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Inhibition of nitric oxide is a good therapeutic target for bladder tumors that express iNOS

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ABSTRACT

Bladder cancer is the second cause of death for urological tumors in man. When the tumor is nonmuscle invasive, transurethral resection is curative. On the other hand, radical cystectomy is the treatment chosen for patients with invasive tumors, but still under treatment, these patients have high risk of dying, by the development of metastatic disease within 5 years. It is therefore important to identify a new therapeutic target to avoid tumor recurrences and tumor progression. Nitric oxide (NO) is an important biological messenger known to influence several types of cancers. In bladder cancer, production of NO and expression and activity of inducible NO synthase was associated to recurrence and progression. The objective of this work was to analyze if inhibition of nitric oxide production could be considered a therapeutic target for bladder tumors expressing iNOS. Using a bladder cancer murine model with different invasiveness grade we have demonstrated that NO inhibition was able to inhibit growth of bladder tumors expressing iNOS. Furthermore, invasive properties of MB49-I orthotopic growth was inhibited using NO inhibitors. This paper also shows that levels of NO in urine can be correlated with tumor size.

In conclusion, inhibition of NO could be considered as a therapeutic target that prevents tumor growth and progression. Also, urine NO levels may be useful for measuring tumor growth.

Introduction

Bladder cancer (BC) ranks fifth in malignant tumor frequency and second as cause of death from genitourinary cancer in men [1]. Transitional bladder tumors originated from urothelial cells, are the most frequent type of BC, and are classified as non muscle invasive (NMI) when tumors are confined to mucosa (pTa) or to lamina propria (pT1), and as muscle invasive when the detrusor muscle is affected. At the time of diagnosis 80% of tumors are NMI and if the adjacent and distant urothelium is histological normal, transurethral resection is curative. However, recurrences are common in BC, and in many cases progression to muscle invasion may occur. The invasive bladder tumors (pT2–pT4) require more aggressive therapies involving radical cystectomy and/or chemo and radiotherapy [2].

Although many groups have attempted to clarify the biological mechanisms that lead to tumor invasion [3,4], these mechanisms are not yet fully known. Deregulation of extra cellular proteolysis involving plasminogen activator system, matrix metalloproteinase (MMPs) and Cathepsins among others [5,6] are implicated in the invasion process. Recently, our laboratory has developed an orthotopic invasive BC murine model useful to study mechanisms involved in tumor progression. Orthotopic NMI and invasive bladder tumors are generated by electro-cauterization of the bladder wall and subsequent instillation of MB49 and MB49-I bladder cancer cells, respectively. *In vitro*, the MB49-I cell line showed higher invasive properties than MB49 associated with an increase of proteolytic enzymes such as metalloproteinase 9 (MMP-9) and urokinase-type plasminogen activator (uPA) [7]. Both tumor cell lines were able to produce nitric oxide (NO) and express the inducible NO synthase (iNOS) isoform; however, the invasive MB49-I showed higher iNOS expression than the MB49 cell line [8].

NO has been shown to have dichotomous effects on cell proliferation, apoptosis, migration, invasion, angiogenesis and many

Abbreviations: BC, bladder cancer; NMI, nonmuscle invasive; MMP, matrix metalloproteinase; uPA, urokinase-type plasminogen activator; NO, nitric oxide; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; L-NAME, N-nitro-L-arginine-methyl-esterHCl; CM, conditioned medium; DAPI, 4′,6-diamino-2-phenylindole.

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other important processes involved in cancer biology. It has shown to be both pro- and anti-tumorigenic, depending on its concentration, time of exposure and tumor microenvironment [9]. The two isoforms that are constitutively expressed (neuronal, nNOS and endothelial, eNOS) produce nano molar NO levels. By the contrary, iNOS isoform, produces higher NO levels, in a micro molar order, in response to inflammatory stimuli [3]. In physiological conditions when the stimuli stop the expression and activity of iNOS decreases. However, if iNOS activity is prolonged in time, carcinogenesis induction, tumor growth and/or tumor progression may occur [10]. Concordantly, it is known that a continuous overexpression of iNOS leads to DNA damage, increased cell proliferation, tumor vascularity and metastatic potential [11].

We previously reported that iNOS, that was not expressed in human normal bladder epithelium, has been found in almost 50% of patients with bladder cancer and, associated with early recurrences [12]. Furthermore, iNOS expression was associated with invasive human BC [8]. Taking into account the relation between invasion and iNOS expression in BC, and the fact that NO could be involved in the activation of MMPs and angiogenesis activities [13,14], it is reasonable to hypothesize that the inhibition of NO should be a good approach in the treatment of patients with bladder tumors expressing iNOS.

