate (PC-3) cancer cell lines. BJcuL lectin-inhibited cell proliferation in a dose-related manner for these cancer cell lines. For most of the cancer cell lines, a transient stimulation of cell proliferation was observed at low doses (<0.5 μM) of BJcuL lectin. Incubations reported in these studies were carried out in 5% FBS. Incubation with 10% FBS decreased the inhibitory activity of BJcuL lectin by approximately 50% (data not shown). The potency of BJcuL lectin as an inhibitor of cell proliferation is reflected by the IC_{50} values in the eight cancer cells lines shown in Table I. The IC_{50} values in renal cancer cell lines were 1.06 μM for Caki-1, 1.63 μM for A-498 and 7.10 μM for Caki-2. BJcuL lectin was also a potent inhibitor of the pancreas (CFPAC-1) and prostate (PC-3) cancer cell lines. On the other hand, BJcuL lectin was not as effective as a growth inhibitor of melanoma (Wm-115), colon (Caco-2) and breast (MCF7) cancer cell lines (IC_{50} values < 8.5 μM).

Discussion

Lectins are multivalent carbohydrate-binding proteins that are widely distributed in plants and animals (1,6). Previous reports have shown that, depending on the tissue target, type and concentration of the lectin, they may either stimulate or inhibit cell growth (9). Most studies of the effect of lectins on cancer cell growth have focused on plant lectins. PNA (peanut lectin) inhibited cultured breast cancer cell proliferation (7), but stimulated benign and malignant colorectal cells (HT29 and Caco-2) (13) as well as smooth muscle and pulmonary artery cells (14). A more recent study assessed the effect of five plant lectins on the growth of three colorectal cancer cell lines (LoVo, HCT-15 and SW837) (15). Wheat germ (WGA) and concanavalin A (Con A) had significant inhibitory effects on growth of all three cell lines

whereas PNA had modest stimulatory effects on cell growth. Similar results were reported in three melanoma cell lines (SK-MEL-28, HT-144 and C32) where four plant lectins inhibited and PNA stimulated cell growth (16).

We report here the first evidence that snake-venom lectins were potent inhibitors of cell growth in selected cancer cell lines. Lectin-like proteins have been found in the venom of four snake species (Elapidae, Viperidae, Crotalidae and Bothrops) (2). Snake venom lectins have properties intermediate between the S and C families of animal lectins. They are CA^{2+}-dependent galactose-binding proteins that show a common n-terminal amino acid sequence. Our data clearly demonstrated a marked inhibition of cell proliferation in renal (Caki-1 and A-498) and pancreas (CFPAC-1) cancer cell lines (IC_{50} values = 1-2 μM) by BJcuL lectin. It was also an effective inhibitor in renal (Caki-2), melanoma (Wm-115) and prostate (PC-3) cell lines. However, BJcuL lectin did not show significant inhibition in breast (MCF7) and colon (Caco-2) cancer cells. The lack of inhibition of estrogen positive breast cancer (MCF7) proliferation by BJcuL lectin suggests that its mechanism of action differs from PNA. A high correlation has been shown between estrogen receptor (ERα). PNA binding and inhibition of proliferation in MCF7 breast cancer cells by PNA (7). The growth response of colon (Caco-2) cancer cell lines to BJcuL lectin was similar to plant lectins.

In the A-498 cells, the higher cell concentrations of BJcuL lectin (10 μM) resulted in increased cell proliferation (Figure 1). This observation is difficult to explain. We hypothesize that BjcuL lectins stimulated cell proliferation. In fact, lectins have been shown to enhance the secretion of cytokines (16). Other investigators have reported a significant serum protective effect on cell toxicity in plant lectins (9,16). The explanation for this effect is that FBS contains significant amounts of glycoligands specific for the plant lectins that interfere with the binding of the plant lectins to the cell surface (16). BJcuL lectin showed high levels of cell toxicity when incubated in the presence of 5% FBS. This is 5-fold higher than the plant lectins which were incubated in 0 or 1% FBS. This suggests that FBS contains much less BJcuL-specific glycoligands compared to plant glycoligands. Thus, the BJcuL lectin is more available for binding to the cancer cells.

