Mycobacterium bovis bacille Calmette-Guérin (BCG) enhances human β-defensin-1 gene transcription in human pulmonary gland epithelial cells[1]

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ABSTRACT

AIM: To examine the stimulatory effect of bacille Calmette-Guérin (BCG) cell wall components on human β-defensin-1 (hBD-1) gene expression and analyze the response element in the 5'-flanking region of the gene. **METHODS:** BCG cell wall proteins were fractionated by Sephadex G-150 chromatography. Using reverse-transcription polymerase chain reaction (RT-PCR) and Northern hybridization analysis, hBD-1 mRNA expression was detected in a human pulmonary gland epithelial cell line SPC-A-1 cells. Progressive deletions of 5'-flanking region of hBD-1 gene were produced by PCR and ligated into promoterless chloramphenicol acetyltransferase (CAT) expression plasmid to construct pCAT reporter plasmids. Reporter gene expression was determined by ELISA. **RESULTS:** There was an obvious enhancement of hBD-1 mRNA expression after stimulation with heat-inactivated BCG whole cells (50 mg/L), or the cell wall components with a molecular weight of 18-30 kDa (3 mg/L) for 8 h. The upstream sequence between -314 bp and +54 bp had the inducible activity by BCG, which contained CCAAT/enhancer binding protein-β (C/EBPβ), activator protein-1 (AP-1), and CP2 *cis* element. **CONCLUSION:** BCG cell wall components (18-30 kDa) can stimulate hBD-1 mRNA expression in pulmonary gland epithelial cells. The sequence (-314 /+54) containing C/EBPβ, AP-1, and CP2 binding sites in the upstream of hBD-1 is involved in this induction.

INTRODUCTION

Antimicrobial peptides are key components of innate immunity[1]. β-Defensin (hBD-1) is one of subclasses of antimicrobial peptides, and primarily expressed in various epithelial tissues including skin, lung, and intestine[1,2]. In addition to their striking microbicidal properties, mammalian defensins have chemotactic activity for immature dendritic cells and memory T cells[3]. Human hBD-1 was first isolated from human hemofiltrate obtained from patients with end-stage of renal disease[4]. It widely expresses in secreted glands and epithelial cells including pulmonary submucosal gland epithelia[5,6]. The compromising of its activity leads to susceptibility to the airway infection in cystic fibrosis, suggesting the crucial role of hBD-1 in airway defense against infections[6].

It has been demonstrated that some β-defensins such as human β-defensin-2 (hBD-2) and bovine tra-

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cheal antimicrobial peptide (TAP) expression can be induced in epithelial cells by LPS, IL-1β, and TNF-α through Toll/NF-κB pathway[7, 8]. However, someone thought that hBD-1 gene could not response to inflammatory stimuli[9].

Mycobacterium tuberculosis including bacille Calmette-Guérin (BCG) has long been found to have powerful immunologic adjuvant activity, not only augment both cell-mediated and humoral immune response but also initiate innate defense mechanisms[10]. We therefore examined the stimulatory effect of BCG cell wall components on hBD-1 gene expression in human pulmonary gland epithelial cells and preliminarily analyzed the response element in the upstream region of the gene.

MATERIALS AND METHODS

Materials Human pulmonary gland epithelial SPC-A-1 cell line was obtained from Shanghai Institute of Cell Biology and kept in our laboratory. BCG vaccine Shanghai line (D_2-PB302 line) was obtained from Chengdu Institute of Biological Products. DNA polymerase, endonuclease, and one step RT-PCR kit were purchased from Takara Company (Dalian, China). pCAT Basic and pSV-β-Galactosidase control vector were from Promega Company (USA). DIG DNA labeling and detection kit, Dorsper liposomal transfection reagent, CAT ELISA, and β-Gal ELISA assay kit were purchased from Roche Company (Germany). BCA Protein Assay Kit was from Pierce Company (USA). DNA sequencing was performed by Shanghai Genecore Company.

