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Dissertação

CLONAGEM DO GENE QUE CODIFICA PARA A LISTERIOLISINA EM BCG *ΔleuD* E AVALIAÇÃO DA ATIVIDADE ANTITUMORAL EM CÉLULAS DE CÂNCER DE BEXIGA

KAREN SILVA LEAL

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RESUMO

LEAL, Karen. **CLONAGEM DO GENE QUE CODIFICA PARA A LISTERIOLISINA EM BCG *ΔleuD* E AVALIAÇÃO DA ATIVIDADE ANTITUMORAL EM CÉLULAS DE CÂNCER DE BEXIGA** 2013. 42f. Dissertação (Mestrado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

O *Mycobacterium bovis* BCG é utilizado mundialmente como vacina contra tuberculose há mais de meio século, com altos níveis de segurança. O BCG possui características que o tornam um promissor vetor para a produção de vacinas recombinantes multivalentes. Modificações genéticas podem aprimorar as propriedades imunogênicas e adjuvantes do BCG. Uma dessas modificações permite que o BCG escape do endossoma de células apresentadoras de antígenos. Desta maneira, os imunógenos de BCG acessam vias de apresentação cruzada de antígenos, ampliando a sua imunogenicidade. Este trabalho teve como objetivo desenvolver uma cepa de BCG *ΔleuD* expressando listeriolisina (Hly) de *Listeria monocytogenes* e avaliar seu potencial citotóxico na linhagem celular 5637 de câncer de bexiga. Para isso, o BCG *ΔleuD* foi transformado com o plasmídeo recombinante pUP410 contendo o gene *hly*. A cepa obtida demonstrou um nível de citotoxicidade similar à cepa BCG Pasteur parental, quando avaliada na linhagem celular de câncer de bexiga. Esta cepa necessita ser avaliada em outras linhagens de câncer para comprovar seu possível efeito citotóxico potencializado.

Palavras chaves: *Mycobacterium bovis BCG , ΔleuD,* listeriolisina, câncer de bexiga.

ABSTRACT

LEAL, Karen. **CLONING OF THE GENE THAT CODES FOR LISTERIOLYSIN ON BCG Δ*leu*D AND EVALUATION OF THE ANTITUMORAL ACTIVITY ON BLADDER CANCER CELLS** 2013. 42f. Dissertação (Mestrado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Mycobacterium bovis BCG has been used worldwide as a vaccine against tuberculosis for over fifty years with an outstanding safety record. BCG has features that make it a promising vector for production of recombinant multivalent vaccines. Genetic modification can enhance the adjuvant and immunogenic properties of BCG. One of these modifications would allow BCG to escape the endosome of antigen presenting cells. Therefore, the immunogens of BCG can access antigens cross priming routes, increasing their immunogenicity. The aim of this work was to develop a strain of BCG Δ*leu*D expressing listeriolysin (Hly) of *Listeria monocytogenes* and evaluate its cytotoxic potential on cell line 5637 of bladder cancer. Thus, BCG ΔleuD was transformed with recombinant plasmid pUP410 containing the *hly* coding sequence. The recombinant BCG strain expressing listeriolysin showed a similar level of cytotoxicity when compared to the parental BCG Pasteur. This strain needs to be evaluated in other cancer lines in order to confirm its possible potentiated cytotoxic effect.

Keywords: *Mycobacterium bovis BCG*, *ΔleuD,* listeriolysin, bladder cancer;

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LISTA DE ABREVIATURAS E SIGLAS

BAAR- Bacilo Alcool-Ácido Resistente

BCG - Bacillus Calmette-Guérin

rBCG – BCG recombinante

Hly- Listeriolisina

HPV- Human Papiloma Virus

PSA - Prostate Specific Antigen

RT – PCR - Reverse-Transcription Polymerase Chain Reaction

S1PT- Subunidade 1 da toxina de Bordetela pertussis

TB- Tuberculose

SUMÁRIO

1. INTRODUÇÃO

O *Mycobacterium bovis* bacilo Calmette-Guérin (BCG) é um bacilo alcool-ácido resistente (BAAR) não esporulado, do grande grupo das micobactérias (PARISH; STOKER, 1998). Sua parede celular é composta majoritariamente por lipídeos, o que lhe proporciona muitas das propriedades antigênicas, adjuvantes, anti-tumorais e de virulência (FORRELLAD et al., 2013). Apesar disso, as micobactérias não possuem fatores de virulência clássicos, como toxinas, que são típicas de outros patógenos bacterianos. Dessa maneira, outros fatores de virulência como enzimas de várias rotas lipídicas, proteínas de superfície celular, sistemas de transdução de sinal e proteínas reguladoras permitem sua sobrevivência no interior dos macrófagos, como a inibição da fusão do fagossomo com o lisossomo e escape do ambiente ácido (FORRELLAD et al., 2013).