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Capítulo 3

Efeito de BJcuL (uma Lectina do Veneno da Serpente *Bothrops jararacussu)* na Adesão e Crescimento de Células Tumorais e Endoteliais

Artigo Publicado

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Effect of BJcuL (a lectin from the venom of the snake *Bothrops jararacussu*) on adhesion and growth of tumor and endothelial cells

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Abstract

Lectins are polyvalent carbohydrate-binding proteins of non-immune origin. Recently, we have isolated and characterized a lectin from the venom of the snake *Bothrops jararacussu*. This lectin (BJcuL) has been shown to bind to lactose moieties and induce agglutination of erythrocytes. In the present work, we observed that cells from human metastatic breast cancer (MDA-MB-435) and human ovarian carcinoma (OVCAR-5) cell lines adhere, although weakly, to BJcuL. However, BJcuL did not inhibit adhesion of these cells to the extracellular matrix proteins fibronectin, laminin and type I collagen. Importantly, viability of these tumor cells and cells from other human tumor cell lines and a bovine brain endothelial cell line was suppressed by BJcuL. These findings suggest that the lectin BJcuL may serve as an interesting tool for combating tumor progression by inhibiting tumor cell and endothelial cell growth.

Keywords: Snake venom; Lectin; BJcuL; Cytotoxicity; Tumor cells; Endothelial cells

1. Introduction

Lectins are proteins or glycoproteins with one or more carbohydrate-binding site(s) per subunit. They play a key role during the initiation of infection, in the altered behavior of cells during metastasis, and in the protection of neonates against environmental antigens. The specificity of lectins for certain carbohydrates has allowed their use as probes to detect cell surface carbohydrates, glycoproteins, immunoglobulins, and to identify tumorigenic cells (Singh et al., 1999).

Numerous studies have demonstrated that carbohydrates serve as determinants mediating cell–molecule and cell–cell interactions. Lectin–glycoligand interactions are associated with a variety of biological processes, and recognition of carbohydrates by lectins is also involved in cancer metastasis (Varki, 1997). The ability of lectins to bind selectively to glycoconjugates has made them valuable tools in the characterization of normal and abnormal carbohydrate structure in human cells (Kim and Varki, 1997).

Previous studies have suggested that cell surface gangliosides (sialic acid-containing glycolipids) play a major role in the attachment of human melanoma and neuroblastoma cells to various extracellular matrix (ECM) components (Cheresh et al., 1987). The expression of proteoglycans (glycosaminoglycan-containing proteins) is known to modulate adhesion, proliferation and metastatic potential of melanoma cells (Burg et al., 1998). Thus, lectin–carbohydrate interactions may impact on the activity of oligosaccharide-containing proteins by lectin binding to the glycosidic portion of the cell surface proteins.

Lectin-like proteins have been found in the venom of *Elapidae*, *Viperidae* and *Crotalidae* snakes. These proteins bind to lactose moieties and induce agglutination of erythrocytes (Ogilvie and Gartner, 1984) and aggregation of platelets (Ogilvie et al., 1989). Recently, we have purified a lectin from the venom of the snake *Bothrops jararacussu* (BJcuL) (Carvalho et al., 1998). BJcuL has been identified as a disulfide-linked dimer composed of

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Table 1
Tumor and endothelial cell lines used in this study

Cell line	Name	Medium	Source
OVCAR-5	Ovarian carcinoma	RPMI—10% FBS	Dr T. Hamilton, Fox Chase Cancer Center, Philadelphia, PA, USA (Hamilton et al., 1984)
MDA-MB-435	Breast carcinoma	DMEM—10% FBS	Dr J. Price, University of Texas MD Anderson Cancer Center, Houston, TX, USA (Price et al., 1990)
A-172	Glioblastoma	DMEM—10% FBS	Dr T. Chen, University of Southern California Keck School of Medicine, Los Angeles, CA, USA (Giard et al., 1973)
U87	Glioblastoma	DMEM—10% FBS	Dr. T. Chen (Ponten and Macintyre, 1968)
T24 (ECV-304)[a]	Bladder carcinoma	DMEM—5% FBS	American Type Culture Collection (ATCC) Rockville, MD, USA (Bubenik et al., 1970)
BBMEC	Bovine brain microvascular endothelial	M199/F12—10% FBS[b]	Dr K. Kim, Childrens Hospital of Los Angeles, CA, USA (Stins et al., 1997)
K-562	Leukemia	RPMI—10% FBS	ATCC (Lozzio and Lozzio, 1975)

[a] Formerly identified as human endothelial cells (ECV-304) by ATCC, but recently shown to be T24 cells,
[b] Medium also contained 1 mM sodium pyruvate and 5 U/ml heparin.

