Available online at www.sciencedirect.com
ScienceDirect
Nitric Oxide 15 (2006) 337–343
NITRIC OXIDE Biology and Chemistry
www.elsevier.com/locate/yniox

Enhanced formation of nitric oxide in bladder carcinoma in situ and in BCG treated bladder cancer

Abolfazl Hosseini [a,1], Lotta Renström Koskela [a,1], Ingrid Ehrén [a], Miguel Aguilar-Santelises [a], Allan Sirsjö [b], N. Peter Wiklund [a,*]

[a] *Department of Molecular Medicine and Surgery, Section of Urology, Karolinska University Hospital, Stockholm, Sweden*
[b] *Center for Molecular Medicine, Karolinska University Hospital, Stockholm, Sweden*

Received 16 November 2005; revised 18 January 2006
Available online 6 March 2006

Abstract

The purpose of the study was to analyze endogenous nitric oxide (NO) formation and NO-synthase (NOS) gene expression in the urinary bladder from patients with urinary bladder cancer and to investigate the relationship between local NO formation, treatment with Bacillus Calmette Guerin (BCG) and clinical stage in bladder cancer patients. One hundred and three patients with bladder cancer were studied. Endogenous formation of NO was measured in 72 patients, including 6 patients with BCG treated bladder cancer and 6 tumor free control subjects. iNOS expression was analyzed at transcriptional and protein level in biopsies from 31 patients with bladder cancer by real time polymerase chain reaction (PCR) and Western blot (WB), respectively. Three patients in this group had received BCG treatment. Eight biopsies from normal bladder served as control for PCR and WB analysis. Patients with carcinoma in situ (CIS) had higher iNOS expression ($p < 0.01$) and NO formation ($p < 0.01$) than control subjects and patients with papillary tumors without concomitant CIS. Markedly increased iNOS expression ($p < 0.05$) and NO formation ($p < 0.001$) were also found in patients treated with BCG as compared to the other groups. In conclusion, the presence of elevated NO concentration and iNOS expression in the urinary bladder from BCG treated patients and patients with CIS further supports the notion that NO may be an important factor in bladder cancer biology and that the BCG effect on superficial bladder cancer may partly be due to stimulation of local NO formation.

Keywords: Bladder cancer; Nitric oxide; CIS; BCG

Urinary bladder cancer is one of the most frequent cancers in the western world [1]. The predominant histological type is the transitional cell carcinoma, which accounts for more than 90% of all the bladder tumors in Europe and USA [2]. The most common form is a low-grade papillary transitional tumor that seldom progresses to invasive growth. Carcinoma in situ (CIS) is a less common form accounting for 1–4% of the primary tumors and occurs concomitantly with a papillary tumor in 13–20% of the bladder cancer patients [3,4]. CIS is a high-grade lesion with high risk of developing invasive growth. Approximately 50% of patients with untreated CIS develop invasive cancer within 5 years [5].

Treatment with Bacillus Calmette Guerin (BCG) infused directly into the bladder is an established treatment modality for superficial urinary bladder cancer and considered the golden standard for treating CIS [6]. Although the antitumor mechanisms following this treatment remain largely unknown, there is substantial evidence for a strong nonspecific inflammatory reaction and T-cell response involvement. Several cytokines that can be found in urine from BCG treated patients [7–9] are able to stimulate nitric oxide (NO) synthesis, and NO is one of the main factors responsible for the cytotoxic activity that macrophages exert against tumor cells [10]. NOS has been found in various types of tumors [11–13] suggesting that NO may be

* Corresponding author.
E-mail address: peter.wiklund@karolinska.se (N.P. Wiklund).
[1] These authors contributed equally to this work.

1089-8603/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.niox.2006.01.007

produced in tumor tissue. In some studies, NO appears to enhance tumor proliferation and angiogenesis [14] while in others, an increased NO-synthase (NOS) activity correlates with a diminished metastatic potential [15,16], or has no apparent effect on tumor growth [13].

NO is produced by three different NOS. The constitutively expressed endothelial NOS (eNOS) and neuronal NOS (nNOS) are induced by intracellular Ca^{2+} fluxes and produce small amounts of NO while the inducible NOS (iNOS) is Ca^{2+}-independent and produces large amounts of NO [17]. NO can stimulate cell growth and cell differentiation when present at low concentrations, whereas high concentrations often result in cytotoxic effects [18].

