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iNOS EXPRESSION AND NO PRODUCTION CONTRIBUTE TO THE DIRECT EFFECTS OF BCG ON UROTHELIAL CARCINOMA CELL BIOLOGY

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Abstract

OBJECTIVES—Evidence suggests that oxidative stress occurring as a consequence of inducible nitric oxide synthase/nitric oxide (iNOS/NO) contributes to the biologic effects of Bacille Calmette Guérin (BCG). Objective of the current study is to examine iNOS expression, NO production and the biologic impact of NO, on established intermediate end points for the human urothelial carcinoma cell response to BCG.

MATERIALS AND METHODS—Quantitative rt-PCR and real time measurement of NO was used to assess iNOS and NO production respectively, in two human urothelial carcinoma (UC) cell lines, in response to BCG. The effect of blocking NO production using the specific iNOS inhibitor 1400W was determined for multiple intermediate end points characterizing BCGs direct effects on tumor cell biology. Activation of NF- κ B and NRF2 signaling pathways, transactivation of genes including p21, CD54, IL6, IL8, CXCL1, CXCL3, CCL20 and cytotoxicity as measured by vital dye exclusion, lactate dehydrogenase (LDH) release and MTT assay were measured in response to BCG with and without iNOS inhibition.

RESULTS—Exposure of UC cells to BCG significantly increased both iNOS expression and NO production. Inhibition of iNOS activity with 1400W significantly inhibited BCG's direct biologic effect on UC cells for all of the end points evaluated.

CONCLUSIONS—iNOS expression, NO production and the associated oxidative stress play a central role in the response of UC cells to BCG exposure. Manipulation of oxidative stress may afford an opportunity to enhance the antitumor effects of BCG.

Keywords

Bladder cancer; iNOS; NO; BCG

1. Introduction

While its precise mechanism of action remains incompletely defined, the attenuated mycobacterium *Bacille Calmette Guérin* (BCG) remains the most effective agent currently available for the treatment of non-muscle invasive bladder cancer (NMIBC) [1–3]. One of BCG's recognized mechanisms is the induction of inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production in urothelial cells [4–6]. This effect appears to contribute to cellular cytotoxicity as well as facilitating a host cellular immune response [7, 8].

iNOS, a member of the NOS family, is an inducible, soluble, Ca^{++} independent enzyme expressed by a wide variety of cell types. iNOS functions to generate the free radical NO as a product of its enzymatic activity on L-arginine [9]. iNOS and NO production are important contributors to cellular oxidative stress [10, 11]. The consequences of iNOS associated oxidative stress are widely recognized. Reactive oxygen and nitrogen species activate signaling pathways, gene expression and can culminate in cytotoxicity. At the extreme, oxidative stress induced cytotoxicity can lead to cell death [11].

At present, data is lacking as to the specific contribution of iNOS/NO to the multiple direct biologic effects which characterize the consequences of urothelial carcinoma (UC) cells post-BCG exposure. Given the growing body of evidence that iNOS/NO contribute to BCG anti-tumor activity, this study set out to define the role of iNOS in mediating the direct effects of BCG on UC cells. BCG internalization in UC cells results in oxidative stress which activates signaling pathways leading to expression of iNOS and NO generation. This NO contributes more oxidative stress, leading to further activation of signaling pathways, gene transcription of cytokines and chemokines and phenotypic changes to elicit a host cellular immune response targeting the site of injury. These elements combine to culminate in an anti-tumor response (Figure 1). Using a robust panel of immediate end points of the direct tumor response to BCG, coupled with specific inhibitors of iNOS, our results demonstrate a central role of iNOS/NO stress in mediating the UC cell response to BCG. Inhibition of iNOS activity is associated with significant inhibition of BCG induced signaling, gene expression and alterations in the cellular cytotoxic response to BCG. These findings support an important role for iNOS/NO in mediating BCG's direct biologic effects, and offer insight into opportunities for new strategies to serve as alternatives to, or potentiators of, intravesical BCG therapy. In conclusion, manipulation of the iNOS/NO pathway may represent a strategy for improving the treatment efficacy of BCG.

2. Materials and methods

2.1 Cell Lines

The human UC cell line T24 was obtained from American Type Cell Culture (Rockville, MD). The 253J cell line was a kind gift of Dr. Richard Williams (University of Iowa). Cells were maintained at 37°C, 5% CO_2 in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin (Complete media).