Preparation of BCG cell wall protein fractions BCG were cultured in Sauton medium. Dry cells (300 mg) were suspended in 5 mL of phosphate-buffered saline (PBS, pH7.4) 0.02 mol/L containing phenylmethylsulfonyl fluoride 4 mmol/L, Teween-80 0.05 %, and ethylene diaminetetraacetic acid 1 mmol/L, and sonicated with 20 kHz on ice for 2 min×5 times at 1-min interval each. The sonicate was centrifuged at 27 000×*g* for 30 min. The pellet was suspended in 2 mL of the phenylmethylsulfonyl fluoride-containing PBS buffer, layered over a discontinuous sucrose gradient composed of sucrose 27 %, 39 %, 49 % (w/w) and centrifuged at 100 000×*g* for 2 h. The particulate band at the interphase between 27 % and 39 % sucrose contained most of the cell walls of BCG[11]. Having been washed with PBS, the cell wall pellet was suspended in 2 mL of PBS 0.02 mol/L containing SDS 2 %. The proteins were extracted at room temperature for 2 h followed by centrifugation at 27 000×g for 30 min. The supernatant was subjected to Sephadex G-150 column (900 mm×16 mm), and proteins were eluted at a flow rate of 6 mL/h with PBS 0.02 mol/L containing SDS 0.5 %. Fractions were pooled based on the A_{280} readings detected by UV /VIS spectrometer (Lambda Bio40, Perkin Elmer, USA). SDS was removed by dialysis against superpured water at room temperature for 72 h. The relative molecular weight was determined by Tricine-SDS-PAGE (15 %). Protein concentration was measured by BCA Protein Assay Kit.

Cell culture and hBD-1 mRNA determination SPC-A-1 cells were grown in RPMI-1640 medium at 37 ºC in 5 % CO_2 air, and were seeded into 35-mm plate one day before experiments at a concentration of 3×10^5 cells per well. After incubation with or without BCG for 8 h, Epithelial cells were washed with PBS and total RNA was isolated using the TRIzol reagent. RNA concentration was measured by UV Spectrometer. Total RNA (1 μg) served as a template in a one-step reverse transcribed polymerase chain reaction (RT-PCR) with an internal control of β-actin. Four intron spanning specific primers were designed. Two of the primers for hBD-1 amplification were as follows: R1, 5'-TTG TCT GAG ATG GCC TCA GGT GGT AAC; R2, 5'-ATA CTT CAA AAG CAA TTT TCC TTT AT. The others for β-actin were: A1, 5'-GCG GGA AAT CGT GCG TGA CAT T; A2, 5'-GAT GGA GTT GAA GGT AGT TTC GTG. The thermocycling program of the one step RT-PCR consisted of a reverse transcription at 50 ºC for 30 min, an initial denaturation at 94 ºC for 2 min, 25 to 35 cycles (denaturation at 94 ºC for 30 s, annealing at 58 ºC for 30 s, and elongation at 68 ºC for 45 s), and a final 7-min elongation at 68 ºC. PCR products were subjected to electrophoresis on an 1.5 % agarose gel. For Northern hybridization analysis, total RNA (about 4 μg) were separated by electrophoresis on agarose gel containing formaldehyde, then transferred to positively charged nylon membranes. The hBD-1 and β-actin RT-PCR products were used as probes. According to the instruction of DIG DNA labeling and detection kit, probe labeling and detection were performed.