15 kDa subunits and shown to have a high affinity for glycoproteins containing β-D-galactosides (Carvalho et al., 1998). Furthermore, this lectin has been demonstrated to be a potent inhibitor of renal and pancreatic tumor cell growth in vitro (Pereira-Bittencourt et al., 1999).

The aim of the present work is to examine the in vitro effect of the *B. jararacussu* lectin (BJcuL) on: (i) adhesion of human ovarian and breast cancer carcinoma cells to immobilized ECM proteins, and (ii) viability of these cell lines, as well as of human glioblastoma, human bladder carcinoma, human leukemia and bovine brain endothelial cells.

2. Materials and methods

2.1. Cell culture

BJcuL was purified as previously described (Carvalho et al., 1998), extensively dialyzed against water and lyophilized. The cell lines and media used in this work are summarized in Table 1. Media were supplemented with 10% FBS (fetal bovine serum; 5% for T24) and 1 mM glutamine, 100 U/ml streptomycin, and 100 U/ml penicillin (Sigma, St Louis, MO, USA). The cells were incubated at 37°C with 5% CO_2.

2.2. Cell adhesion assay

Wells of 96-well flat bottom plates (Immulon II; Dynex Technologies Inc., Chantilly, VA, USA) were coated with fibronectin (0.5 μg/well), laminin (0.5 μg/well), type I collagen (0.6 μg/well) or BJcuL (0–10 μg/well) overnight at 4°C. The ECM proteins were obtained from Becton Dickinson, Mountain View, CA, USA. Excess proteins were washed off three times with PBS, followed by blocking with 1% BSA/PBS for 2 h. For the adhesion assay, MDA-MB-435 and OVCAR-5 cells (1×10^5 cells/well) were allowed to adhere to immobilized BJcuL (1 μg/well) for 1 h. Cell adhesion to fibronectin-coated wells (1 μg/well) was considered 100%. For the inhibition assay, MDA-MB-435 and OVCAR-5 cells were pretreated with BJcuL (0–10 μM) for 1 h at room temperature and allowed to adhere to fibronectin, type I collagen, or laminin for another 1 h. For both assays, the wells were washed twice with PBS to remove unbound cells and cell debris. Viable cells, that have adhered to immobilized proteins, were colorimetrically quantified by use of Cell Titer 96™ Aqueous Proliferation Assay Kit (Promega, Madison, WI, USA) and reading absorbance at 490 nm on a plate reader (Dynatech Laboratories, Chantilly, VA, USA).

2.3. Cytotoxicity assay

The cytotoxic effect of BJcuL was examined using several human tumor cell lines and a bovine brain microvascular endothelial cell line (Table 1). The cells were harvested and adjusted to a density of 1×10^4 cells/ml with serum free medium. After centrifugation of cell suspension (100 μl), the pellet was resuspended in medium containing 5% FBS {10% FBS for bovine brain microvascular endothelial cells (BBMEC)} and BJcuL at concentrations ranging from 0 to 10 μM. Untreated and treated cells were added to wells of 96-well plates and incubated at 37°C for 96 h (144 h for BBMEC). After incubation, viable cells were quantified colorimetrically as described above. The results are given as mean ± SD and are expressed as the percentage in the rate of cell viability as compared to the control value (untreated cells) arbitrarily defined as 100%.

3. Results

3.1. Adhesion of MDA-MB-435 and OVCAR-5 cells to BJcuL

We have previously shown that human breast carcinoma (MDA-MB-435) and ovarian carinoma (OVCAR-5) cells

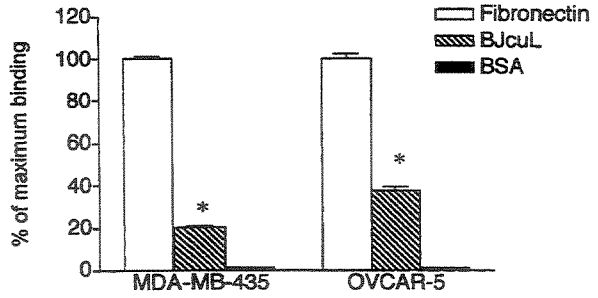

Fig. 1. Adhesion of MDA-MB-435 and OVCAR-5 cells to immobilized BJcuL and BSA. 1×10^5 MDA-MB-435 or OVCAR-5 cells were plated onto immobilized fibronectin (1 μg/well), BJcuL (1 μg/well), or BSA (1 mg/well). The cells were allowed to adhere to fibronectin, BJcuL, or BSA for 1 h at 37°C. Adhesion of MDA-MB-435 and OVCAR-5 to fibronectin was considered 100%. Data are mean ± SD from three experiments performed in triplicate. $^*P < 0.0001$ (cell binding to fibronectin vs. cell binding to BJcuL; Student's t test).

