ELSEVIER

Biochemical Pharmacology 63 (2002) 273–282

Biochemical Pharmacology

The signalling pathway for BCG-induced interleukin-6 production in human bladder cancer cells

Ye Zhang[a], Ratha Mahendran[b], Lai Lai Yap[a], Kesavan Esuvaranathan[b], Hoon Eng Khoo[a,*]

[a]*Department of Biochemistry, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore*
[b]*Department of Surgery, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore*

Received 4 December 2000; accepted 24 September 2001

Abstract

Intravesical bacillus Calmette-Guérin (BCG) is currently the therapy of choice for superficial bladder cancer with a 60–70% response rate. Induction of cytokine production (e.g. IL-6, etc.) by BCG has been found in patient's urine *in vivo* as well as bladder cancer cell lines. However, the signalling mechanisms are still unclear. In this study, we investigated the effect of BCG on cAMP production and its role in regulating interleukin-6 expression in the human bladder cancer cell line, MGH. After 1 hr exposure to BCG, IL-6 gene expression in MGH cells increased by 2.5–3-fold and cAMP production increased by 8–10-fold in a time- and dose-dependent manner. BCG-induced cAMP production was inhibited by both antifibronectin antibody and an adenylate cyclase inhibitor, SQ22536 in a dose-dependent way. In the presence of SQ22536, IL-6 expression in MGH cells was also greatly reduced. Furthermore, cAMP-dependent kinase inhibitors H7 and HA1004 also inhibited BCG-induced IL-6 expression in MGH, with HA1004 being much less effective than H7. Thus, BCG induces cAMP production and may regulate interleukin-6 expression partially via a cAMP-dependent pathway in human bladder cancer cells.
© 2002 Elsevier Science Inc. All rights reserved.

Keywords: Bacillus Calmette-Guérin; Interleukin-6; Human bladder cancer cells; cAMP

1. Introduction

Intravesical BCG immunotherapy combined with transurethral resection of tumour is the standard treatment for superficial bladder cancer, with routine complete response rates of 60–70% [1,2]. However, two major problems exist: a significant proportion of patients do not respond to BCG therapy and side-effects are common. Moreover, the mechanisms of BCG action are still not well understood.

Intravesical instillation of BCG causes a non-specific activation of the local cellular immune response. This response is probably T lymphocyte-dependent [3,4] and is modulated by T helper type 1 (Th1-like) cytokines such as interleukin-2 (IL-2), interferon (IFN), tumour necrosis factor alpha (TNF-α) and interleukin-12 (IL-12), and T helper type 2 (Th2-like) cytokines such as interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6) and interleukin-10 (IL-10), which have been identified in patients' urine following instillation of BCG [5]. Furthermore, IL-6 in urine correlates with the stage of bladder cancer and is absent after resection of the tumour [6].

It is now well established that BCG attachment to tumour cells [7–10] and some of its antitumour effects may be fibronectin dependent [7,9]. BCG can be internalised by tumour cells and may have direct effects on cell growth [11]. It was previously shown that several bladder carcinoma cell lines had the capacity to secrete IL-6 after stimulation with BCG [12,13]. It has also been shown that IL-6 is produced by spontaneous transitional cell carcinomas and this production may be up-regulated by intravesical BCG therapy [13]. Recently, Bevers *et al.* reported that in human bladder cancer cell lines T24 and J82, BCG internalisation appeared to result in an up-regulation of IL-6 synthesis. For the T24 cell line, internalisation could be a prerequisite for BCG-induced up-regulation of IL-6 production [14].

Furthermore, recombinant IL-6, TNF-α and IFN have direct antiproliferative effects on transitional cell carcinomas [15–17]. Our previous studies showed that BCG had

* Corresponding author. Tel.: +65-87-43-250; fax: +65-77-91-453.
E-mail address: bchkhe@nus.edu.sg (H.E. Khoo).

Abbreviations: BCG, bacillus Calmette-Guérin; IL, interleukin; Th, T helper; cAMP, 3′,5′-cyclic adenosine monophosphate; IFN, interferon; TNF, tumour necrosis factor; GM-CSF, granulocyte macrophage colony stimulating factor; RT, reverse transcription; PCR, polymerase chain reaction.

0006-2952/02/$ – see front matter © 2002 Elsevier Science Inc. All rights reserved.
PII: S0006-2952(01)00831-0

direct cytotoxic and antiproliferative effects on human bladder cancer cell lines [18]. BCG can also stimulate the production of various cytokines in these cells, namely IL-6, IL-8, TNF-α and granulocyte macrophage colony stimulating factor (GM-CSF) in a tumour grade related manner [19]. However, these events were studied only after 72 hr exposure to BCG *in vitro*. In clinical studies, however, intravesical instillation of BCG was only carried out for 2 hr and after BCG treatment for 4 hr, the production of cytokines *in vivo* was observed.

Despite these observations, mechanisms of the modulatory effect of BCG on cytokine production are poorly understood. In 1979, Lowrie *et al.* reported that when ingested by mouse peritoneal macrophages, live *M. bovis* BCG caused a transient increase in cyclic AMP (cAMP), whereas live *M. lepraemurium* did not [20]. Therefore, in human urothelial cells, BCG might also act via a cAMP pathway and thus regulate the production of cytokines. In the present study, we investigated cAMP production induced by BCG and its role in regulating IL-6 production in a human bladder cancer cell line, MGH.

2. Materials and methods

2.1. Target cells

The human transitional carcinoma cell line, MGH, representing histopathological tumour grade III, was kindly provided by John Masters, Institute of Urology. The cells were routinely grown in RPMI-1640 with 5% (v/v) foetal bovine serum (FBS), 5 mM sodium pyruvate, 4 mM glutamine and 2% (w/v) solution PS (penicillin G 5000 U/mL streptomycin sulphate 5000 μg/mL) (all from Sigma) and incubated at 37° supplied with 5% CO_2.

2.2. BCG

BCG, living organisms of an attenuated strain of *Mycobacterium tuberculosis* (Connaught strain, 2.2×10^8 to 6.4×10^8 colony-forming units per vial, containing 27 mg freeze-dried BCG powder), was obtained from Connaught Laboratories Limited. Before the experiments, BCG was prepared in the diluent (PBS with 0.025% (v/v) Tween 80) obtained from the company and used within 2 hr. For control studies, the BCG supernatant (diluent part) was harvested after centrifuging the BCG solution. Autoclaved BCG was prepared by autoclaving at 121° for 15 min, and after centrifugation, the BCG pellet was re-dissolved.

2.3. Effect of BCG and autoclaved BCG on IL-6 mRNA expression

MGH cells were plated in 6-well plates at 1×10^6 cells per well and incubated for 4 hr to allow for cell attachment. The cells were then incubated with BCG (1000 μg/mL) or autoclaved BCG (1000 μg/mL) in antibiotic-free culture medium for 0.5–4 hr at 37°. At the end of the experiments, the supernatant was harvested by centrifuging at 3000 rpm for 10 min and kept at −70° for ELISA assays. Cells were rinsed with PBS (pH 7.2) and further used for extraction of total RNA.