Construction of hBD-1 reporter plasmids Based on a previously published sequence of the hBD-1 gene[12] and chromosome 8 working sequence (GenBank number: AF298854), progressive deletions of promoter sequence were isolated by PCR amplification. A com-

mon antisense primer, 5'-GTG TCT AGA TTT GGA GGC TGA GCT GAC AG , corresponding to the sequence between positions +54 and +38 relative to transcription start site and containing a *Xba*I restriction enzyme site (underlined) was used in the PCR reaction. The oligonucleotides used as sense primers each contained a *Hin*dIII restriction enzyme site (underlined) and were as follows: -2161 bp, 5'-CAG AAG CTT TCG GCT CAC TAC AAC CAA TG; -575 bp, 5'-GAC AAG CTT GAG GAG TGC CCT TTG GAA AC; -484 bp, 5'-CAG AAG CTT GAT GAG AAG GTA GCC TTG GC; and -314 bp, 5'-GTG AAG CTT CCT CCA TGT GAT CCA GAA GG. Typically, the PCR reaction thermocycling program consisted of initial denaturation at 94 ºC for 3 min, followed by 6 cycles of 20-s denaturation at 94 ºC, 20-s annealing at 65 ºC, and 36-s elongation at 72 ºC, then followed by 26 cycles of 20-s denaturation at 94 ºC, 20-s annealing at 58 ºC, and 36-s elongation at 72 ºC. A final 8-min elongation at 72 ºC was followed. For amplication of the long sequence (-2161/+54), the denaturation and annealing time were prolonged to 30 s, elongation time was extended to 2 min in every cycle. The PCR fragments were digested with *Hin*dIII and *Xba*I, and ligated into the *Hin*dIII and *Xba*I sites of a promoterless pCAT Basic Vector. One deletion construct, pCAT -69 plasmid, was generated from pCAT -575 plasmid by *Hin*dIII and *Sal*I digestion. Each construct was verified by sequencing.

Transfection and CAT assay SPC-A-1 cells were plated at 3×10^5 cells per 35-mm dish one day before transfection. When the cells were 60 % to 80 % confluent, growth media were replaced with serum-free culture medium. By using Dosper Liposomal transfection reagent, the hBD-1 CAT reporter constructs (2.0 mg per well) were introduced into SPC-A-1 cells co-transfected with internal control pSV-β-galactosidase control plasmid (0.3 μg per well). After incubation for 6 h the transfection media were replaced with 2 mL of fresh growth medium and the cells were further incubated with or without BCG cell wall components for 39 h. CAT and β-Gal concentrations of the cell lysate were assayed by ELISA following the manufacturer's instruction. β-Gal concentration values were used to normalize CAT activity between different transfections for transfection efficiency.

Statistics Data were presented as mean±SD and compared with paired *t*-test.

RESULTS

BCG enhanced hBD-1 mRNA expression in SPC-A-1 cells Whole BCG cells were inactivated by boiling for 10 min and used to stimulate SPC-A-1 cells. RT-PCR detection indicated that an obvious enhancement of hBD-1 mRNA expression in SPC-A-1 cells was observed after incubation with 50 mg/L of whole BCG cells for 8 h (Fig 1A). Different PCR cycles and Northern hybridization analysis confirmed this finding (Fig 1B). BCG-induced enhancement of hBD-1 mRNA expression was time- and dose-dependent[13].