A utilização do BCG como vacina foi iniciada em 1921 e, desde então, mais de três bilhões de pessoas já foram vacinadas, sendo considerada atualmente a vacina mais administrada no mundo (BLOOM; FINE, 1994). Em 2011 a Organização Mundial da Saúde estimou 8,7 milhões de casos de tuberculose no mundo (WHO; 2012). O BCG é a única vacina usada na prevenção da tuberculose (TB) em humanos (VON REYN; VUOLA, 2002) e oferece vantagens únicas: não é afetado por anticorpos maternais; pode ser administrada logo após o nascimento; uma dose única é segura, estável, possui baixo custo na produção em relação a outras vacinas vivas e confere uma imunidade prolongada (STOVER et al., 1991; (BASTOS et al., 2009a).

1.2 BCG recombinante

Avanços na área da tecnologia do DNA recombinante, bem como o conhecimento do genoma e o desenvolvimento de sistemas de expressão de antígenos heterólogos em BCG, fizeram com que essa bactéria se tornasse um excelente candidato a vetor de vacina recombinante multivalente expressando antígenos heterólogos de outros microrganismos (JACOBS et al., 1987; (BASTOS et al., 2009b). A vacina com rBCG confere vantagens em relação ao BCG selvagem, como uma maior segurança, além de causar menos efeitos colaterais e ser capaz de se manter a longo prazo no organismo inoculado (WANG et al., 2012). A indução de uma resposta imune contra antígenos heterólogos pela inoculação de rBCG e a primeira evidência de proteção foram descritas por STOVER et al.,1991.

Desde então, inúmeros outros estudos confirmam o uso do rBCG contra agentes parasitários, bactérias, e antígenos virais (BASTOS et al., 2009b). Para a obtenção de uma resposta protetora satisfatória, a via de administração é um fator importante para garantir o sucesso na vacinação, pois a imunogenicidade de algumas vacinas é alterada quando não for administrada adequadamente (VARALDO et al., 2004).

A construção de cepas recombinantes que proporcionem maior estímulo do sistema imune e aumento do efeito antitumoral da bactéria constitui um dos principais focos de pesquisas em melhorias imunoterapêuticas do BCG (AMIRKHAH et al., 2009). A utilização dos rBCG para antígenos heterólogos de diferentes bactérias, bem como a expressão de combinados antigênicos, também demonstram eficácia aumentada frente a tumores, tanto em ensaios *in vitro* quanto em modelos animais (ANDRADE et al., 2010;CHUNG et al.,

2003).O rBCG tem sido, sem dúvida, uma das terapias com mais sucesso diante de tumores de bexiga (HERR et al., 2008).

1.3 Sistema auxotrófico Δ*leu*D

Um mutante de BCG Pasteur denominado BCG Δ*leu*D foi desenvolvido por nosso grupo de pesquisa. Esse sistema de seleção é baseado no uso de uma cepa auxotrófica para o aminoácido leucina e na complementação com o gene *leuD* inserido no vetor plasmidial pUP410. Esse sistema possui vantagens como: promover uma seleção *in vivo* ativa, não necessitando de vetores que contenham genes de resistência a antibióticos (BORSUK et al., 2007).

Estudos realizados por (BANGE; BROWN; JACOBS, Jr., 1996) mostraram que o crescimento de *M. bovis* BCG auxotrófico para leucina era restrito no interior de macrófagos. Quando este mutante foi complementado com o gene *leuD* de *Escherichia coli* os níveis de crescimento no interior dos macrófagos foram restabelecidos, demonstrando a eficiência desse sistema de seleção. Um estudo prévio comprovou também a eficácia de um BCG Δ*leu*D expressando o antígeno 85B quando submetido a um teste desafio com bovinos. Este se manteve de forma estável mesmo sem a presença de um marcador de resistência a antibiótico (RIZZI et al., 2012). Segundo (BEGNINI et al., 2013) este BCG Δ*leu*D/*Ag85*B também se mostrou eficaz na imunoterapia atuando na inibição do crescimento de células de câncer de bexiga.

1.4 Imunoterapias e câncer

A imunoterapia tem como objetivo a estimulação do sistema imune, alterando a resposta biológica mediada por células ou interferindo na interação antígeno-anticorpo, sendo esta uma promissora estratégia potencialmente eficaz para o tratamento do câncer (ALDRICH et al., 2010). O sistema imunológico é capaz de reconhecer e extinguir lesões pré-cancerosas e cancerosas (SHENG; HUANG, 2011). Esse reconhecimento é obtido principalmente através de vacinação com peptídeos antigênicos ou através de células dendríticas ativadas (DRANOFF, 2004).