2.4. Extraction of total RNA and reverse transcription (RT)

Total RNA was extracted from cells with 1 mL of Trizol reagent per well (Gibco BRL) according to the manufacturer's protocol. The RNA integrity was checked on a 1% (w/v) agarose-formaldehyde gel. RT was carried out using oligo-$dT_{(16)}$ and MMLV-reverse transcriptase (Stratagene). RT was performed with 2 μg of total RNA in a 20 μL cocktail mixture of 10× RT buffer, dNTPs (2.5 mM each), 10 μM oligo-$dT_{(16)}$, 50 U/μL MMLV-reverse transcriptase and 30 U/μL rRNasin for 1 hr at 37° following 5 min at 95° in an automated DNA thermal cycler (Hybaid Omn-E). The cDNA obtained was stored at −20°.

2.5. Polymerase chain reaction (PCR)

PCR was performed in a final volume of 25 μL containing 2.5 μL of cDNA (diluted 20-fold after RT), dNTPs (2.5 mM each), 10× PCR buffer, 2 U/μL Taq polymerase (Finnzymes) and 10 μM gene specific sense and antisense primers (Table 1). The cycle program was carried out in an automated DNA thermal cycler (Hybaid Omn-E) for 30 cycles with the following parameters: denaturation at 95° for 30 s, annealing at 60° for 30 s and extension at 72° for 30 s. The PCR products were then analysed together with a molecular weight marker (KB DNA ladder, Gibco BRL) on 1.5% (w/v) agarose gel containing 0.4 mg/mL ethidium bromide. The products amplified by using specific IL-6 and β-actin primers were confirmed to be those of human IL-6 and β-actin by gene sequencing (data not shown). β-Actin served as a control for comparison of mRNA expression between samples. PCR without templates and PCR using the total RNA as template were performed as negative controls. The relative intensity of specific PCR bands was measured by using the BioProfile software of a photoimager (Vilber Lourmat). Relative mRNA levels of the samples were determined by comparison to the corresponding β-actin levels.

Table 1
Sequence of primers used for RT-PCR

Product		Sequence of the primer	Size (bp)
IL-6	5′-Primer	5′-ATGAACTCCTTCTCCACAAGCGC-3′	628
	3′-Primer	5′-GAAGAGCCCTCAGGCTGGACTG-3′	
β-Actin	5′-Primer	5′-AGCACTGTGTTGGCGTACAG-3′	277
	3′-Primer	5′-AAATCGTGCGTGACATTAAGG-3′	

2.6. Determination of the effects of BCG on cellular cAMP levels

As a preliminary experiment, MGH cells were plated in 24-well plates at 5×10^5 cells per well. After 4 hr incubation for cell attachment, cells were incubated with BCG (1000 μg/mL) in the presence or absence of 50 μM RO20-1724 (a phosphodiesterase inhibitor) for 30 min. RO20-1724 (50 μM) was used for accumulating cAMP to a detectable level for further experiments. As controls, MGH cells were treated with the BCG supernatant (matched volume) or autoclaved BCG for 30 min.

In time- and dose-dependent studies, MGH cells were treated with BCG (1000 μg/mL) for 0–30 min, or with BCG (0–2000 μg/mL) for 30 min, respectively. To determine the effects of antifibronectin antibody and SQ22536 (an adenylate cyclase inhibitor) on cAMP levels, MGH cells were pre-treated with antifibronectin antibody (0–0.5 μg/mL) for 30 min, and then incubated with BCG (1000 μg/mL) for another 30 min. The same procedure was adopted for the control antibody. In some experiments, MGH cells were incubated with BCG in the presence of SQ22536 (0–400 μM) for 30 min to investigate the effects of SQ22536 on BCG-induced cAMP production.

2.7. Determination of cAMP accumulation levels by radio-immunoassay kit

At the end of each treatment, the medium was quickly removed and the cells were rinsed twice with Tris–EDTA buffer (50 mM Tris and 4 mM EDTA), followed by adding 50 μL of 1 M HCl for 10–15 s to stop the reaction. Tris–EDTA buffer was then added, followed by 50 μL of 1 M NaOH for neutralisation. The cells were then scraped off, transferred to Eppendoff tubes and kept at 4°. Total disruption of cells was achieved by sonication for 30 s. The tubes were then incubated at 95° for 10 min (for coagulation of proteins) and centrifuged at 12,000 *g*, after which aliquots of the supernatant were used to determine cAMP levels by a radio-immunoassay kit (Amersham Corp.).

2.8. Determination of the effects of different inhibitors on BCG-induced IL-6 mRNA expression

MGH cells were plated in 6-well plates at 1×10^6 cells per well. After 4 hr incubation for cell attachment, cells were pre-treated with H7 (0–100 μM) (a cAMP-dependent kinase inhibitor) or HA1004 (0–100 μM) (a cAMP-dependent kinase inhibitor) for 1 hr. Pre-treated cells were further incubated with or without BCG (1000 μg/mL) for another 1 hr in antibiotic-free culture medium at 37°. In some experiments, cells were also treated with BCG and/or SQ22536 (0–200 μM) for 1 hr. At the end of the treatment, the supernatant was harvested and centrifuged at 3000 rpm for 10 min at 4°, and then kept at −70° for ELISA assays. The cells were rinsed with PBS and used for extraction of total RNA.

2.9. Determination of the effects of inhibitors or antifibronectin antibody on BCG-induced IL-6 protein secretion

As a preliminary experiment, direct cytotoxicity of SQ22536, H7 and HA1004 on MGH cells was determined by the lactate dehydrogenase release assay. The results showed that all these inhibitors had negligible cytolytic effects on MGH cells (data not shown).

MGH cells were plated in 6-well plates at 1×10^6 cells per well. After 4 hr incubation for cell attachment, cells were pre-treated with antifibronectin antibody (or control antibody (mouse IgG1)) (0.5 μg/mL) for 30 min, H7 (0–100 μM) or HA1004 (0–100 μM) for 1 hr. Pre-treated cells were further incubated with or without BCG (1000 μg/mL) for another 4 hr in antibiotic-free culture medium at 37°. In some experiments, cells were treated with BCG and/or SQ22536 (0–200 μM) for 4 hr. At the end of the treatment, the supernatant was harvested and centrifuged at 3000 rpm for 10 min at 4°, and then kept at −70° for ELISA assays.

2.10. Determination of the effects of RO20-1724 or forskolin on IL-6 protein secretion

MGH cells were plated in 6-well plates at 1×10^6 cells per well. After 4 hr incubation for cell attachment, cells were treated with RO20-1724 (50 μM) or forskolin, an adenylate cyclase activator (50 μM), for another 4 hr. At the end of the treatment, the supernatant was harvested and centrifuged at 3000 rpm for 10 min at 4°, and then kept at −70° for ELISA assays.

2.11. ELISA essays

IL-6 protein levels in the supernatant were determined with the ELISA immuno-assay Kit (R&D). The supernatants were thawed to room temperature, and assayed in duplicates with a standardised laboratory technique according to the manufacturer's instructions.