2.2 Bacille Calmette- Guérin (BCG)

TICE BCG, living organisms of an attenuated, bacillus of Calmette and Guérin strain of *Mycobacterium bovis* were used in these experiments (Organon Inc., West Orange, NJ). Freeze dried BCG was reconstituted in complete media at an estimated concentration of 2.5×10^7 viable organisms/ml. (dilution assumed average viability of 4×10^8 organisms per vial based upon manufacturer's specified range of 1 to 8×10^8 per vial).

2.3 Real Time Measurement of Nitric Oxide

253J and T24 cells were plated at 1×10^4 cells/well in 96-well plate. Twenty-four h later, BCG (1:50 cells:BCG), 1400W, or a combination of 1400W and BCG, was added to the cultures. 1400W is a potent and selective inhibitor of iNOS [12]. 1400W was used at 500 μ M concentration based on dose dependent response (data not shown). The cells were incubated for 6 h and washed twice with sterile PBS. Intracellular NO levels were measured using fluorescence probe DAF-2DA (Calbiochem, Darmstadt, Germany) with excitation at 485 nm and emission at 535 nm.

2.4 Luciferase Reporter Assays

UC cell exposure to BCG has been shown to increase the activation of intracellular signaling pathways and p21 expression. The effect of iNOS inhibition on signaling pathway activation and p21 expression in response to BCG was measured as reported earlier [13]. The effect of BCG on signaling pathway activation was measured as follows: 253J and T24 cells were plated at 1×10^5 cells/well in 24-well plate. Twenty-four h later, the cells were transiently transfected with previously described NF- κ B and NRF2 plasmid reporter constructs using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Twenty-four h after transfection, cells were left untreated or treated with BCG (1:50 cells: BCG), 1400W and BCG with 1400W. Six h later, the cells were then washed with PBS and lysed with 1X reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured using a luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activities were normalized to protein concentration as measured by BCA protein assay kit (Pierce, Rockford, IL).

2.5 Quantitative rtPCR

Prior work has demonstrated that UC cell exposure to BCG increases the expression of cell cycle regulatory and immune response genes. Q-rtPCR was used to measure the expression of iNOS, IL-6, IL-8, CXCL11, CXCL13, CCL20, CD54 and p21. The effect of iNOS inhibition on gene expression in response to BCG was measured as previously described [14]. qRT-PCR was performed using the LightCycler® 480 Real-Time PCR System (Roche Applied Science, IN). β -actin gene expression was used to normalize the data in q RT-PCR experiments.

2.6 Dye Exclusion Assay for Cell Viability

Cytoplasmic membrane integrity, as measured by the ability of the cell to exclude vital dyes is used as a measure of cellular injury. The effect of iNOS inhibition on vital dye exclusion

in response to BCG was measured as described earlier [14]. Experimental groups included BCG (1:50 cells: BCG) 1400W or a combination of 1400W and BCG.

2.7 LDH Release Assay

LDH is a stable cytosolic enzyme, which is released upon cell lysis and/or injury to the cytoplasmic membrane. The effect of iNOS inhibition on LDH release in response to BCG was measured as follows: 1×10^5 253J or T24 cells were seeded in 24-well plate. The next day, the medium was replaced and BCG (1:50 cells: BCG), 1400W or a combination of 1400W and BCG was added to the cultures. The plate was incubated at 37°C for 24 h. Released LDH in culture supernatants was measured with CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI) by following manufacturer's instruction.

2.8 Cell Proliferation

BCG is known to exert an antiproliferative effect on UC cells. The effect of iNOS inhibition on BCG's anti-proliferative effect was measured using the MTT assay as previously described [15].

2.9 Statistical Analysis

All experiments were performed in triplicate at three different times. The data from individual experiments for assays employing luciferase reporter constructs was subject to arithmetic normalization relative to the highest values among the corresponding replicate experiments. Data was analyzed using two way ANOVA for repeated measures. Results were considered significant at $p < 0.05$. Graphical representation of the data is shown as the mean + S.E.

3. Results

3.1 iNOS expression

Two UC cell lines 253J and T24 were treated with BCG (1:50 ratio) for 6 h which resulted in up regulation of iNOS expression as measured by quantitative rtPCR. iNOS mRNA levels were significantly increased 8.9-fold and 4.7-fold in response to BCG in 253J and T24 cells, respectively, relative to controls (Figure 2, $p < 0.001$). As reported earlier, pretreatment of cells with 1400W inhibited BCG induced iNOS expression [16, 17]. Compared to BCG, cells treated with 1400W prior to BCG exposure exhibited significantly decreased iNOS expression (7.5- and 4-fold in 253J and T24 cells, respectively, $p < 0.0001$).