Desse modo, o uso do BCG para terapia de câncer foi proposto em 1930. No entanto pouco foi feito até os anos 1950 e 1960, quando foram diagnosticados outros tipos de tumores, como leucemia, câncer colo-retal, câncer de pulmão e melanomas. Os resultados não impressionavam muito, exceto por um estudo promissor de Mathé no tratamento de leucemia linfoblástica em 1969 que outros pesquisadores não tinham confirmado até então (LOCKYER et al., 2001). O advento da quimioterapia e radioterapia reduziu o entusiasmo com o BCG. Enquanto isso, em 1966, estudos de Coe & Feldman mostraram uma reação de hipersensibilidade na bexiga de cobaios ao serem administrados com BCG. Com isso, foram capazes de explorar a resposta imune quando obtiveram o êxito no tratamento câncer (LOCKYER et al., 2001).

A utilização do BCG na imunoterapia para câncer de bexiga teve início baseando-se principalmente em três razões: no tratamento de *carcinoma-in-situ*; na redução da frequência de tumores recorrentes e também na profilaxia da doença (KAWAI et al., 2013). A partir de então, muitas pesquisas tem se

mostrado eficientes nas imunoterapias contra vários tipos de câncer (WALLECHA et al., 2011; KAWAI et al.,2013).

NASCIMENTO e colaboradores, construíram uma cepa de BCG recombinante em fusão com subunidade 1 da toxina de Bordetela pertussis (S1PT) e demonstraram que essa vacina é capaz de estimular uma resposta imune Th1 mais forte que o BCG selvagem.Em outro estudo *in vivo*; (ANDRADE et al., 2010) confirmam que no ensaio de citotoxicidade, camundongos tratados com rBCG-S1PT foram mais eficazes em destruir células da linhagem MB49 de tumores de bexiga. É provável que as citocinas secretadas tenham ativado uma maior produção de linfócitos e células efetoras durante o contato do rBCG com células tumorais. Esses estudos também constataram uma redução significante em tumores, aumentando assim a taxa de sobrevivência dos camundongos em relação ao grupo controle vacinados somente com BCG.

Em outro trabalho, a utilização de uma cepa recombinante de BCG superexpressando o antígeno 85B também se mostrou eficiente em ensaios citotóxicos com a linhagem de carcinoma de bexiga 5637. Foi constatado um aumento da morte celular quando estas foram tratadas com BCGΔ*leu*D/ag85B em relação a células tratadas apenas com o controle BCG Pasteur que é usado no tratamento convencional (BEGNINI et al., 2013). Estes dados comprovam a eficiência gerada pelo rBCG, enfatizando seu uso na imunoterapia de câncer de bexiga, aumentando as perspectivas do emprego de cepas geneticamente modificadas para aprimorar o tratamento já existente.

1.5 Listeriolisina e câncer

A bactéria *Listeria monocytogenes* vem se destacando na imunoterapia para tumores, como o câncer de próstata, câncer de colo de útero, fazendo uso de modificações genéticas para a expressão de antígenos heterólogos (SHAHABI et al., 2008 WALLECHA et al., 2011). A *L. monocytogenes* é atrativa para o desenvolvimento de imunoterapias, pois gera uma forte resposta imune inata e adaptativa. Ela é uma bactéria intracelular que acomete macrófagos e células dendríticas. Quando secreta uma proteína nativa, a listeriolisina consegue formar poros no fagolisossomo, escapando para o citosol, onde consegue acessar outras vias de apresentação de antígenos, ampliando sua imunogenicidade (WALLECHA et al., 2011). Alterações genéticas no BCG expressando listeriolisina podem ser alternativas para aprimorar as imunoterapias de câncer já existentes. Na Figura 1 está representado o mecanismo de ação do BCG recombinante expressando a listeriolisina:

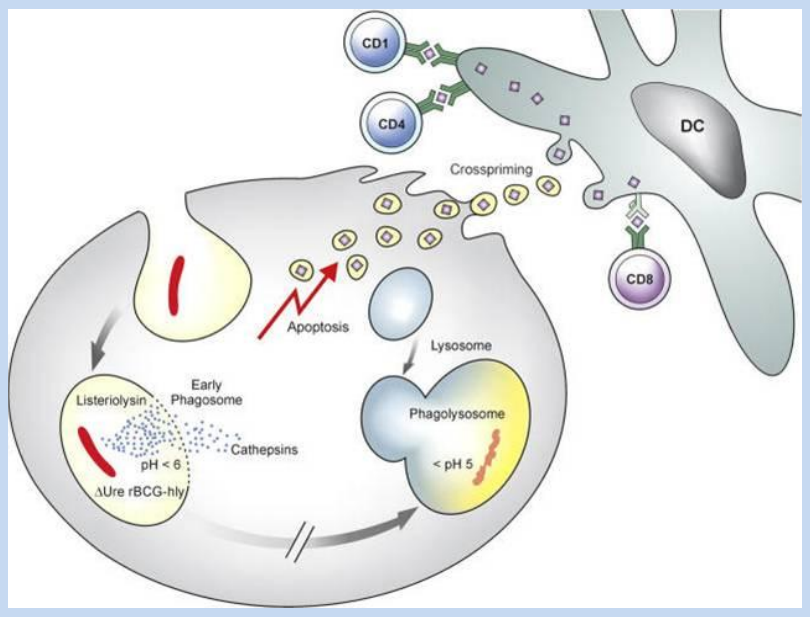

Figura 1: Descrição esquemática do sistema empregando BCG recombinante expressando listeriolisina, acessando outras vias de apresentação de antígenos, aumentando a imunogenicidade da vacina (Imagem concedida por Stefan Kaufmann).