2.12. Statistical methods

The data were analysed using the Mann–Whitney test. Each experiment was repeated thrice for determining IL-6 mRNA expression and at least twice for determination of cAMP levels and ELISA assays. The results were expressed as the mean ± SEM.

3. Results

3.1. Effects of BCG and autoclaved BCG on IL-6 mRNA expression

Our earlier *in vitro* experiments showed that several cytokines (IL-6, IL-8 and GM-CSF) in grade III cell lines such as MGH, J82 and SD were elevated after 72 hr

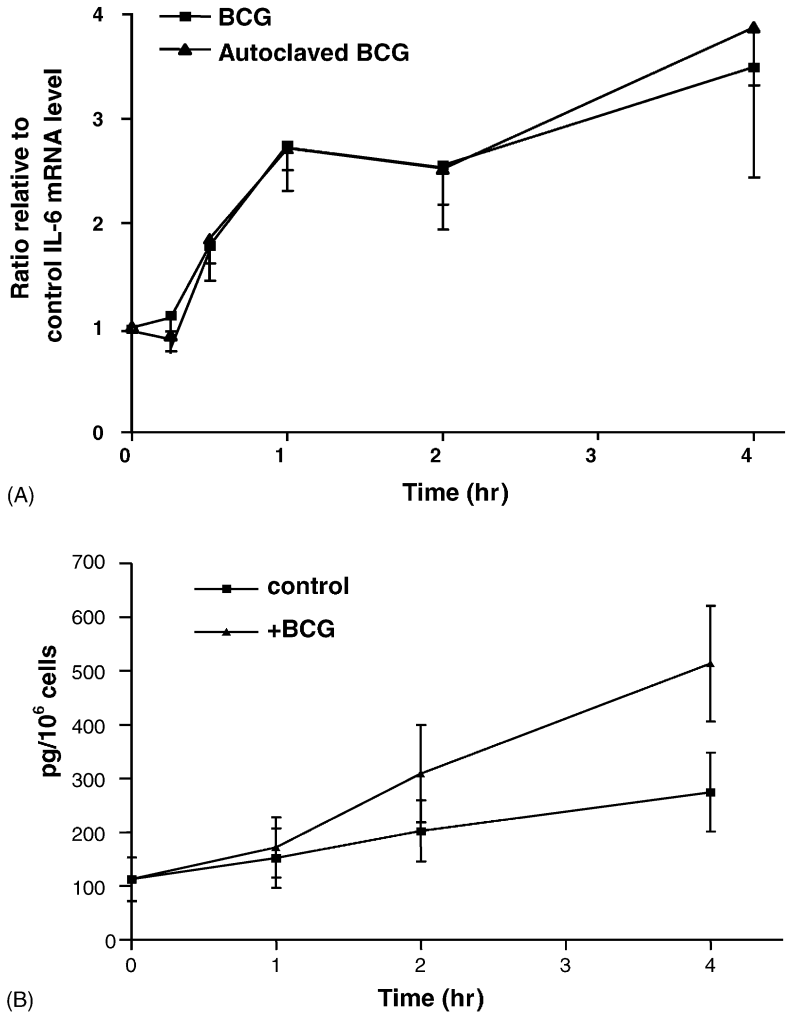

Fig. 1. (A) Early expression of IL-6 mRNA in MGH after treatment with BCG (1 mg/mL) or autoclaved BCG (1 mg/mL). Relative IL-6 mRNA expression was compared with control levels after treatment with different agents for 0.5, 1, 2 and 4 hr. Results are presented as a percentage relative to control levels (mean ± SEM) from three independent experiments. (B) Early IL-6 protein release from MGH cells after treatment with BCG (1 mg/mL) for 0–2 and 4 hr. Results are shown as mean ± SEM from two independent experiments, each in duplicate.

stimulation with BCG [18,19]. The effects of live BCG (1000 μg/mL) or autoclaved BCG (1000 μg/mL) on IL-6 mRNA expression were investigated in MGH cells after treatment for 0.25, 0.5, 1, 2 and 4 hr by quantitative RT-PCR, using primers specific for β-actin and IL-6.

IL-6 expression levels were significantly increased after 30 min incubation with BCG, and the levels increased with increasing BCG incubation time ($P < 0.05$, Fig. 1A). Compared to the control cells, the expression of IL-6 mRNA was increased by 2.5–3-fold after 1 hr exposure to BCG. The treatment of cells with autoclaved BCG also resulted in significantly higher IL-6 mRNA levels compared to the control cells, but was not significantly different from that induced by live BCG (Fig. 1A).

3.2. Effects of BCG on IL-6 protein secretion

IL-6 protein secretion in MGH cells was determined after treatment with BCG (1000 μg/mL) for 1, 2 and 4 hr by an ELISA assay. IL-6 secretion was significantly increased by 10% after 1 hr exposure to BCG ($P < 0.05$). With increasing incubation time, the level of IL-6 was further increased. Compared to the control cells, IL-6 secretion from BCG-treated cells was increased by 2-fold after 4 hr treatment (Fig. 1B).

3.3. Effects of BCG on cAMP production

The effects of BCG on the accumulation of intracellular cAMP in MGH cells were also studied in the absence or presence of RO20-1724, a phosphodiesterase inhibitor.

Stimulation of MGH cells with BCG for 30 min resulted in a significant increase in cAMP production compared to the control cells, although at a low level ($P < 0.01$, Fig. 2A). In the presence of 50 μM RO20-1724, the accumulation of cAMP in BCG-treated MGH cells was significantly increased 8–10-fold compared to both the control cells and the cells treated with BCG supernatant ($P < 0.001$, Fig. 2A and B). Autoclaved BCG had similar effects on cAMP production in MGH cells (Fig. 2B).

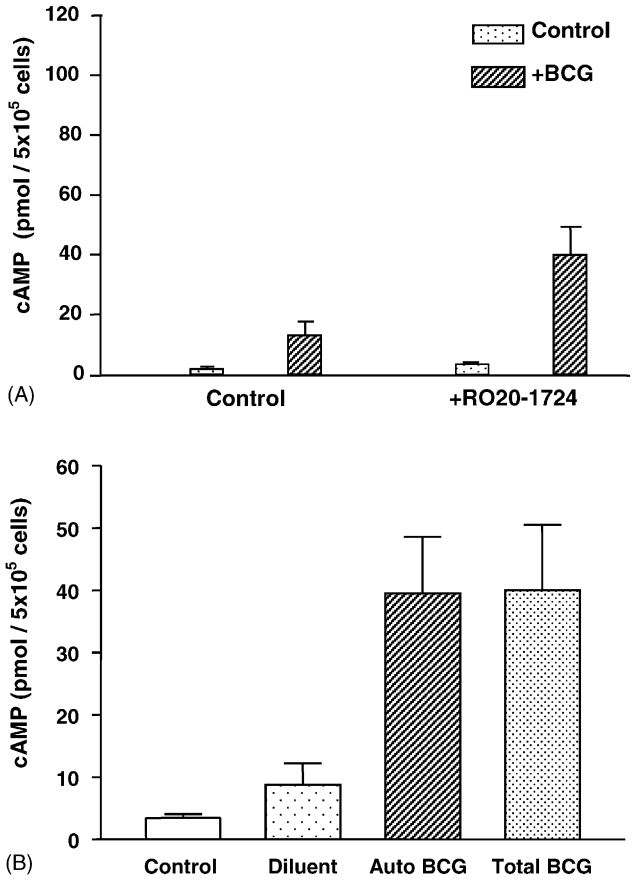

Fig. 2. (A) cAMP production levels in MGH cells after stimulation with BCG (1 mg/mL) in presence or absence of RO20-1724 for 30 min. (B) Effects of autoclaved BCG (1 mg/mL) and BCG supernatant (matched volume) on cAMP accumulation levels in MGH cells after 30 min treatment in the presence of RO20-1724. Results are shown as mean ± SEM from at least two independent experiments, each in duplicate.