3.2 NO production

Both 253J and T24 cell lines were treated with BCG (1:50) with or without 1400W for 6 h for real time measurement of NO. The cellular NO production significantly increased following BCG exposure in both cell lines (Figure 3, $p < 0.005$). iNOS inhibition with 1400W significantly decreased NO production in 253J and T24 cells in response to BCG exposure (253J $p < 0.001$, T24 $p < 0.05$).

3.3 Activation of Intracellular Signaling pathways

Exposure of UC cells to BCG (1:50) for 6 h resulted in the activation of the NF- κ B and NRF2 intracellular signaling pathways. BCG significantly increased activation of both NF- κ B and NRF2 luciferase reporter construct ($p < 0.0001$). Relative to BCG, pretreatment of cells with the iNOS inhibitor 1400W significantly reduced NF- κ B reporter activation to 65% and 67% of that observed in response to BCG in 253J and T24 cells, respectively (Figure 4, $p < 0.01$). Treatment with 1400W prior to BCG exposure significantly reduced NRF2 activation to control levels, representing approximately 50% of the response to BCG alone in both cell lines ($p < 0.0001$). NF- κ B and NRF2 activation in response to BCG in 1400W treated cells was not significantly different than cells treated with 1400W alone (Figure 4).

3.4 Gene Expression

p21 expression in response to BCG has been shown to be necessary for non-apoptotic cell death and HMGB1 release [18]. Figure 5 demonstrates the effect of iNOS inhibition on activation of a p21 promoter-reporter construct in response to BCG treatment (1:50) of UC cells for 6 h. UC cells showed a significant increase in activation of p21 promoter reporter construct post-BCG exposure ($p < 0.0001$). Cells pretreated with 1400W showed significantly reduced p21 reporter activation in response to BCG relative to BCG alone ($p < 0.0001$). p21 activation in response to BCG in 1400W treated cells was not significantly different than cells treated with 1400W alone. Similar results were obtained with quantitative rtPCR for p21 (Figure 5).

Previous studies have shown that BCG exposure results in the up regulation of genes with potentially important roles in host immune response [14]. Figure 5 illustrates the results of quantitative rtPCR for genes including the cytokines/chemokines IL6, IL8, CXCL1, CXCL3 and CCL2 as well as the white blood cell receptor CD54. The results are shown as the fold-increase in specific mRNA relative to untreated controls. BCG exposure resulted in a significant increase in activation of “immune response” genes in both cell lines. (ANOVA, $p < 0.01$). Relative to controls BCG in combination with iNOS inhibition (1400W) also resulted in significantly increased expression of the six evaluated genes relative to controls (ANOVA, $p < 0.01$). Expression of the six genes in response to BCG+1400W, compared to BCG alone, was significantly lower in both 253J and T24 cell lines representing 37% and 33% of BCG values respectively (253J $p < 0.01$; T24 $p < 0.05$).

3.5 Effect of iNOS inhibition on BCG cytotoxicity

BCG treatment significantly increased the number of trypan blue positive cells in both 253J and T24 cell lines following BCG treatment (1:50) for 72h (Figure 6; ANOVA, $p < 0.0001$). Relative to controls BCG in combination with iNOS inhibition (1400W) also resulted in significantly increased trypan blue uptake (ANOVA, $p < 0.001$). BCG cytotoxicity was significantly lower in cells treated with 1400W relative to BCG alone. The percentage of trypan blue positive cells in the BCG+1400W group was 45% and 39% of the BCG group in 253J and T24 cells, respectively ($p < 0.001$). Exposure of UC cells to BCG significantly increased LDH release compared to controls representing 54% and 55% of maximal release (cell culture lysis) in 253J and T24 cells, respectively ($p < 0.0001$). This was reduced to 39% and 43% of maximal release, respectively, by 1400W pretreatment, a significant decrease

relative to BCG alone ($p < 0.05$). Of note, 1400W alone resulted in an increase in LDH relative to control cells. When the 1400W group is used as the baseline, the addition of 1400W to BCG decreased LDH release by 50% and 36%, respectively (Figure 7). Relative to 1400W alone, LDH release was significantly increased in the BCG+1400W group ($p < 0.001$).

As measured by the MTT assay, exposure of UC cells to BCG for 72 h significantly inhibited cell proliferation compared to controls representing 46% and 45% of control values in 253J and T24 cells, respectively ($p < 0.0001$). Treatment of cells with 1400W prior to BCG treatment inhibited BCG's antiproliferative/cytotoxic effect by 41% and 51%, respectively, in 253J and T24 cells ($p < 0.01$). Proliferation in response to BCG in 1400W treated cells was significantly lower than cells treated with 1400W alone (Figure 8; $p < 0.05$).