adhered to various extracellular matrix proteins, including fibronectin, vitronectin, type I collagen, and laminin (Zhou et al., 2000, and unpublished observation). It was of interest to test whether these tumor cells also bind to the lectin BJcuL. As shown in Fig. 1, both tumor cell lines adhere to BJcuL, but significantly weaker when compared to fibronectin ($P < 0.0001$; binding of the cells to fibronectin vs. cell binding to BJcuL). Since BSA was used to block unspecific cell binding, binding of both tumor cell lines to BSA was tested and found to be negligible (Fig. 1).

3.2. Effect of BJcuL on adhesion of MDA-MB-435 and OVCAR-5 to fibronectin, laminin, and type I collagen

To test whether BJcuL exerts an inhibitory effect on adhesion of MDA-MB-435 and OVCAR-5 cells to fibronectin, laminin, and type I collagen, the tumor cells were pretreated with BJcuL before being plated onto immobilized ECM proteins. As demonstrated in Fig. 2, BJcuL was ineffective in blocking adhesion of MDA-MB-435 (Fig. 2(a)) and OVCAR-5 (Fig. 2(b)) to fibronectin, laminin, and type I collagen.

3.3. Cytotoxic effect of BJcuL on tumor and endothelial cells

Pereira-Bittencourt et al. have recently demonstrated that BJcuL inhibits growth of a number of human tumor cell lines (Pereira-Bittencourt et al., 1999). We tested the same effect of this lectin using other tumor cell lines and a bovine brain microvascular endothelial cell line (BBMEC). Cells were exposed to BJcuL over various incubation periods (48, 72 and 96 h). Cells did not receive fresh medium during these periods of incubation. After 48 h, BJcuL was not cytotoxic to the tumor cell lines and the bovine endothelial cell line used (data not shown). However, when the cell lines MDA-MB-435, U87, A-172 and OVCAR-5 were exposed to BJcuL

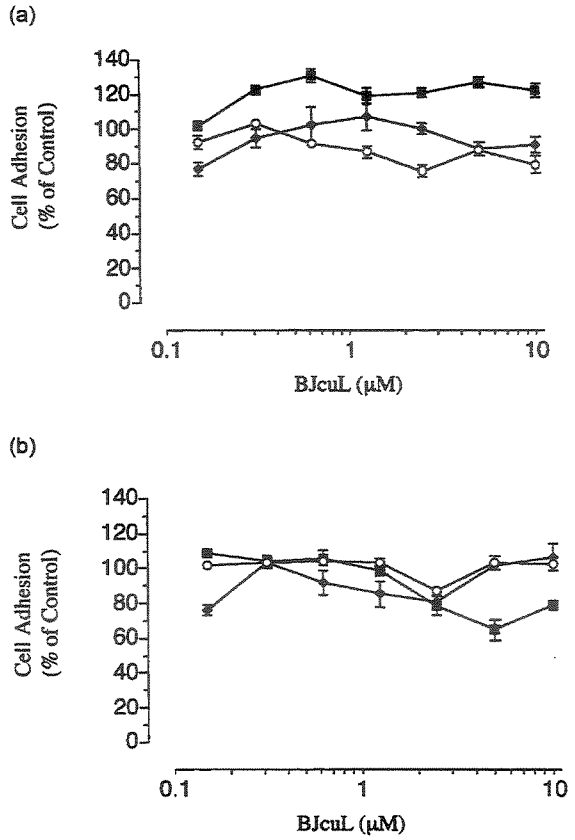

Fig. 2. Effect of BJcuL on adhesion of MDA-MB-435 and OVCAR-5 cells to ECM proteins. 1×10^5 MDA-MB-435 or OVCAR-5 cells were treated with BJcuL at different concentrations (0–10 μM) for 1 h and then plated onto immobilized fibronectin (■), laminin (◆), or type I collagen (○). Binding of untreated cells to each ECM protein was considered 100%. The adhesion assay was carried out as described in the Materials and Methods section. For MDA-MB-435 (a), the concentration of fibronectin and laminin was 0.5 μg/well. For OVCAR-5 cells (b), the concentration of fibronectin and laminin was 1 μg/well. For both cell lines, the concentration of type I collagen was 0.6 μg/well. Data are mean ± SD from three experiments done in triplicate.