Outras propriedades da *L. monocytogenes* indicam que quando fusionados com uma forma truncada da listeriolisina O, antígenos expressados e secretados pela *L. monocytogenes* foram significantemente mais imunogênicos. Em estudos prévios, uma cepa recombinante de *L. monocytogenes* foi capaz de causar a regressão de determinados tumores como em câncer de próstata (SHAHABI et al., 2008). Também foi avaliado o potencial de listeriolisina O para erradicar tumores induzidos por HPV (WALLECHA et al., 2011).

2. OBJETIVO

2.1 Geral

Construir uma cepa de *M. bovis* BCG *Δleu*D recombinante contendo o gene que codifica para a listeriolisina de *L. monocytogenes* e avaliar seu potencial citotóxico na linhagem 5637 de células de câncer de bexiga.

2.2 Específicos

1- Obter a sequência codificadora da listeriolisina e clonar no vetor de expressão pUP410;

2- Construir a cepa recombinante e confirmar a expressão da listeriolisina por RT-PCR;

3- Avaliar o potencial da cepa recombinante em ensaios de citotoxicidade em linhagens de células de câncer de bexiga

3. ARTIGO

CLONING OF THE GENE THAT CODIFIES FOR LISTERIOLYSIN ON BCG Δ*leu*D AND EVALUATION OF THE ANTITUMORAL ACTIVITY ON BLADDER CANCER CELLS

(Artigo científico escrito sob formato do periódico Current Microbiology)

CLONING OF THE GENE THAT CODIFIES FOR LISTERIOLYSIN ON BCG Δ*leu*D AND EVALUATION OF THE ANTITUMORAL ACTIVITY ON BLADDER CANCER CELLS

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ABSTRACT

Mycobacterium bovis BCG has been used worldwide as a vaccine against tuberculosis for over fifty years with an outstanding safety record. BCG has features that make it a promising vector for production of recombinant multivalent vaccines. Genetic modification can enhance the adjuvant and immunogenic properties of BCG. One of these modifications would allow BCG to escape the endosome of antigen presenting cells. Therefore, the immunogens of BCG can access antigens cross priming routes, increasing their immunogenicity. The aim of this work was to develop a strain of BCG Δ*leu*D expressing listeriolysin (Hly) of *Listeria monocytogenes* and evaluate its cytotoxic potential on cell line 5637 of bladder cancer. Thus, BCG ΔleuD was transformed with recombinant plasmid pUP410 containing the *hly* coding sequence. The recombinant BCG strain expressing listeriolysin showed a similar level of cytotoxicity when compared to the parental BCG Pasteur. This strain needs to be evaluated in other cancer lines in order to confirm its possible potentiated cytotoxic effect.

Keywords: *Mycobacterium bovis*, *ΔleuD,* listeriolysin, bladder cancer;

1. INTRODUCTION

The *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) strain is used worldwide as a vaccine against tuberculosis [3]. It has also been used as a vector for the development of recombinant and multivalent vaccines against many infectious diseases and cancer immunotherapy [14,16,4]. BCG has adjuvant properties, is not affected by maternal antibodies, a single dose confers a long immune response, is stable and safe, can be administered orally and has a low production cost compared to other live vaccines [14].

Various expression systems for exogenous antigens in BCG have been developed to enable the construction of recombinant BCG (rBCG) [19]. Most of these systems employs integrating vectors or episomal vectors containing antibiotics resistance genes as selection markers, which respectively confer low levels of protein expression and absence of in vivo selection, hence low vector stability [6,3]. Our research group has developed a BCG Δ*leu*D expression system, which allows for stable genetic transformation of exogenous genes without the use of antibiotics as selection markers. This system employs the complementary auxotrophic selection leucine marker, allowing the production of safe and stable recombinant strains, in vivo, resulting in high levels of protein expression [5].

An improvement on the ability of the current BCG to induce a broader immune response can be achieved by allowing the escape of the BCG from the endosome of antigen-presenting cells. This results in cross-presentation of antigens, increasing their immunogenicity [13,16]. The listeriolysin (Hly) is secreted by the intracellular bacterium *Listeria monocytogenes* and is responsible for the release of this

microorganism from the phagosomes into the cytoplasm of infected cells [8]. Besides its use as a vaccine, BCG has also been exploited in anti-tumour therapy. BCG expressing foreign antigens or overexpressing some of its own antigens is a promising approach to improve the performance of BCG anti-tumour therapy [2]. Current studies are developing rBCG strains to further improve the effectiveness of the therapy [4,7].