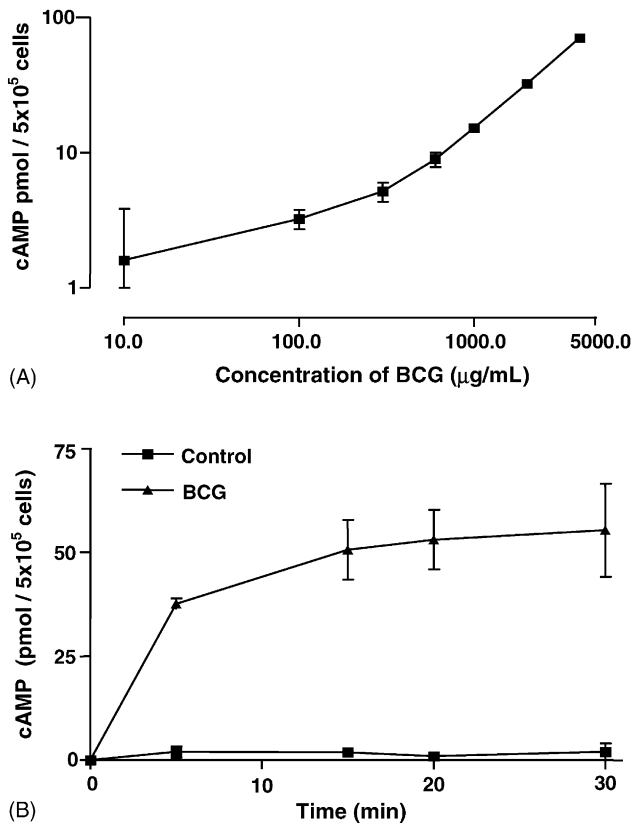

Fig. 3. (A) Dose-dependent effects of BCG (0–4000 μg/mL) on cAMP accumulation levels in MGH cells after 30 min treatment. (B) Time-dependent effects of BCG (1 mg/mL) on cAMP accumulation levels in MGH cells after stimulation in the presence of RO20-1724 for 5, 10, 20 and 30 min. Data are presented as mean ± SEM from two independent experiments, each in triplicate.

3.4. Dose-dependent and time-course effects of BCG on cAMP levels

The dose- and time-dependent effects of BCG on cAMP production in MGH cells were also investigated upon treatment with BCG for 5, 15, 20 and 30 min.

BCG induced the production of cAMP in a dose- and time-dependent manner in MGH cells. The results indicated that BCG had a stimulatory effect on cAMP levels at a concentration of 100 μg/mL and the cAMP levels rose with increasing concentrations of BCG (Fig. 3A). Furthermore, cAMP levels in MGH cells were significantly increased as early as 5 min after stimulation with BCG ($P < 0.005$), after which the increase was more gradual (Fig. 3B).

3.5. Effects of antifibronectin antibody on BCG-induced cAMP production and IL-6 secretion

It has previously been shown that fibronectin may be implicated in mediating the attachment and internalisation of BCG, and that blocking the binding of fibronectin could significantly reduce BCG-induced antitumour effects [8–10].

To investigate the role of fibronectin in BCG-induced cAMP production, antifibronectin antibody was used for blocking fibronectin activities. The results showed that antifibronectin antibody significantly blocked BCG-induced cAMP production in MGH cells by up to 40% in a dose-dependent manner ($P < 0.005$, Fig. 4A).

The effects of antifibronectin antibody on IL-6 protein production were also determined by measuring the secretion of IL-6 from MGH cells after 4 hr treatment with BCG in the presence of 0.5 μg/mL antifibronectin antibody or control antibody (IgG1). The presence of antifibronectin antibody significantly decreased BCG-induced IL-6 protein secretion by about 5–10% ($P < 0.05$, Fig. 4B) whereas cells treated with antifibronectin antibody alone showed no difference in IL-6 secretion (data not shown). There was also no significant difference in BCG-induced IL-6 protein secretion in the presence of control mouse IgG1 antibody (Fig. 4B).

3.6. Effects of SQ22536 on BCG-induced cAMP production

To determine the role of adenylate cyclase in the stimulation of cAMP production by BCG treatment, a

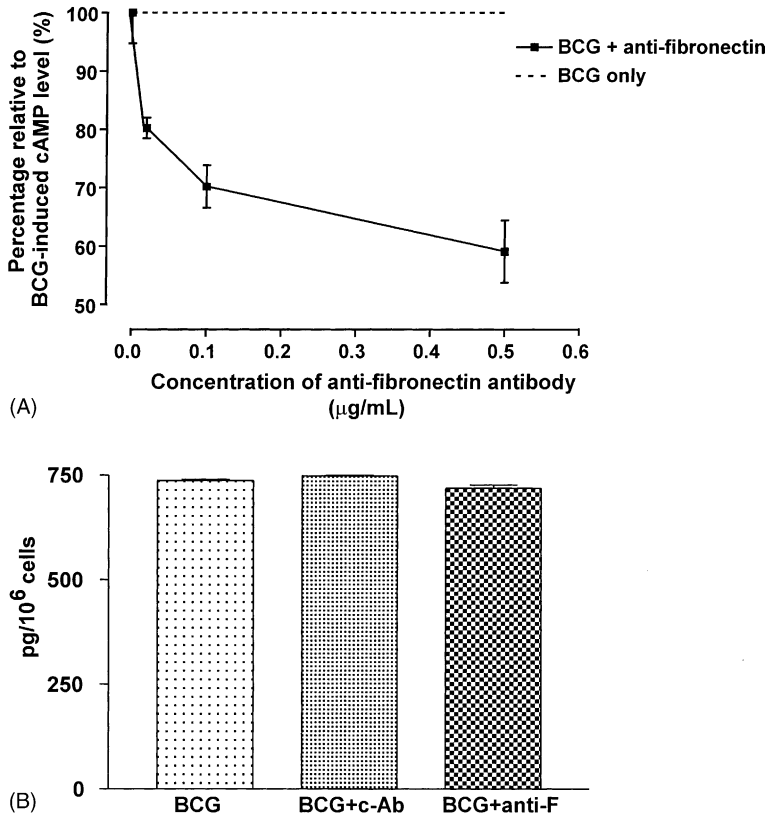

Fig. 4. (A) Effects of antifibronectin antibody (0–0.5 μg/mL) on BCG-induced cAMP accumulation in MGH cells. Data are presented as a percentage relative to BCG-stimulated levels (mean ± SEM) from two independent experiments, each in triplicates. (B) Effects of antifibronectin antibody [anti-F] (0.5 μg/mL) and control antibody [c-Ab] (0.5 μg/mL) on BCG-induced IL-6 protein secretion in MGH cells after treatment with BCG for 4 hr. Results are shown as mean ± SEM from two independent experiments, each in duplicate.

specific adenylate cyclase inhibitor, SQ22536 was used. The BCG-induced cAMP levels decreased with increasing concentrations of SQ22536 (0–400 μM, $P < 0.005$). The concentration of SQ22536 required for total inhibition of BCG-induced cAMP levels to the basal level in control cells was found at 200 μM (Fig. 5).