for 96 h, a cytotoxic effect of this lectin could be seen (Fig. 3). In contrast to untreated cells, the number of viable BJcuL-treated cells dramatically decreased after 96 h incubation. A 144-h incubation with BJcuL was employed for BBMEC to demonstrate the extreme cytotoxic effect of BJcuL on these cells. Despite the long period of incubation (144 h) without adding fresh medium, untreated BBMEC survived as well as those cells from a shorter period of incubation (data not shown). As shown in Fig. 3, BJcuL had a differing effect on viability of tumor cells, depending on its concentration. BJcuL at concentrations lower than 1 μM did not have a cytotoxic effect on the cells even at 96 h, but was cytotoxic to the cells at concentrations higher than 1 μM. In T24 cells, BJcuL had no effect since BJcuL-treated cells were as viable as untreated

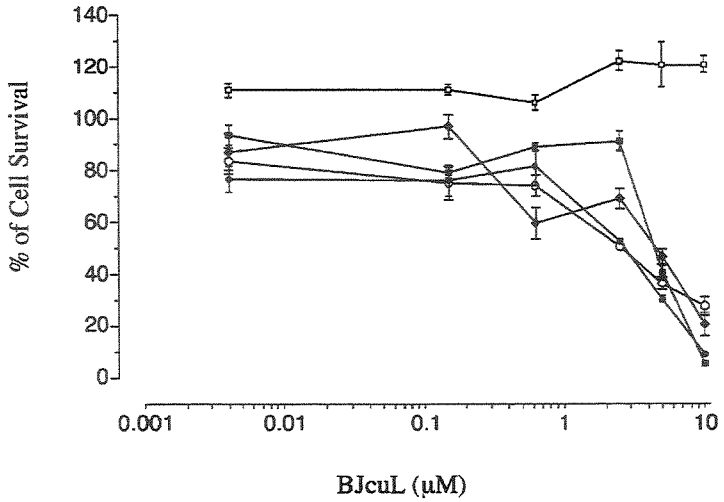

Fig. 3. Effect of BJcuL on viability of tumor cells. Harvested tumor cells were suspended at a density of 1×10^4 cells/ml in medium containing 5% FBS and BJcuL (0–10 μM). After incubation for 96 h at 37°C, viable cells were quantified colorimetrically as described in the Materials and Methods section. Cell lines used were T-24 (□), OVCAR-5 (◆), U87 (■), A-172 (●), and MDA-MB-435 (○). The data are expressed as the survival rate of BJcuL treated cells in % in comparison with the survival rate of untreated cells which is defined as 100%. Error bars show mean ± SD from three experiments performed in triplicate.

cells at any BJcuL concentration tested. This was also true for the human leukemia (K-562) cell line (Table 2). When using BBMEC, BJcuL was extremely cytotoxic (Table 2). The cytotoxic effect (IC_{50}) of BJcuL on tumor and endothelial cells is summarized in Table 2.

3.4. Effect of FBS on the cytotoxicity of BJcuL

Using OVCAR-5 cells, the effect of FBS in the medium on BJcuL cytotoxicity was clearly demonstrated in Fig. 4. The lectin was not cytotoxic to these cells when 10% FBS was used. BJcuL exerted a higher cytotoxic effect on the cells suspended in medium containing 5% FBS than on those suspended in medium containing 2.5% FBS. Incubation of the cells with BJcuL at a concentration of 10 μM resulted in a decrease of cell survival of approximately 20 or 95% when 2.5 or 5% FBS was used, respectively.

Table 2
Cytotoxic effect (IC_{50}) of BJcuL on tumor and endothelial cells. Cells were incubated with BJcuL at different concentrations for 96 h (144 h for BBMEC) at 37°C (see Materials and Methods section). Data are mean ± SD from three experiments performed in triplicate

Cell line	IC_{50} (μM)
OVCAR-5	6.00 ± 0.3
MDA-MB-435	6.37 ± 0.5
A-172	4.36 ± 0.4
U87	5.40 ± 0.4
BBMEC	0.09 ± 0.02
T24	≫10
K-562	≫10

4. Discussion

Lectins are carbohydrate-binding proteins that agglutinate erythrocytes and aggregate normal and tumor cells, as well as microorganisms (Sharon, 1993). Lectin binding to cell surface carbohydrates elicits various effects on cell processes, such as proliferation, apoptosis and mitosis.