The aim of this study was to construct a BCGΔ*leu*D/hly secreting lysteriolysin and to evaluate the cytotoxic effect in 5637 bladder carcinoma cells line.

2. MATERIALS AND METHODS

2.1 Coding sequence of the listeriolysin and primer design

A synthetic gene containing the coding sequence for the Hly protein was constructed based on the sequence deposited in GenBank (NC 003210) derived from *Listeria monocytogenes* EGD-e. To facilitate secretion of the protein by BCG, the synthetic gene had a region corresponding to the listeriolysin signal peptide replaced by Ag85B signal peptide, a protein normally secreted by *M. bovis*. The primers used in PCR amplification were (5'-ACTGGGGTACCGGCCGGTGAACTTC-3' and 5'-GGGGTACCGGGTGCGCGGTCTGCCA-3'), designed using *Vector NTI* 11,0 (Invitrogen ™, Carlsbad, CA, EUA).

2.2 Cloning of the hly gene into the expression vector

The PCR product was digested with *Kpn*I enzyme (Promega), and cloned into pUP410 mycobacterial expression vector [5], which had previously been digested with the same restriction enzyme (Figure 1). Competent *E. coli* was transformed with

ligation product and a recombinant plasmid (pUP410/hly) was identified and verified by restriction enzyme digestion and PCR.

2.3 Construction of rBCG*Δleu*D containing *hly*

Electrocompetent BCG Pasteur *ΔleuD* was transformed with pUP410/hly and grown on Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI, USA) supplemented with 0.5% glycerol, 0.05% Tween 80, 10% oleic albumin-dextrose-catalase (OADC) and supplemented with 100 mg/ml L-leucine (Sigma). The recombinant BCG was grown to an optical density at 600 nm (OD_{600}) of 0.6 in selective 7H9 media, the culture (10 mL) was centrifuged (4,000g for 10 min). The detection of mRNA was confirmed by Reverse-Transcription PCR (RT-PCR). BCG Δ*leu*D/hly was grown in liquid culture and then tested regarding its cytotoxicity on a bladder carcinoma cell line.

2.4 Reverse-Transcription PCR (RT-PCR)

The *hly* expression profiles was investigated by RT-PCR. Total RNA was isolated with TRIzol® Reagent (Invitrogen™, Carlsbad, USA). DNase treatment of RNA samples was conducted with a DNA-free® Kit (Ambion™, USA) following the manufacturer's protocol. First-strand cDNA was performed with 2μg of RNA using SuperScript™ III Reverse Transcriptase (Invitrogen™, Carlsbad, USA) according to the manufacturer's protocol.RT-PCR reaction was run using PCR Master Mix (Applied Biosystems, UK) and the primers used were (5'- GCAAGCCTACCCGAATGTGT- 3' and 5'GTCGGTTCGTTGACGTTGAC-3').

2.5 Cell culture

The human bladder carcinoma cell line 5637 was obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil). Cells were grown at 37 °C in an atmosphere of 95% humidified air and 5% CO_2. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS). The experiments were performed with cells and bacteria in the logarithmic phase of growth and all experiments were performed in triplicate.

2.6 Determination of cytotoxicity

The viability of the 5637 cell line was determined by measuring the reduction of soluble MTT [3-(4,5-dimethylthiazol- 2-yl)-2,5-diphe-nyltetrazolium bromide] to water insoluble formazan [1]. Cells were seeded at a density of 2×10^4 cell per well in a volume of 100 μL in 96-well plates, and grown at 37 °C in a 5% CO_2 atmosphere for 24 h before being used in the cell viability assay. Cells were then incubated with the BCG strain (BCG *Δleu*D- BCG *Δleu*D/hly1, BCG *Δleu*D/hly2 and BCG *Δleu*D/hly3) (at a concentration of 4.8×10^6 CFU for 48 h.The cells were washed and 180 μL of medium and 20 μL of MTT (5 mg MTT/mL solution) were added to each well. The plates were incubated for 3 h, the cells were washed and 200 μL of DMSO was added to each well, and the formazan was solubilized on a shaker for 15 min at 150 rpm[4]. The absorbance of each well was read on a microplate reader at a test wavelength of 492 nm. The inhibition (%) of cell proliferation was determined as follows: inhibitory growth (1-Abs492 treated cells/Abs492 control cells) x 100% [24]. All observations were validated by three independent experiments in triplicates for each experiment.