In this study, using the MB49/MB49-I murine model, which mimics human disease, we have investigated the role of tumor cell derived iNOS on cell proliferation, migration, proteolytic activity, as well as tumor growth, angiogenesis, and experimental metastasis.

Materials and methods

Cell culture

The murine BC cell line, MB49, the invasive cell line MB49-I and MBT2, were cultured in RPMI-1640 (GIBCO 31800-14). Culture media were supplemented with 2 mM L-glutamine, 80 μg/ml gentamicin and 10% fetal bovine serum FBS as described [15].

Cell viability assay

5×10^4, MB49, MB49-I or MBT2/ml were cultured in 96-well plates with RPMI1640 + 10% FBS. After 24 h incubation, N-nitro-L-arginine-methyl-esterHCl (L-NAME, Sigma) (2 mM) in RPMI supplemented with 2% FBS was added. Cells were cultured for 48 h and cell viability was determined by the MTS assay (Promega, G5421).

Conditioned medium (CM), tumor homogenate preparation and cell lysates

Semiconfluent monolayers growing in 35 mm plates were treated or not with L-NAME (2 mM) for 24 h. Culture medium was replaced by serum-free medium. After 24 h CM was harvested and the numbers of cells in the remaining monolayers were quantified. CM was stored at −80 °C. Tumors or subconfluents monolayers were homogenized in buffer containing 50 mM tris–HCl (pH 8), 100 μg/ml NaCl and 1% Triton. Tissues were homogenized twice for 10 s at 70% power using an Ultra-Turrax® T25. Protein content was determined by the Bradford method.

Western blot assay

Proteins (80 μg) from the different homogenates were electrophoresed on 10% of SDS–PAGE and then transferred to PVDF membranes, as previously [16]. iNOS (1:200) (Abcam 15323), pERK (1:200) (sc-7383), ERK (1:200) (sc-135900), pAKT (1:1000) (sc-7985-R), AKT (1:1000) (sc-8312) primary antibodies were used. Densitometric units of pAKT or pERK were referred to corresponding band of AKT or ERK respectively. Densitometric units of iNOS were referred to the correspondent band of beta-actin (1:20,000) (Sigma A5441). Values were referred as folds of change of control.

MMP and uPA activity

MMP and uPA were determined in CM as previously described [17]. MMP activity was expressed in arbitrary units (AU)/10^6 cells. Caseinolytic uPA activity was referred towards the urokinase standard curve (range 0.1–10 IU/ml). Results are shown in urokinase international units (IU) /10^4 cells.

Cell migration

To analyze the effect of NOS inhibition on cell migration, 1×10^5 cells were seeded on 6-well plates. After 24/48 h, when confluence was reached, a wound with a tip was done in the monolayer and then photographed. Monolayers were then treated or not with L-NAME (2 mM) in RPMI + 2% FBS and after 18 h the same area was photographed. Cell migration was assessed by determining the covered area by the difference between final and initial wound, considering 20 fields per plate.

Nitric oxide (NO) determination

Subconfluent MB49, MB49-I or MBT2 monolayers were treated with or without 2 mM L-NAME. NO was determined in culture supernatant 24 h later using the Griess reagent, as described previously [18].

Immunofluorescence assay

MB49 or MB49-I cells were grown in chamber slides. Both cell lines were treated or not with L-NAME (2 mM) for 24 h. Immunofluorescence was performed as previously described [16] using iNOS (Abcam 15323) (1:200) antibody and rabbit IgG as isotype control. Anti-rabbit-Alexa-488 conjugate (Invitrogen) (1:1000) was used as secondary antibody. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma).

Heterotopic Tumor growth

MB49 or MB49-I cells (2.5×10^5 cells in 0.1 ml) and MBT2 (2×10^6 cells in 0.1 ml) were subcutaneously (s.c) injected into the left flank of syngeneic mice (C57BL/6 J or C3H, respectively) as previously described [18]. Animals were randomly divided into groups that received no treatment (control) or those receiving L-NAME in drinking water (0.5 g/L) (8 mice per group). Tumor growth was registered twice a week by measuring two perpendicular diameters, and the formula $(D \times d)^{1/2}$ (mm) was used to calculate tumor growth, where D is the longer diameter and d is the shorter one. C57BL/6 J mice (approximately 6 weeks old) were obtained from animal facility of the Institute of Oncology Angel H. Roffo (Buenos Aires, Argentina) and C3H mice were obtained from National Academy of Medicine (Buenos Aires, Argentina).

Orthotopic Tumor Growth

Orthotopic MB49 or MB49-I (2.5×10^4 cell/mouse) tumors were developed as described [7] in the bladder of C57BL/6 J female mice. Animals were randomly divided into groups that received no treatment (control) or those receiving L-NAME in drinking water (0.5 g/L) (8 mice per group). Mice were monitored twice weekly for hematuria. Urine was collected at the end of the experiment,