Cheresh et al. (1987) have shown that cell surface sialogangliosides play a major role in the attachment of human

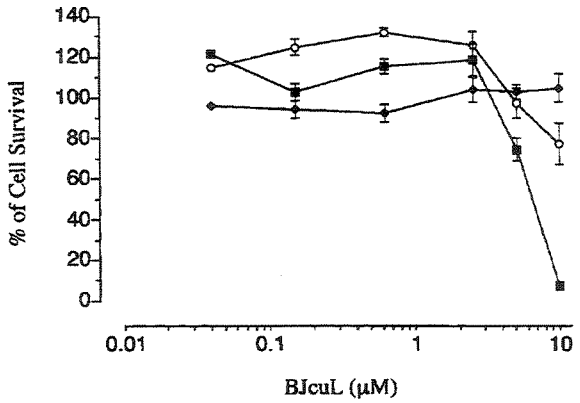

Fig. 4. Effect of FBS on the cytotoxicity of BJcuL. OVCAR-5 cells at a density of 1×10^4 cells/ml were suspended in medium containing BJcuL (0–10 μM) and 2.5% (○), 5% (■), or 10% (◆) FBS. After incubation for 96 h, cell viability was determined colorimetrically as described in the Materials and Methods section. The data are expressed as the viability of BJcuL treated cells in % in comparison with that of untreated cells which is defined as 100%. Error bars show mean ± SD from three experiments performed in triplicate.

melanoma cells to RGD-containing substrates, such as fibronectin. Furthermore, NG2 proteoglycan (molecules composed of 95% of polysaccharides and 5% of proteins) was shown to be capable of modulating the adhesion potential of melanoma cells (Burg et al., 1998). Therefore, we examined the ability of two tumor cell lines (MDA-MB-435 and OVCAR-5) to adhere to BJcuL and the effect of BJcuL on adhesion of these tumor cell lines to the ECM proteins fibronectin, laminin and type I collagen. BJcuL was found to bind to the tumor cells, but did not inhibit adhesion of these cells to fibronectin, laminin and type I collagen. These findings suggest that BJcuL does not interfere with ECM protein-binding cell surface receptors such as integrins. Many integrins recognize the Arg-Gly-Asp (RGD) sequence which is present in ECM proteins (Ruoslahti, 1991).

Previous studies have shown that lectins act as stimulators or inhibitors of cell growth, depending on cell types. The peanut lectin PNA, for instance, which binds to galactose-β1,3-*N*-acetylgalactosamine, inhibits proliferation of breast cancer cells like ZR-75.1 and 734-B (Marth and Daxenbichler, 1988) and stimulates growth of smooth muscle and pulmonary cells (Sanford and Harris-Hooker, 1990). Furthermore, a galactose-specific lectin from mistletoe induces reduction in the tumor mass of glioma in rats and in humans (Lenartz et al., 1996, 1998). Other lectins like *Griffonia simplicifolia* (GSA), concanavalin A (Con A), wheat germ agglutinin (WGA), and *Phaseolus vulgaris* (PHA-L) exert various effect on growth of human colorectal cancer cells (Kiss et al., 1997). A recent study demonstrated that the lectin from the venom of the snake *B. jararacussu* (BJcuL) inhibited growth of several human tumor cell lines (Pereira-Bittencourt et al., 1999). In the present study, it was observed that BJcuL also negatively affects viability of several other tumor cell lines, including human breast carcinoma (MDA-MB-435), ovarian carcinoma (OVCAR-5), glioblastoma (U87 and A-172), and a bovine brain microvascular endothelial cell line (BBMEC). Of all cell lines tested in this work, BBMEC appeared to be the most sensitive to BJcuL. Furthermore, BJcuL did not inhibit or stimulate growth of bladder carcinoma (T24) or leukemia (K-562) cells, suggesting that these cells may not express a counter-receptor for this lectin.

Using OVCAR-5 cells, the cytotoxic effect of BJcuL was dramatically modulated according to the concentration of fetal bovine serum (FBS) in the medium. The lectin was highly cytotoxic to these cells when incubated in the presence of 5% FBS, and had no inhibitory effect on cell growth when 10% FBS was used. These findings are in good agreement with a previous study described by Pereira-Bittencourt et al. (1999). However, 5% FBS had a lower 'protective' effect on the lectin cytotoxicity compared to 2.5% FBS. It has been reported that the presence of fetal bovine serum (FBS) at low concentrations may actually promote cell entry into the cell cycle in slowly dividing cells. However, the mechanism by which serum acts to modify the cell cycle of cultured cells is unclear and difficult to address due to the complexity of its composition (Esher et al., 1973). Inhibition of DNA synthesis as well as an increased rate of long-lived cellular protein degradation has been reported in fibroblasts exposed to serum-deficient medium (Warburton and Poole, 1977). Taken together, the metabolic changes in a cell caused by low concentration of FBS in the medium, could modify the exposure of cell receptors to BJcuL. This may explain why the cytotoxic effect of BJcuL on OVCAR-5 cells in 2.5% FBS was almost totally diminished.