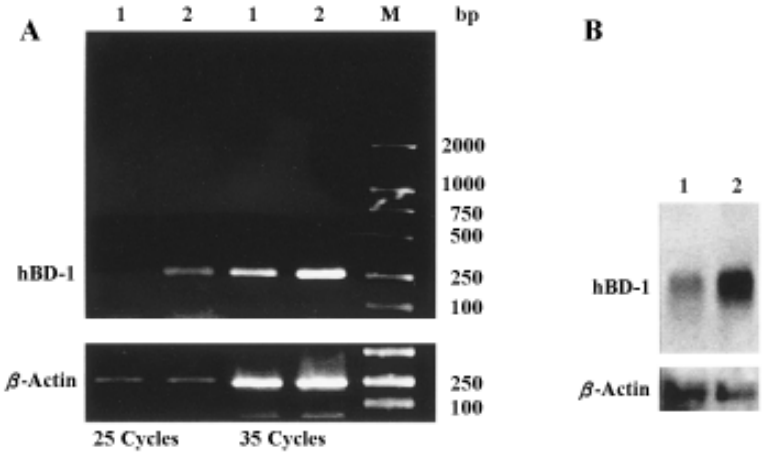

Fig 1. BCG-induced enhancement of hBD-1 mRNA expression in human pulmonary gland epithelial cells. SPC-A-1 cells were incubated with or without BCG whole cell (50 mg/L) for 8 h. β-Actin served as an internal control. (A) RT-PCR detection. Total RNA (1 μg) was applied for one step RT-PCR with different cycles. (B) Northern hybridization. The total RNA (4 μg) was used for Northern hybridization. Lane 1: unstimulated; Lane 2: stimulated with BCG; Lane M: marker. This was the representative of 3 independent experiments.

In search for the effective components of BCG, the cell wall proteins were isolated and fractionated by Sephadex G-150 chromatography. Two fractions of the cell wall proteins were obtained (Fig 2A). The second fraction with a range of molecular weight from 18 kDa to 30 kDa seemed to be more effective in the stimulation of hBD-1 mRNA expression detected by RT-PCR (Fig 2B).

Analysis of BCG response element in the 5'-flanking region of hBD-1 gene Firstly we constructed pCAT -575 reporter plasmid. Its transfection experiment showed that the -575/+54 sequence of hBD-1 gene could drive CAT gene transcription and expression in SPC-A-1 cells after incubation with the second fraction of BCG cell wall proteins (Fig 2C). The -2161/+54 sequence had almost the same transcription activity. The BCG cell wall proteins inducibility remained in all constructs pruned to -69 bp (Fig 3B). Using MatInd and MatInspector analysis programs[14], the -575 bp/+54 bp sequence of the hBD-1 gene was searched for potential transcription factor sites in a Transfac transcription factor database (http://tansfac.gbf.de/TRANSFAC). Multiple high homologous *cis* elements were found among the sequence from –575 bp to the first extron (Tab 1).

DISCUSSION

Our environment is contaminated by an enormous number and variety of microorganisms and we have developed many host defenses to contend with the invaders. The epithelial lining of our skin, gastrointestinal and genitourinary tracts, and tracheobronchial tree produces a number of antimicrobial peptides, and our phagocytic neutrophils rapidly ingest and enzymatically degrade invading organisms, as well as produce peptides and enzymes with antimicrobial activities. Some of these antimicrobial molecules also appear to alert host cells involved in both innate host defense and acquired immune response[15]. hBD-1 is the most important antimicrobial peptide in human epithelial defense against infection[6]. Study of hBD-1 gene expression regulation would not only contribute to understanding the mucosal defense mechanisms, but also have a potential