4. Discussion

Prior reports have demonstrated that iNOS is expressed in response to bacterial pathogens as part of a cellular defense mechanism [19]. iNOS functions to generate the free radical NO as a product of its enzymatic activity on L-arginine [9]. NO is a freely diffusible small molecule that has been shown to have a direct role in both intra- and intercellular signaling, as well as the regulation of a number of important physiologic processes. NO promotes the expression of inflammatory cytokines and can contribute to inflammatory disease processes. Simultaneously, excessive production of NO can have serious detrimental impact at both the cellular and organismal level [11]. As a consequence of free radical interaction with cellular proteins and lipids, excessive production of NO can damage cells and may contribute to carcinogenesis [20].

BCG is recognized as contributing to iNOS/NO production in the clinical setting. Studies have shown that increases in both iNOS and NO are observed in urothelial carcinomas in laboratory models and human bladders following the intravesical instillation of live BCG [4, 5, 8]. Several prior publications have reported increased bladder tissue expression of iNOS and NO production following the intravesical administration of BCG [5, 6]. Subsequent reports demonstrated persistent increases in NO production in the bladder for up to 6 months following BCG administration [21]. Similar responses have been reported in animal model systems [4].

In *in vitro* settings the manipulation of iNOS/oxidative stress has been demonstrated to influence BCG's treatment effect. Antioxidants or inducers of iNOS had a positive impact on the *in vitro* and *in vivo* effects of BCG respectively [7, 8, 22]. While one study has suggested that inhibition of iNOS may improve the tumor response to BCG [23], more studies have suggested that increasing iNOS production may be beneficial [7, 8, 22, 24]. Using BCG having constitutive expression of the iNOS inducer IFN gamma, Arnold et al. demonstrated improved response rates in an animal model system relative to conventional BCG [24].

Our prior work has demonstrated that the direct response of UC cells to BCG treatment as measured via multiple intermediate end points is remarkably similar to that observed in response to other types of oxidative stress. BCG induces the activation of multiple signaling pathways known to be oxidative stress sensitive including NF- κ B and NRF2 [14]. Increased intracellular signaling is associated with the downstream up regulation of multiple genes coding for chemokines and cytokines as well as the cyclin dependent kinase inhibitor p21. In a fraction of BCG exposed cells, these changes culminate in non-apoptotic cell death with the associated release of HMGB1 [25]. These observations parallel the types of responses observed in other biologic systems subject to “oxidative stress”.

The nature of the UC cell response to BCG prompted our group to study the role of iNOS/NO production in greater detail. The results of our current work demonstrate a contributing role for iNOS/NO production in all of the intermediate end points characterizing the UC cell response to BCG. BCG induced activation of intracellular signaling pathways involving NF- κ B and NRF2, expression of chemokines and cytokines including IL-6, IL-8, CXCL1, CXCL3, CCL20, and CD54, as well as direct cytotoxicity were all iNOS/NO dependent. Its effect on p21 expression is particularly notable, given prior work demonstrating that p21 expression is required for BCG’s direct cytotoxicity [18]. Overall, the iNOS/NO pathway appear to be a key contributor to BCG’s direct effect on urothelial carcinoma cell biology.

The insight provided by these observations serves as the basis for additional important questions. Specifically, what is the mechanism through which BCG generates oxidative stress resulting in iNOS expression and NO production? Can manipulation of BCG induced iNOS/NO production lead to enhanced BCG treatment efficacy? From a therapeutic standpoint, is more or less BCG induced oxidative stress valuable? Clearly, further study of the role of iNOS in BCG associated oxidative stress is warranted. Multiple pharmacologic agents are available to either potentiate or decrease iNOS/NO activity. These agents afford an opportunity for the clinical translation of these observations.

5. Conclusions

Exposure of UC cells to BCG up regulates iNOS expression and NO production. iNOS/NO increases correlate with multiple BCG induced changes in UC biology, which are inhibited by pretreating cells with the specific iNOS inhibitor 1400W. This data supports early studies in suggesting that manipulation of the iNOS/NO pathway may represent a strategy for improving the treatment efficacy of BCG.

Abbreviations

ANOVA	analysis of variance
BCG	Bacillus Calmette Guérin
CD54	intercellular adhesion molecule 1
CM	complete medium

CXCL1	chemokine (C-X-C motif) ligand 1
CXCL3	chemokine (C-X-C motif) ligand 3
CCL20	chemokine (C-C motif) ligand 20
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
IL6	interleukin 6
IL8	interleukin 8
iNOS	inducible nitric oxide synthase
LDH	lactate dehydrogenase
mRNA	messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	nuclear factor kappa B
NO	nitric oxide
NRF2	nuclear factor (erythroid-derived 2)-like 2
P21	cyclin dependent kinase inhibitor p21 (cip1; waf1)
PBS	phosphate buffered saline
RNA	ribonucleic acid
rtPCR	reverse transcriptase polymerase chain reaction
q rtPCR	quantitative reverse transcriptase polymerase chain reaction
UC	urothelial carcinoma

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Figure 1.