In conclusion, the present study demonstrates that the lectin from the venom of the snake *B. jararacussu* (BJcuL) is a potent inhibitor of growth of some tumor cell lines and an endothelial cell line, and may thus be of eventual clinical use in slowing tumor progression. However, further investigations into intracellular mechanisms of BJcuL action are needed to explore the possible apoptotic and/or anti-angiogenic effect of this lectin.

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Conclusões Gerais

O presente trabalho permitiu concluir que:

1. A seqüência dos resíduos de aminoácidos da lectina do veneno da serpente *Bothrops jararacussu* revelou que esta molécula pertence à superfamília de lectinas dependentes de cálcio, as lectinas tipo-C (Drickamer, 1999). As lectinas de veneno têm mostrado alta homologia entre si, e podemos encontrar na seqüência de BJcuL alguns resíduos altamente conservados nos CRD tipo-C; como Cys (posição 3), Gly (12), Cys (14), Trp (24), Cys (31), Glu (48), Trp (67), Ile (68), Gly (69), Leu (70), Trp (92), Pro (97), Glu (104), Cys (106), Gly (114), Trp (118), Asp (120), Cys (123) e Cys (131). Da mesma forma, a posição conservada das meias cistinas na estrutura primária de BJcuL indica a formação de 4 pontes dissulfeto intramoleculares; entre Cys (3) e (14); Cys (31) e (131); Cys (38) e (133); e Cys (106) e (123). Estas ligações dissulfeto são altamente conservadas entre as lectinas de venenos.

2. A lectina do veneno da serpente *Bothrops jararacussu* é capaz de inibir a proliferação de certas linhagens de células tumorais. Foi demonstrado que a presença de BJcuL interfere drasticamente na proliferação de células tumorais, principalmente de rins, pâncreas e gliomas. Até o presente momento nenhuma lectina de veneno de serpente tinha sido descrita apresentando efeitos antiproliferativos sobre células tumorais. Por outro lado, BJcuL em concentrações de até 10 μM não reduz significantemente a proliferação de células de carcinoma cólon-retal e de mama (MCF-7).

As diferentes sensibilidades das diversas células à BJcuL observadas nos experimentos pode ser explicado pelos diferentes padrões de glicosilação nas superfícies celulares de células normais e neoplásicas (Kobata, 1998), já que a glicosilação é um processo específico de cada célula e tecido (Lis & Sharon, 1993).

3. A presença do Soro Fetal Bovino (SFB) é de crucial importância para o crescimento e desenvolvimento das células, por outro lado, a presença de SFB implica no que diz respeito a quantidades significantes dos açúcares peculiares às lectinas. O SFB pode interferir na concentração de BJcuL capaz de inibir 50% da proliferação celular (IC_{50}). A interferência do SFB nos efeitos induzidos por lectinas já foi descrita por vários autores (Glad & Borrebaeck, 1984; Milton & Rhodes, 1995), alguns deles descreveram que a isolectina PHA-E (hemaglutinina de amendoim) possui um forte efeito mitogênico para linfócitos quando todas as glicoproteínas do soro são removidas, e que outra lectina, a jacalina, só inibe a proliferação de células tumorais cólon-retais (HT29) quando em meio livre de soro.

   Em nossos experimentos, a redução dos níveis de soro para 2,5% na cultura de células OVCAR-5, não aumentou o efeito de BJcuL sobre este tipo celular, fato a ser investigado. Com a quantidade usada para cultivo das células – 10% - o efeito de BJcuL sobre as células tumorais de ovário é praticamente anulado, desta forma, o SFB interfere na concentração citotóxica ideal da BJcuL, pois é necessária uma concentração superior para sobrepor o efeito "protetor" do SFB.