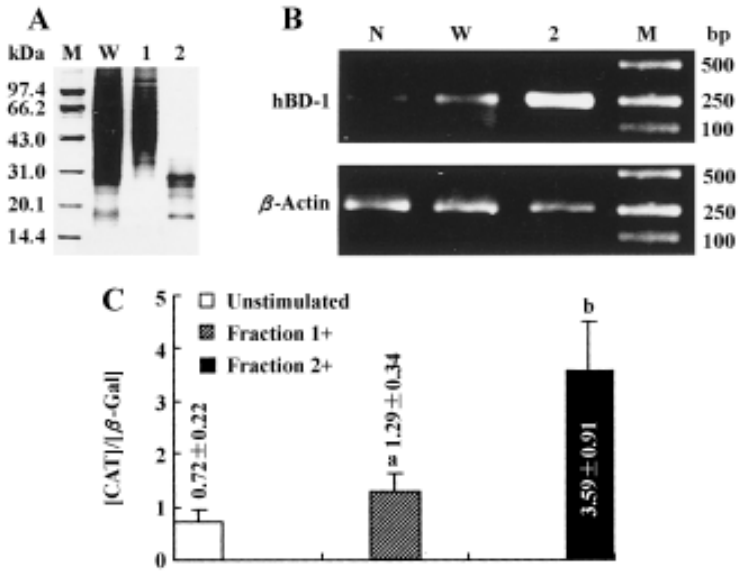

Fig 2. BCG cell wall components induced hBD-1 expression in SPC-A-1 cells. (A) Tricine-SDS-PAGE (15 %) of BCG cell wall protein fractions isolated by Sephadex G-150. Lane M: protein marker; Lane W: whole BCG cell wall proteins; Lane 1: fraction 1; Lane 2: fraction 2. (B) RT-PCR detection of hBD-1 mRNA expression. SPC-A-1 cells were incubated with or without BCG for 8 h. Total RNA (1 μg) of which was applied for one step RT-PCR (30 cycles) with β-actin as an internal control. Lane M: marker; Lane N: nonstimulation; Lane W: whole cell stimulation (50 mg/L); Lane 2: the fraction 2 stimulation (3 mg/L). This was the representative of three independent experiments. (C) CAT activity determination of hBD-1 reporter plasmid pCAT –575. pCAT –575 construction was shown in Fig 3A. SPC-A-1 cells were co-transfected with pCAT -575 (2.0 μg per well) and pSV-β-galactosidase control plasmid (0.3 μg per well), then incubated with or without BCG cell wall protein fractions (3 mg/L) for 39 h. CAT and β-Gal concentrations of the cell lysate were determined by ELISA. [CAT]/[β-Gal] represents CAT activity. *n*=3 independent experiments. Mean±SD. $^{a}P>0.05$, $^{b}P<0.05$ *vs* unstimulated.

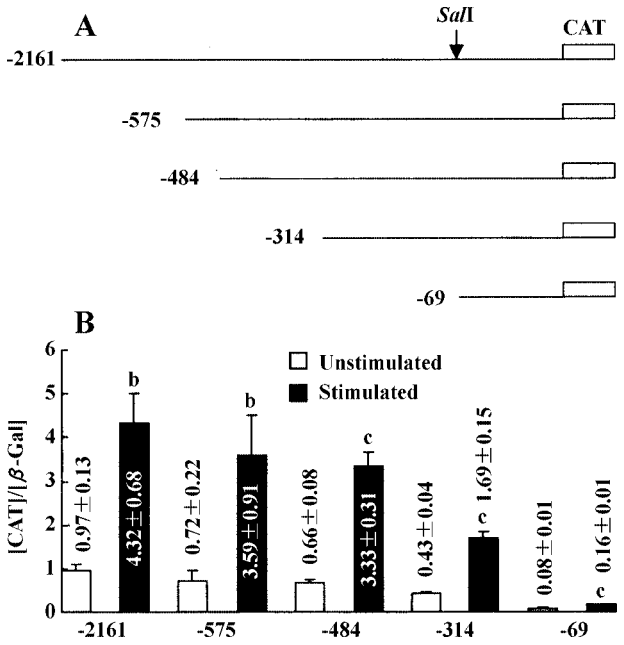

Fig 3. Analysis of BCG response sequence in the 5'-flanking region of hBD-1 gene. (A) Diagram of hBD-1 reporter gene construction. Progressive deletions of 5'-flanking region in hBD-1 gene were isolated by PCR and ligated into pCAT Basic vector to construct a series of hBD-1 CAT reporter plasmids. pCAT –69 was generated from pCAT –575 by digestion with *Sal*I. (B) CAT activity determination of the reporter plasmids. SPC-A-1 cells were co-transfected with the hBD-1 CAT constructs (2.0 μg per well) and pSV-β-galactosidase control plasmid (0.3 μg per well), then incubated with or without the BCG cell wall components (18-30 kDa, 3 mg/L) for 39 h. CAT and β-Gal concentrations of the cell lysate were determined by ELISA. [CAT]/[β-Gal] represents CAT activity. *n*=3 independent experiments. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* unstimulated construct.

application in prevention and treatment of respiratory, genitourinary, and gastrointestinal infections. The most significant progress in the regulation of antimicrobial peptide gene expression is the finding of inducible epithelial antimicrobial peptides such as TAP and hBD-2 by LPS, IL-1β, and TNF-α via Toll/NF-κB pathway[7,8]. Our study and other's[16,17] provide evidence that hBD-1 gene dose response to some bacterial factors or inflammatory stimuli although someone thought that the expression of hBD-1 did not appear to be affected by bacterial factors and proinflammatory mediators[9, 17].