3.7. Effects of SQ22536 on BCG-induced IL-6 mRNA expression

To determine the relationship between elevated IL-6 production, increased cAMP levels and adenylate cyclase, MGH cells were treated with BCG in the presence of SQ22536. The IL-6 mRNA expression was evaluated by quantitative RT-PCR. The concentrations of SQ22536 used were 1, 10, 50 and 200 μM and these resulted in 20, 70, 85 and 100% inhibition of cAMP production, respectively (Fig. 5).

The results indicated that upon treatment with SQ22536, IL-6 mRNA expression levels in control cells remained unchanged, whereas cells stimulated with BCG (1000 μg/mL) for 1 hr expressed twice the amount of IL-6 mRNA as compared to the control. In the presence of SQ22536, a significant decrease in BCG-induced IL-6 mRNA expression was attained at a concentration of 10 μM SQ22536 ($P < 0.005$) and this further decreased with increasing SQ22536 concentrations (Fig. 6a and b). These inhibitory effects of SQ22536 on IL-6 mRNA expression was dose-dependent and had similar inhibitory effects on cAMP production ($P < 0.03$, Figs. 5, 6a and b).

3.8. Effects of SQ22536 on BCG-induced IL-6 protein secretion

To investigate if SQ22536 had similar effects on IL-6 protein secretion in MGH cells, IL-6 secretion was mea-

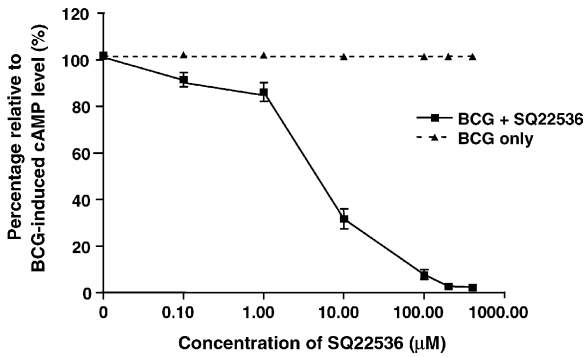

Fig. 5. Inhibitory effect of SQ22536 on BCG-induced cAMP production in MGH cells in the presence of BCG (1 mg/mL) and RO20-1724 after 30 min treatment. Data are presented as a percentage relative to BCG-stimulated level (mean ± SEM) from two independent experiments, each in triplicate.

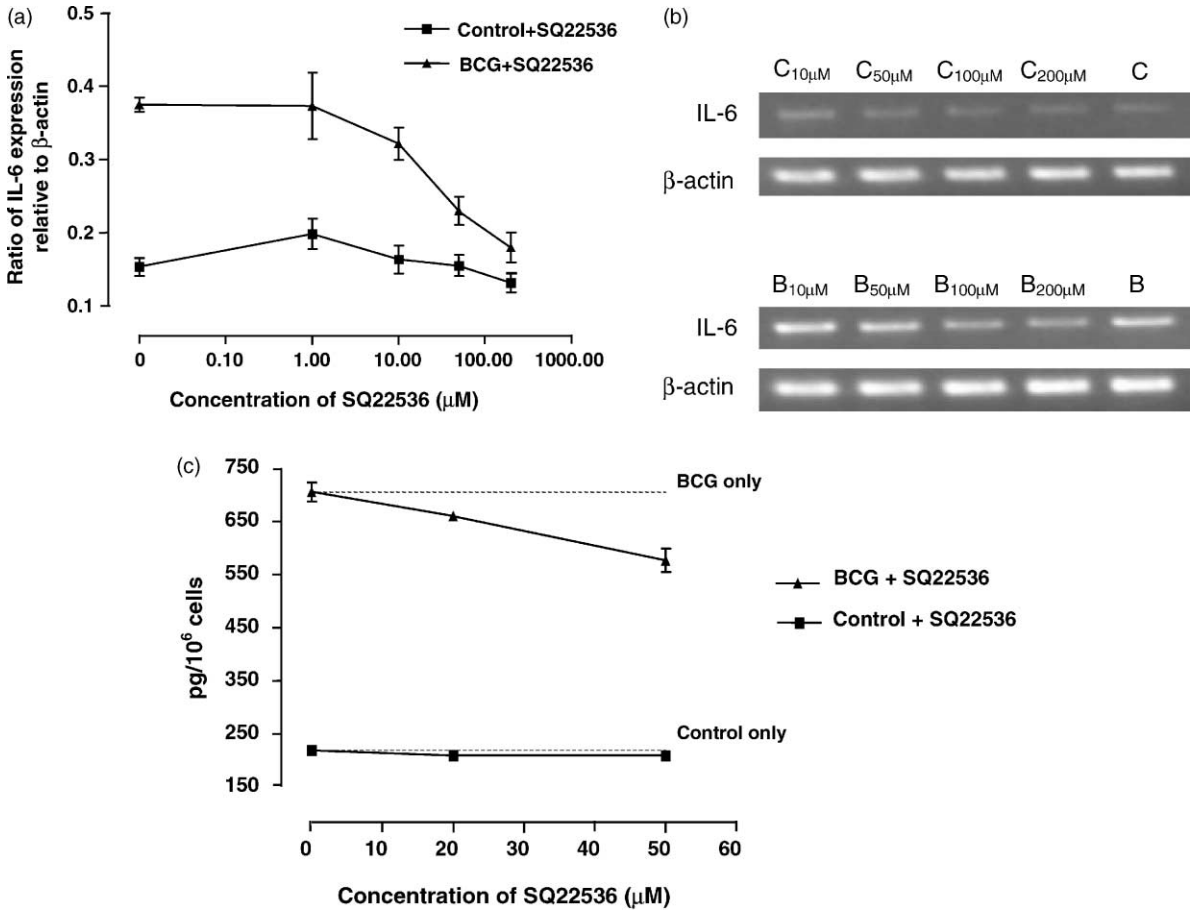

Fig. 6. (a) Effects of SQ22536 (0–200 μM) on IL-6 mRNA expression after treatment with or without BCG (1 mg/mL) for 1 hr. Results are presented as a ratio of IL-6 to β-actin (represented as a standard expression) with mean ± SEM from three independent experiments. (b) Gel samples: C, control MGH cells; B, MGH cells treated with BCG (1 mg/mL), with the subscripts indicating the concentrations of SQ22536 (M) used. IL-6 expression in control MGH cells is shown on the upper panel, whereas IL-6 expression in MGH cells after stimulation with BCG is shown on the lower panel. (c) Effects of SQ22536 (0–50 μM) on BCG-induced IL-6 protein secretion in MGH cells after treatment with BCG for 4 hr. Results are shown as mean ± SEM from two independent experiments, each in duplicate.

sured by ELISA after 4 hr treatment with BCG in the presence of 0–50 μM of SQ22536.