4. As células tumorais de ovário e mama, OVCAR-5 e MDA-MB-435, respectivamente, se aderem a BJcuL imobilizada. Porém, a ligação das células tumorais a BJcuL não é tão potente quando comparada à ligação das células a proteínas da matriz extracelular, como a fibronectina. A ligação de BJcuL nestes tipos celulares não influenciou a adesão destas células às proteínas da matriz extracelular, como laminina, fibronectina e colágeno tipo I. A lectina do veneno da serpente *Bothrops jararacussu* se liga, provavelmente, a sítios diferentes dos das integrinas, receptores das proteínas de matriz extracelular responsáveis pela adesão das células (Cheresh e cols, 1987).

5. BJcuL demonstrou inibir a proliferação de células endoteliais. Em testes preliminares, tanto a viabilidade de células endoteliais microvasculares HMEC, quanto a viabilidade da linhagem de células endoteliais de cérebro bovino (BBEC), foram reduzidas em 50% na presença de BJcuL em concentrações de 5,3 μM e 0,09 μM, respectivamente. Ao contrário do que foi descrito por Sanford & Harris-Hooker, (1990), onde algumas lectinas específicas para galactosídeos estimulavam a mitose em células vasculares, nossos resultados demonstraram uma atividade inibitória de BJcuL sobre a proliferação de células endoteliais. Os dados mostrados sugerem que as linhagens celulares HUVEC e BBEC expressam em sua superfície glicoconjugados que contêm unidades de carboidratos que são reconhecidos por BJcuL. A especificidade de BJcuL por galactosídeos (Carvalho e cols, 1998) sugere a ligação desta molécula aos resíduos gal α1-3 gal presentes nos terminais dos glicoconjugados de superfície de células endoteliais (Palmetshofer e cols, 1998).

Abreviações Para Aminoácidos

Aminoácidos	Três Letras	Uma Letra
Alanina	Ala	A
Arginina	Arg	R
Asparagina	Asn	N
Ácido Aspártico	Asp	D
Cisteína	Cys	C
Glutamina	Gln	Q
Ácido Glutâmico	Glu	E
Glicina	Gly	G
Histidina	His	H
Isoleucina	Ile	I
Leucina	Leu	L
Lisina	Lys	K
Metionina	Met	M
Fenilalanina	Phe	F
Prolina	Pro	P
Serina	Ser	S
Treonina	Thr	T
Triptofano	Trp	W
Tirosina	Tyr	Y
Valina	Val	V

Segundo nomenclatura IUPAC

Perspectivas

O trabalho realizado com a lectina do veneno da serpente *Bothrops jararacussu* nos forneceu base para diversos estudos:

1- O seqüenciamento da BJcuL como o primeiro passo na caracterização da estrutura terciária por meio da cristalização da mesma. A determinação da estrutura tridimensional será muito útil no esclarecimento da função inibidora da proliferação de células endoteliais e tumorais, indicando semelhanças ou diferenças dos mecanismos de ação de proteínas citotóxicas já caracterizadas.

2- A caracterização da atividade inibitória sobre a proliferação de células tumorais e endoteliais, abre caminho para estudos mais aprofundados acerca do mecanismo de ação desta proteína, relacionando sua estrutura molecular com sua capacidade citotóxica.

3- A partir dos resultados obtidos, e juntamente com a necessidade de uma maior quantidade de proteína para testes biológicos, foi iniciada a clonagem e expressão desta lectina.

4- Aprofundamento dos estudos em cultura de células, no que diz respeito à atividade de BJcuL sobre o crescimento, proliferação e migração de células endoteliais, caracterizando os componentes celulares nos quais a lectina age.

Comunicações em Congressos Relacionadas ao Projeto de Tese

D.D. Carvalho, J.C. Novello and F.S. Markland . *In vitro* effect of BJcuL (*Bothrops jararacussu* Lectin) on the proliferation of cancer cells. XXIX Reunião Anual da SBBq (Sociedade Brasileira de Bioquímica e Biologia Molecular), 23 a 26 de maio de 2000, Caxambu-MG, Brasil.

D.D. Carvalho, S. Marangoni and J. C. Novello. Molecular Characterization of a Cytotoxic Snake Venom Lectin. 18th International Congress of Biochemistry and Molecular Biology - Beyond the Genome. Birmingham, UK, 16-20, July, 2000.

Outras Comunicações em Congressos

E. Rego, **D.D. Carvalho**, R. Benatti, S. Marangoni; B. Oliveira and J.C. Novello. Isolation and Partial Characterization of a Lectin From Leguminoseae *Crotalaria mucronata* Seeds. XXVII Reunião Anual da SBBq (Sociedade Brasileira de Bioquímica e Biologia Molecular). 23-26 maio de 1998, Caxambu-MG, Brasil.

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