2.7 Flow cytometry

The Guava Nexin assay (Guava Technologies) was conducted following the manufacturer's instructions. Briefly, 2.0×10^4 to 1.0×10^6 of the treated 5637 cells (100 μL) was added to the Guava Nexin reagent. Cells were incubated in the dark at room temperature for 20 min and samples were acquired on flow cytometry Guava EasyCyte System. In this assay, an annexin V-negative and 7-AAD-positive result indicated nuclear debris, an annexin V-positive and 7-AAD-positive result indicated late apoptotic cells, while an annexin V-negative and 7-AAD-negative result indicated live healthy cells and annexin V-positive and 7-AAD-negative result indicated the presence of early apoptotic cells.

2.8 Data analysis

Data sets were analyzed using one-way ANOVA. Significance was considered at $P<0.05$ in all analyses. Data were expressed as mean±SEM.

3. RESULTS

3.1 Construction of rBCG*Δleu*D

The fragment amplified by PCR, containing the entire coding sequence of the *hly*, plus the Ag85B signal peptide was cloned into pUP410 cloning vector. The fragment of 2.371 bp was successfully cloned. The insert was fully characterized by restriction enzyme digestion.

3.2 RT-PCR detection the mRNA

The presence of Hly mRNA was confirmed by RT-PCR, and the amplicon size (200bp) was confirmed by agarose gel electrophoresis at a concentration of 0.8% (Fig 2).

3.3 Determination of cytotoxicity

Human bladder carcinoma cells were incubated with the BCG strains (BCG Pasteur, BCG Δ*leu*D, BCG Δ*leu*D/hly1, BCG Δ*leu*D/hly2 and BCG Δ*leu*D/hly3) for 48 h. As demonstrated in Fig. 3, the recombinants BCG/Δ*leu*D strains (BCG Δ*leu*D/hly1, BCG Δ*leu*D/hly2 and BCG Δ*leu*D/hly3) did not demonstrate a significant *in vitro* cytotoxic activity after 48 h of treatment. The treatment with BCG/Δ*leu*D Pasteur induced cell growth of 19%, for BCG/Δ*leu*D treatment the growth inhibition was 16% and for BCG Δ*leu*D/hly1, BCG Δ*leu*D/hly2 and BCG Δ*leu*D/hly3 treatments it was 18%, 19% and 14% respectively (Fig 3). This percentage of growth inhibition is not different from the BCG Pasteur control.

3.4 Flow Cytometry

The annexin-PE staining assay was performed to characterizetreatments with the recombinant BCG inducing apoptosis in 5637 cell line after 48h of exposure. Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic feature of cells entering apoptosis. The results showed that the BCGΔ*leu*D/hly treatments were little effective in causing cell death through apoptosis.BCGΔ*leu*D strain showed an early apoptosis ratio of 3.18%, while BCG Δ*leu*D/hly1, BCG Δ*leu*D/hly2 and BCGΔ*leu*D/hly3 showed 3.64%, 3.70% and 3.13% of early apoptosis respectively (Fig4). For late apoptosis, the percentage ratio of

BCGΔ*leu*D treatment was 1.93%, for BCG Δ*leu*D/hly1 it was 2.16%, for BCG Δ*leu*D/hly2 it was 2.26% and for BCG Δ*leu*D/hly3 it was 2.12%.There are no statistical differences between control and BCG strains treatment.

4. DISCUSSION

The *Mycobacterium bovis* strain Bacillus Calmette-Guerin (BCG) is used as a vaccine against tuberculosis[3], is one of the most widely administered vaccines worldwide [10]. Moreover, *M. bovis* BCG-based therapy is also the treatment of choice for superficial bladder cancer [18]. Another way to improve BCG is by introducing genetic modifications for superior targeting of essential immune pathways [11]. Studies demonstrated that rBCG has a great potential as a vaccine vector, and a recombinant vaccine able to protect against tuberculosis and several other diseases is conceivable [3]. Recently, a work demonstrated an overexpression with Ag85B in a BCG Pasteur strain, using an expression system based on the use of a auxotrophic strain for the leucine amino acid, and complemented with *leuD* inserted into the plasmid vector (pUP410) This recombinant BCG strain was evaluated as a vaccine against bovine tuberculosis, and showed protection against a *M. bovis* challenge at higher level than its parental strain [14,12]. This recombinant strain demonstrated a significant in vitro cytotoxic activity after 48 h of treatment, inhibiting more than 50% of tumour cells. The inhibition of cell proliferation following BCG Δ*leu*D/Ag85B treatment was 77.8%, while for the other strains it was 28% (BCG Δ*leu*D) and 38.1% (BCG Pasteur) [4] .

Intensive research has been carried out to find less toxic and more potent therapeutic agents for the immunotherapy of bladder tumors [22]. In a previous study, *Listeria monocytogenes*-based vaccines limited autochthonous tumor growth and broke immunologic tolerance in human papillomavirus [17,21]. A Molecular strategy for

some types of cancer treatment is the use of a important protein of the bacterium *L. monocytogenes*, called listeriolysin, with acts forming pores on the membrane of the early phagosome and allows leakage of the BCG immunogen from the phagosome into the cytoplasm of infected host cells[8,15]. Several groups have shown recombinant *L. monocytogenes* to be an effective agent for immunotherapy against infection and cancer [15].