IL-6 protein secretion from MGH cells was significantly increased by 2-fold after 4 hr treatment with BCG. In the presence of SQ22536, IL-6 protein secretion from control cells showed no significant change, whereas BCG-induced IL-6 secretion was significantly inhibited in a dose-dependent fashion ($P < 0.05$, Fig. 6c).

3.9. Effects of H7 and HA1004 on BCG-induced IL-6 mRNA expression

The role of cAMP-dependent kinase in the signalling pathway for BCG-induced IL-6 production was investigated by using two inhibitors, H7 and HA1004. H7 is a potent inhibitor of both protein kinase C and cAMP-dependent protein kinase, whereas HA1004 is a potent inhibitor of cAMP-dependent protein kinase. Because HA1004 has a low affinity for protein kinase C, it serves as a negative control for H7.

The results showed that upon treatment with H7 or HA1004, control cells exhibited a decrease in constitutive mRNA expression of IL-6. After treatment with BCG, the IL-6 levels in MGH cells were about 3-fold higher to the control cells. In the presence of H7, a significant decrease in BCG-induced IL-6 mRNA expression was attained at 10 μM H7 ($P < 0.005$). This inhibitory effect of H7 was dose-dependent. The total inhibition of BCG-induced IL-6 mRNA expression was achieved at 50 μM H7 ($P < 0.005$, Fig. 7A and B). However, HA1004 was less efficient as compared to H7 even at its highest concentration of 100 μM, in which HA1004 had only a partial inhibitory effect on IL-6 mRNA expression in BCG-treated MGH cells (Fig. 7A–C).

3.10. Effects of H7 and HA1004 on BCG-induced IL-6 secretion

IL-6 protein secretion was determined after 4 hr treatment with BCG in the presence of 0–50 μM H7 or HA1004. Both H7 and HA1004 inhibited IL-6 protein secretion in control cells. IL-6 secretion was significantly increased by about 2-fold upon treatment with BCG for 4 hr. However, upon incubation with H7 or HA1004, BCG-

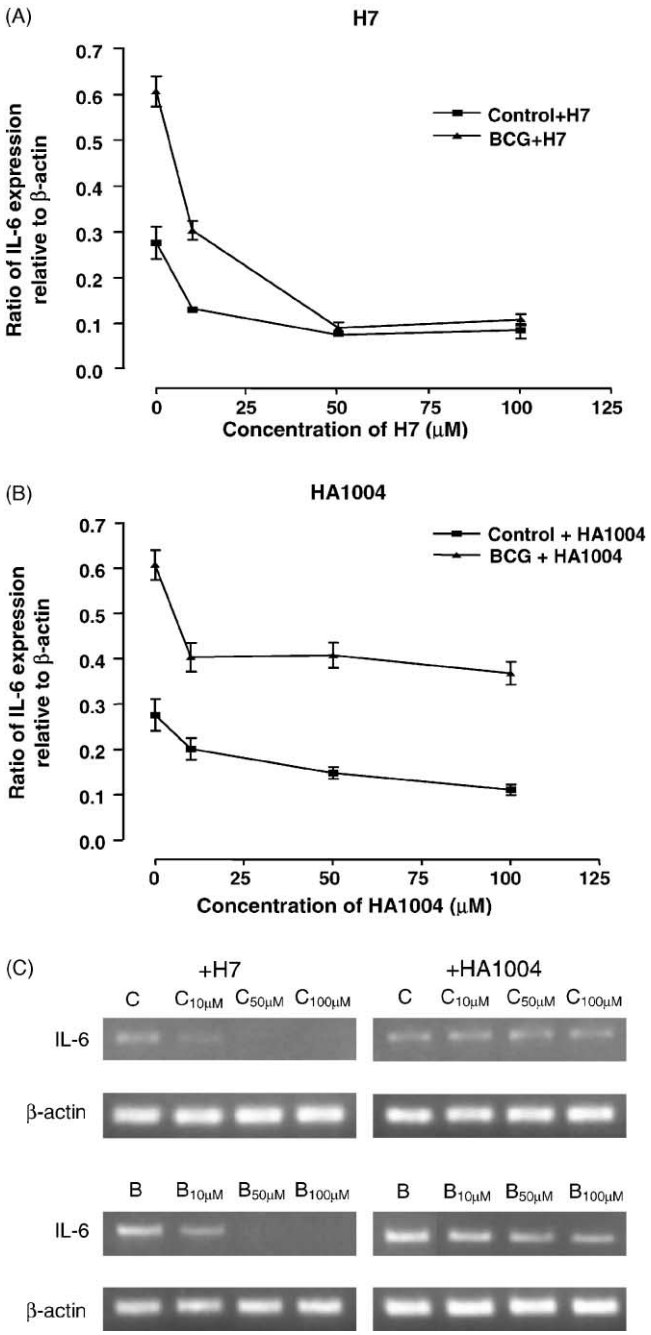

Fig. 7. Effects of (A) H7 (0–100 μM) and (B) HA1004 (0–100 μM) on IL-6 mRNA expression after treatment with or without BCG (1 mg/mL) for 1 hr. Data are represented as a ratio of IL-6 to β-actin (represented as a standard expression) with mean ± SEM from three independent experiments. (C) Gel samples: C, control MGH cells; B, MGH cells treated with BCG (1 mg/mL), with the subscripts indicating the concentrations of H7 and HA1004 (M) used, respectively. IL-6 mRNA expression in the presence of H7 is shown on the left panel, whereas IL-6 mRNA expression in the presence of HA1004 is shown on the right panel. In each case, the upper panel represents the control MGH cells, while the lower panel represents MGH cells treated with BCG (1 mg/mL).

induced IL-6 protein secretion was inhibited in a dose-dependent manner. Compared to HA1004, H7 was more potent at inhibiting IL-6 secretion, and dramatically inhibited IL-6 protein secretion in BCG-treated cells to a level similar to that in control cells ($P < 0.05$, Fig. 8A and B).