Previous data showing increased NO concentrations in the urinary bladder from patients treated with BCG [19–22] suggested that NO may be a critical factor in the BCG-mediated anti-tumor effect. Thus, with the aim to gain further knowledge on the participation of NO as effector molecule in bladder cancer biology, we have measured the endogenous NO formation and iNOS gene expression at transcriptional and protein level in the urinary bladder from patients with bladder cancer of different grade and stage.

Table 1A
Study population for measurement of endogenous NO formation

Histological type	Number of patients	Age of patients
Papillary tumors without concomitant CIS	$n = 53$	68 ± 14
G I tumors	$n = 24$	65 ± 10
G II tumors	$n = 21$	69 ± 12
G III tumors	$n = 8$	72 ± 14
Papillary G III tumors with concomitant CIS	$n = 7$	73 ± 7
Carcinoma in situ alone(CIS)	$n = 6$	62 ± 10
Patients treated with BCG	$n = 6$	75 ± 3
CIS	$n = 3$	
Recurrent Ta–TI GII	$n = 3$	
Control subjects	$n = 6$	43 ± 13

Table 1B
Histological stage of papillary tumors in patients used for measurement of endogenously formed NO

Histological stage	Number of patients
Papillary tumors	$n = 53$
Ta tumors	$n = 45$
T1 tumors	$n = 7$
>T1 tumors	$n = 1$

Materials and methods

NO determinations in human urinary bladder

NO concentration in the urinary bladder was determined prior to cystoscopy with biopsy and/or transurethral tumor resection in 72 patients with bladder cancer, including 6 patients who had received treatment with intravesical BCG instillations. Tumors were categorized according to the WHO 1999 classification system [23] and the patients were grouped according to tumor grade (G) and stage (T). NO concentration was also measured in the urinary bladder from 6 patients with stress incontinence, which were used as control subjects. The NO measurements in patients who had received BCG treatment were made 1–4 weeks after the last treatment. All patients had received at least an induction treatment with six weekly instillations. The cause for BCG treatment was in three patients CIS and in three patients recurrent Ta-T1 GII tumors. The final study population for endogenously formed NO is seen in Tables 1A and 1B. Reagent strip urine analysis for urinary tract infection (UTI) was negative in all patients.

A 100% silicon catheter (Argyle, Sherwood Medical) was introduced into the bladder and 25 mL room air was infused into the catheter balloon. After 5 min incubation, the air was aspirated into a syringe and injected into a chemiluminescense NO analyzer (CLD 700, Eco Physics, Dürnten, Switzerland) to register peak levels of NO. Air from the examination room was also collected and analyzed to determine the NO concentration in the bladder by subtracting the NO level in the room air from the peak value in the air incubated in the catheter balloon. According to our previous studies, the NO levels were also corrected for an 80% recovery of NO in to the silicon catheter balloon [24]. NO concentrations were analyzed within a few minutes after collecting the samples. NO is fairly stable in the gaseous phase and it has an oxidation rate of 25% after 2.3 h at a NO level of 10,000 parts per billion (ppb) in air containing 20% oxygen [25]. The detection limit for NO was 1 ppb and the analyzer was calibrated at known concentrations of NO in N_2, using an electromagnetic controller. This chemiluminescence assay is highly specific for NO and there is no interference from other nitrogen oxides [26].

Tissue collection

Samples were obtained from patients undergoing transurethral surgery. Biopsies were taken from 28 patients with bladder cancer, 3 patients with bladder cancer treated with BCG and 8 tumor free control subjects. The biopsies were snap frozen in liquid nitrogen and stored at −70 °C until analyzed. Corresponding material from the tumors and the surrounding mucosa was taken and sent for histopathological examination. In 22 of the patients with bladder cancer, biopsies were taken from the tumor lesion and/or the surrounding mucosa. Four of these patients had CIS lesions, two of those were primary CIS lesions in patients without papillary tumors and two were concomitant and found in the surrounding mucosa of patients with papillary GIII tumors. In 6 of the patients with bladder cancer biopsies were only taken from the surrounding mucosa, since the tumors were very small and had to be sent for histopathological examination. In 2 of these cases CIS was found in the surrounding mucosa. The final study population is shown in Tables 2A and 2B.