In the present study, we successfully performed the cloning of the hly gene into BCG Pasteur *ΔleuD* using pUP410/hly as cloning vector. The transcription of the synthetic gene was confirmed by RT-PCR. BCG *ΔleuD/hly* liquid culture (OD_{600}) was used in the bacterial preparation and it was incubated with bladder cancer cells (5637 line) to conduct a preliminary test of cytotoxicity level. The initial results showed that there was no statistical difference between BCG Δ*leu*D/*hly* and conventional treatment using BCG Pasteur for this cell line. In a previous work, rBCGΔ*leu*D overexpressing Ag85B demonstrated enhanced cytotoxicity on superficial bladder cancer cells in vitro and this effect may be due to apoptosis and cell-cycle arrest [4]. These data corroborate that thedevelopment of immunotherapy based on recombinant BCG (rBCG) overexpressing antigens or expressing foreign antigens is a promising approach to improve the performance of BCG anti-tumour therapy [2].

The listeriolysin expressed in rBCGΔ*leu*D/hly can allow access to both phagosomal and cytosolic compartments and BCG antigens may be presented in the context of both MHC I and II molecules, resulting in strong cellular immune responses [8]. Previous studies have indicated that when fused to a truncated form of listeriolysin, antigens expressed and secreted are significantly more immunogenic than when they are secreted as stand-alone proteins [9,16]. Moreover, a recombinant *L. monocytogenes*, expressing and secreting the human PSA protein fused to Hly, was able togenerate an

effective and durable anti-tumor response in mice [15]. Based on this, we constructed a new recombinant BCG strain, comprised of an rBCG*ΔleuD* which expresses and secretes the Hly. Our preliminary evaluation demonstrated that the rBCG strain had no enhanced cytotoxic activity on bladder cancer cells hly, however, this strain may be evaluated in other assay using other bladder cell lines or different kinds of cancer, and the result might be different.

Tumor suppression frequently involves the modulation of signal transduction pathways, leading to alterations in gene expression, arrest in cell cycle progression or apoptosis [23]. Apoptosis is considered a highly regulated process that allows a cell to self-degrade in order to eliminate an unwanted or dysfunctional cell [20]. Anticancer treatments kill tumor cells primarily by the induction of apoptosis through activation of the intrinsic pathway [20]. In this work, we performed flow cytometry assay to verify apoptosis level in bladder cancer cell. The results showed that the level of apoptosis induced by BCG Δ*leu*D/hly was similar to BCG Pasteur employed on the conventional therapy in bladder cancer cells. There were no significant differences in comparison to the control group.

In conclusion, the preliminary data here presented indicate that a recombinant BCG Δ*leu*D/hly, can generate an anti-tumor response similar to the BCG Pasteur in the treatment of bladder cancer cells. Our findings indicate that it maintains the same cytotoxicity levels in vitro. Further investigation of the rBCG strain may be evaluated as a new immunotherapeutic agent against other kinds of cancer.

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Figures

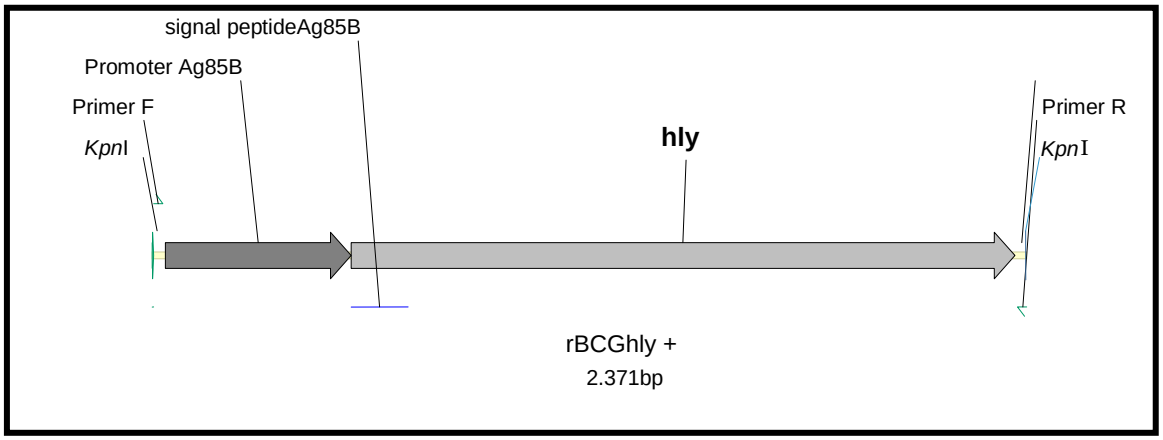

Figure 1.Schematic representation of the synthetic gene cloned in vector pUP410.