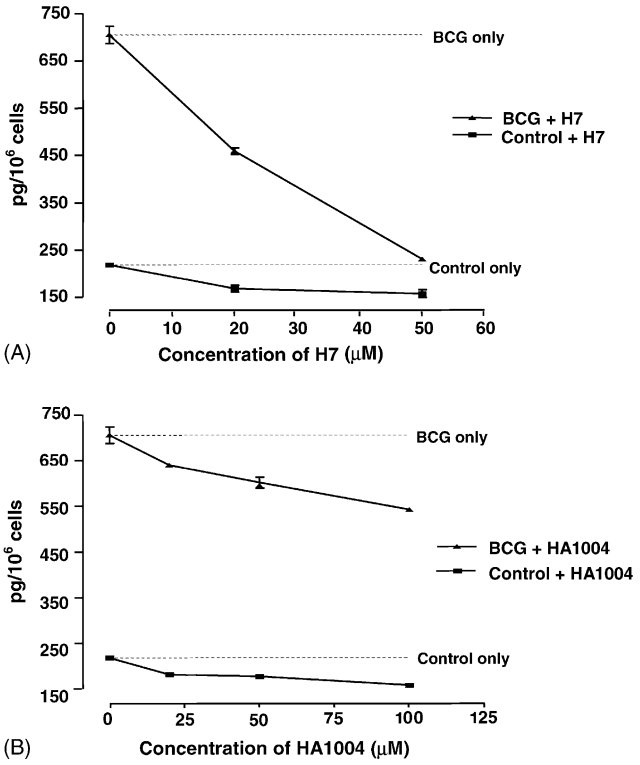

Fig. 8. Effects of (A) H7 (0–50 μM) and (B) HA1004 (0–100 μM) on BCG-induced IL-6 protein secretion in MGH cells after treatment with or without BCG (1 mg/mL) for 4 hr. Results are shown as mean ± SEM from two independent experiments, each in duplicate.

Table 2
IL-6 protein secretion in MGH cells upon treatment with RO20-1724 or forskolin[a]

Treatment	IL-6 secretion ($pg/10^6$ cells)	±SEM
Control	274.57	73.12
RO20-1724 (50 μM)	750.07	4.75
Forskolin (50 μM)	722.62	5.36

[a] Results are shown as mean ± SEM from two independent experiments, each in duplicate.

3.11. Effects of RO20-1724 and forskolin on IL-6 secretion

To investigate whether cAMP-increasing agents would mimic BCG-induced IL-6 expression, MGH cells were treated with 50 μM RO20-1724 or forskolin for 4 hr. IL-6 protein secretion was increased upon treatment with RO20-1724 or forskolin as compared to control cells without treatment (Table 2).

4. Discussion

Although the mechanism of antitumour activity of BCG in transitional cell carcinoma of the urinary bladder has not

been fully elucidated, it is believed that BCG immunotherapy for bladder cancer involves a local non-specific T cell-dependent immunological reaction modulated by Th1- and Th2-like cytokines [4,5]. The attachment of BCG to the urothelium or bladder cancer cells has been identified as the initial step for successful BCG immunotherapy [7,9], and attention is now focused on the direct response of the urothelium and bladder cancer cells to BCG stimulation.

The initiating event of BCG attachment to the urothelium is mediated through fibronectin as an opsonin, but it is not well-understood whether fibronectin just acts as an attachment matrix or is actively involved in the antitumour response [7,8]. The internalisation of BCG by urothelium cells occurs via β1α5 integrin receptors upon attachment [11]. To combat infection, malignant or normal urothelial cells may show phenotypic changes like up-regulation of various proteins such as cytokines. Various urinary cytokines can be detected after intravesical BCG instillation, including IL-1β, IL-6, IL-8, IL-10 which can be detected after the first instillation, while IL-2, TNF-α and IFN are detectable only after several intravesical BCG instillations [5]. Sander *et al.* suggested that certain cytokines might be located in the endothelium, malignant urothelium and leukocytes although no phenotypic staining of the cytokine positive cells was performed [21].

In our previous *in vitro* studies, 72 hr stimulation with BCG resulted in an increase in IL-6, IL-8 and GM-CSF secretion from histopathological tumour grade III cell lines such as MGH, J82 and SD [18,19]. Time course studies (0.25–4 hr) of IL-6 and GM-CSF mRNA expression were performed in MGH cells to investigate whether BCG-induced cytokine expression is an early event of cellular response to BCG. BCG up-regulated both IL-6 mRNA expression and secretion as early as 30 min, whereas GM-CSF expression can only be detected after 2 hr [18,19]. Ratliff *et al.* had reported that the attachment of BCG occurred within 30 min of treatment [8]. Thus, our current results suggested that the up-regulation of IL-6 expression may be one of the early events stimulated by BCG.

IL-6, a proinflammatory cytokine induced by a variety of stimuli, is involved in the induction of IL-2 production, T cell proliferation, cytotoxic T cell differentiation and the acute-phase reaction. The effects of IL-6 are synergistic with IL-1 and TNF-α [22]. Cytokines, such as IL-6, produced by the tumour cells could attract immunocompetent cells such as macrophages and lymphocytes to the tumour sites and stimulate them. This could be one of the reasons why BCG needs to come into contact with tumour cells *in vivo*, to elicit an optimal antitumour response. Furthermore, although it has not been demonstrated whether secreted cytokines can inhibit or stimulate the growth of bladder cancer cells, recombinant IL-6 has been shown to have direct antiproliferative effects on bladder cancer cells *in vitro* [16]. Stimuli like IL-1, TNF-α, substance P (SP) and histamine up-regulate IL-6 expression through different pathways, e.g. cAMP-dependent pathway, protein kinase C pathway and mitogen-activated kinase (MAP kinase) pathway in different cell types. Our present study showed that cellular cAMP production in MGH cells was dramatically increased by 8–10 times in a dose- and time-dependent manner upon treatment with live BCG. We also found that autoclaved BCG also caused a similar increase in cAMP levels. This suggested that autoclaved BCG may retain its ability to attach to the bladder cells and be internalised via the integrin receptors. The autoclaved BCG may still contain antigenic epitopes needed to elicit the same immune response as live BCG which are presented by antigen-presenting cells (APCs).

The role of fibronectin-mediated BCG attachment in BCG-induced cAMP production in MGH cells was also investigated. Antifibronectin antibody was used to block the binding of fibronectin, resulting in the inhibition of cAMP production in a dose-dependent fashion with a maximum of 40% inhibition at the concentration of 0.5 μg/mL. Correspondingly, about 5–10% inhibition of IL-6 protein secretion from BCG-treated MGH cells was also observed at the same concentration of antifibronectin antibody. Thus, it is possible that the effects of BCG on cAMP induction and IL-6 production in MGH cells were triggered by fibronectin-mediated attachment of BCG. Moreover, it also implied a possible correlation between increased cAMP production and up-regulated IL-6 expression. However, the inhibitory effect was only partial, suggesting that the internalisation of BCG may not be mediated by fibronectin alone, and that some other receptors or opsonins may also be involved.