In our study, several detections such as RT-PCR, Northern hybridization, and reporter gene experiments indicated that BCG, especially its cell wall components (18-30 kDa) enhanced hBD-1 gene expression at transcription level in human pulmonary gland epithelial cells.

Unlike TAP and hBD-2, there is no transcription factor NF-κB binding site in the upstream region of hBD-1 gene[12]. We therefore preliminarily analyzed its upstream sequence responsible for the induction of hBD-1 gene expression by BCG. A series of progressive deletion sequences in the upstream region of hBD-1 gene were produced by PCR and their CAT reporter plasmids were constructed. The transfection experiments indicated that the BCG cell wall protein inducibility remained in all constructs from -2161 to -69 bp, suggesting that -69/+54 sequence be essential for the induction of hBD-1 gene expression by BCG cell wall proteins. Consensus match analysis revealed that the region from -63 to -50 bp was homologous to CCAAT/enhancer binding protein-β (C/EBPβ) motif. C/EBPβ (alternative name, NF-IL6) is a member of C/EBP family[18], which is involved in regulation of cellular differentiation, inflammation, and immunity. Accumulating data suggest that C/EBPβ may play an important role in the induction of inflammatory response. C/EBPβ binding motif has been identified in the functional regulatory region of various genes including acute phase proteins, IL-6, IL-8, IL-12, nitric oxide synthase, and TAP[8,18,19].

Because the fold-increase in the transcripition activity of -69 construct was lower than that of -314 and other constructs, multiple transcription factors might cooperate in BCG-mediated up-regulation of hBD-1 gene

Tab 1. Consensus match analysis for potential transcription factor binding sites in hBD-1 gene 5'-flanking region.

Matrix Name	Position of Matrix	Core Similarity	Matrix Similarity	Sequence
NFAT	-566	1.000	0.948	ctttgGAAAcgt
IK2	-540	1.000	0.954	tagtGGGAaaag
NFAT	-541	1.000	0.935	agtggGAAAaga
IRF2	-534	1.000	0.915	gaaaagaGAAAag
MZF	-494	1.000	0.954	taaGGGGa
NF-1	-471	1.000	0.929	cctTGGCcagaggaagaa
CP2	-194	1.000	0.935	gctaaatCCAG
AP-1	-141	1.000	0.934	gcTGACtgctc
C/EBPβ	-63	1.000	0.984	aggttgtGCAAtcc
TATA	-42	1.000	0.941	ttaTAAAtacagtga

Note: 1, Matrix positions correspond to sense strand numbering relative to transcription start site. 2, Capital letters within the sequence indicate the core string. 3, NFAT: nuclear factor of activated T-cells; IRF2: interferon regulatory factor 2; MZF: myeloid zinc finger protein; NF-1: nuclear factor-1; AP-1: activator protein-1; C/EBPβ: CCAAT/enhancer binding protein-β.

expression in human gland epithelial cells. The -314 sequence also contained potential transcription factor activator protein-1 (AP-1) and CP2 sites. AP-1 is another important transcription factor to regulate inflammation reaction[20,21]. For example, it dominantly participates in the regulation of inducible expression of granulysin, an antimicrobial protein, after stimulation with *Acholeplasma laidlawii*[21]. CP2 is found to regulate the expression of IL-4[22].

In conclusion, BCG cell wall components (18-30 kDa) can up-regulate hBD-1 gene expression at transcription level in human pulmonary gland epithelial cells. The sequence containing C/EBPβ, AP-1, and CP2 sites in the upstream of hBD-1 gene is involved in this induction.

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