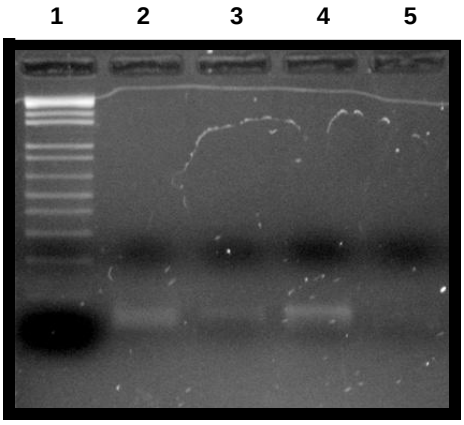

Figure 2.(A) Electrophoresis in a 0.8% agarose gel. Lane 1,1 kb Plus DNA Ladder (Invitrogen®); Lane 2, rBCG/hly1; Lane 3,rBCG/hly2; Lane 4,rBCG/hly3; and Lane 5, negative control;

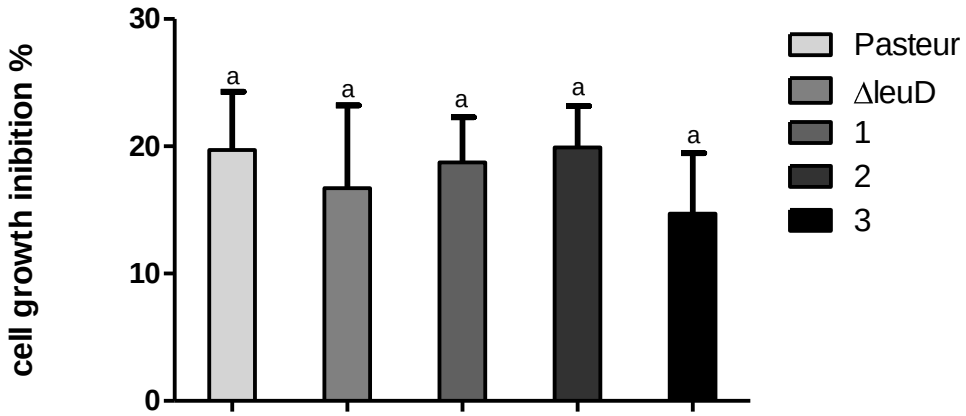

Figure 3.Human bladder carcinoma cells (5637) growth inhibited after 48h of treatment with BCGΔ*leu*D/hly. Cell proliferation was investigated by MTTassay. Data are expressed as means±SEM from three independent experiments.

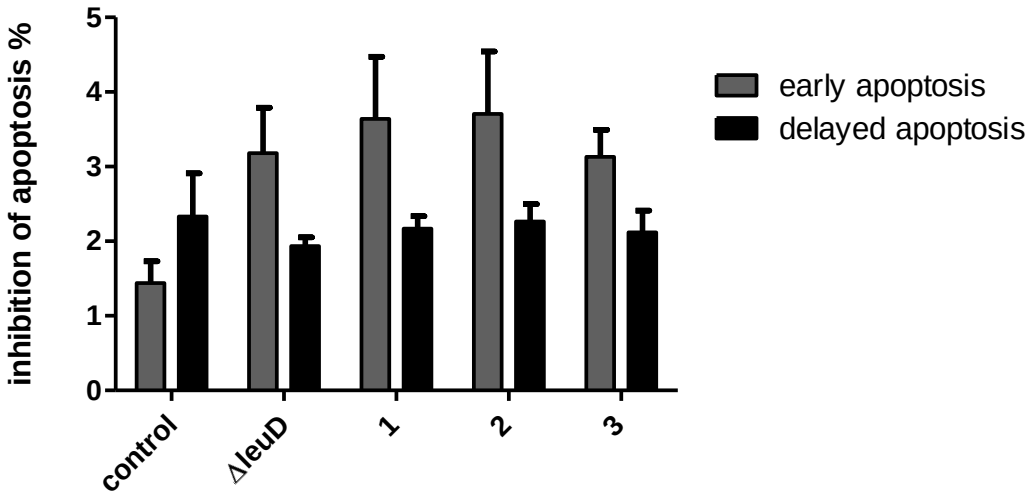

Figure 4. Inhibition rate of early and delayed apoptosis, in treated BCG/Δ*leu*D/hly; data are expressed as means±SEM from three independent experiments.

4. CONCLUSÕES

- A cepa auxotrófica BCG Δ*leu*D/hly apresenta um nível de citotoxicidade similar à cepa BCG Pasteur utilizada no tratamento convencional;
- A expressão do gene que codifica para a listeriolisina não confere um aumento na atividade citotóxica do BCG em linhagem celular de câncer de bexiga, conforme era esperado.

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