To investigate the relation between increased cAMP production and augmented IL-6 expression, we measured BCG-induced cAMP production in the presence of a specific adenylate cyclase inhibitor, SQ22536. As expected, the production of cAMP in BCG-treated MGH cells was reduced with increasing concentrations of SQ22536. Similarly, IL-6 mRNA expression and protein secretion in BCG-treated cells were also inhibited in the presence of SQ22536. Thus, BCG may be up-regulating IL-6 expression via a cAMP-dependent pathway, which could probably involve cAMP-dependent protein kinase. However, the cAMP-dependent pathway may not be the only pathway involved since SQ22536 was more potent at reducing cAMP accumulation than IL-6 expression. In addition, when MGH cells were treated with forskolin (an adenylate cyclase activator) or RO20-1724 alone in the absence of BCG, cAMP levels were also increased with corresponding augmented IL-6 protein secretion as compared to control cells (Table 2), similar to that observed when BCG was present.

Furthermore, H7, a potent inhibitor of protein kinase C ($K_i = 6.0$ μM) and cAMP-dependent protein kinase ($K_i = 3.0$ μM), was used for inhibiting downstream protein kinases that are activated by cAMP. As a negative control, HA1004, a potent inhibitor of cAMP-dependent protein kinase ($K_i = 2.3$ μM), was used due to its low

affinity for protein kinase C ($K_i = 40\ \mu M$). Our results showed that both H7 and HA1004 had inhibitory effects on BCG-stimulated IL-6 production, and subsequently inhibited IL-6 secretion. However, the inhibitory effect of H7 appeared to be more potent than that of HA1004, suggesting that BCG-induced IL-6 production in MGH cells is only partially mediated via a cAMP-dependent protein kinase pathway, and that other signal transduction pathways involving other protein kinases (such as protein kinase C) may also be involved. Therefore, it is possible that the regulatory effects of BCG on IL-6 production may be mediated via multiple second messengers and their downstream pathways.

Acknowledgments

We are grateful to Schering-Plough Pte. Ltd., Malaysia and Connaught Laboratories Limited, Canada for the gift of INTRON A and Immucyst. We also thank Hon Wei Min, Pook Sim Hwee and Carmel Lawrencia for their help. Ye Zhang is the recipient of a National University of Singapore postgraduate research scholarship. This work was funded by Grant RP960352N from the National University of Singapore.

References

[1] Morales A, Eidinger D, Bruce AW. Intracavitary bacillus Calmette-Guérin in the treatment of superficial bladder tumours. J Urol 1976;116:180–3.
[2] Brosman SA. Bacillus Calmette-Guérin immunotherapy: techniques and results. Urol Clin North Am 1992;19:557–64.
[3] Bohle A, Gerdes J, Ulmer AJ, Hofstetter AG, Flad HD. Effects of local bacillus Calmette-Guérin therapy in patients with bladder carcinoma on immunocompetent cells of the bladder wall. J Urol 1999;144:53–8.
[4] Ratliff TL, Ritchey JK, Yuan JJ, Andriole GL, Catalona WJ. T-cell subsets required for intravesical BCG immunotherapy for bladder cancer. J Urol 1993;150:1018–23.
[5] Jackson AM, Alexandroff AB, Kelly RW, Skibinska A, Esuvaranathan K, Prescott S, Chisholm GD, James K. Changes in urinary cytokines and soluble intercellular adhesion molecule-1 (ICAM-1) in bladder cancer patients after bacillus Calmette-Guérin (BCG) immunotherapy. Clin Exp Immunol 1995;99:369–75.
[6] Seguchi T, Yokokawa K, Sugao H, Nakano E, Sonoda T, Okuyama A. Interleukin-6 activity in urine and serum in patients with bladder carcinoma. J Urol 1992;148:791–4.
[7] Akaza H, Iwasaki A, Ohtani M, Ikeda N, Niijima K, Toida I, Koiso K. Expression of antitumor response: role of attachment and viability of bacillus Calmette-Guérin to bladder cancer cells. Cancer 1993;72:558–63.
[8] Ratliff TL, Kavoussi LR, Catalona WJ. Role of fibronectin in intravesical BCG therapy for superficial bladder cancer. J Urol 1998;139:410–4.
[9] Hudson MA, Ritchey JK, Catalona WJ, Brown EJ, Ratliff TL. Comparison of the fibronectin-binding ability and antitumor efficacy of various mycobacteria. Cancer Res 1990;50:843–7.
[10] Coplen DE, Brown EJ, McGarr J, Ratliff TL. Characterization of fibronectin attachment by a human transitional cell carcinoma line, T24. J Urol 1991;145:1312–5.
[11] Becich MJ, Carroll S, Ratliff TL. Mycobacterial proliferation in macrophages is prevented by incubation with lymphocytes activated in vitro with a mycobacterial antigen complex. J Urol 1991;145:1316–24.
[12] De Reijke TM, Vos PC, De Boer EC, Bevers RF, De Muinck Keizer WH, Kurth KH, Schamhart DH. Cytokine production by the human bladder carcinoma cell line T24 in the presence of bacillus Calmette-Guérin (BCG). Urol Res 1993;21:349–52.
[13] Esuvaranathan K, Alexandroff AB, Mcintyre M, Jackson AM, Prescott S, Chisholm GD, James K. Interleukin-6 production by bladder tumors is upregulated by BCG immunotherapy. J Urol 1995;154:572–5.
[14] Bevers RF, de Boer EC, Kurth KH, Schamhart DH. BCG-induced interleukin-6 upregulation and BCG internalization in well and poorly differentiated human bladder cancer cell lines. Eur Cytokine Netw 1998;9:181–6.
[15] Borden EC, Groveman DS, Nasu T, Renzikoff C, Bryan GT. Antiproliferative activities of interferons against human bladder carcinoma cell lines in vitro. J Urol 1984;132:800–3.
[16] Jackson AM, Alexandroff AB, Fleming D, Prescott S, Chisholm GD, James K. Bacillus Calmette-Guérin (BCG) organisms directly alter the growth of bladder tumour cells. Int J Oncol 1994;5:697–703.
[17] Niell HB, Maue AM, Rademacher D. Cytotoxic effects of alpha- and gamma-interferon and tumor necrosis factor in human bladder tumor cell lines. Urol Res 1994;22:247–50.
[18] Zhang Y, Khoo HE, Esuvaranathan K. Effects of bacillus Calmette-Guérin and interferon α-2b on human bladder cancer in vitro. Int J Cancer 1997;71:851–7.
[19] Zhang Y, Khoo HE, Esuvaranathan K. Effects of bacillus Calmette-Guérin and interferon α-2b on cytokine production in human bladder cancer cell lines. J Urol 1999;161:977–83.
[20] Lowrie DB, Aber VR, Jackett PS. Phagosome–lysosome fusion and cyclic adenosine 3′:5′-monophosphate in macrophages infected with *Mycobacterium microti*, *Mycobacterium bovis* BCG or *Mycobacterium lepraemurium*. J General Microbiol 1979;110:431–41.
[21] Sander B, Damm O, Gustafsson B, Andersson U, Hakansson L. Localization of IL-1, IL-2, IL-4, IL-8 and TNF in superficial bladder tumors treated with intravesical bacillus Calmette-Guérin. J Urol 1996;156:536–41.
[22] Thomson AW. The cytokine handbook. London: Academic Press, 1994.