Physiological events post-BCG exposure: UC cell exposure to BCG results in $\alpha 5\beta 1$ receptor mediated BCG internalization involving $\alpha 5\beta 1$ cross linking. Following internalization, BCG generates oxidative stress which activates signaling pathway including NF- κ B, NRF2, gene transcription, and phenotypic changes. NF- κ B signaling pathway activates iNOS which generates NO contributing to further oxidative stress. $\alpha 5\beta 1$ cross linking also activates NF- κ B signaling pathway. NF- κ B also activates expression of various cytokines and chemokines to elicit a host cellular immune response.

Figure 2. BCG up regulates iNOS expression

UC cell exposure to BCG resulted in significant up regulation of iNOS expression as measured by quantitative rtPCR in both cell lines (** $p < 0.001$). Pretreatment of cells with the pharmacologic iNOS inhibitor 1400W significantly decreased iNOS mRNA expression by 253J and T24 UC cells in response to BCG exposure (*** $p < 0.0001$).

Figure 3. BCG increases intracellular nitric oxide production

Exposure of UC cells to BCG significantly increased NO production in both cell lines (* $p < 0.05$). Pretreatment of cells with the pharmacologic iNOS inhibitor 1400W significantly decreased NO production by 253J and T24 UC cells in response to BCG exposure (253J ** $p < 0.001$, T24 * $p < 0.05$)

Figure 4. The effect on iNOS inhibition on NF- κ B, NRF2 and p21 activation in response to BCG Exposure of UC cells to BCG significantly increased activation of an NF- κ B, NRF2 and p21 luciferase reporter construct (** $p < 0.0001$). Pretreatment of cells with the iNOS inhibitor 1400W significantly reduced activation in response to BCG relative to BCG alone (NF- κ B * $p < 0.01$, NRF2, p21 # $p < 0.0001$). NF- κ B, NRF2 and p21 activation in response to BCG in 1400W treated cells was not significantly different than cells treated with 1400W alone.

Figure 5. Effect of iNOS Inhibition on UC cell Gene Expression in Response to BCG

Exposure of UC cells to BCG significantly increased the activation of a panel of “immune response” genes in both 253J and T24 cells (ANOVA, ** $p < 0.01$). Relative to controls BCG in combination with iNOS inhibition (1400W) also resulted in significantly increased expression of the six evaluated genes relative to controls (ANOVA, $p < 0.01$). Expression of the six genes in response to BCG+1400W, relative to BCG alone, was significantly lower in both 253J and T24 cell lines representing 37% and 33% of BCG values respectively (253J $p < 0.01$; T24 $p < 0.05$).

Figure 6. Effect of iNOS inhibition on BCG cytotoxicity

Exposure of UC cells to BCG significantly increased percentage of cells staining positive for trypan blue in both 253J and T24 cells (ANOVA, *** $p < 0.0001$). Relative to controls BCG in combination with iNOS inhibition (1400W) also resulted in significantly increased trypan blue uptake (ANOVA, ** $p < 0.001$). BCG cytotoxicity was significantly decreased in cells treated with 1400W relative to BCG alone. The percentage of trypan blue positive cells in the BCG+1400W group was 45% and 39% of the BCG group in 253J and T24 cells respectively ($p < 0.001$).

Figure 7. The effect on iNOS inhibition on LDH release in response to BCG

Exposure of UC cells to BCG significantly increased LDH release compared to controls ($p < 0.0001$). Pretreatment of cells with the iNOS inhibitor 1400W significantly reduced LDH release in response to BCG relative to BCG alone ($p < 0.05$). LDH release in response to BCG in 1400W treated cells was significantly higher than cells treated with 1400W alone ($p < 0.001$).

Figure 8. The effect on iNOS inhibition on the antiproliferative effect of BCG

Exposure of UC cells to BCG significantly inhibited cell proliferation compared to controls as measured by the MTT assay (*** $p < 0.0001$). Pretreatment of cells with the iNOS inhibitor 1400W significantly reduced BCG's antiproliferative effect relative to BCG alone (** $p < 0.01$). Proliferation in response to BCG in 1400W treated cells was significantly lower than cells treated with 1400W alone (* $p < 0.05$).